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# **PREDICTION AND PREVENTION OF VENOUS THROMBOSIS IN PREGNANCY**

BY

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MBBChBAO, MRCPI

A thesis presented to the University College Cork for the degree of Doctor of Philosophy

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To Nizam

## ABSTRACT

**Background:** Venous thromboembolism (VTE) remains the leading cause of maternal morbidity and mortality. Delayed diagnosis, inadequate treatment and inadequate thromboprophylaxis account for the majority of deaths associated with this disease. Recent triennial reports identified obese pregnant women and women post caesarean section (CS), as two groups particularly at risk where further research is required. Although there are accepted risk factors for thromboembolism during pregnancy, these risk factors are absent in a significant number of cases indicating the need for a simple and effective screening tool for pregnancy. Perturbation of the uteroplacental haemostasis has been implicated in placenta mediated pregnancy complications in thrombophilic women. Despite scientific uncertainty, LMWH have been employed for the prevention of placenta mediated pregnancy complications.

This thesis had 4 main aims:

- 1)** To investigate the full anticoagulant effects over 24 hours following a fixed prophylactic dose of tinzaparin (4,500 iu/day) in healthy women post elective caesarean section.
- 2)** To evaluate the CAT assay as a potential predictive tool for thrombosis in pregnancy.
- 3)** To compare the anticoagulant effects of fixed dose versus weight adjusted LMWH dose used for thromboprophylaxis in morbidly obese pregnant women.
- 4)** To investigate the LMWH effects on human haemostatic gene and antigen expression in placental tissue and plasma from the uteroplacental, maternal and fetal circulation from women treated with LMWH prophylaxis.

**Methods:** Tissue factor pathway inhibitor (TFPI), thrombin antithrombin (TAT) and calibrated automated thrombogram (CAT) and anti-Xa levels were measured in twenty healthy women who received 4,500iu tinzaparin 6 hours post CS (CS1), twenty women who received 4,500iu tinzaparin 10 hours post delivery (CS2) and twenty women post spontaneous vaginal delivery (SVD). In Study 2, twenty patients with documented VTE in pregnancy or postpartum and 61 controls were identified from the SCOPE cohort (total of 5000 pregnant women). CAT assay with and without presence of thrombomodulin was performed on citrated plasma collected at 15 weeks gestation, prior to any thrombotic event.

Twenty morbidly obese pregnant women were started on a fixed dose of tinzaparin (4,500 iu/day) in Study 3, at 32 weeks gestation and then changed to a weight adjusted dose (75iu/kg/day) after 48 hours of wash-out period, for the remainder of their pregnancy. Four hour post dose venous

blood were taken after each initial dose and repeated every 2 weeks until delivery. TAT, TFPI, CAT and anti-Xa assays were performed and levels were compared with twenty normal weight women at the same gestation.

In the final study, eight women on antenatal LMWH prophylaxis (tinzaparin 75 IU/kg) due to moderate risk of VTE undergoing CS and a control group of 15 healthy pregnant women undergoing CS had venous blood taken from the peripheral and uterine vein before delivery of placenta. Simultaneously, cord venous blood and placental biopsy was collected. TFPI, TAT and CAT were measured. Real-time PCR and ELISA were used to quantify mRNA and protein expression of TFPI and TF in placental tissue.

**Results:** In Study 1, prior to initiation of LMWH, TAT levels at 6 hours post delivery were significantly higher in the CS1 and CS2 groups than the SVD group ( $P<0.002$ ); TAT levels were significantly reduced up to 24 hours post LMWH treatment despite declining anti-Xa levels ( $P<0.001$ ). In CS1, peak thrombin and ETP were significantly reduced following LMWH prophylaxis ( $P<0.0001$ ;  $P<0.002$ ) and reverted to pre-delivery levels 10hours post LMWH. TFPI levels mirror anti-Xa levels during the 24 hours following LMWH treatment in CS1 group with peak levels coinciding with peak anti-Xa levels 4 hours post injection.

In venous blood collected prior to thrombotic event using the SCOPE data and biobank, there were no significant difference in ETP, peak thrombin, lagtime or time to peak or normalised thrombomodulin sensitivity ratio using the CAT parameters between pregnant women who eventually developed VTE and controls.

Before LMWH prophylaxis, TFPI levels in the obese group at 30 weeks were significantly lower ( $p<0.001$ ) and ETP and peak thrombin levels in obese group were significantly higher compared with controls ( $P<0.0001$ ;  $P<0.001$ ). Within the obese group, there was no significant difference between ETP levels before and after fixed LMWH dose. However, ETP levels were significantly lower post weight-adjusted dose (75iu/kg tinzaparin) compared with post fixed dose. There was a significant effect of LMWH on TFPI levels, ( $p<0.0001$ ). ETP correlated positively with total body weight at fixed dose ( $r=0.578$ )( $p<0.05$ ). Anti-Xa did not correlate with total body weight at both fixed dose or weight adjusted dose.

TAT levels within uterine vein are significantly higher compared to maternal peripheral circulation in both the control group ( $P<0.0001$ ) and LMWH group ( $P<0.02$ ). In the LMWH group, TAT is reduced compared with controls in the uterine vein ( $P<0.001$ ). ETP and TFPI within uterine

circulation is reduced significantly in the LMWH group ( $P<0.05$ ) and ( $P<0.02$ ) respectively. Down-regulation of placental TFPI and TFPI<sub>2</sub> mRNA expression was also found ( $p<0.05$ ). Placental TF mRNA expression in LMWH group showed a non significant increase compared to control and this is replicated in placental TF antigen expression.

**Conclusion:** In women post CS, anti-Xa levels do not reflect the full anticoagulant effects of LMWH. LMWH thromboprophylaxis in this healthy cohort of patients appears to have a sustained effect in reducing excess thrombin production post elective CS. The results of this study suggest that predicting VTE in pregnant women using CAT assay is not possible at present time. The prothrombotic state in pregnant morbidly obese women was substantially attenuated by weight adjusted but not at fixed LMWH doses. LMWH may be effective in reducing *in- vivo* thrombin production in the uteroplacental circulation of thrombophilic women. All these results collectively suggest that at appropriate dosage, LMWH is effective in attenuating possible excess thrombin generation, be it in low risk pregnant women post caesarean section or moderate to high risk pregnant women who are morbidly obese or tested positive for thrombophilia. The results of the studies provided data to inform evidence-based practice to improve the outcome for pregnant women at risk of thrombosis.

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## DECLARATION

I declare that this thesis contains my own work and that the work has not been submitted to any other university.

I declare that full and informed consent was obtained from all participating patients.

I agree that the Librarian of University College Cork may lend or copy this thesis on request.

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Siti Khadijah Ismail

I declare that the work in this thesis was undertaken by Dr Siti Khadijah Ismail

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## PUBLICATIONS AND PRESENTATIONS

### Publications

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- Ismail SK, Higgins JR. Semin Thromb Hemost. 2011 Mar;37(2):111-7. **Hemostasis in pre-eclampsia.**
- [http://www.rcpi.ie/Faculties/Institute of Obstetricians and Gynaecologists Down/Thromboprophylaxis in Pregnancy - Clinical Practice Guidelines](http://www.rcpi.ie/Faculties/Institute%20of%20Obstetricians%20and%20Gynaecologists/Down/Thromboprophylaxis%20in%20Pregnancy%20-%20Clinical%20Practice%20Guidelines)

### Abstract Publications

- Ismail SK, Norris L, Kelly L, Higgins JR. Reproductive Sciences (Conference Abstracts: Society for Gynecologic Investigation 58<sup>th</sup> Meeting). March 2013. Low molecular weight heparin and the uteroplacental haemostasis.
- Ismail SK, Norris L, O'Shea S, Higgins JR. Reproductive Sciences (Conference Abstracts: Society for Gynecologic Investigation 58<sup>th</sup> Meeting). March 2013. Low molecular weight heparin and the uteroplacental haemostasis.
- Ismail SK, Norris L, O'Shea S, Higgins JR. Thromb Res. Papers and Abstracts of The 5<sup>th</sup> International Symposium on Women's Health Issues in Thrombosis and Haemostasis Vienna, Austria. 2013 January; 131 (Suppl 1): S76. OC-20 Effects of LMWH prophylaxis on morbidly obese pregnant women.
- Ismail SK, Norris L, Kelly L, Higgins JR. Thromb Res. Papers and Abstracts of The 5<sup>th</sup> International Symposium on Women's Health Issues in Thrombosis and Haemostasis Vienna, Austria. 2013 January; 131 (Suppl 1): S72. OC-06 Effects of low molecular weight heparin on the uteroplacental unit.
- Ismail SK, Norris L, Kelly L, Higgins JR. Obstetric Medicine (Conference Abstracts: International Society of Obstetric Medicine). July 2012. Effect of LMWH on the Uteroplacental Unit.
- Ismail SK, Norris L, Kelly L, Higgins JR. Reproductive Sciences (Conference Abstracts: Society for Gynecologic Investigation 58<sup>th</sup> Meeting). 2012 March. Effect of LMWH on the Uteroplacental Compartment.

- Ismail SK, Norris L, Muttukrishna S, Higgins JR. Reproductive Sciences (Conference Abstracts: Society for Gynecologic Investigation 57<sup>th</sup> Meeting). 2011 March. Effect of LMWH Prophylaxis on Thrombin Generation in Post Caesarean Section Women.
- S.K. Ismail, L. Norris, S. Muttukrishna, J.R. Higgins. Thromb Res. Papers and Abstracts of The 4th International Symposium on Women's Health Issues in Thrombosis and Haemostasis Berlin, Germany. 2011 Feb; 127 (Suppl 3): S137-S138. P.36 Effect of LMWH prophylaxis on thrombin generation post-caesarean section

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- **Effects of LMWH on Uteroplacental Haemostasis**  
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- **Effects of LMWH on the Morbidly Obese Pregnant Women**  
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Irish Congress of Obstetrics and Gynaecology, December 2012, Glen Druids, Wicklow.
- **Effects of LMWH on the Morbidly Obese Pregnant Women**  
Irish Congress of Obstetrics and Gynaecology, December 2012, Glen Druids, Wicklow.
- **Effects of LMWH on the Uteroplacental Compartment**  
International Society of Obstetric Medicine, July 2012, Keble College, Oxford, London
- **Effects of LMWH on Thrombin Generation Post Caesarean Section.**  
Junior Society of Obstetricians and Gynaecologists Meeting. November 2011. Dublin
- **Thrombin Generation in Post Caesarean Section Women and Effects on LMWH.**  
Irish Perinatal Society Meeting, November 2010, Royal College of Physicians of Ireland, Dublin.

## GLOSSARY OF ABBREVIATIONS

ACCP	American College of Chest Physicians
aCL	Anticardiolipin antibody
a $\beta_2$ GP <sub>I</sub>	Anti-beta-2-glycoprotein-I antibody
APC	Activated protein C
aPL	Antiphospholipid antibody
aPT	Anti prothrombin antibody
APTT	Activated thromboplastin time
APS	Antiphospholipid syndrome
BCA	Bicinconinic acid
BMI	Body mass index
BSA	Bovine serum albumin
CAT	Calibrated automated Thrombogram
cDNA	Complementary deoxyribonucleic acid
CS	Caesarean section
C <sub>T</sub>	Cycle threshold
DEPC	Diethylpyrocarbonate
dNTP	deoxyribonucleotide triphosphate
DVT	Deep venous thrombosis
ePCR	Endothelial cell protein C receptor
eNOS	Endothelial nitric oxide synthase
ETP	Endogenous thrombin potential
FDP	Fibrin degradation products
FPA	Fibrinopeptide A
F V	Factor V
F VII	Factor VII

F VIII	Factor VIII
F IX	Factor IX
F X	Factor X
F1.2	Prothrombin fragment F1.2
HESC	Human endometrial stromal cells
HRT	Hormone replacement therapy
HS	Heparin sulphate
LMWH	Low molecular weight heparin
MP	Microparticles
mRNA	Messenger ribonucleic acid
MTHFR	Methylenetetrahydrofolate reductase
n-TMs <sub>r</sub>	Normalised thrombomodulin sensitivity ratio
NAC	No amplification Control
NTC	No template control (negative control)
PAI	Plasminogen activator inhibitor
PAP	Plasmin $\alpha_2$ antiplasmin complex
PBS	Phosphate Buffer Saline
PC	Protein C
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PMC	Placenta mediated pregnancy complications
PSGL	P-selectin glycoprotein
PT	Partial thromboplastin time
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomised controlled trial
RNA	Ribonucleic acid
TAT	Thrombin antithrombin

TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
tPA	Tissue plasminogen activator
UFH	Unfractionated heparin
uPA	Urokinase type plasminogen activator
VTE	Venous thromboembolism
vWF	von Willebrand factor

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# **Chapter One: Introduction**

## **1.1 Venous Thromboembolism**

### **1.1.1 Epidemiology and Risk Factors**

Venous thromboembolism (VTE) is a collective term describing deep vein thrombosis (DVT) and/or pulmonary embolism (PE). VTE is a term describing formation of 'clot' or thrombi within the vein. DVT mainly occurs in the lower limbs whereas PE is a complication of DVT that occurs when part of the thrombus breaks away from the vein in the lower limb and travels up to the lungs, lodging in the pulmonary artery, resulting in the obstruction of normal blood flow[1]. VTE is the third leading cause of death in the general population, after myocardial infarction and stroke. The average annual incidence of VTE among American Caucasians is 108 per 100,000 person-years. Studies have estimated that in the North America, 900,000 patients develop VTE annually and 300,000 of those die due to PE and the incidence of VTE has not changed significantly in the last 25 years [2, 3]. VTE is a major health problem in Europe, with over one million VTE events or deaths per annum [4]. VTE is a disease with episodic recurrence; about 30% of patients develop recurrence within the next ten years [5]. The hazard of recurrence varies with the time since the incident event and is highest within the first 6–12 months. While anticoagulation is effective in preventing recurrence, the duration of anticoagulation does not affect the risk of recurrence once primary therapy for the incident event is stopped [6]. Given the availability of effective VTE prophylaxis, many of these events and deaths could have been prevented. However, the lack of specific predictors and biomarkers of VTE has hampered progress in this area.

#### **Risk Factors for Venous Thromboembolism and Recurrence**

VTE is a multifactorial disease that can be influenced by genetic factors (hereditary thrombophilias such as Factor V Leiden and prothrombin 20210 gene mutation), acquired

factors (e.g antiphospholipid antibodies) and environmental factors. Acquired risk factors would include old age, surgery, trauma, hospital or nursing home confinement, active cancer, obesity, pregnancy, hormone therapy and immobilization [7]. The process of aging is associated with changes to the haemostatic system which contribute to the increase the risk of VTE [8]. Compared to community residents, hospitalized patients have over a 100-fold increased incidence of acute VTE [9]. Hospitalization and nursing home residence together account for almost 60% of VTE events [10]. Thus, VTE prophylaxis during hospital confinement provides an important opportunity to significantly reduce VTE incidence. It is noteworthy that, hospitalization for medical illness and hospitalization for surgery account for almost equal proportions of venous thromboembolism (22% and 24%, respectively), emphasizing the need to provide thromboprophylaxis to both of these risk groups.

### **Surgery**

Surgery and immobilisation are usually treated as a combined risk factor for VTE. Surgery can increase VTE risk particularly in patients undergoing lower limb surgery such as knee or hip replacement surgery. Additionally, the recovery for these surgeries is typically involves long periods of immobilisation, which can lead to DVT [11]. The risk among surgical patients can be further stratified based on patient age, type of surgery and the presence of active cancer [11, 12]. The incidence of postoperative venous thromboembolism is increased with advancing patient age. High-risk surgical procedures include neurosurgery, major orthopaedic surgery of the leg, thoracic, abdominal or pelvic surgery for malignancy, renal transplantation, and cardiovascular surgery [11]. After controlling for the type of surgery and active cancer, additional independent risk factors for VTE within three months after major surgery include increasing body mass index, intensive care unit admission for six days or longer, a central venous catheter, prolonged immobility, varicose veins and infection [13].

## **Obesity**

Obesity is also a risk factor for VTE. Increased lipid levels are usually seen in these patients leading to vascular damage and activation of the haemostatic system, surgery such as gastric bypass can also exacerbate VTE risk [14]. In a review of hospital records, the risk of DVT and PE was 2.2 and 2.5 times higher respectively, in patients who were obese [15]. The relationship between excess body weight and VTE recurrence was found to be linear. The adjusted hazard ratio for each 1-point increase in BMI was 1.044 (95% confidence interval [CI] 1.013-1.076) ( $P < 0.001$ ). Four years after discontinuation of anticoagulant therapy, the probability of recurrence was 9.3% (95% CI, 6.0%-12.7%) among patients of normal weight and 17.5% (95% CI, 13.0%-22.0%) among overweight and obese patients, respectively. Compared with patients of normal weight, the hazard ratio of recurrence adjusted for age, sex, factor V Leiden, prothrombin G20210A mutation, high factor VIII levels, and type of initial venous thromboembolic event was 1.6 (95% CI, 1.1-2.4) ( $P = .02$ ) among obese individuals [16].

## **Women and Venous Thromboembolism**

Among women, additional risk factors for VTE include oral contraceptive use and hormone therapy (HRT) [17] including therapy with the selective oestrogen receptor modulators. Both pregnancy and the postpartum period are associated with increased VTE particularly following caesarean section delivery [18]. First and third generation oral contraceptives convey higher risk than second generation oral contraceptives. Hormone therapy is associated with a 2- to 4-fold increased risk of VTE, but the risk may vary by type of oestrogen [17]. The overall incidence of pregnancy-associated VTE is about 200 per 100,000 woman-years [18]. Compared to non-pregnant women of childbearing age, the relative risk is at least two to five-fold [19]. The risk during the postpartum period is about 5-fold higher per day than the risk during pregnancy [18].

## **Thrombophilia**

Recent family-based studies indicate that VTE is highly heritable and follows a complex mode of inheritance involving environmental interaction [20, 21]. Inherited reductions in plasma natural anticoagulants (e.g., antithrombin, protein C, or protein S) have long been recognized as uncommon but potent risk factors for VTE. More recent discoveries of impaired down regulation of the procoagulant system (e.g., activated protein C resistance, Factor V Leiden), increased plasma concentrations of procoagulant factors (e.g., factors I [fibrinogen], II [prothrombin], VIII, IX, and XI), increased basal procoagulant activity and impaired fibrinolysis, have added new paradigms to the list of inherited or acquired disorders predisposing to thrombosis (thrombophilia). Inherited thrombophilias interact with environmental risk factors such as OCP use, pregnancy, hormone therapy, and surgery to increase the risk of incident VTE. Similarly, genetic interaction increases the risk of recurrent VTE. These findings support the hypothesis that an acquired or inherited thrombophilia may help predict the subset of persons who, when exposed to common risk factors, will actually develop symptomatic venous thromboembolism [22].

## **Recurrent Venous Thromboembolism**

Independent predictors of recurrence include male gender [23], increasing patient age and body mass index, neurological disease with leg paresis, and active cancer [5, 24]. Additional predictors include unexplained venous thromboembolism, a lupus anticoagulant or antiphospholipid antibody, antithrombin, protein C or protein S deficiency [25], and possibly persistently increased plasma fibrin D-dimer [26] and residual DVT [27]. Systemic review of 10 studies involving 3104 patients with first-ever VTE revealed that factor V Leiden gene mutation (both homozygous and heterozygous) was present in 21.4% of patients and associated with increased odds of recurrent VTE of 1.41 [28]. The same review revealed that prothrombin G20210A gene mutation was present in 9.7% of patients with first-ever VTE and associated with an increased odds of recurrent VTE of 1.72. While patients with

recurrent VTE are more likely to have heterozygous factor V Leiden or prothrombin G20210A gene mutations than those without recurrence, the magnitude of increased risk is modest.

### 1.1.2 Pathogenesis – Virchow’s triad and Thrombus Formation

The pathogenesis of VTE is centred on three key factors known as Virchow’s triad, (a) the hypercoagulability or thrombogenicity of circulating blood, (b) changes in the vessel wall such as endothelial injury and (c) stasis causing abnormal blood flow (see Figure 1.1)[29]. More recently stasis is thought to be a more permissive factor and blood constituents, including inflammatory mediators and changes in the vascular endothelium are considered more important[30]. Unlike in arterial thrombosis where platelets are the core component, in venous thrombi the main constituent is fibrin which facilitates the thrombus attaching to the vessel wall.

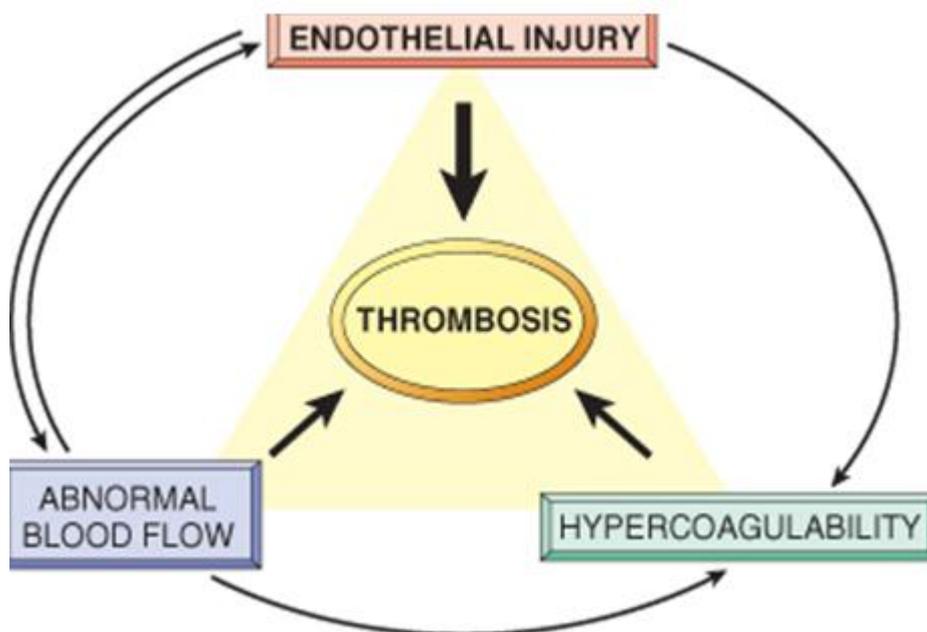
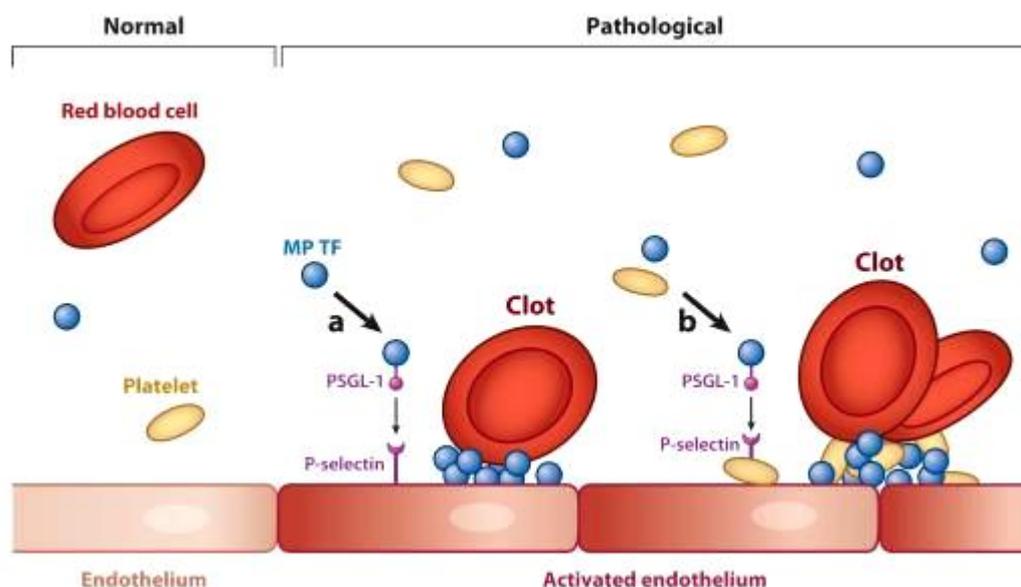


Figure 1.1. Virchow’s Triad [31].

The processes that initiate the formation of such thrombi are uncertain but inflammation and stasis play a major role. Inflammation activates the endothelium which in turn causes the release of Weibel-Palade bodies containing von Willebrand factor and p-selectin which can facilitate the binding of leucocytes to the area. Inflammation also causes the release of inflammatory mediators which can down regulate anticoagulant pathways hereby promoting thrombus formation at a site of endothelial damage. Stasis can also activate the endothelium but in a different manner [32]. For example, the risk for DVT is increased in bedridden patients and also those on long haul flights. This occurs as a result of low flow rate of blood causing a build up of prothrombotic components such as thrombin that would normally be inactivated by regular flowing blood [33]. Venous thrombosis is believed to be initiated at the venous valves [34], stasis and hypoxia may occur at these valves increasing the thrombotic risk. Hypoxia can also lead to a hypercoagulable state in the blood by increasing the availability of tissue factor and P-selectin [30]. Venous thrombi are fibrin-rich clots that may develop in the absence of gross endothelial damage (Figure 1.2)[35].



**Figure 1.2. Formation of Venous Clot [35].** In a healthy vein (*left*), high levels of tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), and endothelial cell protein C receptor (EPCR) maintain an antithrombotic phenotype (normal endothelium). In pathological conditions (*right*), elevated levels of tissue factor (TF)-positive microparticles (MPs) are

present in the blood, and reduced blood flow, activation of the venous endothelium, and deposition of platelets may conspire to trigger formation of a thrombotic clot. Two potential mechanisms that initiate activation of the coagulation system includes (a) TF-positive MPs expressing P-selectin glycoprotein-1 (PSGL-1) docking to an activated endothelium expressing P-selectin, and (b) TF-positive MPs binding to activated platelets that adhere to the activated endothelium. In both scenarios, the presence of MP TF serves as a potent trigger of coagulation activation, and the continuous delivery of TF-positive MPs may enhance propagation of the thrombus.

### **1.1.3 Activation of the haemostatic system**

#### **Haemostasis in a Healthy Individual**

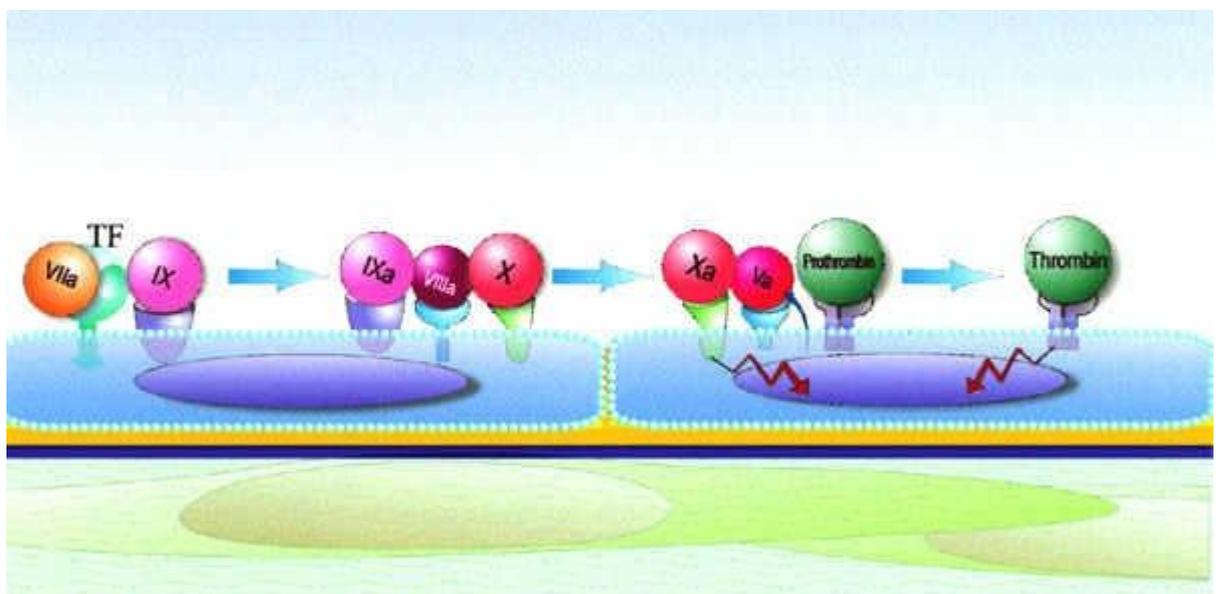
In a healthy individual blood circulates as a liquid which can gel rapidly when necessary to form a fibrin clot. Formation of fibrin clot in response to tissue injury is the most clinically relevant event of haemostasis under normal physiological conditions. The explosive activation of the haemostatic system occurs as a result of the coagulation cascade, in which inactive zymogens and cofactors are sequentially activated by proteolytic cleavage. The resulting fibrin produced stimulates the fibrinolytic system, limiting fibrin deposition to the site of injury and a system of naturally occurring anticoagulants feedback and prevent further activation of the coagulation pathway. Therefore coagulation is a complex system of soluble enzymes and substrates which ultimately lead to the conversion of fibrinogen to fibrin. Fibrin acts locally at the site of injury to stabilise a clot before it is naturally dissolved by the process of fibrinolysis.

#### **Coagulation**

The coagulation cascade (Figure 1.3) was previously believed to be organised into separate pathways, the intrinsic and the extrinsic pathway. Coagulation was initiated by factor XII (FXII) of the intrinsic pathway and activated factor VII/tissue factor (FVIIa/TF) complex of the extrinsic pathway. These pathways then converged at the prothrombinase complex (FXa/FVa). Most publications have changed this view to one that suggests there is no exclusion between the two and they work synergistically [36, 37].



Thrombin can also convert FVIII to its activated form FVIIIa via a feedback mechanism. FVIIIa forms a complex with activated FIX, the tenase complex, the result of which substantially increases the amount of FXa generated. FXa is a poor enzyme without association with its cofactor active factor V (FVa), the prothrombinase complex, and through the action of this complex, alongside calcium and phospholipids, converts its substrate prothrombin to active thrombin which leads to an explosive generation of thrombin and formation of a fibrin clot (Figure 1.4). The large-scale thrombin generated through the interaction of FV, FVIII, FXI and the action of platelets, is part of the amplification and propagation steps of blood coagulation [36]. Thrombin generation occurs through a number of steps guided by the tenase complex. The end product, active enzyme  $\alpha$ -thrombin, leads the conversion of fibrinogen to fibrin. Fibrinogen is a plasma protein synthesised in the liver. Converted by thrombin to fibrin, it forms an insoluble polymer that seals the site of injury by forming a haemostatic plug [36].



**Figure 1.4 Thrombin Formation on Perturbed Endothelial Cells [42].**

#### **1.1.4 Inhibition of the haemostatic system**

The coagulation system is regulated by a series of anticoagulant pathways that ensure a localized response. This is necessary to avoid unwanted activation of the coagulation pathway and therefore excess fibrin generation and deposition and vessel occlusion.

##### **TFPI pathway**

The initiation of coagulation through TF/FVIIa is shut down through the action of tissue factor pathway inhibitor (TFPI) (Figure 1.5). TFPI is predominantly produced from the microvascular endothelium and is found in three distinct regions. The first is in the circulation and contains both free TFPI and TFPI that is bound to plasma lipoproteins. The second is found within platelets, and the largest 80% is bound to the endothelium; ten percent of the total TFPI is contained within platelets and is released in response to thrombin and other stimulants [43]. TFPI is a three Kunitz domain glycoprotein which inhibits and control thrombin generation [44]. The first Kunitz domain binds and inhibits FVIIa, the second FXa and the third is proposed to bind to the vessel wall [45]. TFPI inhibits the Xa/TF/FVIIa complex in a 2-stage process. First, TFPI binds and inactivates FXa. Secondly, TFPI/FXa forms a quaternary complex with TF/FVIIa, thus inhibiting thrombin generation. TFPI promotes the degradation of monocytes and the internalisation of TF/FVIIa complexes on the cell surface [46]. Heparin induces the release of cell surface-associated TFPI [47, 48].

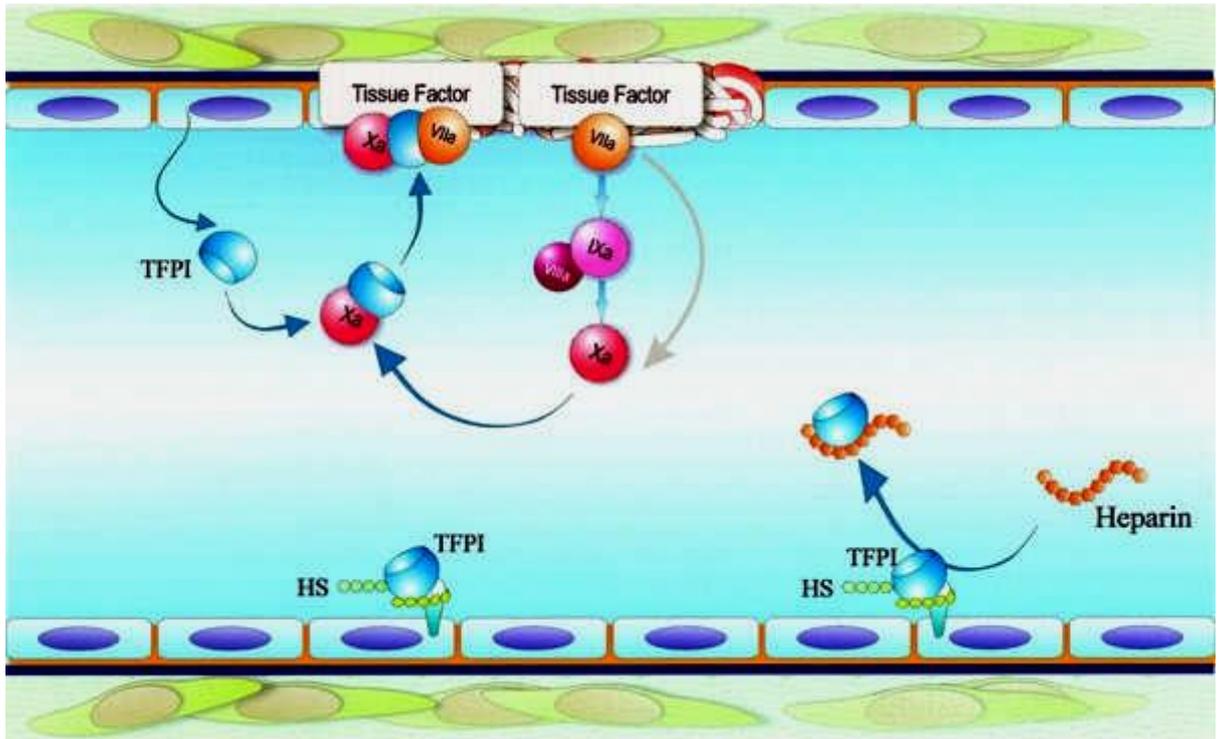


Figure 1.5 The TFPI pathway [42].

### Protein C pathway

Thrombin is not only procoagulant as described above but it can also activate the protein C anticoagulant pathway (Figure 1.6). When thrombin binds to the cellular receptor thrombomodulin, then FV, FVIII, FXI and fibrin are no longer preferred substrates. When thrombomodulin binds with thrombin, it acquires the ability to cleave protein C (PC) to its active form. This is enhanced by the binding of PC to endothelial cell protein C receptor (ePCR) [49, 50]. Activated protein C (APC) becomes dissociated from EPCR and binds to its cofactor protein S. Protein S is a vitamin-K-dependant plasma protein that is inactive until bound to APC. APC along with its cofactor protein S can prevent thrombin generation by inactivating the cofactors FVa and FVIIIa, therefore shutting down the prothrombinase and tenase complexes. With protein S acting as catalyst, APC activity increases 10-20 fold[51]. APC cleaves two sites on FVIIIa to inactive tenase activity[52].

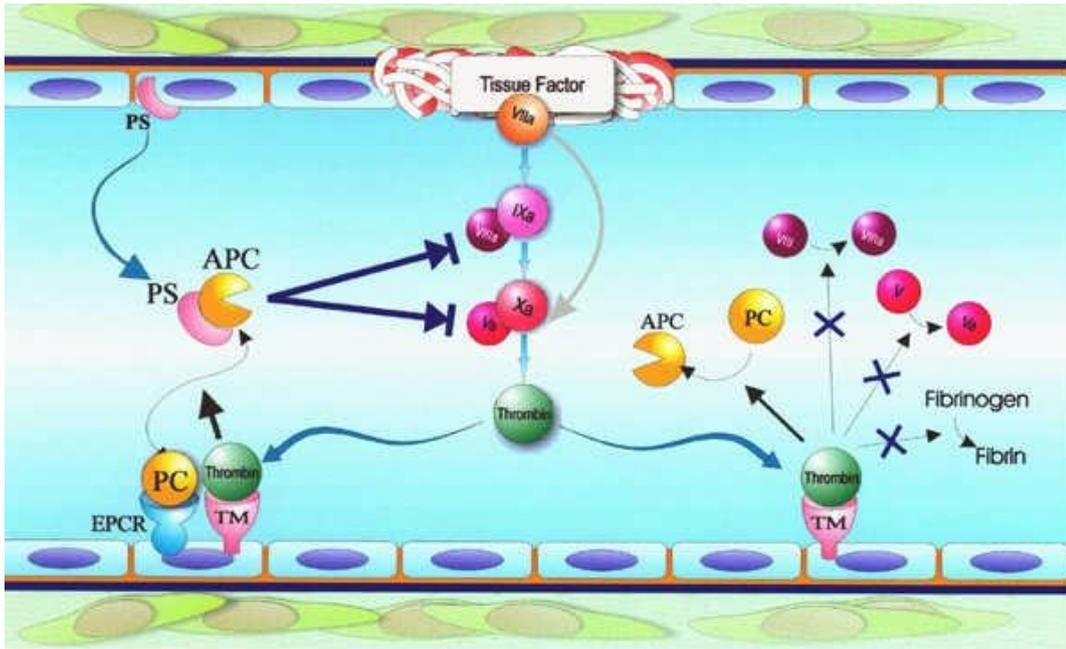


Figure 1.6 Protein C Pathway [42].

### Antithrombin

Antithrombin III is the primary inhibitor of activated serine proteases related to the coagulation cascade such as thrombin, factor IXa, factor Xa and TF:VIIa complex. Antithrombin inactivates free thrombin and Xa more efficiently than thrombin and Xa bound to activation complexes (Figure 1.7). This effectively removes free thrombin and Xa from general circulation and limits thrombotic effects on injury sites [39].

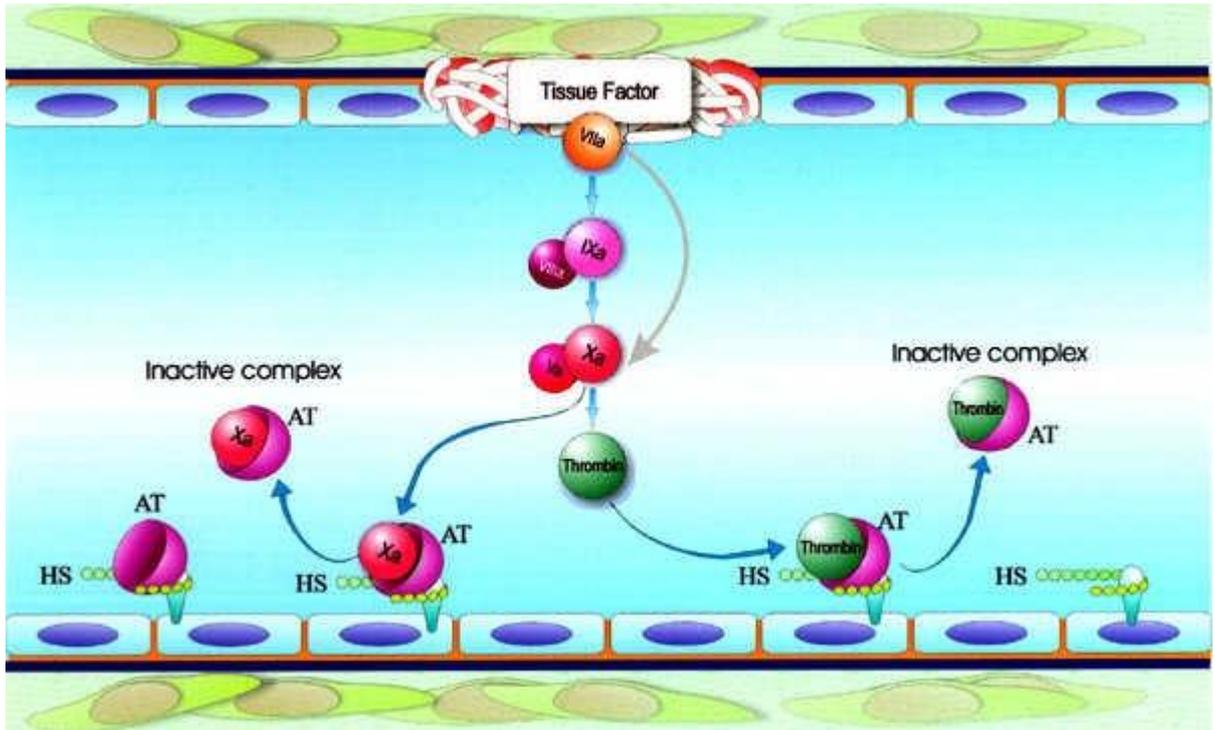


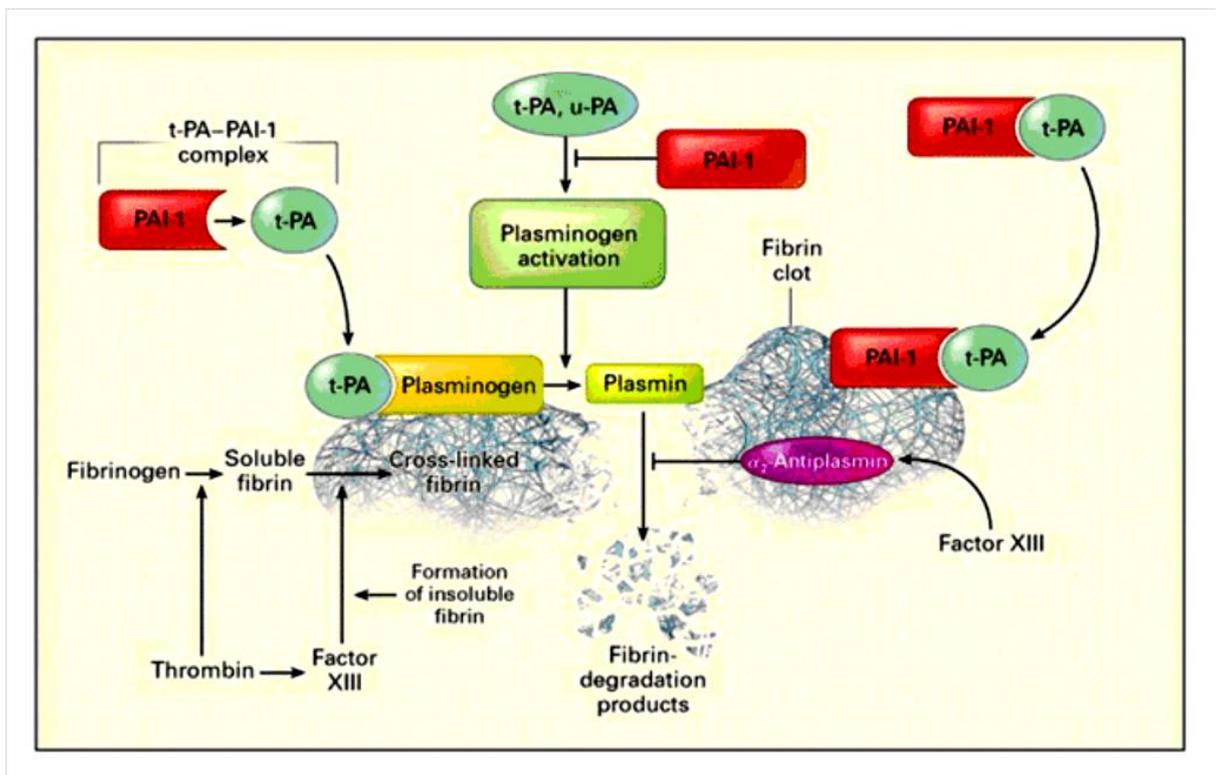
Figure 1.7 The Antithrombin Pathway [42].

Antithrombin binds to thrombin creating a stable thrombin-antithrombin complex (TAT), which is also used as a surrogate marker for coagulation activation. While antithrombin levels decrease, TAT complex has been observed to be increased in pregnancy. In normal pregnancy, thrombin-antithrombin III complex and soluble fibrin levels were significantly higher in the uterine vein than in the antecubital vein [53].

### 1.1.5 Fibrinolysis

Fibrinolysis is a mechanism that limits the formation of fibrin clots. The fibrinolytic system is a series of enzymes which is initiated after the formation of fibrin, and functions by breaking down this protein into fibrin degradation products [54] (Figure 1.8). Fibrinolysis also plays a role in ovulation, embryo implantation, tissue remodelling and inflammation [55].

The active enzyme of the fibrinolytic pathway responsible for fibrin breakdown is plasmin. Plasmin is formed by the activation of the zymogen plasminogen. The formation of plasmin can occur through various different activators. These plasminogen activators along with their inhibitors play a crucial role in controlling fibrinolysis. FXIIa, FXIa and kallikrien are all capable of converting plasminogen to plasmin. The main activator is t-PA, a serine protease produced by the vascular endothelium. The tPA-mediated activation of plasminogen is slow until accelerated by the presence of fibrin. Conversion of fibrinogen into fibrin is accompanied by conformational changes that result in the exposure of multiple binding sites and modulation of various activities. Urokinase-type plasminogen activator (uPA) also functions as a plasminogen activator. This is a naturally occurring enzyme found in human urine, blood and on the extracellular matrix via the urokinase receptor[55].



**Figure 1.8 The Fibrinolytic System [54].** Tissue plasminogen activator (tPA), urokinase type plasminogen activator (uPA), Plasminogen activator inhibitor 1 (PAI-1).

### 1.1.6 Thrombophilic Mutations of the Haemostatic System

Following the first description of antithrombin deficiency as a cause of familial thrombophilia by Egeberg in 1965 [56], there were increasing reports of inherited risk factors for VTE in the past two decades [57]. The risk for a first deep vein thrombosis (DVT) was 1.52% to 1.90% per year for those with deficiencies of antithrombin, protein C or protein S, and 0.34% to 0.49% per year for those with factor V Leiden or prothrombin 20210A gene mutation [58] (Table 1.1).

Thrombophilic Mutations	Annual risk of first DVT	Relative risk	Risk of recurrence
Antithrombin deficiency	1.52-1.90%	15-19 fold	40% at 5 years
Protein C deficiency			55% at 10 years
Protein S deficiency			
Factor V Leiden	0.34-0.49%	3-5 fold	11% at 5 years
Prothrombin 20210A			25% at 10 years

**Table 1.1 Risk of Venous thromboembolism in common thrombophilias [58]**

#### **Factor V Leiden Mutation and APC Resistance**

APC resistance is the term used to describe the reduced sensitivity of the coagulation pathway to the inhibitory actions of protein C. APC resistance can occur as a result of a thrombophilic mutation on Factor V (Factor V Leiden) or can be acquired[52]. APC cleaves FVa in a two-phase process which in patients with the factor V Leiden mutation cannot be completely accomplished leaving FVa active, an increased risk of thrombosis and these patient phenotypes being APC resistant[52]. APC resistance was first described by Dahlback in 1993[59] and in most cases involves a single gene mutation in the coagulation factor V leading to replacement of arginine (R) at position 506 with a glutamine (Q) [FV Q506, FV Leiden (FVL)] (factor V Leiden gene mutation)[60]. Risk of VTE for Factor V Leiden

heterozygous is 4-7 fold higher than non carriers and this VTE risk increased to 80-fold for Factor V Leiden homozygotes[61]. In pregnancy, there is a 3% increased risk of VTE for Factor V Leiden heterozygotes and 10% increased risk in pregnant homozygotes. Compared with women without thrombophilia, VTE occurred significantly more often in the homozygous women; 4.2% during pregnancy and 4.7% after delivery or termination of pregnancy [62]. Clinically, APC activity is expressed by APC sensitivity ratio (APCsr) and women with APCsr below the 95<sup>th</sup> centile are regarded as APC resistant [63, 64]. In normal pregnancies, there is up to 50% reported acquired APC resistance by the third trimester [65].

### **Protein C mutations**

The gene for protein C is located on the long arm of chromosome 2 and nearly 200 pathogenic mutations of this gene have been described [66, 67]. Heterozygous protein C deficiency is inherited in an autosomal dominant fashion. These mutations are divided into 2 types; on the basis of whether they cause a quantitative (type I) or functional (type II) deficiency of protein C [67].

#### *i) Type I Protein C deficiency*

Type I protein C deficiency refers to a quantitative deficiency in the plasma protein C concentration. There is marked phenotypic variation among families with heterozygous type I protein C deficiency, from complete asymptomatic to severe thrombotic tendency [68]. The presence of a second thrombophilic mutation such as factor V Leiden has been associated with a more severe phenotype in some protein C-deficient individuals [69].

#### *ii) Type II Protein C deficiency*

Type II protein C deficiency is less common than type I disease, and is associated with decreased functional activity and normal levels of protein C. A number of point mutations within the protein C gene giving rise to this disorder have been described [66].

### **Protein S mutations**

Two genes for human protein S have been identified and both are linked closely on chromosome 3p11.1-3q11.2. One gene is the active gene, *PROS* -b (ie, *PROS1*), and the other, *PROS*- a, is an evolutionarily duplicated nonfunctional pseudogene, containing multiple coding errors (eg, frameshifts, stop codons). Molecular studies into the genetic causes of protein S deficiency are complicated by the presence of the pseudogene, *PROS*- b, and phenotypic variation. Deletions of large portions of the *PROS*- a gene are associated with protein S deficiency and thrombophilia [57]. Family members with either deletion exhibit protein S deficiency and thrombophilia, however, subsequent studies indicate that the most common genetic defects in the protein S gene are point mutations rather than gene deletions [70]. Phenotypic variation has been observed in protein S deficiency. Type I protein S deficiency is a reduction in the level of free and total protein S. Type III deficiency is a reduction in the level of free protein S only. Type II deficiency is a reduction in the cofactor activity of protein S, with normal antigenic levels [71].

### **Prothrombin G20210A mutation**

The human prothrombin gene spans 21 kb on chromosome 11p11-q12. A report published in 1996 identified a transition (guanine to adenine) at nucleotide 20210 in the 3'

untranslated region of the prothrombin gene as a risk factor for thrombosis [72]. Heterozygous carriers have 30 percent higher plasma prothrombin levels than controls [72].

G20210A is a functional polymorphism as well as the association of this polymorphism with an increased susceptibility to thrombosis [73]. The molecular mechanism by which the nucleotide G20210A transition raises plasma prothrombin levels may result from altering the efficiency of mRNA processing and/or the decay rate of prothrombin mRNA [74, 75].

### **Antithrombin mutation**

Inherited antithrombin deficiency was first described 1965 and was the first inherited trait associated with thrombophilia [76]. It is classified as either type I or type II, based upon functional and immunochemical antithrombin analyses [77]. Typically as a result of type I or type II antithrombin deficiency, functional antithrombin levels are reduced to below 50% of normal [78].

#### *i) Type I antithrombin deficiency*

Type I antithrombin deficiency is characterized by a decrease in both antithrombin activity and concentration in the blood of affected individuals. The antithrombin of subgroup Ia individuals showed a normal affinity for heparin while the antithrombin of subgroup Ib individuals showed a reduced affinity for heparin [79]. Most cases of type I deficiency are due to point mutations, deletions or minor insertions within the antithrombin gene [80].

#### *ii) Type II antithrombin deficiency*

Type II antithrombin deficiency is characterised by normal antithrombin levels but reduced activity in the blood of affected individuals. It is subdivided into subgroups: type II PE, where mutations has pleiotrophic effect leading to decreased thrombin inactivation, decreased

factor Xa inactivation and decreased heparin affinity, with type II RS, where mutations affect the reactive site and type II HBS, were mutations effect the antithrombin heparin binding site [81]. Type IIc (Budapest) involves defects at the heparin binding site [82] and this has a potential role for intervention with anticoagulants.

### **Antiphospholipid Syndrome**

Traditionally, antiphospholipid syndrome (APS) is defined as an acquired autoimmune disease characterised by clinical symptoms associated with Antiphospholipid antibody (aPL) family. Clinical symptoms may involve venous, arterial and microvascular thrombotic features or pregnancy complications (early pregnancy losses or placenta mediated late complications). The aPLs which are detectable includes lupus anticoagulant (LA), anticardiolipin antibodies (aCL), and anti- $\beta_2$ -glycoprotein-I (a $\beta_2$ GP<sub>I</sub>). Although APS is conventionally looked upon as one entity, the mechanism of aPL mediated thrombosis and pregnancy loss are different [83].

#### *i) Thrombotic APS*

Increased endothelial cell activation and plasma levels of adhesion molecules in APS causes up-regulation of TF expression and inhibits endothelial nitric oxide synthase (eNOS) leading to increased leucocyte endothelial cell adhesion and thrombus formation [84]. Anti prothrombin antibody (aPT) can be detectable in APS and thrombotic patients [85]. Increased anti-FII was reported in venous and arterial thrombosis event in APS [86]. However, its clinical value needs prospective evidence [87].

## ii) *Obstetric APS*

The causal link between aPLs and pregnancy complications are not clear, hence the obstetric APS diagnosis criteria is lacking in biological specificity [83, 88]. Despite evidence for an inherited predisposition to recurrent miscarriages, there is a lack of genomic studies on this condition [89]. aPL-mediated complement activation generates C5a, which induces TF expression on maternal neutrophils; this TF-FVIIa-PAR<sub>2</sub> mediated neutrophil activation is critical to pathogenesis of aPL related fetal loss [90]. In APS murine model, the fetal loss could be prevented by heparins inhibiting complement activation [91]. aβ<sub>2</sub>GP<sub>1</sub> were found to be correlated with LA in women with pregnancy loss [92]. aPT was strongly associated with intrauterine death [93]. There is increased venous and arterial thrombosis in obstetric APS despite primary prophylaxis with low dose aspirin [94]. Therefore, obstetric APS must be considered a thrombotic syndrome.

### **1.1.7 Haemostatic Markers and Venous Thrombosis Risk**

VTE constitutes a major health care cost problem due to its high morbidity and mortality. After a first episode of VTE, about one-third of patient will develop a recurrence of either DVT or PE within ten years [5]. Hence, the presence of a reliable biomarker to i) triage patient to high or low risk of VTE, ii) allow swift and definite diagnosis and iii) effectively monitor anticoagulated patients on treatment would be extremely helpful. Several studies have attempted to identify biomarkers of thrombin and fibrin production in an attempt to measure the *in vivo* level of coagulation activation which may be predictive of VTE risk.

#### **D-Dimer**

Plasma D-Dimer is a degradation product that is formed after thrombin-generated fibrin clots are degraded by plasmin. It reflects activation of blood coagulation and

fibrinolysis. D-Dimer levels rise during acute VTE event, making it the most investigated biomarker of VTE. D-Dimer is widely used as an initial assessment of suspected VTE in non-pregnant population due to its high sensitivity (up to 95%) and a negative predictive value of nearly 100% using commercially available D-Dimer assays [95], making it possible to rule out both DVT and PE with a negative result. Thus, D-Dimer has been integrated into diagnostic algorithms in management of patients with suspected VTE. However, due to its poor specificity, sequential clinical probability assessments and objective testing with imaging studies are still needed to diagnose VTE.

Elevated D-Dimer levels may suggest a hypercoagulability state. Increased D-Dimer to >70<sup>th</sup> percentile of levels in healthy individuals measured 6 month after first VTE event is associated with 2.2-fold increased risk of recurrent thrombosis [96]. D-Dimer measurement also has a pivotal role in decision making on duration of oral anticoagulation after first VTE event[97]. Patients who had first unprovoked VTE, with normal D-Dimer levels (<500ng/ml), measured three to four weeks after discontinuation of oral anticoagulant had a lower risk of VTE recurrence [98, 99]. The cumulative probability of VTE recurrence after 2 years was 11.5% (compared with 3.5% in normal D-Dimer levels) in individuals with higher D-Dimer levels. This fact is even more pronounced in patients with congenital thrombophilia such as prothrombin gene variant or Factor V Leiden where the hazard ratio for VTE recurrence is 8.3 (CI 2.7-17.4)[100]. D-Dimer is an independent risk factor for VTE recurrence and the presence of co-morbidities such as obesity did not increase the risk of recurrence associated with an abnormal post-anticoagulation D-Dimer levels [101].

Currently, D-Dimer testing is a mainstay for initial diagnostic assessment for suspected VTE patients. It also plays a role in detecting hypercoagulable states and guides duration of anticoagulation for secondary VTE prophylaxis and help determine risk of cardiovascular events and occult cancer after a first episode of idiopathic VTE [102].

### **Prothrombin Fragment F1.2**

The prothrombinase-mediated conversion of prothrombin to thrombin is a key step within the coagulation cascade. Prothrombin is cleaved into two peptides, the active thrombin and the prothrombin fragment F1.2 (F1.2). Thrombin reacts with its inhibitor antithrombin III, and generates the thrombin–antithrombin III complex (TAT). Therefore F1.2 and TAT can be considered as markers of *in vivo* hemostasis activation [103, 104]. The value of F1.2 as a predictor of recurrent VTE was evaluated in a prospective study comparing the F1.2 levels of thrombosis patients without a defined thrombophilia compared with those of Factor V Leiden patients with a history of venous thrombosis and compared with those of healthy controls. Before or at several time points after oral anticoagulants, no significant difference in F1.2 levels was found in patients with and without recurrent thrombosis [105]. F1.2 levels at 3 weeks and prior to recurrence were not significantly different in both patient groups. No difference in F1.2 level was seen between patients with and without Factor V Leiden. The authors concluded that F1.2 is not predictive of and not suitable for identification of individuals at risk of recurrent venous thrombosis. However, a permanent haemostatic system activation is detectable both in patients with and without thrombophilia after a thrombotic event.

### **Thrombin antithrombin (TAT) Complex**

TAT is a marker of thrombin generated *in-vivo*. Immediately after thrombin is formed, it is inactivated through 1:1 binding to antithrombin. This chemical reaction produces a stable stoichiometric complex, thrombin antithrombin complex (TAT) [106]. Many studies have investigated TAT levels in many prothrombotic states to detect evidence of increased thrombin generation to limited success. Patients with factor V Leiden mutation was demonstrated to have high levels of TAT [107]. On the other hand, a population-based study failed to find an association between TAT and factor V Leiden [108]. Another study has

shown that increased TAT level is present in <80% of patients with confirmed venous thrombosis and increased plasma FVIII:C levels [109]. This is a much higher prevalence and higher level of TAT than has been found in other prothrombotic states. The clinical significance of this observation remains unclear, but it may be useful in understanding the nature of the prothrombotic state. In patients with previous venous thrombosis in whom no thrombophilic trait was identified, TAT was also increased in approximately 30% of patients. Although the frequency and intensity of TAT formed were significantly less than that observed among patients with elevated FVIII:C levels ( $P < 0.001$ ), they were significantly higher than age- and sex-matched controls ( $P < 0.01$ ) [109]. Thus, the relationship between increased TAT and the prothrombotic state is unclear.

### **Fibrinopeptide A**

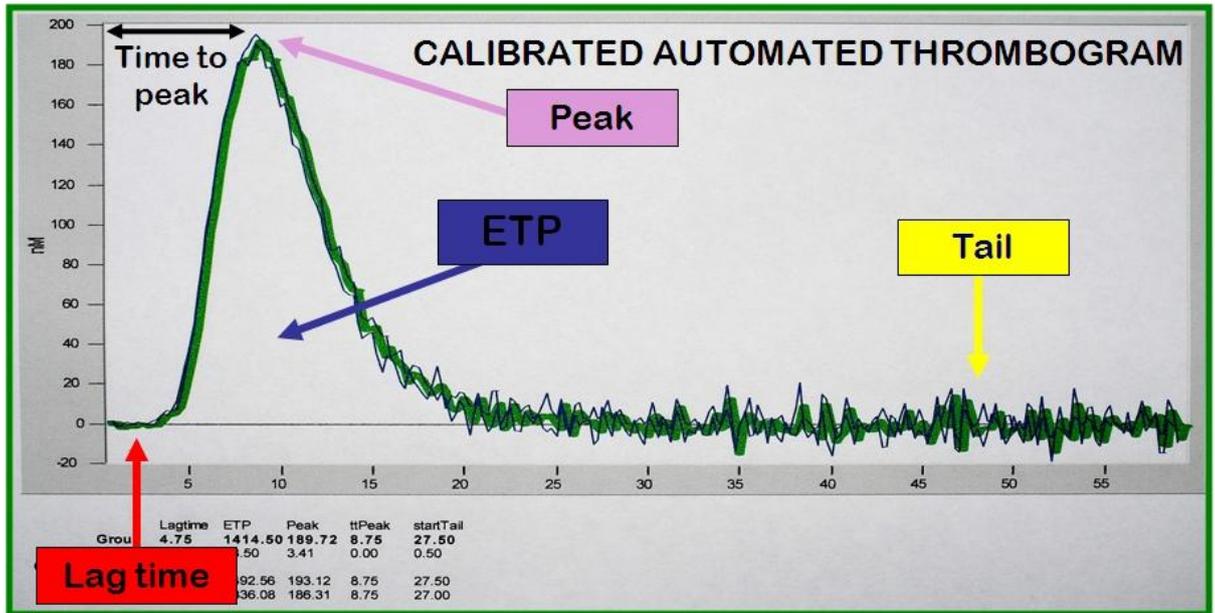
Thrombin cleaves fibrinopeptides A (FPA) and B from the NH<sub>2</sub>-terminal end of the fibrinogen molecule during fibrin formation. Hence, measurement of fibrinopeptide levels in plasma may provide a direct index of thrombin action [110]. Many clinical conditions are associated with high plasma levels of FPA, reflecting increased activity of the enzyme thrombin. These include not only conditions where thrombosis is evident such as VTE and disseminated intravascular coagulation, but also some where thrombosis may not be evident (coronary heart disease, malignancies, sepsis, systemic lupus erythematosus) [111, 112]. The clinical applicability of this method for the diagnosis of a prethrombotic state is limited by the high probability of thrombin activation *in-vitro* during venepuncture and plasma processing, with subsequent spurious generation of the peptide. Despite meticulous blood collection and processing and the use of special anticoagulant solutions to prevent *ex vivo* activation of coagulation, even in expert laboratories high plasma peptide levels are not

infrequently encountered which are held to be artefactual and not related to any underlying clinical condition [113].

### **Calibrated Automated Thrombogram (CAT)**

The ability of a particular individual to generate thrombin is a crucial measure of the integrity of the coagulation system and may correlate with either a risk of bleeding or thrombosis. Historically tests such as the PT and APTT ratios have been used. However, although widely performed they have a number of intrinsic problems 1) they look at the clotting cascade in isolation, 2) they are non-physiological, 3) they show a poor correlation with the clinical phenotype (the PT or APTT may be prolonged but this does not necessarily predict the bleeding phenotype) and 4) they are, in general, insensitive to prothrombotic states [114].

The Endogenous Thrombin Potential (ETP) was a term introduced by Hemker in 1986 and refers to the total amount of thrombin generated in plasma following activation by TF [115]. The continuous measurement of thrombin was achieved using a slow acting fluorogenic substrate. Furthermore, the signal from the fluorogenic substrate was not quenched by turbidity and so thrombin generation assays could be performed in platelet rich plasma. However, there is no direct correlation between thrombin activity and fluorescent signal intensity; to overcome this, the splitting of the fluorogenic substrate is compared to a constant known thrombin activity in a parallel non-clotting sample the so called calibrated automated thrombogram (CAT) [116]. Variables from the CAT include lag time, peak thrombin, time to peak thrombin and endogenous thrombin potential (Figure 1.9).



**Figure 1.9 Parameters of the Calibrated Automated Thrombogram [117].**

Several recent studies have reported that ETP and peak thrombin were increased in patients with history of VTE compared to healthy individuals. Hron *et al* reported that there is a relationship between recurrent VTE and peak thrombin. They concluded that patients in lower quartile with a peak thrombin of less than 300nmol/L had a significantly lower risk of recurrent VTE compared to patients with peak thrombin above the upper quartile of 400nmol/L [118]. The relationship between ETP and peak thrombin with risk of recurrent VTE is also supported by other prospective studies [119-121]. CAT assay evaluating an overall haemostatic profile of an at-risk patient may be a valuable tool for prediction of VTE and possibly for the clinical management of patients in long-term anticoagulation. Recently, elevated peak thrombin (>611nM thrombin, or 75<sup>th</sup> percentile) had been shown to be predictive of VTE in patients with malignancies in The Vienna Cancer and Thrombosis Study [122]. Furthermore, the probability of developing recurrent VTE after 6 months was significantly higher in patients with elevated peak thrombin. Large prospective interventional studies are needed to assess whether thrombin generation assessment via

means of CAT assay may be clinically useful for triaging cancer patients according to their VTE risk.

Normalised thrombomodulin sensitivity ratio (N-TMSr) is defined as the ratio of endogenous thrombin potential determined in presence and absence of thrombomodulin which was normalized against the same ratio determined in normal control plasma. The thrombin generation parameters with and without thrombomodulin (by means of n-TMSr) has been reported to be highly predictive (OR 8.3 95%CI 1.9-36.9) in the detection of factor V Leiden or other prothrombotic states in first degree relatives of patients with venous thromboembolism and factor V Leiden [123]. Measurements of thrombin generation parameters in the presence and absence of thrombomodulin have an added value because they improve sensitivity of tests to the protein C pathway which are otherwise dampened in pregnancy [124].

## **1.2 Venous thromboembolism in Pregnancy**

Venous thromboembolism (VTE) is a leading cause of maternal death in the developed world, causing 1.2 to 4.7 deaths per 100,000 pregnancies [125]. Symptomatic VTE is estimated to occur antepartum (from conception to delivery) in 5 to 12 per 10,000 pregnancies, and postpartum (up to 6 weeks after delivery) in 3 to 7 per 10,000 deliveries [126]. Compared with age-matched, non-pregnant controls, this translates into a per-day risk that is increased 7- to 10-fold for antepartum VTE and 15- to 35-fold for postpartum VTE [18]. After delivery, the clinical risk of VTE diminishes rapidly to antepartum level 3 weeks postpartum, before returning to the non-pregnant level after 6 weeks postpartum [61, 127].

### 1.2.1 Worldwide Incidence Pregnancy-Associated Venous Thromboembolism and Maternal Mortality

Pregnancy amplifies VTE risk 7-10 fold to that of non-pregnant women of child-bearing age [18]. While the worldwide leading cause of maternal death is haemorrhage [128], in the developed world (Table 1.2), where modern obstetric practices curtail the haemostatic challenge of delivery, VTE is the leading cause of maternal mortality [129, 130]. Epidemiologic studies in Caucasian population reported annual incidence of VTE in pregnancy (per 10,000 deliveries) was 16.7-19.98 in North America [18, 131, 132], 8.5 in the United Kingdom [126], 8.87 in Denmark [133], 10 in Norway [127] and 13 in Sweden [134]. A large population based study in Asia reported that incidence of VTE is about one-tenth to one-fifth of that reported in the Caucasian population [135]. This is reflected on the incidence of pregnancy related VTE of only 0.82 per 10,000 deliveries in Korea [136].

	Developed Countries	Africa	Asia	Latin America and the Caribbean
Number of maternal deaths	2823	4508	16089	11777
Haemorrhage	13.4%	33.9%	30.8%	20.8%
Hypertensive disorders	16.1%	9.1%	9.1%	25.7%
Sepsis/Infections	2.1%	9.7%	11.6%	7.7%
Abortion	8.2%	3.9%	5.7%	12.0%
Obstructed labour	0.0%	4.1%	9.4%	13.4%
Embolism	14.9%	2.0%	0.4%	0.6%
Anaemia	0.0%	3.7%	12.8%	0.1%
HIV/AIDS	0.0%	6.2%	0.0%	0.0%
Ectopic pregnancy	4.9%	0.5%	0.1%	0.5%
Other direct causes	21.3%	4.9%	1.6%	3.8%
Other indirect causes	14.4%	16.7%	12.5%	3.9%
Unclassified deaths	4.8%	5.4%	6.1%	11.7%

**Table 1.2 Worldwide causes of Maternal Death from WHO [128]**

Maternal death from pulmonary embolism in Europe and United States is estimated at 1.1 to 1.5 per 100,000 deliveries [132, 137]. The Confidential Enquiry into Maternal and Child Health reported that, VTE accounts for about one-third of maternal deaths in the UK [137]. Delayed diagnosis, inadequate treatment and inadequate thromboprophylaxis

account for the majority of deaths. In Japan there was a 6.5-fold increase of PE in pregnancy between 1991 and 2000; PE occurred in 0.003% after vaginal delivery compared to 0.06% after caesarean delivery resulting in mortality rate from VTE of 2.5 per 100,000 deliveries [138].

### **1.2.2 Clinical Predictors of Venous Thromboembolism in Pregnancy**

Pregnancy is a hypercoagulable state. Increased fibrin turnover and decreased levels of coagulation inhibitors combine to promote thrombus formation. In addition, the reduced venous flow velocity, which occurs in the legs in late pregnancy and the puerperium, contributes to the prothrombotic state [19, 125]. Common additional risk factors include, smoking, older age (>35 yrs), parity >3, obesity and caesarean delivery [125]. Approximately 50% of VTE in pregnancy is associated with inherited thrombophilia [19]. However some of the main thrombophilias (*e.g.* Factor V Leiden) are relatively common in European populations and are asymptomatic in many individuals [139]. For this reason, genetic screening for thrombophilias in pregnancy is not cost effective. Although FDP-D-Dimer testing is useful in diagnosis [140], it is of limited use in predicting VTE, and to date no one laboratory assay has been shown to be useful for prediction of VTE in pregnancy.

#### **Risk Factors for VTE during Pregnancy: Epidemiological Evidence**

One of the most important risk factors for VTE in pregnancy is a history of thrombosis. Up to 25% of thromboembolic event in pregnancy are recurrent events. The risk of recurrent VTE in pregnancy is increases 3 to 4 fold [141, 142]. Thrombophilia is a well known risk factor for VTE and the risk varies by type of thrombophilia. Odds ratio for different medical conditions and pregnancy complications from 14 335 records (National Inpatient Sample, USA) are as listed in Table 1.3 below [132].

The Registry of Patients with Venous Thromboembolism (RIETE) [143] is an on-going multicentre observational registry designed to gather data on clinical characteristics, treatment patterns and outcome in patients with symptomatic, objectively confirmed acute VTE. Up to Dec 2008, 25066 patients enrolled in RIETE, 2816 (<55y), 173 (6.1%) pregnant, 135 (4.8%) post partum and 798 (28%) hormonal contraceptive users. Of the pregnant women, 39% developed VTE in first trimester, 18% in second and 43% in third trimester. 65% had no additional risk factors. Moreover, 60% of post-partum women who developed VTE underwent caesarean delivery.

<b>Risk Factor</b>	<b>Odds Ratio</b>	<b>Confidence Interval</b>
<b>Medical Conditions</b>		
<b>Thrombophilia*</b>	51.8	38.7- 69.2
<b>History of thrombosis</b>	24.8	17.1- 36.0
<b>Heart Disease</b>	7.1	6.2 - 8.3
<b>Sickle Cell Disease</b>	6.7	4.4 – 10.1
<b>Lupus</b>	8.7	5.8 -13.0
<b>Obesity</b>	4.4	3.4-5.7
<b>Anaemia</b>	2.6	2.2 – 2.9
<b>Diabetes</b>	2.0	1.4 -2.7
<b>Hypertension</b>	1.8	1.4 -2.3
<b>Smoking</b>	1.7	1.4 -2.1

\*Risk varies by type of thrombophilia

<b>Complications of Pregnancy and Delivery</b>	<b>Odds ratio</b>	<b>Confidence Interval</b>
<b>Multiple gestation</b>	1.6	1.2 -2.1
<b>Hyperemesis</b>	2.5	2.0 -3.2
<b>Fluid and electrolyte Imbalance</b>	4.9	4.1 -5.9
<b>Antepartum haemorrhage</b>	2.3	1.8 – 2.8
<b>Caeserean Delivery</b>	2.1	1.8 – 2.4
<b>Post partum infection</b>	4.1	2.9 – 5.7
<b>Post partum haemorrhage</b>	1.3	1.1 – 1.6
<b>Transfusion</b>	7.6	6.2 – 9.4

**Table 1.3 Medical Risk Factors for thrombosis in Pregnancy**

Specific risk factors have been identified for antepartum and postpartum VTE. Some of these risk factors can be easily explained by the pathophysiology of VTE such as hypercoagulability (thrombophilia), venous stasis (immobilisation) and vascular injury (operative delivery). The mechanism of other risk factors is still poorly explained.

#### **Antenatal Risk Factors for VTE**

A recent case control study involving 559 pregnant women with confirmed first lifetime VTE showed that risk factors differ significantly in antenatal and postpartum period [144]. In antenatal VTE, risk factors includes assisted reproductive technique (ART), antepartum immobilisation, cigarette smoking and slight weight gain (<7kg). Multiple pregnancy and conception following ART has an additive effect while high body mass index (BMI) and antepartum immobilisation has a multiplicative effect [127].

#### **Post partum Risk Factors for VTE**

Risk factors for postpartum VTE include cigarette smoking, antepartum immobilisation, fetal growth restriction, pre-eclampsia, emergency caesarean section, postpartum haemorrhage, infection, surgery, age and high parity. High BMI and reoperation (bleeding) showed multiplicative effects on postpartum VTE [144].

#### **Thrombophilia and pregnancy**

Thrombophilic mutations have been suggested as a cause of higher VTE risk in pregnancy. While only a small proportion are affected by thrombophilia, about 50% of pregnant women who developed VTE are thrombophilic [145]. This is especially true in the Caucasian

population whereby incidence of pregnancy-associated VTE in Asian population is only about 10% to that of Caucasians [136].

Out of patients with confirmed VTE associated with pregnancy, 50% are reported to have a heritable thrombophilia, however 28% of pregnancy-associated VTE are not associated with either an established clinical risk factor for thrombosis or a thrombophilic defect [146]. VTE is significantly associated with all types of heritable thrombophilia except (methylenetetrahydrofolate reductase) MTHFR homozygosity, whereby in contrast to non-pregnant population, there was no increased risk to VTE [147]. As listed above, thrombophilia in general confers an odds ratio of 51.8 (95% CI 38.7 -69.2) for developing VTE. The odds ratio of VTE by type of thrombophilia is summarised in Table 1.4 [147]. The highest risk for VTE was observed with Factor V Leiden homozygosity with an odds ratio of 34.4 (95% CI 9.86-120.05) and Prothrombin Gene Mutation homozygosity 23.36 (95% CI 1.24 -559.29). It has to be noted that while these relative risks are significantly higher compared to non-thrombophilic women, the absolute risk remains modest, 3.4% and 2.3% respectively (incidence of VTE in pregnancy is approximately 1:1000) [147].

<b>Thrombophilia</b>	<b>Odds Ratio (CI)</b>
<b>Factor V Leiden homozygosity</b>	34.40 (9.85 – 120.05)
<b>Factor V Leiden heterozygosity</b>	8.32 (5.44 – 12.70)
<b>Prothrombin Gene Mutation homozygosity</b>	23.36 (1.24 -559.29)
<b>Prothrombin gene mutation heterozygosity</b>	6.80 (2.46 – 18.77)
<b>Protein C deficiency</b>	4.76 (2.15 – 10.57)
<b>Protein S Deficiency</b>	2.19 (1.48 – 6.00)
<b>Antithrombin deficiency</b>	4.76 (2.15 -10.57)

**Table 1.4 Risk of VTE in Pregnancy by type of Thrombophilia**

## **Obesity**

The World Health Organisation (WHO) has recognised obesity as a ‘pandemic nutritional disorders’ which represents a rapidly growing threat to the health populations worldwide [148]. This is directly mirrored in the high prevalence of obesity in antenatal population more than doubled compared to previous decade [149]. Obesity is a significant and ever increasing risk factor for thrombosis [144]. A triennium report highlighted that more than a third (38%) of women who died from PE are clinically obese (BMI>35) [137]. The obese pregnant woman is particularly at risk in late pregnancy, when immobility and its associated complications may arise [143]. According to the RIETE Registry, an ongoing, multicentre observational registry of patients with confirmed acute VTE, most (43%) VTE in pregnancy occurs in the third trimester [143]. A recent population-based cohort study reported the rate of VTE during the third trimester was six times higher than any time outside pregnancy [150]. In contrast, both the first (IRR = 1.6) and second (IRR = 2.1) trimesters conferred little increase in risk [150]. There is a paucity of data with regards to thromboprophylaxis in obese pregnant population.

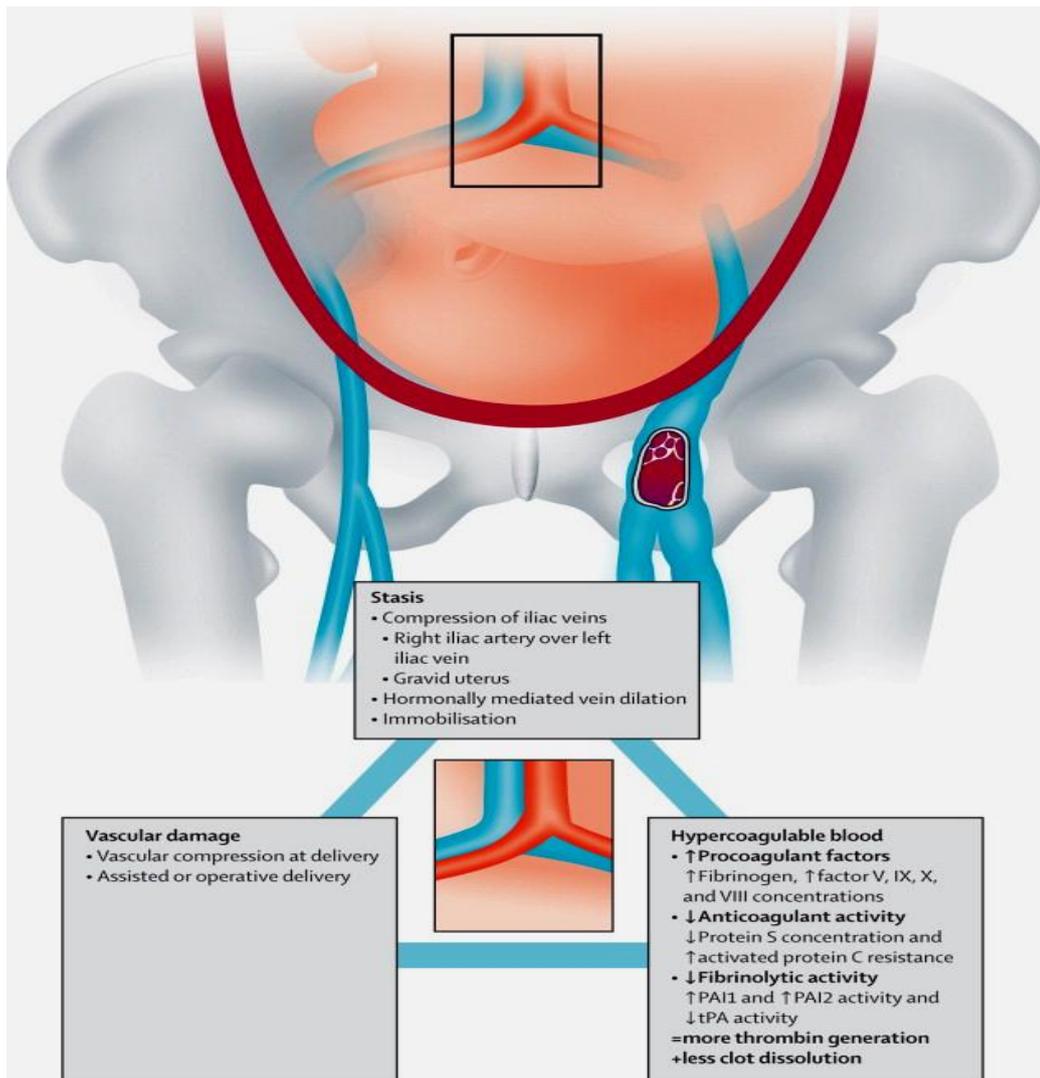
## **Caesarean Delivery**

Caesarean section (CS) is associated with a VTE related mortality up to ten fold higher than vaginal delivery [125]. A Canadian retrospective study spanning 14 years showed that the odds ratio for developing VTE is OR 2.2 (95% CI 1.5-3.2) after a low risk planned CS compared to planned vaginal delivery [151]. While a retrospective case-control study of Norwegian Registry reported that a planned CS had a non-significant adjusted odds ratio (aOR) 1.3 for thrombotic events whereas acute caesarean section has an aOR of 2.7 compared to vaginal delivery [127]. However, it is noteworthy that 95% of all patients post

caesarean delivery in this study had LMWH prophylaxis for 3-7 days. Decision analyses study suggested that even at low incidence of VTE after planned CS, benefits of LMWH exceeds the risk [152]. This study emphasized the need for individual clinical thrombotic risk evaluation after caesarean section. However, our knowledge of post CS risk factors is non-specific and derived from postpartum thrombotic risks based on retrospective studies involving all deliveries. It has also been observed that risk factors were absent in more than one-third of women who died resulting from VTE [137].

### **1.2.3 Effect of Pregnancy and Delivery on Systemic Haemostasis**

The fundamentals of Virchow's triad; venous stasis, vascular damage and hypercoagulability are all present in pregnancy and post-partum [129] (Figure 1.10). Venous stasis commences as early as pregnancy starts and peaks at 36 weeks of gestation. This is thought to be due to a combination of progesterone-induced venodilation, venous compression by the gravid uterus and pulsatile compression of the left iliac vein by the right iliac artery [153]. Normal vaginal delivery, assisted or operative vaginal delivery may result in damage to the pelvic vessels and musculature [129].



**Figure 1.10 Virchow's Triad in Pregnancy [129].**

Pregnancy is a hypercoagulable state and is thought to be a physiological adaptation in preparation for delivery, by which maternal blood flow up to 700ml per minute within the maternal-placental interface has to be promptly stopped at placental separation [154]. Changes in coagulation and fibrinolytic profiles are common during pregnancy to allow the crucial migratory and invasive trophoblast functions to proceed. Hypercoagulable indicators such as prothrombin fragment 1.2 (F1.2), thrombin-antithrombin complex (TAT) and D-Dimer levels in pregnancy have been observed to be comparable to the levels found in

acute thrombotic events [155]. This progressively activated haemostatic system in pregnancy is explained in detail below.

### **Procoagulants**

#### ***Coagulation factors***

Coagulation factor changes in normal pregnancy includes increase in prothrombin (factor II), factor VII, factor VIII, factor IX factor X and factor XII [156, 157] (Table 1.5). Factor V levels increase in early pregnancy before it stabilizes [156]. In contrast, factor V coagulation activity (Vc), shows rising levels throughout pregnancy [51]. Furthermore there is a up to 74% rise of factor VII [158]. In early stages of pregnancy factor XIII, fibrin stabilising factor, increases before returning to non-pregnant values in third trimester. von Willebrand factor(vWF) serves as a carrier for factor VIII and plays a role in platelet adhesion. Significant increase of vWF antigen was noted with rising factor VIII however, in the third trimester the ratio of vWF antigen to factor VIII changes. This reflects the selective thrombotic effect on factor VIII in the later stage of pregnancy [159]. Factor XI, which activation is key to thrombin generation, is shown to decrease gradually in normal pregnancy [160]. It has been suggested that this is due to increase factor XI consumption in pregnancy. It is also possible that factor XI levels are physiologically reduced to balance the increase of other procoagulants. Total fibrinogen doubles in pregnancy even after allowing for plasma volume expansion [156]. Animal studies suggest this is due to increase synthesis in the liver.

Procoagulant	Changes in normal Pregnancy	Reference
<b>Tissue factor</b>	Unchanged	[160]
<b>Monocyte tissue factor</b>	Increases	[160]
<b>Prothrombin (Factor II), Factor VII, VII, IX, X, Fibrin stabilising factor</b>	Increased	[146, 156]
<b>Factor V</b>	Increased	[146, 157]
<b>Fibrinogen</b>	Increased	[146]
<b>Factor XI</b>	Decrease throughout pregnancy	[159]
<b>Von Willebrand factor</b>	Increases throughout pregnancy and postpartum	[158]

**Table 1.5 Procoagulant Changes in Pregnancy**

### ***Tissue Factor***

The primary initiator of blood coagulation cascade is tissue factor (TF) which forms a proteolytically active complex with factor VII (TF-VIIa); a potent activator of factor IX and X. This in turn starts a domino effect which results in thrombin formation and the subsequent conversion of fibrinogen to fibrin. TF is expressed by non-vascular cells and atypically on monocyte and endothelial cells when induced by cytokines, endotoxin, CRP and homocysteine. It is also largely expressed by syncytiotrophoblast cells and is essential for maintaining haemostasis in the placenta. TF concentration remains constant throughout pregnancy. In contrast, monocyte TF expression is lower in pregnancy before returning to non-pregnant levels by the third post partum day [161]. This may play an important role in protecting women from VTE despite the described hypercoagulable state in pregnancy [146].

### **Anti-coagulation changes in pregnancy.**

Normal pregnancy involves a physiological decrease of natural anticoagulants to meet the haemostatic challenges of delivery and to maintain placental function. Changes in the coagulation inhibitors in normal pregnancy are summarized in Table 1.6 below.

<b>Coagulation Inhibitors</b>	<b>Changes in normal Pregnancy</b>	<b>Reference</b>
<b>Antithrombin</b>	Unchanged	[39, 156, 162-165]
<b>TAT complex</b>	Increased	[39, 162, 164, 166]
<b>TFPI-1</b>	Increased	[162]
<b>TFPI-2</b>	Increased	[167]
<b>TM</b>	Increases	[157]
<b>Protein S</b>	Decreased	[157]
<b>Protein C</b>	Unchanged	[157]
<b>APC</b>	Increased	[65]

**Table 1.6 Changes in Coagulation inhibitors in normal pregnancy**

#### ***Antithrombin and TAT***

There are mixed reports over antithrombin levels in pregnancy. Some including recent studies suggests that antithrombin levels remains stable throughout pregnancy [39, 156, 162, 163, 165]. While others, have shown that antithrombin decreased during the third trimester and even further during the postpartum period [168]. Simultaneously, TAT levels increased throughout gestation [39, 53, 162, 166].

#### ***Tissue Factor Pathway Inhibitor***

TFPI levels increased significantly in pregnancy [165]. Two types of TFPI are known; TFPI-1 is found in maternal circulation, fetal blood, endothelial cells and organs [169] while TFPI-2 (first isolated as Placental Protein 5) is mainly found in placenta [170]. Maternal plasma TFPI-2 concentration increases gradually in pregnancy, plateaus around 36 weeks

and subsides after delivery [167]. TFPI is the main inhibitor of TF. Within the maternal plasma, concentration of TFPI is 500-1000 times higher than that of TF [162, 171].

### ***Protein C System***

Total and free protein S levels decreases in first trimester, leading to reduced protein S activity to 46% that of outside pregnancy [163]. The apparent fall in protein S during the first weeks of pregnancy is a major difficulty in diagnosis of inherited protein S deficiency. In contrast, protein C activity increases in the first 22 weeks gestation. The increased levels of thrombomodulin [157, 164] and protein C in the background of falling protein S activity seems to lead to APC resistance in pregnancy [51]. Approximately 30% of APC-resistant pregnant women have been reported not to be carriers of FVL [63]. This is also known as 'acquired' APC resistance.

### **1.2.4 Uteroplacental Haemostasis**

The haemostatic system within the uteroplacental circulation faces three distinctly different physiological challenges as pregnancy proceeds. First, early implantation with trophoblastic invasion and disruption of maternal decidual vessels is associated with an increased risk of haemorrhage which needs to be counterbalanced [172]. Second, during the antenatal period blood fluidity must be maintained as it flows through the low resistance haemochorial system while simultaneously the haemostatic system must be primed for the third challenge around delivery when the significant blood flow into the uteroplacental circulation must be staunches over a matter of seconds [173]. Haemostatic activation within the uteroplacental circulation leaves the system vulnerable to perturbation.

The human placenta is called haemochorial because the highly vascular maternal decidua is disrupted by the invasive fetal extravillous cytotrophoblast cells, bringing maternal blood in direct contact with placental villi. Upon blastocyst implantation, its growth requirements are met by the endometrial glands which secrete their contents into the primitive inter-villous space until 10 weeks of gestation [174]. The cytotrophoblasts form an outer shell positioned to occlude breached capillaries within the decidualised gland stroma [175]. These cytotrophoblasts could interact with decidual cells that express tissue factor and in such, are able to potentially initiate thrombin generation within the haemostatic pathways [176].

#### **Role of tissue factor pathway in placental haemostasis**

Much of our understanding of the role of the haemostatic system in the process of placentation in human pregnancy is based on studies in mice who share a haemochorial placental system [177]. TF appears to be the key pro-coagulant factor in early pregnancy. TF produced by human endometrial stromal cells (HESC) may allow the maternal decidual blood vessels to be disrupted without excessive haemorrhage. Production of TF by HESC cells appears to be under steroid hormone control with increased TF production being demonstrated in cultured HESC cells which have been primed by progesterone [178]. The trophoblast is also a significant source of tissue factor. The haemostatic balance appears to be maintained by a number of factors, among which are tissue factor pathway inhibitor (TFPI), predominantly produced by endothelial cells and TFPI-2, which is uniquely produced in the placenta and is predominantly of trophoblastic origin [179]. TF in placental trophoblast is also effectively counter-balanced by protein C activation via the EPCR [180]. Therefore, human embryogenesis is effectively but tightly controlled by local haemostasis.

While poorly understood there is significant interplay between the process of haemostasis, angiogenesis, embryogenesis and pregnancy-induced inflammation [181-183]. The importance of the haemostatic system has been highlighted in work with knockout mice. Tissue factor gene inactivation ( $TF^{-/-}$ ) results in embryo death with prominent yolk sac vascular defects [183]. TFPI gene activation ( $TFPI^{-/-}$ ) is also frequently lethal *in-utero* [184]. Thrombomodulin ( $TM^{-/-}$ ) knockout mice leads to early mid-gestation growth arrest followed by rapid resorption of all mutant embryos [185].

As pregnancy proceeds, extravillous cytotrophoblast continues to dilate the distal spiral arteries by removing the muscular walls and switch to endothelial-like phenotype where exposed to maternal blood [186, 187]. In healthy circumstances, these cytotrophoblasts are able to suppress pro-inflammatory responses and uteroplacental blood flow increases exponentially in the second trimester allowing more than what is needed for oxygen and nutrient transfer. This renders the villi at low even pressure, in normal healthy individual, and may play a role to effect the Virchow's triad should the tightly regulated placental haemostasis be disturbed [188].

The placenta may become prone to thrombosis due to abnormal differentiation of the placental villi. The underlying decidual vasculopathy, established in early pregnancy, renders the placental villi poorly perfused, inducing static local hypoxia or a process of ischemia-reperfusion injury. The abnormal villi have exposed cytotrophoblasts and basal lamina that trigger local thrombosis [189]. These alterations in trophoblast cell biology are directly relevant to haemostasis because normal trophoblast differentiation, in both mice and humans, is professed by an endothelial-like anticoagulant phenotype.

### **1.2.5 Haemostatic markers and Venous Thrombosis Risk in Pregnancy**

#### **D-Dimers in pregnancy**

D-Dimers are widely used as a marker for venous thrombosis in non-pregnant population as it has a high negative predictive value. However, during pregnancy, plasma D-Dimer raised gradually as pregnancy progresses, decreases sharply after delivery and returns back to pre-pregnancy levels after 4-6 weeks [190]. Thus, D-Dimer testing during pregnancy and puerperium may not be reliable. A cohort study suggests that D-Dimer assay for suspected DVT in pregnancy has a sensitivity and negative predictive value of 100% [191], however, further assessment is needed before this test can be used to exclude both DVT and PE.

#### **TAT in pregnancy**

Reports have shown the mean TAT concentration in normal pregnancies increases significantly in the second and third trimester compared with the first trimester and in non-pregnant women [166]. Reinhaller et al showed that a distinct increase of TAT concentration occurred within an hour after delivery compared to levels before the onset of labour [166]. However, TAT levels returned to normal by 24 hours after vaginal delivery. After a caesarean delivery, TAT levels remained high and does not return to normal levels until one day after surgery [192]. This supports that the contention that activation of the coagulation system occurs in normal pregnancy and that further activation takes place immediately after delivery especially in surgical delivery.

### **CAT in Pregnancy**

Data on thrombin generation in pregnancy are both limited and conflicting. In uncomplicated pregnancy, many authors have reported that thrombin generation as measured by ETP and peak thrombin increases as pregnancy progresses [165, 193]. Other authors found ETP levels remained unchanged until the third trimester and then significantly decreased [194]. However the predictive value of ETP or peak thrombin in identifying women at risk of venous thromboembolism in pregnancy is unknown.

While all these haemostatic markers were widely researched in non-pregnant population, there is no reliable marker for VTE in pregnancy and puerperium especially in at-risk pregnant women who are thrombophilic, obese or underwent caesarean delivery.

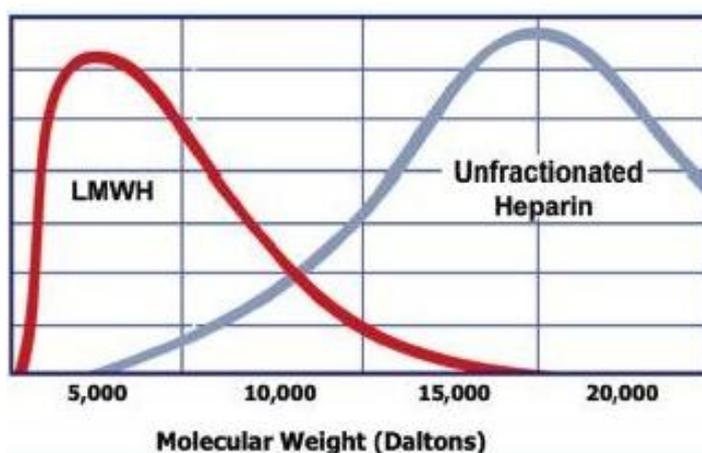
## **1.3 Low Molecular Weight Heparin for Prevention of Venous Thrombosis**

### **1.3.1. Evolution from Unfractionated Heparin to Low Molecular Weight heparin**

Low molecular weight heparin (LMWH) was introduced in the 1990s, offering an attractive option compared to unfractionated heparin (UFH). LMWHs show a more predictive dose response curve, do not require regular monitoring and have a significantly better side effect profile in terms of heparin induced thrombocytopenia or osteoporosis [195]. In the obstetric field, LMWH are mainly indicated for treatment of venous thrombosis, arterial and VTE thromboprophylaxis and prevention of fetal loss in placental dysfunction in thrombophilic women [196].

## Structure and Mechanism of Action

LMWH are polysulfated glycosaminoglycan derived from heparin by chemical or enzymatic depolymerisation resulting in fragments approximately one third the size of heparin with mean molecular weight of 4000 to 5000d and a range of 2000 to 15000d (Figure 1.11)[197].



**Figure 1.11 Molecular weight range of LMWH and UFH [198].**

As LMWH binds less to proteins and cells, it confers a multitude of biological advantages over UFH. Compared to UFH, LMWH have reduced ability to inactivate thrombin because its smaller fragments cannot simultaneously bind to thrombin and antithrombin. This however, is not crucial on anti-Xa activity, so LMWH is able to inactivate factor Xa as competently as UFH [199]. LMWH also binds less to plasma proteins, rendering a more predictable dose-response relationship and clinically making it unnecessary to monitor its anticoagulant effect [200]. Most LMWH preparations have a longer plasma half-life due to lower incidence of binding to macrophages and endothelial cells; this longer bioavailability makes it possible for once daily treatment [201]. Studies have reported that the reduced heparin-dependent antibody due to decreased binding of LMWH to platelets may explain the reduced incidence of heparin-induced thrombocytopenia compared with UFH [202, 203].

Lower incidence of thrombocytopenia with LMWH could be explained by decreased osteoclasts activation resulting from less LMWH binding to osteoblasts [204, 205].

Like heparin, LMWH confers its major anticoagulant effect by activating antithrombin. This is mediated by a specific pentasaccharide sequence[206]. Due to a minimum chain length of 18 saccharides (including the pentasaccharide sequence) is required to form ternary complexes among heparin, antithrombin and thrombin, only 25-50% of LMWH species that are above the critical chain length are able to inactivate thrombin [195, 207]. However, all LMWH containing the high-affinity pentasaccharide has the ability to inactivate factor Xa. Since most heparin molecules have at least 18 saccharides, heparin has an anti-Xa/anti-IIa ratio of 1:1. Alternatively, available LMWHs have anti-Xa/anti-IIa ratios between 2:1 and 4:1 depending on their molecular weight, leading to their minimal anti-IIa activity (Figure 1.12).

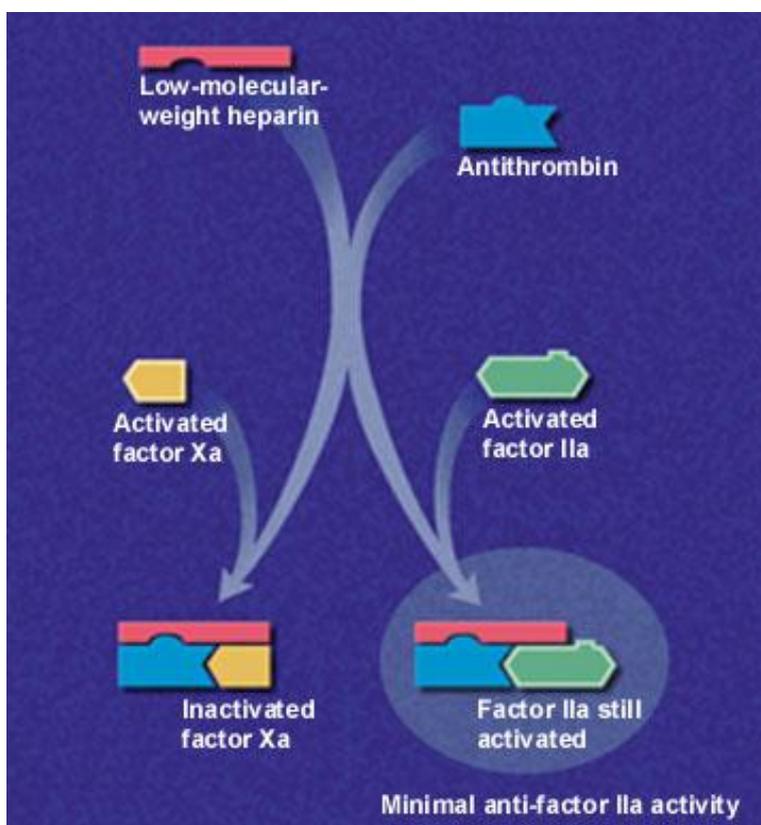


Figure 1.12 Mechanism of action of LMWH [208]

### 1.3.2 Types of LMWH

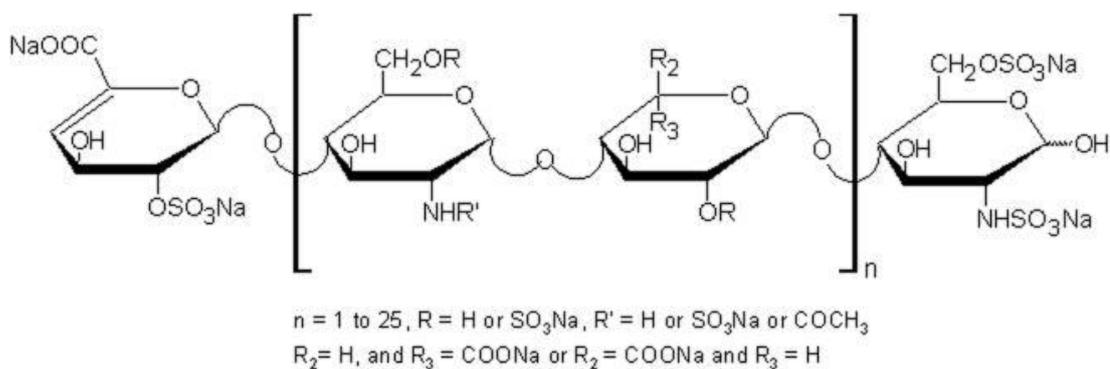
Currently, there are several LMWH approved in Europe, United States and Canada (Table 1.7). They are prepared by different methods of depolymerisation, therefore, they all have different pharmacokinetic properties, anticoagulant profile and are not clinically interchangeable.

Agents	Method of Preparation
Tinzaparin (Innohep®)	Enzymatic depolymerisation with heparinase
Dalteparin (Fragmin®)	Nitrous acid depolymerisation
Enoxaparin sodium (Lovenox/Clexane®)	Benzoylation followed by alkaline depolymerisation
Nadroparin calcium (Fraxiparin®)	Nitrous acid depolymerisation

**Table 1.7 – Methods of Preparation of for LMWHs [197]**

#### Tinzaparin

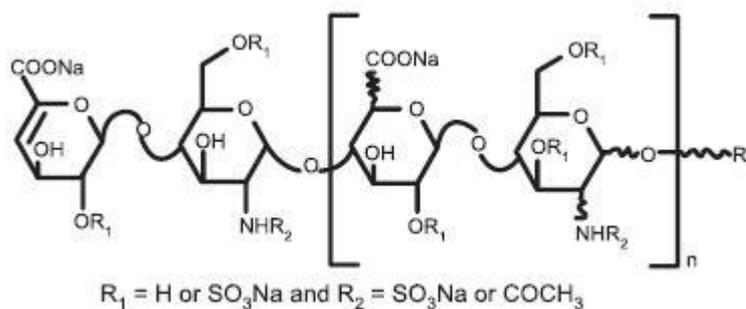
Tinzaparin sodium is the sodium salt of a LMWH obtained by controlled enzymatic depolymerization of heparin from porcine intestinal mucosa using heparinase from *Flavobacterium heparinum*. The majority of the components have a 2-O-sulpho-4-enepyranosuronic acid structure at the non-reducing end and a 2-N,6-O-disulpho-D-glucosamine structure at the reducing end of the chain (Figure 1.13). The average molecular weight ranges between 5,500 and 7,500 daltons. It contains a larger above the critical saccharide chain length rendering it to have lower anti-Xa/anti IIa ratio [209].



**Figure 1.13 Molecular Structure of Tinzaparin [211]**

### Enoxaparin

Enoxaparin sodium is obtained by alkaline depolymerization of heparin benzyl ester derived from porcine intestinal mucosa. Its structure is characterized by a 2-O-sulfo-4-enepyranosuronic acid group at the non-reducing end and a 2-N,6-O-disulfo-D-glucosamine at the reducing end of the chain (Figure 1.14). About 20% (ranging between 15% and 25%) of the enoxaparin structure contains an 1,6 anhydro derivative on the reducing end of the polysaccharide chain. The average molecular weight is about 4500 daltons [212].

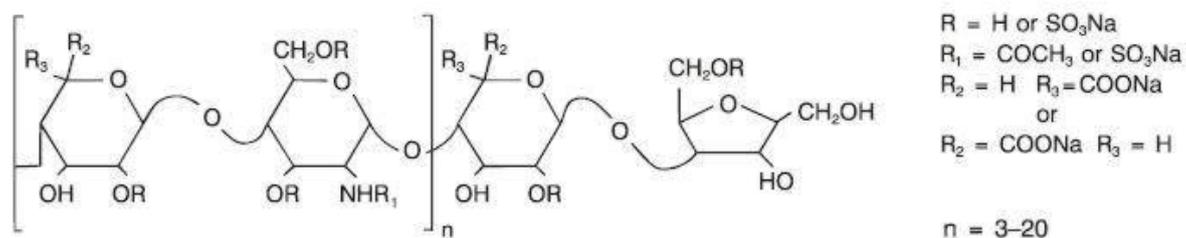


**Figure 1.14 Molecular Structure of Enoxaparin [212]**

### Dalteparin

Dalteparin sodium is produced through controlled nitrous acid depolymerization of sodium heparin from porcine intestinal mucosa followed by a chromatographic purification process. It is composed of strongly acidic sulphated polysaccharide chains (oligosaccharide, containing 2,5-anhydro-D-mannitol residues as end groups) (Figure 1.15) with an average

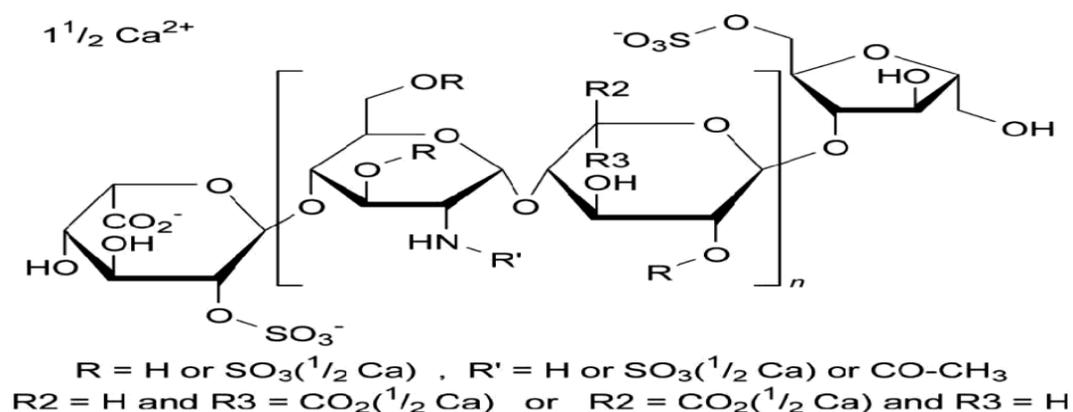
molecular weight of 5000 and about 90% of the material within the range 2000–9000d [213].



**Figure 1.15 Molecular Structure of Dalteparin [213]**

### Nadroparin

Nadroparin calcium is the calcium salt of low-molecular-mass heparin obtained by nitrous acid depolymerisation of heparin from pork intestinal mucosa, followed by fractionation to eliminate selectively most of the chains with a molecular mass lower than 2000d . The majority of the components have a 2-*O*-sulpho- $\alpha$ -L-idopyranosuronic acid structure at the non-reducing end and a 6-*O*-sulpho-2,5-anhydro-D-mannitol structure at the reducing end of their chain (Figure 1.16). The mass-average molecular mass ranges between 3600d and 5000d with a characteristic value of about 4300d.



**Figure 1.16 Molecular Structure of Nadroparin [214]**

### **1.3.3 LMWH Prophylaxis in Pregnancy**

LMWH is widely used in pregnant women and have replaced unfractionated heparin for both the treatment and prophylaxis of VTE as they are considered safe, more user-friendly and do not require frequent monitoring by activated partial thromboplastin time (aPTT) which is less reliable in pregnancy [215, 216]. Despite guidelines from the Royal College of Obstetricians (RCOG)[217] and the American College of Chest Physician (ACCP) [216], there is little consensus internationally, regarding the management of pregnant women receiving LMWH therapy [218]. Of the studies performed to date, the results suggest that pregnant women receiving LMWH thromboprophylaxis may require a higher dose particularly as pregnancy advances [219-221]. Controversy continues regarding once daily versus twice daily injections or whether anti-Xa monitoring is needed [196]. Anti-Xa /anti-IIa ratios differ between commonly used LMWHs, hence data from different LMWHs is not always comparable [222]. In addition, considerable variation exists in the anti-Xa response in pregnant women treated with LMWH. This may be due to the variety of LMWH used and to the poor performance of heparin assays in the diagnostic laboratories [222].

#### **LMWH in Post Caesarean Section**

VTE is a serious complication of CS delivery associated with a mortality up to ten fold higher than vaginal delivery [125]. The RCOG recommends that women undergoing CS, with at least one additional risk factor, be given a fixed dose of LMWH for thromboprophylaxis, 4-6 hours post CS [218] and the ACCP guideline recommend against thrombosis prophylaxis other than early mobilisation for women undergoing caesarean section without additional risk factors [216]. The use of LMWH thromboprophylaxis in low risk women post planned CS is still controversial. In a retrospective case-control study of Norwegian Registry, where 95%

of all CS had LMWH prophylaxis for 3-7 days, stated that a planned CS had a non-significant adjusted odds ratio (aOR) 1.3 for thrombotic events whereas acute CS has an aOR of 2.7 [127] compared to vaginal delivery. They stated that it is difficult to conclude whether a low risk patient who underwent planned CS should be offered prophylactic LMWH. In another retrospective study looking at Canadian registry spanning 14 years showed that the odds ratio for developing VTE is OR 2.2 (95% CI 1.5-3.2) after a low risk planned CS compared to planned vaginal delivery [151]. There is no randomized control trial addressing this issue as currently, they are deemed not feasible. Hence, a decision analyses was performed comparing a 7-day LMWH thromboprophylaxis with none after planned CS. This decision analyses suggested that even at low incidence of VTE after planned CS, benefits of LMWH exceeds the risk [152]. In our centre, women are routinely given a fixed dose of LMWH (tinzaparin 4,500) six hours post CS. There is an absence of data on the effects of LMWH in this risk group. In a comparative study, Ellison *et al* showed that 3 different LMWHs used post CS, generated peak anti-Xa levels similar to those expected in the non pregnant state [223]. At these doses, TAT levels were reduced and TFPI levels were increased; with tinzaparin showing the greatest effect on thrombin generation.

### **LMWH in The Obese Pregnant Women**

Obesity is a significant and ever increasing risk factor for thrombosis [144]. In the obese pregnant women, heparin prophylaxis has been recommended [218], however, little data exists on the anticoagulant effects of heparin in this group. Total body weight fat or body mass index (BMI) were positively associated with lag time, peak thrombin height and ETP in non pregnant women [224]. BMI also demonstrated a positive correlation with risk of post-operative VTE in non-pregnant patients receiving fixed dose enoxaparin for

thromboprophylaxis after total knee or hip replacement [225], suggesting that weight based prophylactic dose might be preferable to fixed dosing in obese patients [195].

Most studies on LMWH in obese patients used total body weight as dose modifier to avoid under dosing (with fixed dose) although there is no linear relationship between intravascular volume with total body weight (hence this could lead to overdosing). In contrast to these assumptions, anti-Xa activity is not increased when a weight adjusted dose LMWHs in as administered to non-pregnant obese patients and increased bleeding complications were not observed [226-229]. Although the current recommendation is to utilise weight adjusted dosing in obese patients, specific dosing guidance has not been provided [11, 230]. A study on morbidly obese non-pregnant patients reported that weight-based single daily dose of LMWH, is feasible and results in a peak anti-Xa levels within recommended range for thromboprophylaxis, without excessive anti-Xa activity [229].

No data exists on the anticoagulant effects of heparin in clinically or morbidly obese pregnant women. In the non pregnant patient, studies using anti-Xa as a marker of activity have shown that individualised weight based therapy generates anti-Xa levels in the required therapeutic range and the data did not support capping doses [231]. Similar pharmacokinetic studies in the obese pregnant woman (using anti-Xa and markers of thrombin generation) would provide important data leading to effective dosing strategies in this group.

### **LMWH in Placenta-Mediated Pregnancy Complications**

Placenta-mediated pregnancy complication (PMC) is a term used to encompass pre-eclampsia, placental abruption, pregnancy loss and fetal growth restriction (FGR). The term is coined due to observation that these myriad of diseases occur in more than 10% of

women in the second half of pregnancy, when placental implantation is complete [232]. Repeatedly, PMC are associated with pathological findings such as placental ischaemic lesions, placental infarction and thrombi within placental decidual blood vessels [233, 234], suggesting that placental thrombosis and insufficient uteroplacental circulation may be central to placenta-mediated pregnancy complications [235]. Despite the inconclusive results of earlier pilot trials on benefits of antithrombotic prophylaxis with heparin [236, 237], over the past decade, obstetricians have advocated heparin prophylaxis in women at risk of PMC. This trend has been further enhanced as evidence has emerged of the excellent safety profile of LMWH in pregnancy [215].

The evidence that LMWH may improve perinatal outcomes in PMC is confined to smaller underpowered trials in thrombophilia negative women [238]. More recent RCTs challenged the role of LMWH prophylaxis to prevent PMC in women without thrombophilia. The HAPPY trial reported that LMWH prophylaxis in addition to medical surveillance failed to decrease the number of late pregnancy complications compared to medical surveillance alone in 135 non-thrombophilic women with previous history of pre-eclampsia, eclampsia, HELLP syndrome, intrauterine death, FGR or placental abruption [239]. They advocate that antithrombotic prophylaxis should not be routinely administered to prevent recurrences of placenta-mediated pregnancy complication and should be restricted to women who have proven benefit from such prophylaxis. One of the few randomised trials in this area has recently reported that LMWH with aspirin reduced recurrence of hypertensive disorders, onset less than 34 weeks, in women with inherited thrombophilia and prior delivery for hypertensive disorders or small for gestation age less than 34 weeks [240].

### **LMWH in Prevention of Recurrent Miscarriages**

Meta analysis combining data from randomised trials testing the efficacy of combination of unfractionated heparin (UFH) and aspirin vs aspirin alone, in patients with antiphospholipid antibodies and recurrent miscarriages, showed that the frequency of live births was significantly higher in the aspirin and heparin group compared with women randomised to receiving aspirin alone [241]. In contrast to UFH, the combination of LMWH and aspirin does not seem to reduce the rate of pregnancy loss compared with aspirin alone [242]. There are few data comparing LMWH and UFH. Although there is limited evidence in efficacy, LMWH has largely replaced UFH in clinical practice for treatment of recurrent miscarriages in women with antiphospholipid syndrome. Pilot studies have shown that combination of LMWH and aspirin is equivalent to UFH and aspirin in preventing recurrent miscarriages [243, 244].

Well-designed trials reported lack of efficacy of LMWH in the prevention of recurrent miscarriages in women without antiphospholipid antibodies or known thrombophilia [245, 246]. This strong evidence leads to a conclusion that, in the management of recurrent miscarriages, LMWH is not indicated in thrombophilia negative women [247]. The evidence however, remains insufficient to conclude the same in thrombophilia positive women. Results of a completed treatment trial (Low Molecular Weight Heparin and/or Aspirin in Prevention of Habitual Abortion; ClinicalTrials.gov number, NCT009596211) that included women with recurrent miscarriage and heritable thrombophilias are awaited.

### **LMWH in Thrombophilic Pregnant Women**

Antepartum and postpartum thromboprophylaxis with LMWH is recommended for pregnant women with known homozygous thrombophilia (Factor V Leiden or prothrombin

20210A mutation) and personal or family history of VTE [216, 217]. This is substantiated by several meta analysis which showed the moderate to high risk of VTE in this at-risk group of pregnant women [147, 248]. For all other thrombophilias, women without personal or family history of thrombosis, clinical vigilance and postpartum LMWH prophylaxis is recommended [248]. The perception of anticoagulant deficiencies of such as antithrombin deficiency are labelled as high-risk of pregnancy-related VTE is based on older studies with methodology limitations whereby all episodes of VTE were not objectively confirmed [249]. More recent studies do not support this [147, 250]. Due to the lack of high-quality evidence of effectiveness and antithrombotic agents in preventing VTE in pregnancy, treatment recommendations were extrapolated from studies of non-pregnant patients undergoing hip-arthroplasty [251]. Therefore, clinicians usually would discuss the risk benefit effect with each patient and agree to a management plan.

A large multicentre observational study on obstetric complications and pregnancy-related VTE on thrombophilia positive women (Factor V Leiden or prothrombin G mutation) reported that prophylactic doses of LMWH had a protective effect on miscarriages (OR 0.52, 95% CI 0.29-0.94) and VTE (OR 0.05, 95% CI 0.01-0.21). Aspirin appeared to have no effect on preventing obstetric complications or VTE in this study. A nested analysis performed on women with two or more obstetric complications showed increased number of live births in the group under LMWH prophylaxis [252]. While not a randomised clinical trial, this is the largest study on thrombophilia-positive women to date.

The epidemiological data linking the inherited thrombophilias with pre-eclampsia has prompted changes in clinical practice [253]. Opinion leaders and clinicians have extrapolated from the data showing the benefit of treating patients with acquired thrombophilia and recurrent pregnancy loss. Many patients with pre-eclampsia who tested positive for inherited thrombophilia are offered and treated with low molecular weight

heparin (LMWH) with or without the addition of aspirin. Undoubtedly one of the issues that have affected the risk-benefit analysis has been the growing awareness that LMWHs have an excellent safety profile in human pregnancy [215]. Furthermore there is an overlap in the use of LMWHs in inherited thrombophilias with the potential therapeutic benefits in VTE prophylaxis.

A recent RCT reported that women with a history of early onset hypertensive disease and/or small-for-gestation infant, with an inheritable thrombophilia have been shown to benefit from a combination of LMWH and aspirin starting before second trimester of pregnancy [240]. Adding LMWH to aspirin at <12weeks gestation reduces recurrent hypertensive disease onset before 34 weeks in women with inheritable thrombophilia, thus reducing considerable financial and emotional burden with such undesirable outcome [240].

#### **1.3.4 Monitoring of LMWH**

Several studies reported that LMWHs has superior pharmacokinetic properties than UFH preparations. LMWHs showed subcutaneous bioavailability approaching 100% at low doses and peak anti-Xa activity at 3-5 hours post subcutaneous injection, with more predictable dose response [254, 255]. Therefore, LMWH usually does not need monitoring except for specific conditions such as renal failure and severe obesity. These pharmacokinetic differences between LMWH and UFH can mainly be explained by the decreased tendency of LMWH to bind to extracellular cells such as proteins, macrophages and endothelial cells as explained above. However, a pharmacokinetic limitation of LMWH is, it is primarily cleared by the renal route and in patients with renal disorders, their half-life may be prolonged.

The chromogenic anti-Xa assay is widely available and recommended by the College of American Pathologists when monitoring LMWH. However, the correlation between anti-

Xa and clinical outcomes is not clear. Although anti-Xa is shown to be inversely related to development of thrombus [256], the minimal effective level remains uncertain . While high anti-Xa levels (>0.8 U/ml) in patients receiving therapeutic doses of intravenous deltaparin have been associated with bleeding [257], others have reported that LMWH given subcutaneously at accepted doses did not show a correlation between anti-Xa level and bleeding [258, 259]. A randomised control trial has showed no benefit of monitoring patients on LMWH therapy [260]. Therefore, laboratory monitoring is generally not necessary for administered LMWH in fixed doses for thromboprophylaxis or weight-adjusted dose for therapeutic effect. However, it has been suggested that monitoring should be considered in special populations such as severe obesity and renal failure [231]. When monitoring with anti-Xa is performed, this is best done 4 hours post LMWH administration as the anti-Xa activity peaks at this time [261, 262]. It should be noted that the measured peak anti-Xa level differs between various LMWH preparations due to variation in their pharmacokinetics [262].

#### **Controversies regarding monitoring LMWH in Pregnancy**

LMWH monitoring is rarely needed in the non-pregnant population with the exception of severe renal disease and obesity [231]. However, in pregnancy, studies have shown that the pharmacokinetics of LMWH are affected and higher doses of LMWH are needed to maintain adequate level of anti-Xa activity compared to non-pregnant population [221]. Studies in pregnancy has reported there is no need in LMWH monitoring in pregnancy especially in thromboprophylaxis doses except in specific cases such as pregnant women with mechanical prosthetic heart valves where they would need judicious monitoring [263-265].

### **Calibrated Automated Thrombogram as a Potential Method of Monitoring LMWH**

Analysis of the propagation phase of thrombin generation suggests that anticoagulants that selectively target Factor Xa or thrombin, may provoke fewer bleeding disorders and therefore, monitoring of anticoagulants may be improved by using CAT, may improve monitoring [266]. Anti-Xa may not reflect the full anticoagulant profile of LMWH. CAT assay, in measuring an individual's capacity to generate thrombin using a well-defined initiator, may give a more global analysis of the coagulation profile and be a better indicator of a haemorrhagic or thrombotic tendencies [267, 268]. CAT also makes it easier to compare efficacy between different types of LMWH. A study reported the concentration-response relation to several LMWHs was very different when the concentrations were expressed in anti-Xa units but became very similar when expressed in anti-thrombin units [269]. The inter-individual variation of the in vitro pharmacodynamics response (using CAT assay) is about 25% for any different types of LMWH [269]. This is in contrast to the general assumption that LMWH need no laboratory monitoring because of their reputedly predictable anticoagulant effect. This questions the rationale for standard dosage, more so as in clinical practice pharmacokinetic variation (e.g. due to body weight) will add to this pharmacodynamic variability. There is no data of LMWH monitoring using CAT in pregnancy.

#### **1.3.5 Effect of LMWH in The Placental Haemostasis**

The placenta is theoretically vulnerable to thrombosis as TF is continually expressed by placental tissue, although the cytotrophoblast cells differentiate and adopt a more thrombo-resistant phenotype [270]. The cytotrophoblast cells strongly express EPCR which will subsequently participate in haemostasis within the intervillous space [270].

LMWH is reported to have antiangiogenic activity which is associated with the release of endothelial TFPI. Previous study reports that the inhibitory effect of the LMWH tinzaparin on endothelial tube formation is associated with stimulation of the release of TFPI, but not to anti-Factor Xa activity [271]. TFPI-2 is a poor inhibitor of the TF/FVIIa complex, compared to TFPI, but a strong inhibitor of factor Xa. TFPI-2 mRNA is especially widely expressed in human placenta [272] . There is a direct correlation between the expression of TFPI-2 with TF and between TFPI or TFPI-2 with PAI-2 in normal human placenta, indicating a possible role for local haemostasis in placentas from normal pregnancies.

## **1.4 Aims of The Study**

The haemostatic effects of LMWH in the morbidly obese pregnant women, women post CS have been identified as areas where further work is needed. The hypothesis of this thesis is that by studying pharmacokinetic anti-Xa and thrombin generation profiles, the optimum prophylactic LMWH dosing regimen in these groups of women can be determined. In this thesis, the effects of tinzaparin, a LMWH used in the majority of maternity centres in Ireland and for which pregnancy data is particularly lacking were studied. The aim of the study is to investigate the full anticoagulant effects of tinzaparin in vulnerable groups of pregnant women identified by the triennium report. Effective prevention of VTE in pregnancy may also be improved by the availability of a predictive test which will identify pregnant women who may develop antenatal and post natal venous thrombosis. An additional aim of this study was to explore the ability of the thrombin generation test to predict venous thrombosis in a population of healthy pregnant women.

LMWH is used widely despite scientific uncertainty, for the prevention of placenta mediated pregnancy complications (pregnancy loss, fetal growth restriction, pre-eclampsia

and abruption) both in thrombophilic and non thrombophilic patients [273]. However, conflicting data has been reported on the efficacy of LMWH in preventing placenta mediated complications [236, 274]. The aim of the final part of the study was to investigate at a local level in uteroplacental compartment, the effects of LMWH on the expression of key genes and proteins from placenta of LMWH treated pregnancies. In order to investigate whether changes in the expression of placental coagulation proteins are translated into altered procoagulant activity, CAT parameters and TAT levels in blood taken from the maternal peripheral vein, uterine vein and fetal umbilical cord vein were also measured.

### **Specific Aims**

1. **Study 1:** To investigate the full anticoagulant effects of LMWH over 24 hours following a fixed prophylactic dose of tinzaparin (4,500 units/day) in healthy women post CS.
2. **Study 2:** To evaluate the CAT assay as a potential predictive tool for thrombosis in pregnancy.
3. **Study 3:** To compare different dosing regimens; fixed dose versus weight adjusted dose on the anticoagulant effects of the LMWH, tinzaparin used for thromboprophylaxis in obese pregnant women.
4. **Study 4:** To compare the haemostatic effects of LMWH
  - a. on the maternal systemic circulation, uteroplacental circulation and fetal circulation of thrombophilic women and
  - b. on human haemostatic gene and antigen expression in placental tissue.



# **Chapter 2:**

# **Patients and Methods**

## 2.1 Ethical Approval

All four studies described below have been granted approval from the Clinical Research Ethics Committee of the Cork Teaching Hospitals on the 7<sup>th</sup> July 2009 (Ref ECM 5 (8) 07/07/09) (Appendices A & B). All patients recruited in all four studies have given written informed consent (Appendices C-G) to participate and are fully aware that they can withdraw from the study at any time, and this will in no way affect their clinical management or care.

## 2.2 Patient Selection

### 2.2.1 Study 1: Effects of Low Molecular Weight Heparin on Thrombin Generation Post Caesarean Section

#### Patient Groups

Sixty healthy women having given full informed written consent were recruited for this study at Cork University Maternity Hospital (CUMH). Forty women undergoing elective CS were recruited. In our centre, patients received 4500IU tinzaparin per day usually at 6 or 10 hours post caesarean delivery, depending on clinician preference. A recent report suggested that ETP levels would be reduced by approximately 15-20% in pregnancy following LMWH treatment [275]. Based on these figures, the sample size required to detect a significant difference in thrombin production between the CS group and the control group would be 17 (in each group).

**Caesarean section groups:** In this study, twenty women received 4,500IU tinzaparin at 6 hours post caesarean section (CS1); the remaining twenty women received 4,500IU tinzaparin at 10-12 hours post delivery (CS2). Women with any additional risk factor for VTE such as personal or family history

of VTE or thrombophilia, BMI>29, cigarette smokers were excluded from this study. Inclusion and exclusion criteria are listed below (Table 2.1). Having given full and informed consent, patients received 4,500 units (fixed dose) (once daily injection) of tinzaparin 6 hours post caesarean section as is the normal practice in CUMH. Approximately 4.5mls of venous blood were taken prior to and at 4, 10, 18 and 24 hours post injection and processed as described below.

Inclusion Criteria	Exclusion Criteria
<ul style="list-style-type: none"> <li>• <b>Pregnant women over 18 years</b></li> <li>• <b>BMI between 19-29</b></li> <li>• <b>Undergoing elective caesarean section</b></li> <li>• <b>No intercurrent fetal or maternal complications</b></li> <li>• <b>No personal or family history of VTE</b></li> <li>• <b>No known personal or family history of thrombophilia</b></li> <li>• <b>Non-smoker</b></li> </ul>	<ul style="list-style-type: none"> <li>• active antenatal or post partum bleeding</li> <li>• increased risk of major haemorrhage (e.g. placenta previa)</li> <li>• bleeding diathesis e.g. von Willebrand's disease, haemophilia, acquired coagulopathy</li> <li>• thrombocytopenia (platelet count &lt; 75 x10<sup>9</sup>)</li> <li>• Acute Stroke in the last 4 weeks (ischaemic or haemorrhagic)</li> <li>• Severe renal disease (GFR&lt; 30 ml/min/1.73m<sup>2</sup>)</li> <li>• Severe liver disease (prothrombin time above normal range or known varices)</li> <li>• Uncontrolled Hypertension (BP &gt; 200mmHg systolic or &gt; 120mmHg diastolic)</li> <li>• Allergy to heparin/bisulfites</li> <li>• History of heparin associated thrombocytopenia</li> </ul>

**Table 2.1: Inclusion and Exclusion Criteria for Caesarean Section Group**

**Control group:** Twenty patients who underwent spontaneous vaginal delivery with no complications were also recruited (SVD group).

These women were matched for age, parity and BMI with the study group. Having given full and informed consent, 4.5 mls of venous blood were taken from each woman at 6, 10, 16, 24 and 30 hours post delivery. Additionally, a control group of healthy women who underwent planned caesarean section but were untreated with tinzaparin were also recruited. Having given full and informed consent, 4.5 mls of venous blood were taken from each woman at 6 and 10 hours post delivery. Women who suffered high blood pressure, diabetes, pre-eclampsia epilepsy, cardiovascular

disease, renal or hepatic dysfunction, previous thromboembolism, known thrombophilia and who are taking any medication known to interfere with haemostasis were excluded.

### **Blood sampling**

At each time point, venous blood (4.5mls) was taken from the ante-cubital fossa with minimum venous stasis using 3.13% sodium citrate as anticoagulant. In the CS1 group, venous blood was also taken at 6, 10, 16, 24 and 30 hours post delivery. This corresponded to pre-dose, 4, 10, 18 and 24 hours post tinzaparin administration. A subset of ten patients had blood sampling pre-delivery. In the CS2 group, venous blood was taken at 6 and 10 hours post delivery. In the SVD group, blood samples were taken at 6, 10, 16 and 24 hours post delivery. Venous bloods were centrifuged at 4°C for 20 minutes at 2000g. The resulting platelet poor plasma was carefully removed, aliquoted into cryogenic microtubes, snap frozen and stored in cryoboxes at -80 °c until assay [276]. All venous blood were processed and stored within 1 hour of phlebotomy.

### **Laboratory assays**

All assays were performed in large batches to minimise intra-assay variation. Anti-Xa, CAT, TFPI and TAT were measured in each sample in the CS groups as described in the Laboratory Methods section. CAT, TFPI and TAT were measured in the control group. Internal quality control was achieved by using quantitative reference plasma. Reproducibility was achieved by triplicating samples for each assay. The Research Coagulation Laboratory in Trinity Centre for Medicine and Health, Dublin is part of the National External Quality Assurance System (NEQAS).

## **2.2.2 Study 2: Thrombin Generation as a Predictive Tool for Venous Thromboembolism In Pregnancy**

### **SCOPE**

Screening for Pregnancy Endpoints (SCOPE) is an international multicenter prospective cohort study for the prediction of pregnancy complications such as pre-eclampsia, fetal growth restriction and preterm birth ([www.scopestudy.net](http://www.scopestudy.net)). It maintains biobank and biodata from over 5000 primiparous women recruited from 2007 to 2011 [277]. The study has ethical approval in each of its eight centres in Europe, Australia and New Zealand.

### **Patient Groups**

Patients with documented VTE in pregnancy or postpartum were identified from the SCOPE cohort, three matched control pregnancies (for age, BMI, ethnicity and smoking status) were selected for each VTE patient. A previous study of normal uncomplicated pregnant women observed a mean peak thrombin level of 500nM (SD100nM)[165] . From the SCOPE databank which has recruited in total of 5000 women, 20 women from the cohort have developed venous thrombosis. Based on these previous findings, it was estimated that for a comparison of means statistical test (probability of Type I error of 0.01, and a probability of Type II error of 0.1)  $n_1=11$  patients in the study group and  $n_2=33$  in the matched control group (matched for age, gestation, BMI, and ethnicity) were required to achieve statistical power of 82%. Patients and controls taking any medication known to interfere with haemostasis, at the time of sampling were excluded from the study. A list of inclusion/exclusion criteria for the study is given in Table 2.2.

Inclusion Criteria	Exclusion criteria
<ul style="list-style-type: none"> <li>• Patients with documented VTE</li> <li>• High probability of VTE by objective testing (Compression Duplex ultrasound, perfusion scan, CT pulmonary angiogram)</li> </ul>	<ul style="list-style-type: none"> <li>• On medication known to interfere with haemostasis</li> <li>• Known thrombophilia (prior to entry into SCOPE)</li> </ul>

**Table 2.2 Inclusion and Exclusion criteria for VTE group**

### **Blood sampling and Laboratory Assay**

Venous blood was collected in 3.13% sodium citrate at booking (15 weeks) for each patient. In the VTE group, all blood samples were taken prior to the thrombotic event. Patients taking any medication known to interfere with haemostasis, at the time of venous blood sampling were excluded from the study. Citrated plasmas (80µl x5) were assayed for CAT in presence and absence of thrombomodulin.

### **2.2.3 Study 3. Antenatal Thromboprophylaxis in The Morbidly Obese Women**

#### **Patient Groups**

**Obese group:** Morbidly obese pregnant women (BMI  $\geq 40$ ) attending the Cork University Maternity Hospital (CUMH) were invited to participate. Women fulfilling the inclusion and exclusion criteria for this group listed in Table 2.3 below were included. All patients had a baseline bioprofile done to check for renal and liver dysfunction prior to inclusion in the study. Using peak anti-Xa levels as an endpoint, and based on previous studies in pregnant women, 75 iu/kg will give peak anti-Xa levels of 0.25+/- 0.10(SD) iu/ml[221]. In order to detect whether the fixed doses will yield anti-Xa levels

lower than 0.2 iu/ml, a sample size of fourteen women in each group was required. Forty patients have been recruited (twenty patients in the obese group, twenty patients in normal BMI group). All patients recruited were non-smokers, underwent singleton pregnancies, have normal creatinine clearance, normal liver function tests, no other contraindications for LMWH and had no personal or family history of VTE or known thrombophilia. None of the patients in this study had any VTE event or bleeding complications following the LMWH prophylaxis.

Inclusion Criteria	Exclusion Criteria
<ul style="list-style-type: none"> <li>• Pregnant women age over 18 years</li> <li>• BMI <math>\geq</math>40</li> <li>• No known thrombophilia</li> </ul>	<ul style="list-style-type: none"> <li>• active antenatal or post partum bleeding</li> <li>• increased risk of major haemorrhage (e.g. placenta previa)</li> <li>• bleeding diathesis e.g. von Willebrand's disease, haemophilia, acquired coagulopathy</li> <li>• thrombocytopenia (platelet count <math>&lt;</math> <math>75 \times 10^9</math>)</li> <li>• Acute Stroke in the last 4 weeks (ischaemic or haemorrhagic)</li> <li>• Severe renal disease (GFR <math>&lt;</math> 30 ml/min/1.73m<sup>2</sup>)</li> <li>• Severe liver disease (prothrombin time above normal range or known varices)</li> <li>• Uncontrolled Hypertension (BP <math>&gt;</math> 200mmHg systolic or <math>&gt;</math> 120mmHg diastolic)</li> <li>• Allergy to heparin/bisulfites</li> <li>• History of heparin associated thrombocytopenia</li> </ul>

**Table 2.3 Inclusion and Exclusion Criteria for Obese Group**

**Control group:** A group of 20 healthy pregnant women with normal BMI were recruited. These women were matched for age and parity with the obese group. Having given full and informed consent, venous bloods were taken from each woman at five stages during late pregnancy from 30-36 weeks gestation.

#### **Fixed Dose Versus Weight Adjusted LMWH Tinzaparin Dose**

Having given full and informed consent, women in the obese group were started on 4500iu tinzaparin once daily injection at 32 weeks gestation. Venous blood (4.5mls) was taken prior to and

4 hours following their injection from the ante-cubital fossa with minimum venous stasis using 3.13% sodium citrate as anticoagulant. At 32 weeks, the tinzaparin dose was then altered to a weight adjusted dose (75iu/kg/day) after a 48 hour wash-out period and the woman were requested to return for blood sampling. Local anti-Xa monitoring was used to check peak levels at 4 hours. Target prophylactic anti-Xa range was defined as 0.2-0.5u/ml. For patients with anti-Xa outside the target range, dose adjustments were discussed with consultant haematologist. Patients continued with the weight adjusted dose until delivery and venous blood were taken again at 34 and 36 weeks. Patients were advised to withhold dose 12 hours prior to planned delivery or if symptoms or signs of labour are present. For the control group, venous bloods were taken at 30, 32, 34 and 36 weeks of pregnancy.

### **Blood Sampling and Laboratory Assays**

Venous blood (4.5mls) was taken from the ante-cubital fossa with minimum venous stasis using 3.13% sodium citrate as anticoagulant and centrifuged at 4°C for 20 minutes at 2000g. The resulting platelet poor plasma was carefully removed, aliquoted into cryogenic microtubes, snap frozen and stored in cryoboxes at -80 °c until assay. All venous blood were processed and stored within 1 hour of phlebotomy.

In the obese group, anti-Xa, TFPI, TAT and ETP were measured in each sample. In the control group, ETP, TFPI and TAT were measured. All assays were performed in large batches to minimise intra-assay variation. All assays measured are described in the Laboratory Methods section.

## **2.2.4 Study 4: Effects of Low Molecular Weight Heparin within the Uteroplacental Compartment.**

### **Patient groups**

**LMWH Group:** Thrombophilic women undergoing elective CS who received antenatal prophylaxis with tinzaparin (4500iu once daily) due to moderate risk of VTE [218] were invited to participate in the study. Study reported the effect of thrombophilia on ETP was 300nM.min and in order to detect this difference, n=6 sample size was needed [267]. Eight thrombophilic women were recruited in this study.

**Control group:** A control group of fifteen healthy women who underwent elective CS were recruited. These women were matched for age, parity and BMI with the study group. Women who suffered renal or hepatic dysfunction, previous thromboembolism, known thrombophilia and who are taking any medication known to interfere with haemostasis were excluded.

### **Blood Sampling and Placental Biopsy**

Having given full and informed consent prior to CS, venous blood were taken from the maternal antecubital fossa and maternal uterine vein (by experienced surgeon) as previously described [53], after delivery of the fetus but prior delivery of the placenta. Simultaneously, venous cord blood sample and placental biopsy were taken [53] .

### **Laboratory assays**

Plasma were prepared and stored as described in the first study. All assays were performed in large batches to minimise intra-assay variation. Anti-Xa, ETP, TFPI and TAT were measured in each sample

from the LMWH group. ETP, TFPI and TAT were measured in the control group. All assays performed are described in the Laboratory Methods section.

### **Placental Biopsy**

Upon delivery of placenta, random 2x2cm<sup>2</sup> placental biopsies were taken, rinsed with sterile water and snap frozen with liquid nitrogen within 30 minutes of placental delivery and stored in -80°C until RNA extraction performed [278].

## **2.3 Statistics**

Statistical analysis software package used in this thesis is Predictive Analytics SoftWare (PASW)/IBM SPSS version 18.

### **2.3.1 Power Calculations**

A previous study of normal uncomplicated pregnant women observed a mean peak thrombin level of 500nM (SD100nM)[165]. Using peak anti-Xa levels as an endpoint, and based on previous studies in pregnant women, 75 IU/kg will give peak anti-Xa levels of 0.25+/- 0.10(SD) IU/ml[221]. The sample size required to detect a significant difference in a specific parameter between the study group and the control group was calculated to achieve probability of Type I error of 0.05, and a probability of Type II error of 0.2. To ensure that all studies were adequately powered, over-recruitment was done to allow for dropouts.

For the predictive study involving SCOPE cohort, it was estimated that for a comparison of means statistical test (probability of Type I error of 0.01, and a probability of Type II error of 0.1) an

$n=11$  patients in the study group and  $n_2=33$  in the matched control group (matched for age, gestation, BMI, and ethnicity) were required to achieve statistical power of 82%. With  $n_1=20$  women with VTE in pregnancy identified for the study group, sufficient statistical power was achieved to allow the study to be performed.

### **2.3.2 t-Test**

Distribution of each parameter was checked for normality using Normal Probability Plot or formal tests such as the Kolmogorov-Smirnov test and the Shapiro-Wilks test. The  $t$  test was used to compare the means between two groups of independent, continuous variable from a set of parametric data. Where a group of non-independent parametric data were involved, a paired  $t$ -test was used. In all circumstances, a  $p$ -value of  $<0.05$  was considered statistically significant.

### **2.3.3 Analysis of Variance (ANOVA) and Post-Hoc Test**

Analysis of variance (ANOVA) estimates the effects of one or more independent factors on a dependent or response variable in a set of parametric data. In cases where the effects of two factors were measured, the interaction between these two factors may also be tested. The  $F$ -test is based on the ratio between groups variability to within groups variability. The ANOVA compare means for a factor by an  $F$ -test and the corresponding  $p$ -value. In all circumstances, a  $p$ -value of  $<0.05$  was considered statistically significant. Post Hoc test was then carried out using Dunnett's Procedure where significant differences were established by ANOVA.

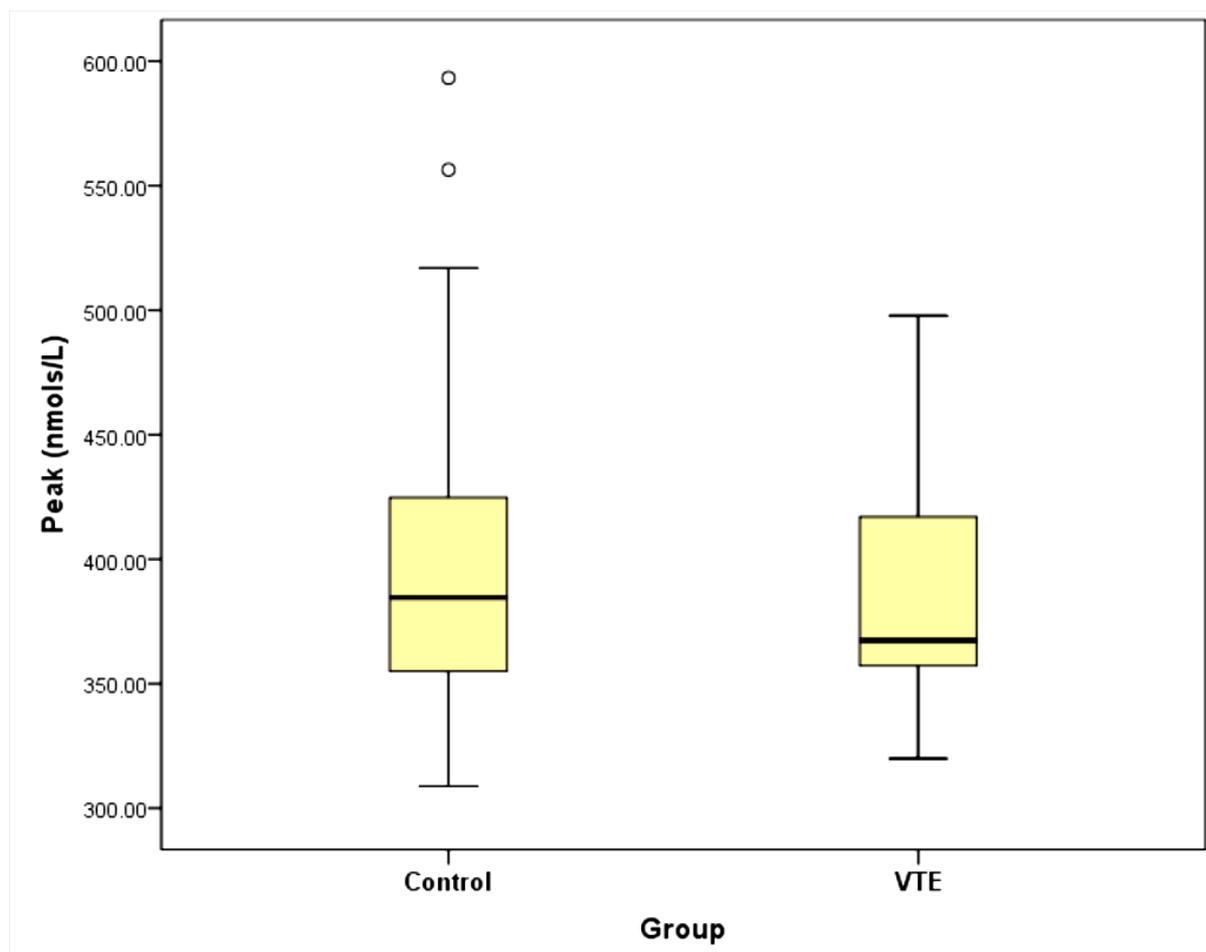
### **2.3.4 Pearson's Correlation Coefficient**

Pearson product-moment correlation coefficient was used a measure of the strength of linear dependence between two variables.

### **2.3.5 Graphical Data Presentation**

#### **Boxplots and Whiskers**

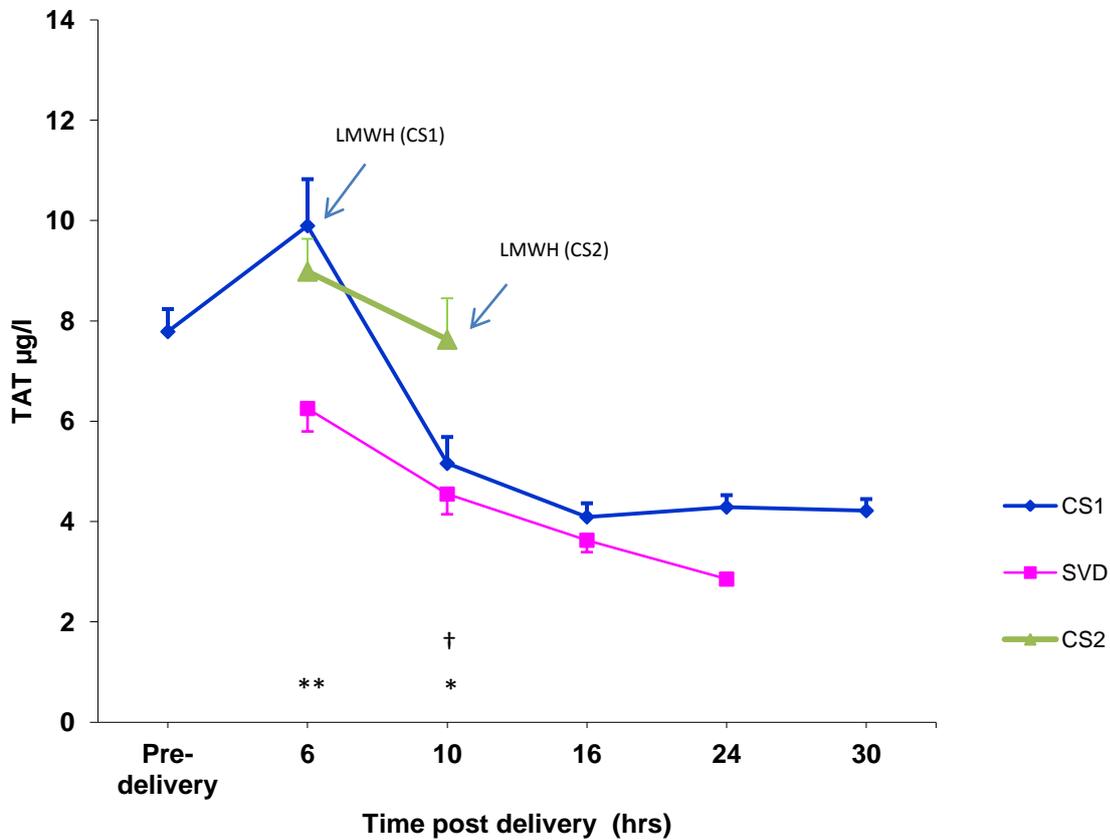
Boxplot (or box-and-whisker diagram) was used to as it is a convenient way of graphically presenting groups of numerical data through their five-number summaries; the smallest observation (sample minimum), lower quartile (Q1), median (Q2), upper quartile (Q3), and largest observation (sample maximum). Boxplots display differences between populations without making any assumptions of the underlying statistical distribution. The spacing between the different parts of the box help indicate the degree of dispersion (spread) and skewness in the data, and identify outliers (See Figure2.1).



**Figure 2.1** A typical example of a boxplot. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

### Line Chart

Line charts were used to display result as a series of data points. This was created by connecting a series of points that represent individual measurements with line segments. It allowed results to be visualized as a trend over intervals of time or a time series. The y-axis represents the dependent variable and the x-axis represents the independent variable . Error bars represented the variability of data and were used on charts to indicate the error, or uncertainty in a reported measurement. Error bars in this thesis represented by standard deviation (Figure 2.2).

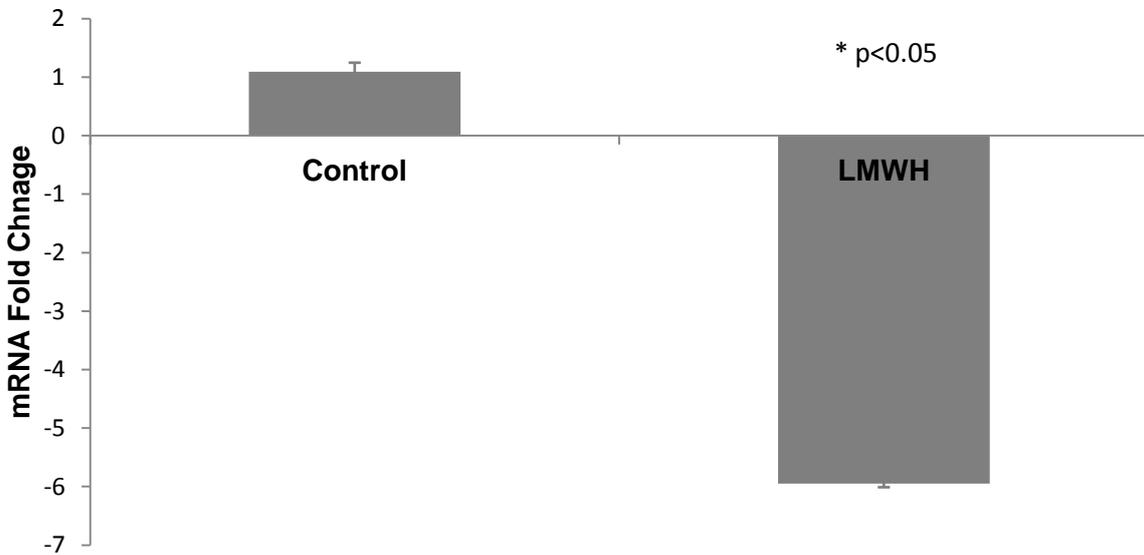


**Figure 2.2** Example of a Line Graph. TAT (ug/l) levels in women post caesarean section (CS1 n= 20), (CS2 n=20) and spontaneous vaginal delivery (SVD n= 20). Blood samples were taken pre-delivery (CS1 only) and at 6, 10 (CS1,CS2 and SVD), 16, 24(CS1 and SVD) and 30 hours (CS1 only)post delivery. Values represent mean  $\pm$  SD. \*\* =  $P < 0.002$  SVD v CS1 and CS2. \* =  $P < 0.03$  SVD v CS2. † =  $P < 0.01$  CS1 v CS2. Arrows show the start of LMWH prophylaxis in the CS1 and CS2 groups.

### Column Charts

A column chart, (or bar chart) were used for illustrating comparisons among categories (groups). The rectangular bars of lengths were proportional to the magnitudes or frequencies of what they represent. The column chart is vertically oriented bars. Categories were organized along the x-axis and the dependent variable along the y-axis. Error bars represented the variability of data and were used on charts to indicate the error, or uncertainty in a reported measurement. Error bars in this thesis represented by standard deviation (Figure 2.3).

## Placental TFPI<sub>2</sub> mRNA Gene Expression



**Figure 2.3 Example of A Column Chart. Placental TFPI<sub>2</sub> Gene and Antigen Expression**

Placental biopsy was taken from all patients and snap-frozen within one hour of delivery. All CS were for obstetric indication. Real-time PCR was used to quantify mRNA expression in placental tissue. LMWH group are thrombophilic pregnant women who were on antenatal LMWH prophylaxis. Control group are healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. Statistical test performed were two- tailed t-test as comparison of means. Error bars represent standard deviation. \*denotes significant result.

## 2.4 Laboratory methods

### 2.4.1 Anti-Xa Chromogenic Assay (Hyphen BioMed, France)

The heparin Anti-Xa assay is a two-stage chromogenic method developed for measuring Anti-Xa activity of homogeneously heparins in plasma or in purified solutions. The method based on inhibiting a constant amount of factor Xa, by the tested heparin, in presence of antithrombin (stage 1) and hydrolysis of factor Xa specific chromogenic substrate 9CS11(65), by the factor Xa in excess (stage 2). pNA is then released from the substrate. The amount of pNA released is then a relation of the residual factor Xa activity. There is an inverse relationship between the concentration of heparin and color development, measured at 405nm.

#### Reagents Required

Reagent 1 (R1) Antithrombin III: Contains 5U/ml of human lyophilised antithrombin.

Reagent 2 (R2) Factor Xa : Contains 25µg of lyophilised purified bovine factor Xa.

Reagent 3 (R3) Chromogenic substrate: Chromogenic substrate specific for FXa(CS-11(65)), lyophilized vial of about 4mg (6µmol) in presence of mannitol.

Reagent 4 (R4) Buffer (Assay reaction buffer): Tris 0.05, NaCl 0.175M, EDTA 0.0075M at pH 8.40, containing PEG at 0.1% and sodium azide as preservative (4 vials of 25mls ready to use).

Acetic acid 20%

Normal reference plasma (Biophen 223201): Obtained in order to avoid any platelet activation, for preparation of the calibration curve.

Heparin Control plasma CI and CII (Biophen Cat 224301 & 224401):

Human control plasmas supplemented with low molecular weight heparin (LMWH) for the quality control of heparin measurements with anti-Xa methods. Control 1 (CI) = 0.22IU/ml LMWH. Control 2 (CII) = 0.43 IU/ml LMWH.

Heparin Standard: Heparin standard used is tinzaparin, the same LMWH being used in patient's sample.

### **Reagents Reconstitution (performed 30minutes before use)**

Reagent 1 Antithrombin III: Each vial is reconstituted with 1ml of distilled water , and allowed to reconstitute for 30 minutes at 18-25°C This is then diluted 1:5 with R4 buffer just before use (4ml of R4 buffer is added to 1ml R1 Antithrombin III).

Reagent 2: Factor Xa: Reconstituted with 1ml of distilled water, allowed to stand for 30 minutes at 18-25°C This is then diluted 1:5 with R4 buffer just before use (4ml of R4 buffer is added to 1ml R2 factor Xa).

Reagent 3: Factor Xa Specific Chromogenic Substrate: Each vial is reconstituted with 5ml of distilled water and allowed to stand for 30 minutes at 18-25 C

Normal Reference Plasma : Reconstituted with 1ml of distilled water

Heparin Control Plasma: Reconstituted with 1ml of distilled water

### **Preparation of Standards**

1. Dilution of 1/100 (100 iu/ml) of low molecular weight tinzaparin was made by adding 990µl of saline with 10µl of tinzaparin 10,000iu/ml.

2. Immediately prior to use, a stock solution was prepared at dilution of 1/10 (10iu/ml). This was made by adding 900µl of saline to 100µl of 100iu/ml from above. This stock solution was then used to prepare the following dilutions as depicted on Table 2.4 below.

Standard	iu/ml	Volume added to 1x diluent	1x diluents (reference plasma)
1	0.8	20µl of standard 1	180µl
2	0.6	20µl of standard 2	180µl
3	0.4	20µl of standard 3	180µl
4	0.2	20µl of standard 4	180µl
5	0.1	20µl of standard 5	180µl
6	0 (blank)	20µl of standard 6	180µl

**Table 2.4 Preparation of Standard Samples for Anti-Xa Assay**

### Procedure

1. 50µls of each standard/control/sample dilution were added in triplicate to a standard ELISA plate
2. The factor Xa and antithrombin was diluted to 1:5 concentration (4 mls of R4 buffer added to the vial to make up 5mls needed for a full ELISA plate).
3. The plate was pre-warmed at 37°C for 5-10 minutes by careful placement in the waterbath.
4. The diluted factor Xa and substrate were pre-warmed at 37°C for 5-10 minutes.
5. Using a multichannel pipette, 50µls of antithrombin was carefully added to each duplicate well, leaving the third well for blanking. Timer was started and the R1 incubated for exactly two minutes.

6. Using a multichannel pipette, 50µls of factor Xa was carefully added to each duplicate well, leaving the third well for blanking. Timer was started and the reagents incubated for exactly two minutes.
7. Using a multichannel pipette, 50µls of substrate were carefully added to each duplicate well, leaving the third well for blanking. Timer was started and the reagents incubated for exactly two minutes.
8. Using a multichannel pipette, 100µls of acetic acid were carefully added to each duplicate well, leaving the third well for blanking. Timer was started and the R1 incubated for exactly two minutes.
9. Plate was removed from the waterbath and the bottom of the ELISA plate was carefully blotted dry.
10. Using a multichannel pipette, 100µls of acetic acid was added to the blank well (third well of the triplicate).
11. Using a multichannel pipette, 150µls of assay buffer (R4) was added to the blank well.
12. The anti-Xa assay program selected on the Revelations© software package.
13. Using the template, controls, standard and samples were entered at the appropriate locations.
14. The plate was read at 405nm using Revelations© software.
15. Standard curve and results printed.

### **Calculations**

The average background value was subtracted from the test values for each sample. A standard curve was constructed using the results from the standards, plotting  $A_{405}$  against the amount of anti-Xa in the standards, as depicted in Figure 2.4. Sample values were interpolated from the curve and corrected for the dilution factor to determine the sample anti-Xa concentrations.

## Absorbance versus Concentration

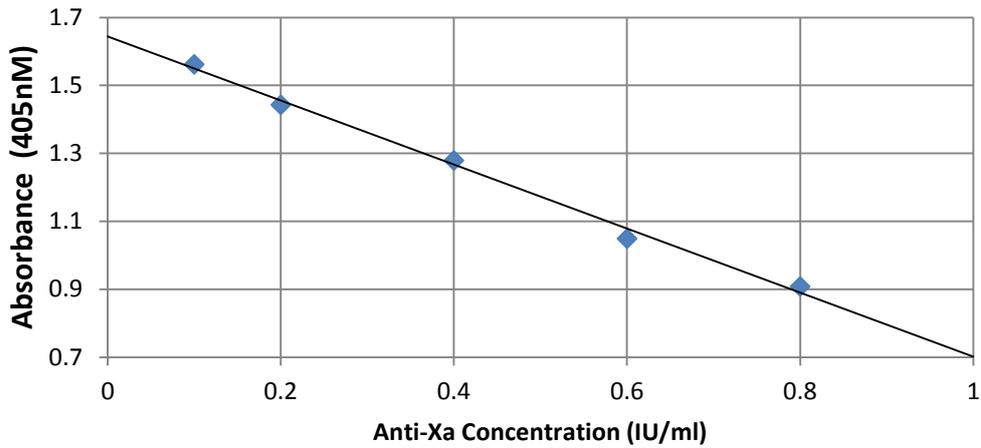


Figure 2.4 A Typical Reference Curve (Absorbance vs Anti-Xa Concentration)

### Quality Control

Use of suitable quality controls of heparin measurements allowed validation of the calibration curve, as well as the homogenous reactivity. They were intended for the assessment of precision and accuracy. Two controls high and low (CI and CII) were processed with known values of LMWH. This standard curve was run for each plate. Results were accepted if co-efficient variation, R value >0.9 and anti-Xa concentration of all duplicates were within ten percent variation. Controls were run with each plate and must be within accepted limits.

## 2.4.2 Calibrated Automated Thrombogram (Thrombinoscope BV)

### Principle

The Calibrated Automated Thrombogram (CAT) assay required two fluorescent measurements in the same plasma. In one well (the measurement well) TF and synthetic phospholipids vesicles were added to plasma to initiate coagulation and induce thrombin formation. In a second well (the calibration well), a known amount of substrate-converting activity (the 'thrombin calibrator') was added to plasma without activating coagulation. The thrombin calibrator consists of thrombin bound to  $\alpha_2$ -macroglobulin ( $\alpha_2$ M-thrombin), a form of thrombin that cannot be inhibited by plasma protease inhibitors. A mixture of  $\text{CaCl}_2$  and fluorogenic substrate was subsequently dispensed in both wells and the developing fluorescence was recorded in real time by a fluorometer. The thrombin generation curve was obtained by taking the first derivative fluorescence recorded in the measurement well after i) correction for inner filter effects and substrate consumption (based on the fluorescence measured in the calibration well) and ii) subtraction of the fluorescence signal deriving from  $\alpha_2$ M-thrombin (based on mathematical algorithm). The thrombin generation curve can be described in terms of lag time, peak time, peak height and area under the curve (endogenous thrombin potential, ETP). A typical thrombin generation curve will be generated as shown in Figure 2.5.

### Reagents Required

Thrombin calibrator: Reconstituted with 1ml of DDD water without vortex

Platelet poor plasma reagent (Tissue Factor 5uM): Reconstituted with 1ml DDD water, shaken carefully.

Fluca substrate and Fluca buffer: The Fluca substrate is stored at 4°C until opened. The Fluca the substrate was incubated at 37°C for 2 minutes before vortexed. 40µls of Fluca was added to the vial

of buffer (1600µls, diluted 40:1). One vial was required as a minimum when using the dispenser on the thrombinoscope instrument.

Normal reference plasma: (Biophen Cat No 223201)

Nunc 96 well round bottom plates (Cat no MPA-510-010V)

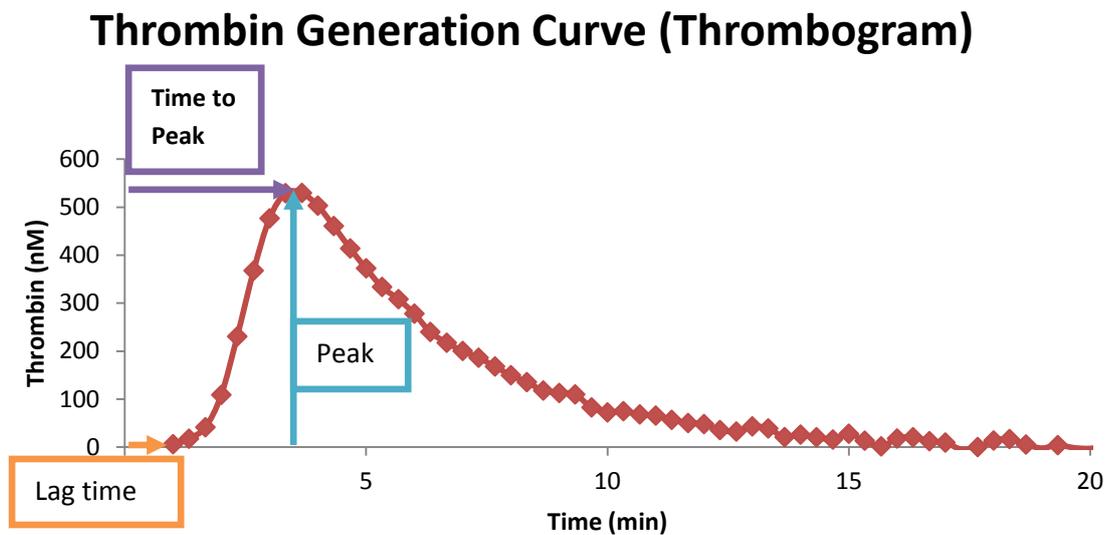
### **Patient Samples**

Citrated plasma was used. For each sample, a triplicate 80µl sample was needed plus another 80µl for the thrombin calibrator. Therefore, frozen aliquots of about 400µls were used for each test sample. Samples were thawed and incubated at 37°C for 5 minutes before assay.

### **Procedure**

1. A fluorimeter with dedicated thrombinoscope software (Fluouriskan Machine) was used. Plasma samples, fluca buffer, and reference plasma were incubated at 37°C for 10-15 minutes.
2. The Nunc plate was set up, each t-cal well labelled to its' appropriate matching sample using the group mode and sample Ids entered.
3. 80µls of plasma was pipetted in triplicate into wells. An additional 80µl plasma sample was added in an adjacent well for thrombin calibration. Caution was noted that fluorimeter works across the plate, i.e wells A1-4 followed by A5-9.
4. The same procedure was repeated for the reference plasma.
5. Thrombin calibrator (20µls) was then added to the calibration well only.
6. 20µls of PPPr was added to the test samples only and not to the t-cal wells.
7. The fluca was made up
8. Plate placed into the fluorimeter, the dispenser was enable and and the cycle started.

9. The dispenser was cleaned as prompted using DDD water. Upon completing the cleaning cycle, the DDD water was replaced with Fluca.
10. The thrombin generated (nM) for each sample was measured by the fluorimeter.
11. A full thrombin generation curve for each sample was available at the end of the run (approximately 50 minutes).



**Figure 2.5 A Typical Thrombin Generation Curve**

- Parameters of thrombogram
  - Lag time
  - Peak
  - Time to peak
  - Endogenous thrombin potential (Area Under the Curve)

## **Modified CAT procedure to determine the effect of Thrombomodulin (probing the APC pathway)**

### **Additional Reagents**

Thrombomodulin (rabbit) RABTM-4202 (Haemtech Biopharma, USA): Final concentration of 10nM (required to inhibit reference plasma by 50%) was calculated. TM was aliquoted into amounts sufficient to be added to 1 ml of PPP and stored at -80 C.

### **Patient Samples**

Citrated plasma was used. For each sample, 5 X 80µl plasma is needed plus 80µls sample plasma for the thrombin calibrator. Therefore, approximately 400µls of frozen citrated sample plasma were required. Samples were thawed and incubated at 37 C for 5 minutes before assay. Triplicate 80µls were required for the ETP assay and duplicate 80µls for ETP in the presence of thrombomodulin. The same sample for the thrombin calibrator was used for both.

### **Protocol (Using a multichannel pipette)**

1. The fluorimeter was switched on. Fluca buffer, plasma samples and reference plasma were placed in an incubator at 37 C for 5-10 minutes
2. The plate was prepared and sample identified on the computer software. Each t-cal well matched to its appropriate samples (all 5 wells used the same t-cal) using group mode.
3. Triplicate of 80µls of plasma were pipette into wells followed by an additional 80µls in duplicate for the TM wells.
4. The same procedure was followed for the reference plasma. In this study, reference plasmas were run at the start and end of the plate (is two reference plasmas for quality control).
5. Thrombin calibrator (20µ) was added to the t-cal well.

6. PPPr (20µls) was added to the triplicate patient samples only and not to the t-cal well.
7. TM treated PPP (20µls) was added to the duplicate wells. Fluca was made
8. Diluted fluca (20µls) was added quickly but carefully using the multichannel pipette to each well.
9. The plate was placed immediately in the machine and the software measuring started.
10. The software will show the peak as it forms for each sample. The results were available at the end of the run at approximately 50 minutes.
11. The files were saved on the computer and exported to a spreadsheet.

### Expression of Results

Results can be expressed for each parameter as for conventional ETP. Alternatively some authors expressed ETP<sub>TM</sub> as **% inhibition (ETP<sub>TM</sub>/ETP x 100)** for each sample.

Less commonly ETP<sub>TM</sub> was expressed as a normalised Thrombomodulin sensitivity ratio (nETP<sub>TMsr</sub>):

$$\frac{\text{ETP}_{\text{TM}}/\text{ETP (patient sample)}}{\text{ETP}_{\text{TM}}/\text{ETP (reference plasma)}} = \text{nETP}_{\text{TMsr}}$$

This method avoids plate to plate variability.

### 2.4.3 Thrombin anti-thrombin complex (TAT) Enzygnost® TAT Micro (Siemens)

#### Principle

Enzygnost®TAT Micro is a sandwich enzyme immunoassay for the in-vitro determination of human thrombin/antithrombin III complex. During the first incubation step the TAT present in the sample binds to the antibodies against thrombin which are attached to the surface of the microtitration plate. Unbound constituents are then removed by washing; the bound enzyme activity is then determined. The enzymatic reaction between hydrogen peroxide and chromogen is terminated by addition of diluted sulphuric acid. The resulting colour intensity, which is proportional to the concentration of TAT, is determined photometrically. The concentration range of 2 to 60µg/L is covered by the standards contained in the kit. For higher concentrations the sample must be diluted with normal plasma.

#### Reagents Required

Enzygnost®TAT micro microtitration plates: coated with rabbit antibodies against human thrombin

Anti-human antithrombin III/POD Conjugate: Rabbit anti-human antithrombin III, peroxidase conjugated (<12mg/L); Preservative: Phenol (<1g/L)

Conjugate Buffer: Tris buffer solution (50mmol/L), bovine serum albumin; Preservative: Phenol (<0.3g/L)

TAT Standard Plasmas S1 to S4 (human)

TAT Control Plasma (human): 9.4 ± 1.9µg/L

Sample Buffer (TAT): tris buffer solution (100mmol/L) Tween (10mL/L), EDTA (37g/L)

Washing solution POD (concentrate): Phosphate buffer solution (90mmol/l) containing Tween (18g/L)

Buffer/Substrate POD: Hydrogen peroxide (0.3g/L) in citrate buffer solution

Chromogen POD: o-phenylenediamine dihydrochloride

Stopping solution POD: 0.5 N sulphuric acid

### Reagent Preparation

Washing Solution POD (Concentrate): 15ml of washing solution is diluted into 300ml of distilled water (sufficient for two test plates)

Working Solution POD (Concentrate): 200µl of anti-human ATIII/POD conjugate is pipetted into a vial of conjugate (reagent) buffer (11ml) and shaken gently 9sufficient for 1 test plate)

### Preparation of Standards

Each standard and control plasma was added with 1ml of distilled water and mixed gently by pipetting. The standards to be used in the assay were then prepared according to Table 2.5

Standard	µg/l	Volume added to 1x diluent	1x diluents
1	2	50µl of standard 1	50µl
2	6	50µl of standard 2	50µl
3	20	50µl of standard 3	50µl
4	60	50µl of standard 4	50µl

**Table 2.5 Preparation of Standard Samples for TAT Assay**

## Procedure

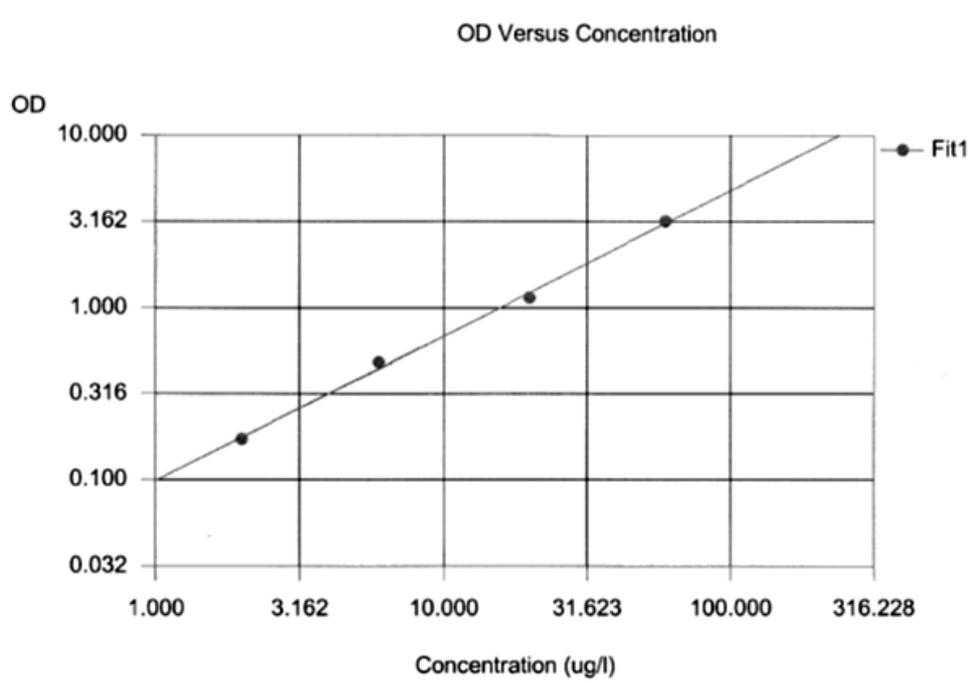
All reagents and samples were placed in the water bath to achieve 15°C to 25°C before the start of the test. All determinations of standard, control and patient samples were performed in duplicates. The samples were pipette within 5 minutes with the aid of a multi-channel pipette in order to assure accuracy and precision. During pipetting, steps were taken to avoid formation or large bubbles. The incubation timing was started when the plate is placed in the water bath.

1. Number of strips required was determined and strips that are not needed are removed from the strip holder and stored well-closed with desiccant in a plastic bag at 2-8°C.
2. Volume of 50µl sample buffer (sample diluents) was pipetted into each well, into which, 50µl of standard, control or sample was then added. The test plates were shaken briefly to ensure thorough mixing.
3. The plate was covered with self-adhesive foil and incubated for 15 minutes at 37°C.
4. Self adhesive foil was removed after incubation, all wells aspirated and filled with 0.3 ml of diluted washing solution and aspirated again. This washing step was repeated twice.
5. Any remaining washing solution was removed by knocking the plate onto cellulose tissue.
6. Then, 100µl of working conjugate solution was pipette into each well, taking care not to wet edges of the wells.
7. Test plate was covered with self adhesive foil and incubated for 15 minutes at 37°C.
8. Working Chromogen Solution was prepared shortly before the end of the incubation period. This was done by transferring 10ml buffer/substrate POD into a vial of chromogen POD and shaken to dissolve.
9. The foil was removed, wells aspirated and washing steps performed three times as described above. Remaining liquid was removed by knocking the test plate onto cellulose tissue.
10. The freshly prepared working chromogen solution was then added (100µl) to each well.

11. The test plate was covered with fresh foil, incubated for 30 minutes at 15-25°C protected from light.
12. The foil was then removed, and 100µl of stopping solution POD was added to each well keeping to the same timing and order as for the dispensing of working chromogen solution.
13. Plate was then measured with a spectrophotometer within one hour, measuring wavelength 492nm (490 -500 nm).

### Calculations

The reference curve was established for each series after calculating the mean absorbance values of standards. An example is given in Figure 2.6. This is done using Relevations Software.



**Figure 2.6 Typical reference Curve (Absorbance vs TAT concentration)**

The TAT concentrations can be read directly from the reference curve (figure 2.6) via their respective absorbance values. Samples which yield absorbance above the uppermost standard were retested at a higher dilution. The TAT content in normal plasma is taken into account in the calculation of TAT concentration of the sample.

## **Quality Control**

Quality control was achieved for each series of measurements using the TAT Control Plasma supplied in the kit. The measurement values of the samples were only used if value for the control is within the confidence interval

### **2.4.4 Human Tissue Factor Pathway Inhibitor Immunoassay: Quantikine® (R&D Systems)**

#### **Principle of Assay**

This is a quantitative sandwich enzyme immunoassay. A monoclonal antibody specific for TFPI has been pre-coated onto a microplate. Standards and samples are pipetted into wells and any TFPI present is bound by the immobilised antibody. After unbound substances are washed off, an enzyme-linked polyclonal antibody specific for TFPI is added to the wells. Following washes to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TFPI bound in the initial step. The colour development is stopped and the intensity of the colour is measured by spectrophotometer.

#### **Reagents Required**

TFPI microplate (Part 893646): 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against TFPI

TFPI Conjugate (Part 893648): 21 ml of polyclonal antibody against TFPI conjugate horseradish peroxidase with preservative

TFPI Standard (Part 893648): 20ng of recombinant human TFPI in a buffered protein base with preservative; lyophilized

Assay Diluent RD1-89 (Part 895881): 11ml of buffered protein base with preservative

Calibrator Diluent RD5-20 Concentrate (part895346): 2 vials (21ml each) of a buffered protein base with preservative.

Wash Buffer Concentrate: (Part 895003): 21ml of a 25-fold concentrated solution of buffered surfactant with preservative.

Colour Reagent A ( Part 895000): 12.5ml of stabilized hydrogen peroxide

Colour Reagent B (Part 895001): 12.5ml of stabilized chromogen (tetramethylbenzidine)

Stop Solution (Part 895032): 6 ml of 2 N sulphuric acid

Plate Covers: 4 Adhesive strips

Human TFPI control (Lot 1154512): 829 -1215 pg/ml

### **Reagent preparation**

Wash Buffer: this was warmed to room temperature ensuring any formed crystals completely dissolved; then, 20ml of Wash Buffer Concentrate was diluted into deionised water to make up 500ml of Wash Buffer.

Substrate Solution: Colour reagents A and B were mixed together in equal volumes within 15 minutes of use; ensuring 200µl of resultant mixture was prepared for each well, and was protected from light using tin foil.

## Sample Preparation

Plasma samples were required to be diluted 100-fold due to high endogenous levels. This was done by adding 10 $\mu$ l of sample into 990 $\mu$ l of calibrator diluents (1:100).

## Preparation of Standards

The TFPI Standard was reconstituted with 1.0ml of deionised water. This reconstitution produces a stock solution of 20,000pg/ml. The standard is allowed to sit for 15 minutes with gentle agitation prior to making dilutions.

- 1) Using polypropylene tubes, 900 $\mu$ l of Calibrator Diluent RD5-20 Concentrate is pipette the tube labelled 2000pg/ml. 500 $\mu$ l of Calibrator Diluent RD5-20 is pipette into six different tubes labelled as Figure 2.7 and Table 2.6.
- 2) The stock solution (20,000 pg/ml) is used to produce a series of dilution as below. Each tube is mixed thoroughly before the next transfer.
- 3) The 2000pg/ml standard serves as the high standard. Calibrator Diluent RD5-20 Concentrate serves as the zero standard (0 pg/ml).

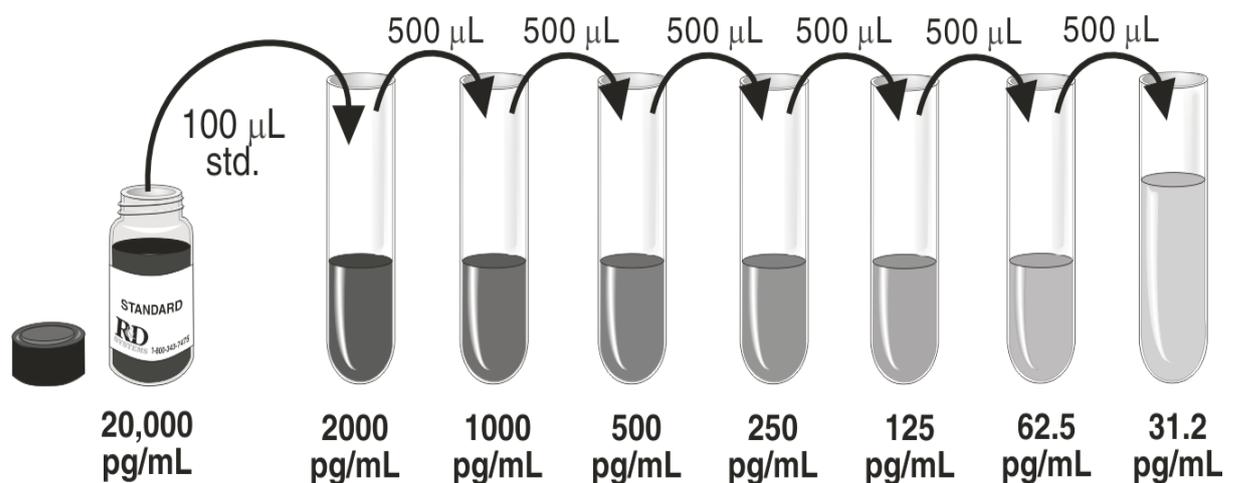


Figure 2.7 Preparation of TFPI Standard

Standard	pg/ml	Volume added to 1x diluent	1x diluents (Calibrator Diluent)
1	2000	100µl of 20,000pg/ml stock	900µl
2	1000	500µl of standard 1	500µl
3	500	500µl of standard 2	500µl
4	250	500µl of standard 3	500µl
5	125	500µl of standard 4	500µl
6	62.5	500µl of standard 5	500µl
7	31.2	500µl of standard 6	500µl
8	0 (blank)	0	500µl

**Table 2.6 Preparation of Standards for TFPI Assay**

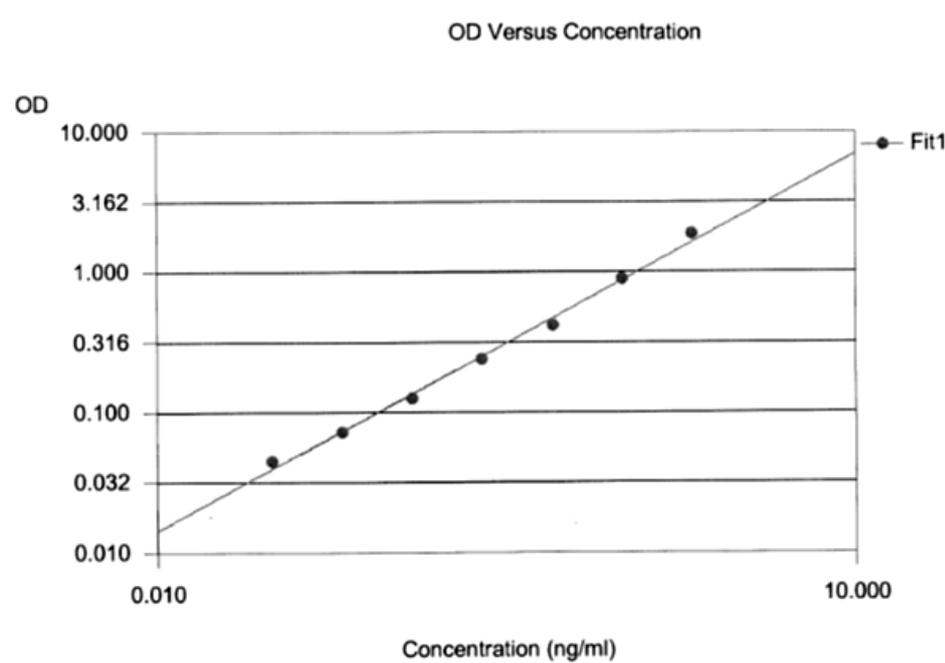
### Procedure

- 1) All reagents, working standards and samples were prepared as explained above.
- 2) Excess microplate strips were removed from the plate frame, returned to the foil pouch containing desiccant pack and reseal.
- 3) 100µl of Assay Diluent RD1-89 was added to each well.
- 4) 50µl of standard, control or sample were pipetted into designated well and cover with adhesive strip provided. Microplate was incubated at 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500±50 rpm. A plate layout was provided to record standards and samples assayed.
- 5) Each well was aspirated and washed with Wash Buffer (400µl) using multichannel pipette. This wash process was repeated three times for a total of four washes. Remains of Wash Buffer were removed by inverting and blotting the plate against clean cellulose tissue.
- 6) TFPI Conjugate (200µl) was added to each well and covered with new adhesive strip. Plate was incubated for 1 hour at room temperature on a horizontal orbital microplate shaker set at 500 ±50 rpm.

- 7) The aspiration/wash step was repeated as in step 5.
- 8) Substrate solution (200 $\mu$ l) was added to each well and incubated for 30 minutes at room temperature on the benchtop, wrapped in tin foil to protected from light.
- 9) Stop Solution (50 $\mu$ l) was added to each well, changing colour of the wells from blue to yellow was observed.
- 10) The optical density of each well was read within 30 min using a microplate reader set at 450nm.

### Calculations

The reference curve was plotted (Figure 2.8) after calculating the mean absorbance values of standards using a log/log plot (Relevations Software).



**Figure 2.8 Typical reference Curve (Absorbance vs TFPI concentration)**

The TFPI concentrations can be read directly from the reference curve via their respective absorbance values. Samples which yield absorbance above the uppermost standard are retested at

higher dilutions. Concentration read from standard curve is multiplied by the dilution factor where samples have been diluted.

## **Gene Expression**

Gene expression was determined by two-step real-time reverse transcription polymerase chain reaction (RT-PCR). Firstly, RNA extracted from the tissue samples was reverse transcribed to cDNA, then Real-Time PCR was performed. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter as an indicator of amplicon production during each PCR cycle (i.e. in “real-time”) as opposed to the endpoint detection.

### **2.4.5 RNA Extraction**

#### **Reagents Required**

TRIzol® Reagent (Invitrogen, USA)

Chloroform without additives

Isopropyl alcohol (Isopropanol)

75% ethanol in DEPC water

DEPC water

RNase free eppendorfs and pipette tips

#### **Protocol**

##### Homogenisation

1. 50-100mg of frozen placental tissue was transferred into a 1.5ml RNase—free eppendorf tube with 1 ml TRIzol®\_reagent.

2. Sample was incubated for 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complex.
3. 200µl of chloroform per 1ml of TRIzol® was added, tubes capped securely and inverted for 15 seconds to ensure adequate mixing.
4. Sample then was centrifuged at 12,000g for 15 minutes at 4°C.
5. Centrifugation resulted in two phases; a lower red organic phase and an upper clear aqueous phase. RNA remained exclusively in the aqueous phase.
6. The aqueous phase was transferred to a fresh eppendorf tube.
7. 500µl of isopropanol per 1 ml TRIzol® was mixed to precipitate RNA and incubated at room temperature for 10 minutes. This was then followed by centrifugation at 12,000g for 10 minutes.
8. RNA precipitate formed as a gel like pellet on the side bottom of the eppendorf tube.
9. The supernatant was removed and the RNA pellet was washed once with 75% alcohol using at least 1ml ethanol per ml TRIzol® used.
10. The RNA pellet and 75% ethanol was mixed by vortex and centrifuged at 7,500g at 4°C for 5 minutes.
11. The supernatant was removed and the RNA pellet was briefly dried on air for 10 minutes.
12. The RNA pellet was dissolved in 50µl DEPC water and incubated at 55°C for 10 minutes.
13. The 50µl RNA solution was collected and 2µl of which was used to measure RNA concentration and quality (A260/280) using NanoDrop 8000 spectrophotometer (Mason Technology, Ireland)

### **Interpretation of results**

A260/230: Ratio of sample absorbance at 260 and 230nm. This is a secondary measure of nucleic acid purity, being usually higher than the respective 260/280 values for “pure” nucleic acids. It is usually in a range of 1.8-2.2; if lower, it may indicate co-purified contamination.

Concentration (ng/μl): Sample concentration in ng/μl based on absorbance at 260nm minus the absorbance at 340nm (i.e. normalized at 340nm) and the selected analysis constant. Good quality RNA is as shown in Table 2.7

Sample	RNA yield (ng/μl)	260:280	260:230
1	1500.0	2.14	1.86
2	1259	1.95	2.18
3	1279	1.96	2.19

**Table 2.7 The typical results from of RNA extraction from the placenta**

#### **2.4.6 cDNA Synthesis**

The High Capacity cDNA Reverse Transcription Kit was used to enable the quantitative conversion of total RNA into single stranded cDNA (Applied Biosystems, CA, USA). Random primers ensure that first strand synthesis occurs efficiently with all species of RNA molecules present. An essential requirement for the relative quantification of cDNA is that the reverse transcriptase reaction generated products in a manner directly dependant on the amount of input RNA template.

#### **Reagents required**

High capacity cDNA kit

10X RT buffer

10X RT Random primers

25X dNTP Mix (100mM)

## Multiscribe™ Reverse Transcriptase 50U/μl

### Protocol

1. 2μg of RNA was reverse transcribed into a final volume of 50μl
2. The appropriate amount of RNA to give 2μg was calculated and added to RNase-free water to produce a final volume of 25μl. A No Template Control (NTC) was also included.
3. The solution was mixed by pipetting.
4. The samples were placed on ice to cool.
5. All kit components were thawed on ice.
6. A 2X RT mastermix was prepared by calculating the volume of each of the components needed for the number of reactions 25μl of 2X master mix was added to each sample and centrifuged briefly to mix then placed on ice before loading on the thermal cycler.
7. The samples were placed on the thermal cycler using the following programme conditions;
  - 10 minutes at 25°C
  - 120 minutes at 37°C
  - 5 seconds at 85°C, then hold at 4°C.
8. When complete the samples were snap-frozen and placed at -80°C for storage.

### Concentration of the resulting cDNA

The concentration of each sample was  $2\mu\text{g}/50\mu\text{l} = 40\text{ng}/\mu\text{l}$ .

## 2.4.7 Taqman<sup>®</sup> Real-Time PCR

### Principle

Real time PCR is used to quantify gene expression; amplified DNA is quantified during the exponential phase of the PCR, allowing the concentration of a particular target DNA or RNA relative to a standard to be quantified. The greater the initial concentration of target sequences in the reaction mixture the fewer the number of cycles required to achieve a particular yield of amplified product. The PCR reaction exploits the 5' nuclease activity of AmpliTaq Gold<sup>®</sup> DNA Polymerase to cleave a TaqMan<sup>®</sup> probe during PCR. The TaqMan<sup>®</sup> probe contains a reporter dye at the 5' end of the probe and a quencher dye at the 3' end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter as shown in Figure 2.9. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

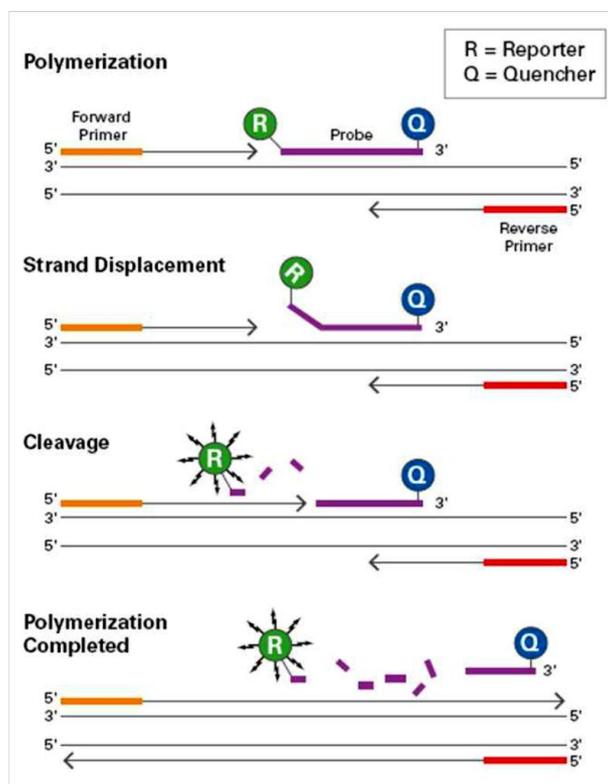


Figure 2.9 5' to 3' Nuclease Activity of AmpliTaq Gold<sup>®</sup> DNA Polymerase

## **Reagents required**

Taqman<sup>®</sup> Universal PCR mastermix (Applied Biosystems, CA, USA).

RNase free water (Qiagen, W.Sussex, UK).

Microamp<sup>®</sup> Optical 96 well reaction plates (Applied Biosystems, CA, USA).

Microamp<sup>®</sup> Optical Adhesive Covers (Applied Biosystems, CA, USA).

Taqman gene expression assay (Applied Biosystems, CA, USA).

## **Controls for Taqman PCR**

It is necessary to include at least three No Amplification Controls (NAC, a minus-reverse transcriptase control) as well as three No Template Controls (NTC, a minus sample control) in each reaction plate. NAC is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase; NTC includes all of the RT-PCR reagents except the RNA template. It is necessary to rule out the presence of fluorescence contaminants in the samples or in the heat block of the thermal cycler so as to avoid false positive results.

An endogenous control is also included so that any skewed results can be corrected. This control must be expressed at a similar level in all study samples, it must also give similar PCR efficiencies when using the comparative (cycle threshold)  $C_T$  method and finally it must be more abundantly expressed than the target gene. 18S was the internal control gene used in all experiment. It is abundant in the tissues and its expression is not affected by treatment with heparins nor pregnancy hormones such as oestrogen and progesterone.

## Procedure

- The preparation of the samples and mastermix was carried out on ice in the laminar flow cabinet. Each cDNA sample was diluted with RNase-free water to give a concentration of 8ng/μl per well.
- For each gene to be measured, 7μls of sample at 8ng/ul is required. Each sample of 40ng/μl was diluted 1:5 with RNase-free water to give a concentration of 8 ng/μl.
- 1μl of sample or control was added in triplicate to each tube of a 72 rotor plate by pipetting into the bottom of the well.
- 1μl of sample #1 was pipetted into the first three tubes (1-3), followed by 1μl of sample #2 into the next three tubes (4-6) and so on until each sample for the first gene had been loaded. This was then repeated for the next gene and continued until all samples had been loaded.
- Next the first gene probe mastermix was added to the first set of samples, the pipette was angled down to the side of the well and the tip contents fully expelled, the pipette tip was changed for each new probe.
- This was continued with the next few probes until all wells had sample plus probe in them.
- The rotor plate was sealed with a plastic thermostable cover (MicroAmp<sup>®</sup> Optical Adhesive film, Applied Biosystems, US) ensuring no gaping areas for evaporation.
- All wells were loaded on the Rotor-Gene 6000™ (Corbett Research, Australia).

## Plate reading

The Rotor-Gene 6000™ was switched on and the computer logged in.

The rotor plate was inserted into the prism in the correct orientation.

The Rotor-Gene 6000™ series software was opened and a new document for absolute quantification in a 72-rotor plate was selected.

The detector was assigned and added to the plate document and the negative control samples were highlighted and NTC was selected.

In the instrument tab, the volume was changed to 10µl (volume in each well), the document was saved, and then the run was started.

### **Reviewing the results of a PCR run**

The data was automatically saved in the hard drive and analysed by the software.

An amplification plot was received and the baseline and threshold values selected, the results were then saved and exported to an excel format.

### Calculation of results

The file was retrieved in Excel and the mean  $C_T$  value of each triplicate sample was calculated.

Results are expressed as the ratio of target gene cDNA to the internal control using the  $2^{-\Delta\Delta C_T}$  method [279].

## **2.4.8 Total protein extraction**

### **Principle**

This tissue protein extraction reagent is for the extraction of total protein from tissue samples. The reagent has a simple composition which is versatile enough to include the addition of inhibitors to assist in the lysis of cells and which may be used in downstream immunoassay analysis.

### **Preparation of Placental Homogenates**

#### **Materials**

Protease Inhibitor cocktail tablet (Roche)

Phosphate buffer saline (PBS) (pH 7.4)

25ml sterile tubes

#### **Procedure**

1. 25x stock solution of protease inhibitor cocktail was prepared by dissolving 1 tablet of protease inhibitor cocktail (Roche) in 1ml 100mM phosphate buffer at pH 7.0.
2. 1ml of stock protease inhibitor cocktail stock was added to 24mls PBS and mix well to make homogenisation buffer.
3. Placental tissue weighed approximately 0.5g.
4. 4 volumes of homogenisation buffer (4ml's buffer to 1gram tissue) added tissue.
5. The homogenising probe cleaned with distilled water.
6. Homogenise the tissue left for 2 minutes on ice.
7. Tissue is ensured to be completely homogenised. If not, the homogenisation process is repeated.

8. The homogenate is freeze and thawed (2-3 times) to make sure all tissue and cells are lysed. If large solid bit are present after the first freezing and thawing procedure, homogenisation is done again and repeat freezing and thawing procedure twice.
9. The homogenate is transferred to eppendorfs and centrifuged at 10,000rpm for 5 min in the micro centrifuge at 4°C.
10. The supernatant is transfered into a new eppendorf and the precipitate discarded.

The supernatant was transferred to a fresh pre-chilled 1.5ml tube and stored at -80°C until used.

#### **2.4.9 Pierce® BCA Protein Assay (Thermo Scientific, USA)**

##### **Assay Principle**

The Pierce® BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the well-known reduction of  $\text{Cu}^{+2}$  to  $\text{Cu}^{+1}$  by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation ( $\text{Cu}^{+1}$ ) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 $\mu\text{g}/\text{mL}$ ). Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration were prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve.

## Reagents required

BCA Reagent A 500mL containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide

BCA Reagent B 25mL, containing 4% cupric sulfate

Albumin Standard Ampules 2mg/mL 1mL ampules, containing bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide

## Preparation of Diluted Albumin (BSA) Standards

A set of protein standards were prepared as shown in Table 2.13.

Dilute The contents of one Albumin Standard (BSA) ampule was diluted into several clean vials.

Vial	Volume of Diluent (μL)	Volume and Source of BSA (μL)	Final BSA Concentration (μg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

**Table 2.8 Preparation of Diluted Albumin (BSA) Standards.** Dilution Scheme for Microplate Procedure (Working Range = 20-2,000μg/mL)

## Preparation of the BCA Working Reagent (WR)

The following formula was used to determine the total volume of WR required:

$(x \text{ standards} + x \text{ unknowns}) \times (x \text{ replicates}) \times (\text{volume of WR per sample}) = \text{total volume WR required}$

For each sample, 200 μl of WR reagent was required in the microplate procedure.

WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). Turbidity was observed when Reagent B was first added to Reagent A. This quickly disappeared upon mixing to yield a clear, green WR. Sufficient volume of WR was prepared based on the number of samples to be assayed.

#### **Procedure (Sample to WR ratio = 1:8)**

1. 25 $\mu$ L of each standard or unknown sample replicate was pipetted into a microplate
2. 200 $\mu$ L of the WR was added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds.
3. The microplate was covered and incubated at 37°C for 30 minutes.
4. Then, the microplate was allowed to cool to room temperature before the absorbance was measured at 562nm on a plate reader.

#### **Calculations**

The reference curve was established by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in  $\mu$ g/mL. This was done using Relevations Software.

**Chapter 3 Result:**  
**Effects of LMWH on Thrombin  
Generation in Women Post  
Caesarean Section**

### 3.1 Background

The risk of developing VTE is increased after an elective CS, (OR 2.2, 95%CI 1.5 -3.2) compared to women who undergo vaginal delivery; mortality associated with post partum VTE following caesarean delivery is increased ten-fold [125, 151]. LMWHs are used extensively for the treatment and prophylaxis of VTE in pregnancy. The use of LMWH thromboprophylaxis in low risk women post elective CS is still debatable. Current guidelines do not recommend LMWH thromboprophylaxis in this setting without any additional risk factors [217] [130]. However, the CEMACH report showed risk factors were absent in more than twenty percent of women who died resulting from VTE [137]. In our centre, women are routinely given a fixed dose of LMWH (tinzaparin 4,500IU) per day post all caesarean delivery.

The aim of this study was to compare thrombin activation in women post elective caesarean delivery with women post vaginal delivery and to determine the full anticoagulant effects of LMWH therapy in the first 24 hours post elective CS. A group of healthy women 6 hours post CS was studied. These women received LMWH prophylaxis tinzaparin 4,500iu per day. Blood samples were taken at 5 points over 24 hours post CS. A subset of patients had blood sampling at pre-delivery. Blood samples were compared with a similar profile from a control group of healthy women following normal vaginal delivery. In addition to anti-Xa assay, Calibrated Automated Thrombogram (CAT), Thrombin-Antithrombin complex (TAT) and the potent inhibitor Tissue Factor Pathway Inhibitor (TFPI) were measured to determine the full anticoagulant activity over 24 hours in the CS group. The ETP, TFPI and TAT levels were compared with levels found in the control group. The results generated a pharmacological profile of the full anticoagulant effects of tinzaparin over a 24 hour period. A recent report suggested that ETP levels would be reduced by approximately 15-20% in pregnancy following LMWH treatment [275]. Based on these figures, the sample size required to detect a significant difference in thrombin production between the caesarean section group and the control group would be 17 (in each group).

## 3.2 Results

### Patients

The patients within these three groups were similar with respect to age, parity, BMI and gestation at delivery as tabulated in Table 3.1. All patients recruited were non-smokers, underwent uncomplicated singleton pregnancies and had no personal or family history of VTE or known thrombophilia. Indications of elective caesarean in CS1 group were repeat caesarean delivery (n=14), breech presentation (n=5) and previous third degree tear (n=1). Indications for elective caesarean in CS2 group were repeat caesarean delivery (n=15) and breech presentation (n=5). None of the patients in this study had any bleeding complications following the LMWH prophylaxis.

Mean ( $\pm$ SD)	CS1 n=20	CS2 n=20	SVD n=20	P- value
Age	31.9 (4.6)	31.4(3.2)	31.5(4.5)	NS
Parity	1.1(0.7)	1.0(0.6)	1.1(0.8)	NS
BMI	23.9(2.2)	23.7(2.3)	23.9(2.6)	NS
Gestation at delivery	39.4(0.5)	39.0(0.5)	39.2(0.6)	NS

**Table 3.1. Patient Demographics .** Values presented as mean(SD). NS-not significant.

### 3.2.1 Thrombin Generation and TFPI levels in Post Elective Caesarean Section and Normal Vaginal Delivery

#### Thrombin Antithrombin (TAT) complex (Figure 3.1)

Repeated ANOVA showed that LMWH prophylaxis ( $p < 0.05$ ), mode of delivery ( $p < 0.001$ ) and time post delivery ( $p < 0.0001$ ) all significantly affected TAT levels in the post partum period. In addition, a significant interaction occurred between LMWH prophylaxis and time post delivery ( $p < 0.01$ ). This means that LMWH prophylaxis altered the profile of TAT levels over the immediate post partum

period. In the SVD group, a significant decrease in TAT levels was observed during the 24 period following delivery ( $p<0.0001$ ) reaching a minimum at 24 hours post partum. Both CS groups showed significantly higher levels of TAT prior to initiation of LMWH prophylaxis compared with the same time points in the SVD group ( $p<0.002$ ) (6 hours post delivery), ( $p<0.03$ ) (10 hrs post delivery). LMWH prophylaxis significantly reduced TAT levels in the CS1 group compared with the CS2 group at 10 hours post delivery ( $p<0.01$ ). Levels remained decreased in the CS1 group compared with pre-LMWH ( $p<0.0001$ ) and were similar to levels observed in the SVD group at the same time points except at 24 hours post delivery. 24 hours post delivery levels were significantly higher in the CS1 group compared with the SVD group ( $p<0.01$ ).

### **Endogenous Thrombin Potential (ETP)(Figure 3.2)**

ETP was significantly affected by LMWH prophylaxis ( $p<0.0001$ ), mode of delivery ( $p<0.03$ ) and time post delivery ( $p<0.002$ ). A significant interaction was also observed between LMWH prophylaxis and post delivery time ( $p<0.0001$ ). Levels of ETP were slightly but significantly lower 6 hours post caesarean section compared with the same time points post vaginal delivery ( $p<0.05$ ). However, at 10 hours similar levels were found in the CS2 and SVD groups. 4 hours post LMWH in the CS1 group, levels of ETP are significantly reduced compared with the same time point in the CS2 group prior to LMWH ( $p<0.0001$ ). Although ETP levels increased in the CS1 group at 16 hours post delivery, they remained lower than the vaginally delivery group from 16-24 hours post delivery ( $p<0.001$ ). ETP levels did not vary significantly post vaginal delivery.

### **Peak thrombin (Figure 3.3)**

Peak thrombin production followed a similar pattern to the ETP results presented above. LMWH prophylaxis ( $p<0.0001$ ), mode of delivery ( $p<0.0001$ ), and time post delivery ( $p<0.0001$ ) all significantly influenced peak thrombin levels with a significant interaction found between LMWH

prophylaxis and post delivery time ( $p < 0.0001$ ). Peak thrombin levels did not change significantly during the first 24 hours following vaginal delivery, however, higher levels of peak thrombin were found in this group at 6 and 10 hours post delivery compared with the equivalent stages in the CS2 group ( $p < 0.0001$ ). LMWH significantly reduced peak thrombin production 10 hours post caesarean section (CS1) compared with the same time point in the other two groups ( $p < 0.0001$ ). In the CS1 group, levels were increased again at 16 and 24 hours post delivery but remained lower than the equivalent stages of the vaginally delivered group ( $p < 0.003$ ;  $p < 0.002$ ).

#### **Lagtime (Figure 3.4)**

ANOVA showed lagtime was significantly affected by LMWH prophylaxis ( $p < 0.0001$ ), mode of delivery ( $p < 0.0001$ ) and time post delivery ( $p < 0.0001$ ). A significant interaction was also observed between LMWH prophylaxis and post delivery time ( $p < 0.0001$ ). There was no significant difference in time to peak at 6 hours post delivery between the three groups. At 4 hours post LMWH in the CS1 group, lagtimes are significantly lengthened compared with the same time point in the CS2 group prior to LMWH ( $p < 0.001$ ). Levels of lagtime in the CS1 group remains plateau from 16-24hours post delivery, at comparable levels to vaginal delivery group. Time to peak levels did not vary significantly post vaginal delivery.

#### **Time to Peak (Figure 3.5)**

Time to peak was significantly affected by LMWH prophylaxis ( $p < 0.0001$ ), mode of delivery ( $p < 0.0001$ ) and time post delivery ( $p < 0.0001$ ). A significant interaction was also observed between LMWH prophylaxis and post delivery time ( $p < 0.0001$ ). There was no significant difference in time to peak at 6 hours post delivery between the three groups. At 4 hours post LMWH in the CS1 group, levels of time to peak are significantly lengthened compared with the same time point in the CS2 group prior to LMWH ( $p < 0.001$ ) and SVD group ( $p < 0.001$ ). Although time to peak levels shortened in

the CS1 group at 16 hours post delivery, they remained longer than the vaginally delivery group from 16-24 hours post delivery ( $p<0.001$ ). Time to peak levels did not vary significantly post vaginal delivery.

### **TFPI (Figure 3.6)**

Repeated ANOVA showed that time post delivery ( $p<0.0001$ ), mode of delivery ( $p<0.01$ ) and LMWH prophylaxis ( $p<0.001$ ) significantly affected TFPI levels post delivery. A highly significant interaction between LMWH prophylaxis and time post delivery ( $p<0.0001$ ) was found. In the 24 hour period post vaginal delivery a gradual significant increase in TFPI levels is observed reaching a maximum at 24 hours post delivery ( $p<0.001$ ). At 6 hours post delivery, levels of TFPI were significantly lower in the vaginal group than in either of the two caesarean section groups prior to LMWH prophylaxis ( $p<0.005$ ). TFPI levels in the CS1 group were significantly increased at 4 hours post LMWH prophylaxis ( $p<0.001$ ) compared with the CS2 group at the same time point post delivery ( $p<0.0001$ ). This peak TFPI level decreased dramatically at 16 hours post delivery (10 hours post LMWH) and was similar to levels found in the SVD group.

### **3.2.2 Relationship between Thombin Production and Anti-Xa in Post Caesarean Section (CS1)**

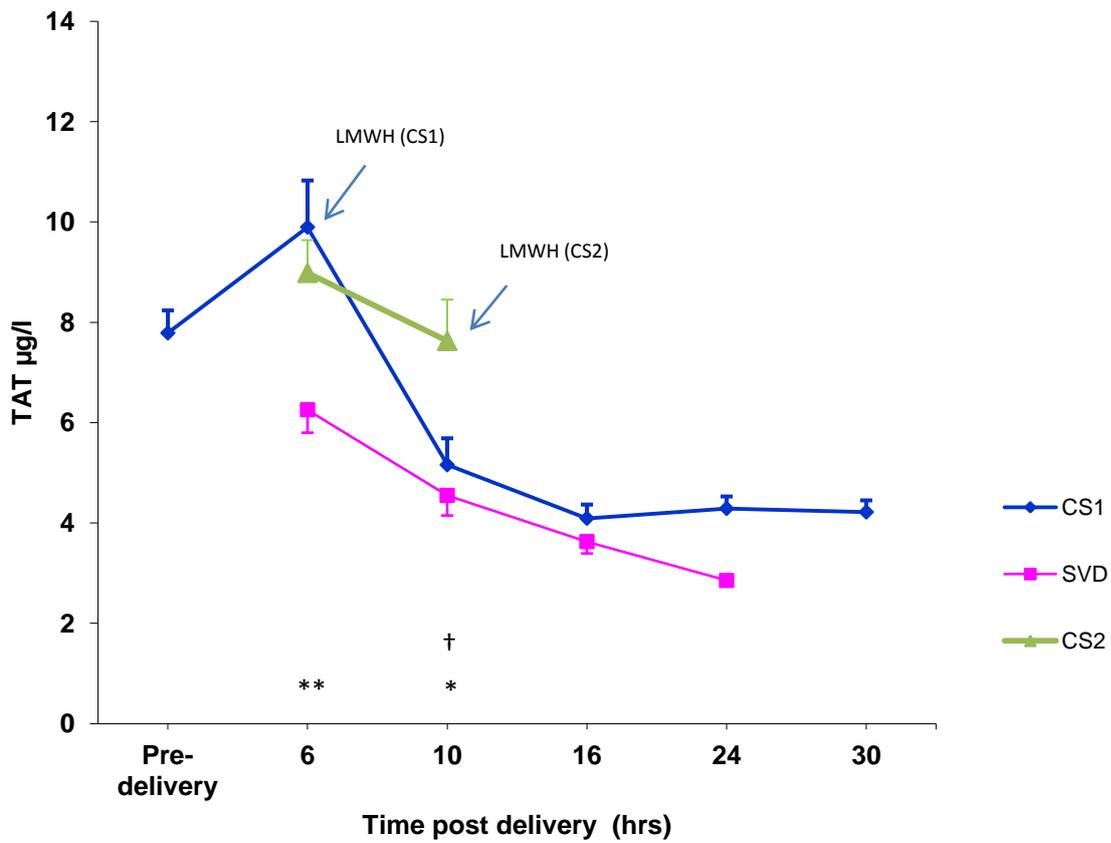
#### **(Figure 3.7)**

As expected peak levels of anti-Xa were observed 4 hours post LMWH prophylaxis which were significantly higher than pre-dose ( $p<0.001$ ), 10, 18 and 24 hours post LMWH. The mean peak anti-Xa level was 0.16 IU/ml (95% CI 0.11-0.2). Levels were below the limit of detection (0.05IU/ml) of the assay at 18 and 24 hours post LMWH dose. *In-vivo* thrombin production (as measured by TAT) is

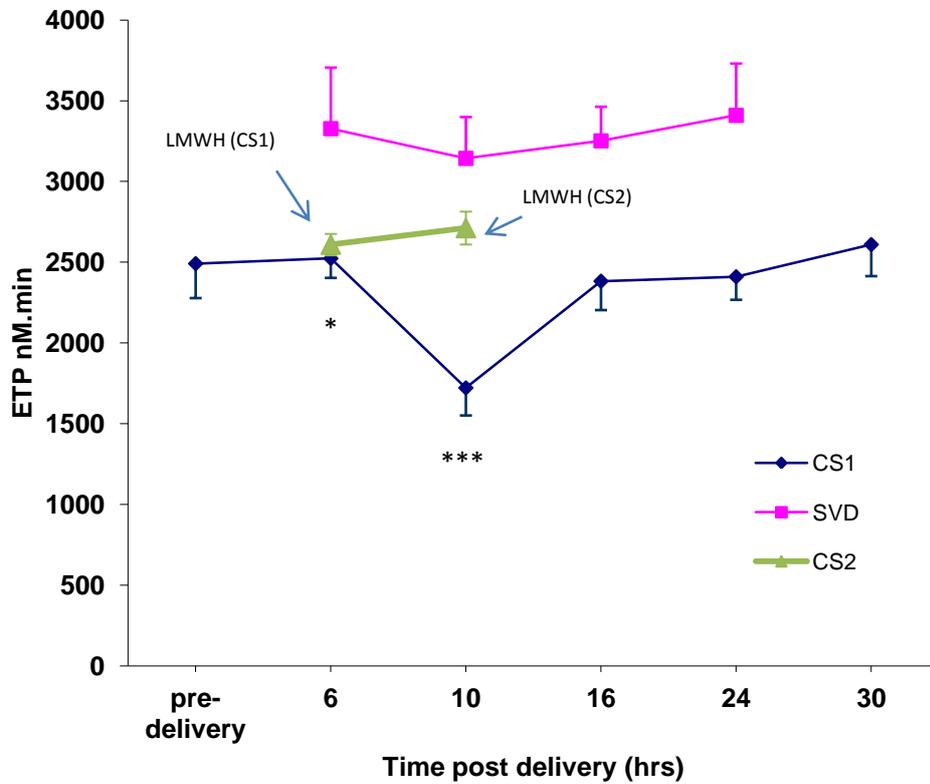
significantly reduced at 4, 10, 18 and 24 hours post LMWH prophylaxis compared to pre LMWH ( $p<0.001$ ) despite declining of anti-Xa levels .

### **3.2.3 Correlation between Anti-Xa and ETP, Peak thrombin and TFPI (CS1 group)**

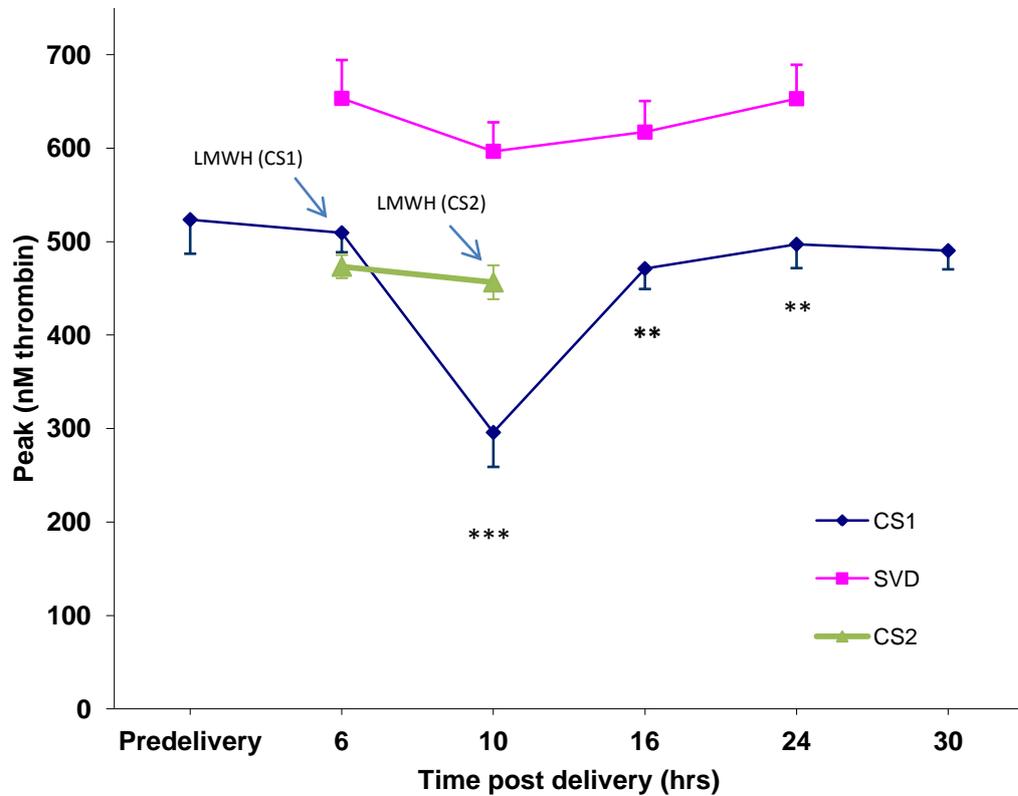
In contrast to TAT, both peak thrombin, ETP and TFPI showed a very similar profile to anti-Xa over the 24 hours post treatment. Correlation analysis (Pearson's correlation) showed a significant negative correlation between anti-Xa and peak thrombin ( $r=-0.695$ ;  $p<0.0001$ ) (Figure 3.8); anti-Xa and ETP ( $r = -0.589$ ;  $p<0.0001$ ) (Figure 3.9). A positive correlation was found between anti-Xa and TFPI (Figure 3.10).



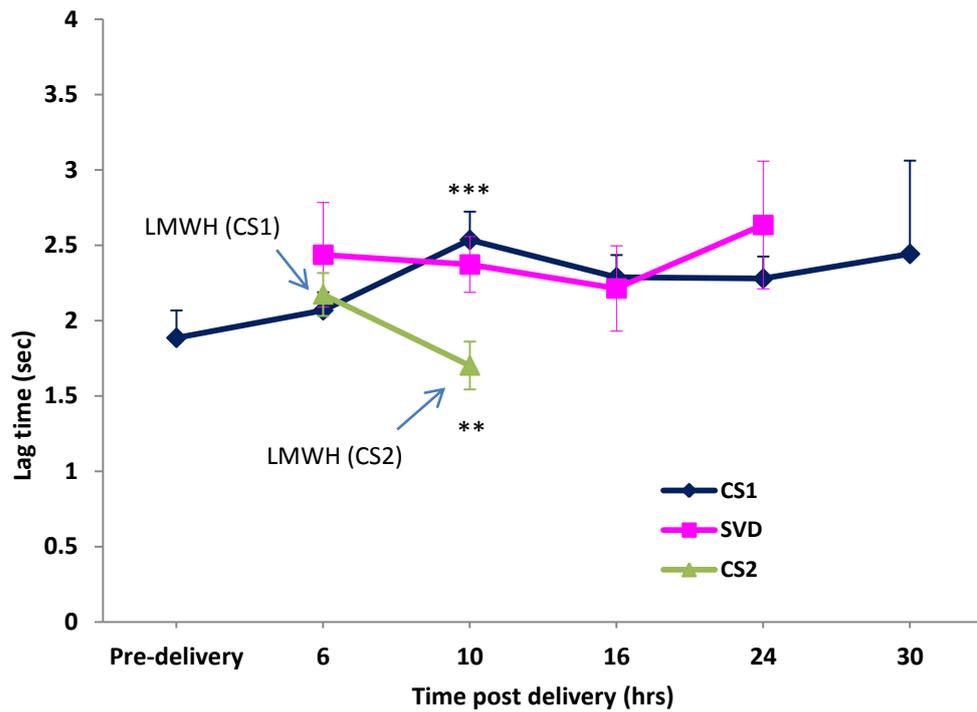
**Figure 3.1.** TAT (ug/l) levels in women post caesarean section (CS1 n= 20), (CS2 n=20) and spontaneous vaginal delivery (SVD n= 20). Blood samples were taken pre-delivery (CS1 only) and at 6, 10 (CS1,CS2 and SVD), 16, 24(CS1 and SVD) and 30 hours (CS1 only)post delivery. Values represent mean  $\pm$  SD. \*\* =  $P < 0.002$  SVD v CS1 and CS2. \* =  $P < 0.03$  SVD v CS2. † =  $P < 0.01$  CS1 v CS2. Arrows show the start of LMWH prophylaxis in the CS1 and CS2 groups.



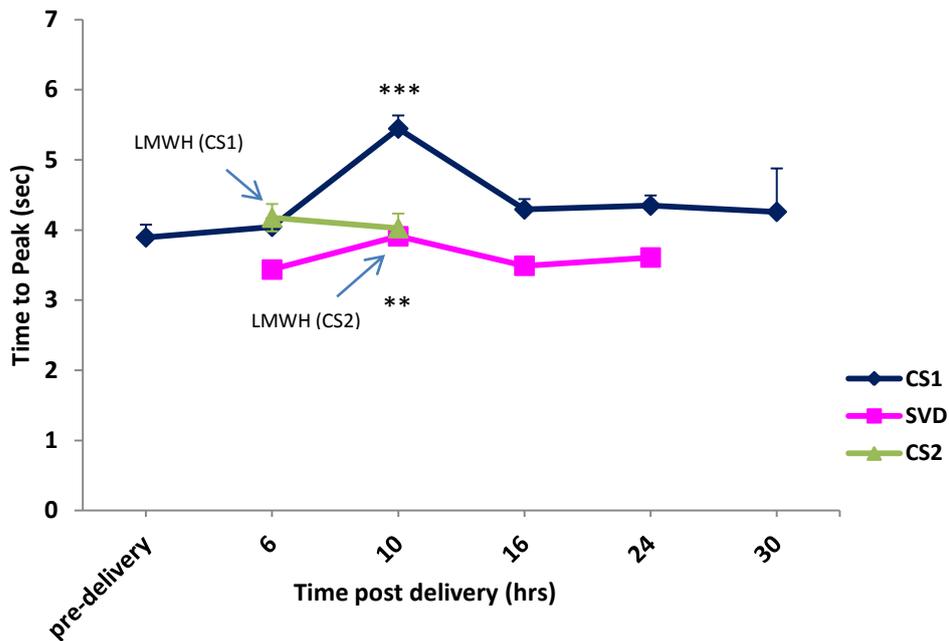
**Figure 3.2.** ETP (nM.min) levels in women post caesarean section (CS1 n= 20), (CS2 n=20) and spontaneous vaginal delivery (SVD n= 20). Blood samples were taken pre-delivery (CS1 only) and at 6, 10 (CS1, CS2 and SVD), 16, 24(CS1 and SVD) and 30 hours (CS1 only)post delivery. Values represent mean  $\pm$  SD. \* =  $P < 0.05$  SVD v CS1 and CS2. \*\*\*=  $P < 0.001$  CS1 v CS2. Arrows show the start of LMWH prophylaxis in the CS1 and CS2 groups.



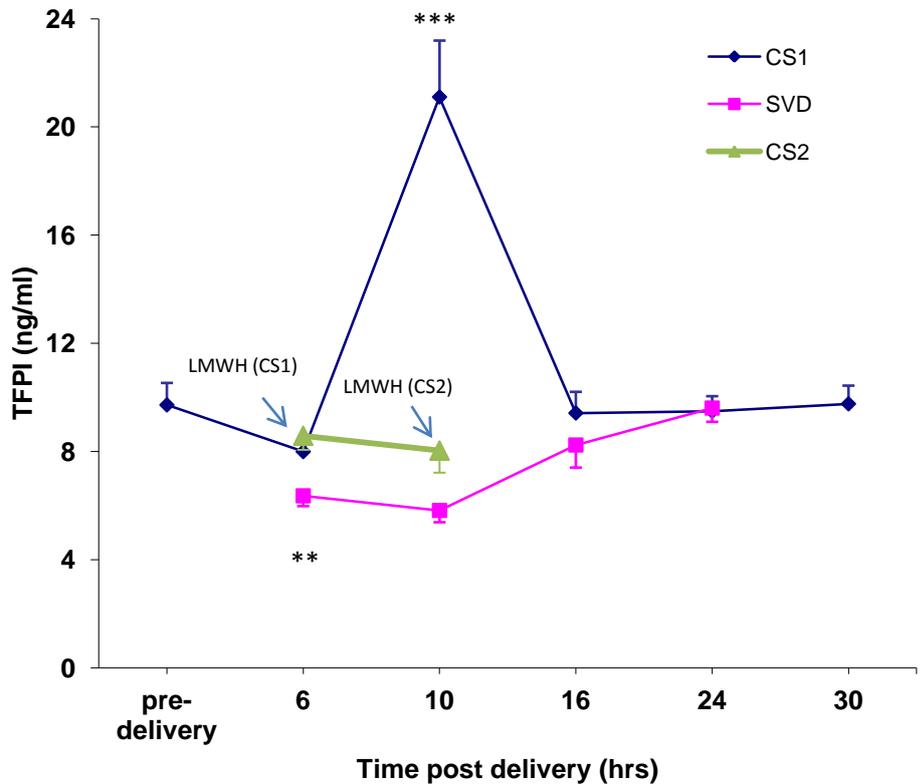
**Figure 3.3** Peak (nM thrombin) levels in women post caesarean section (CS1 n= 20), (CS2 n=20) and spontaneous vaginal delivery (SVD n= 20). Blood samples are taken pre-delivery (CS1 only) and at 6, 10 (CS1,CS2 and SVD), 16, 24(CS1 and SVD) and 30 hours (CS1 only)post delivery. Values represent mean  $\pm$ SD. \*\*\* =  $P < 0.002$  CS1 v CS2. \*\* =  $P < 0.01$  CS1 v SVD. Arrows show the start of LMWH prophylaxis in the CS1 and CS2 groups.



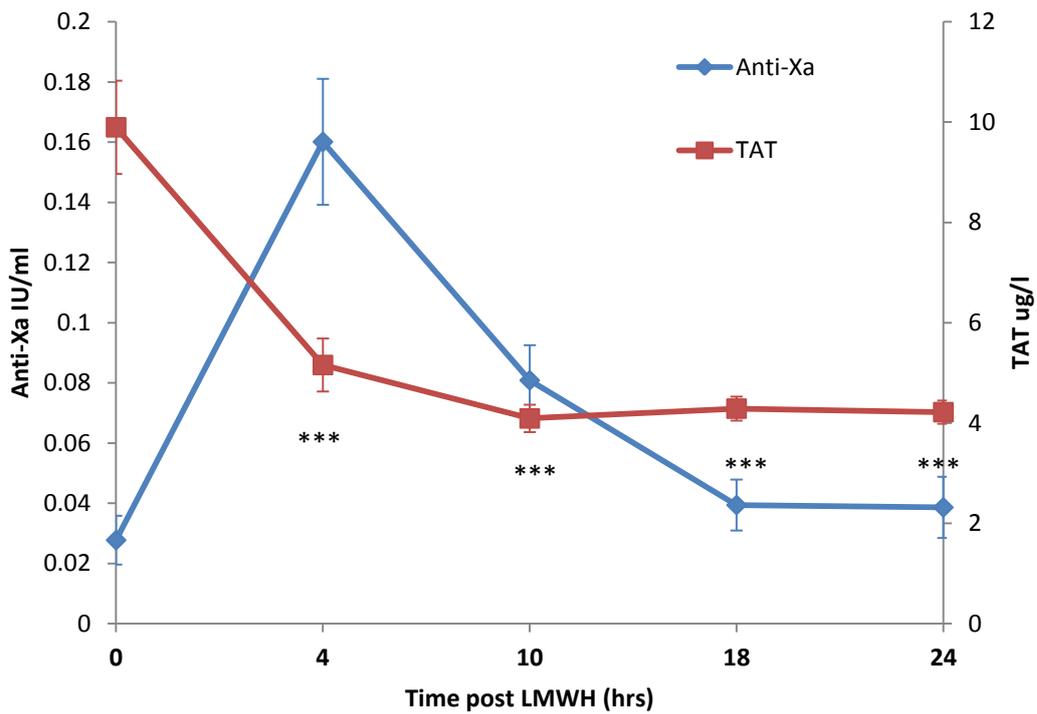
**Figure 3.4** Lagtime levels in women post caesarean section (CS1 n= 20), (CS2 n=20) and spontaneous vaginal delivery (SVD n= 20). Blood samples are taken pre-delivery (CS1 only) and at 6, 10 (CS1,CS2 and SVD), 16, 24(CS1 and SVD) and 30 hours (CS1 only)post delivery. Values represent mean  $\pm$ SD. \*\*\* =  $P < 0.001$  CS1 v CS2. \*\* =  $P < 0.01$  CS2 v SVD. Arrows show the start of LMWH prophylaxis in the CS1 and CS2 groups.



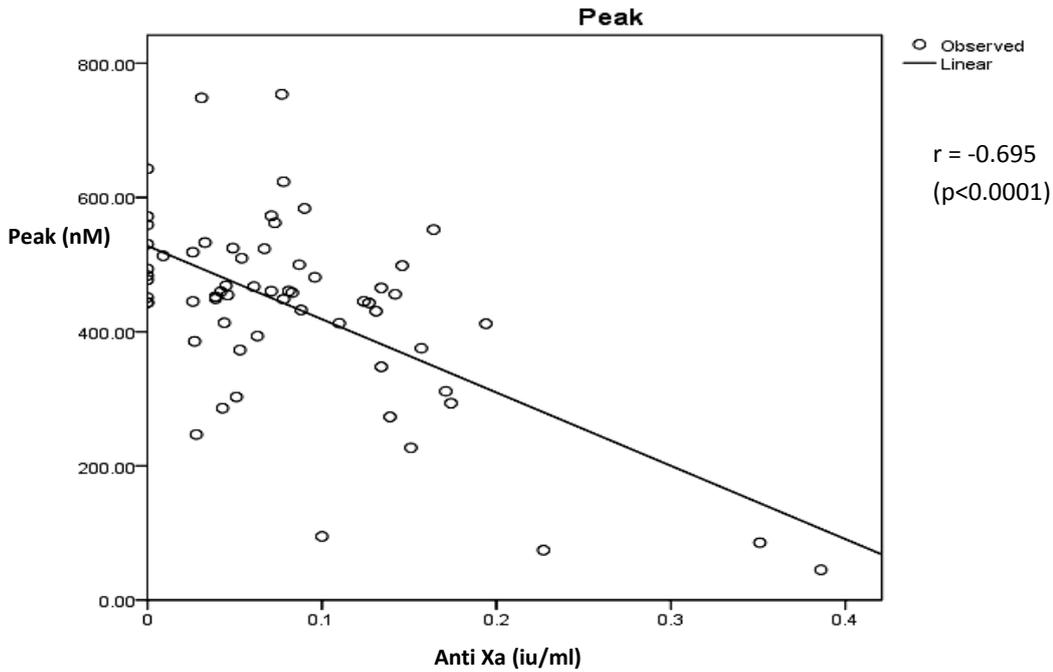
**Figure 3.5** Time to Peak levels in women post caesarean section (CS1 n= 20), (CS2 n=20) and spontaneous vaginal delivery (SVD n= 20). Blood samples are taken pre-delivery (CS1 only) and at 6, 10 (CS1,CS2 and SVD), 16, 24(CS1 and SVD) and 30 hours (CS1 only)post delivery. Values represent mean  $\pm$ SD. \*\*\* =  $P < 0.001$  CS1 v CS2. \*\* =  $P < 0.001$  CS1 v SVD. Arrows show the start of LMWH prophylaxis in the CS1 and CS2 groups.



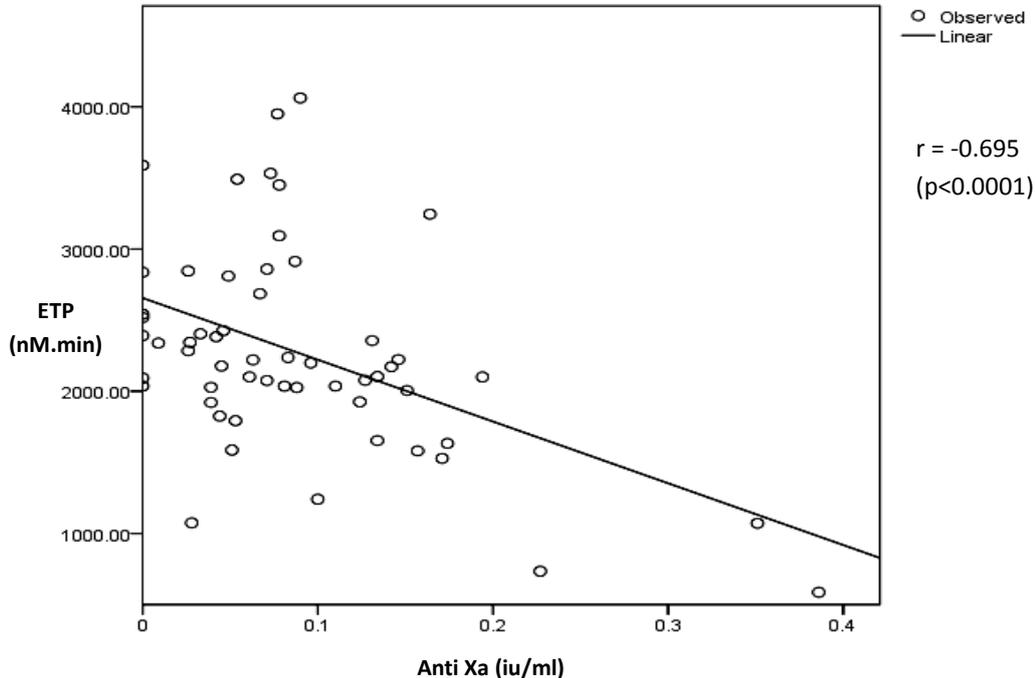
**Figure 3.6** TFPI (ng/ml) levels in women post caesarean section (CS1 n= 20), (CS2 n=20) and spontaneous vaginal delivery (SVD n= 20). Blood samples are taken pre-delivery (CS1 only) and at 6, 10 (CS1, CS2 and SVD), 16, 24(CS1 and SVD) and 30 hours (CS1 only)post delivery. Values represent mean  $\pm$  SD. \*\*\* =  $P < 0.0001$  CS1 v CS2. \*\* =  $P < 0.005$  SVD v CS1 and CS2. Arrows show the start of LMWH prophylaxis in the CS1 and CS2 groups



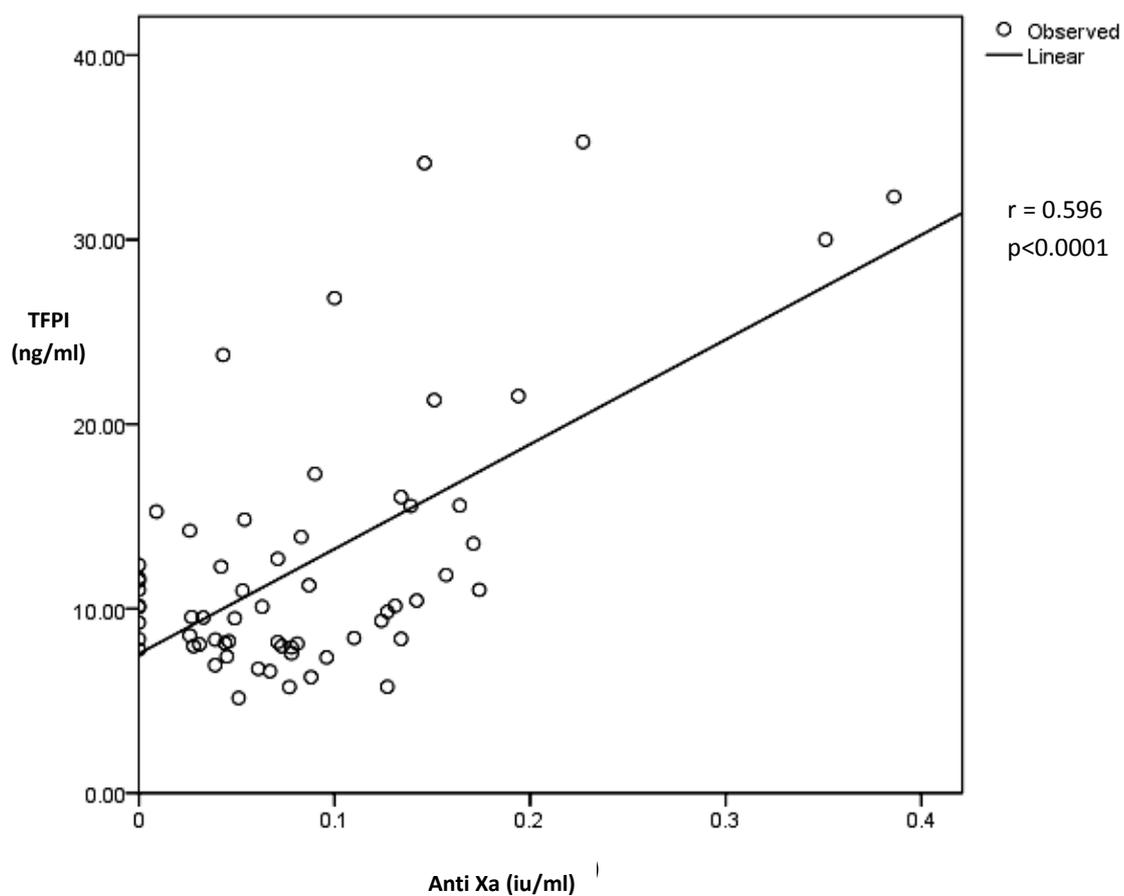
**Figure 3.7** Anti-Xa (IU/ml) and TAT ( $\mu\text{g/l}$ ) levels in women post caesarean section (CS1 n= 20). Blood samples were taken at pre-dose, 4, 10, 18 and 24 hours post LMWH injection (corresponding to 6, 10, 16, 24 and 30 hours post delivery respectively). Values represent mean  $\pm$  SD. \*\*\* =  $P < 0.001$  compared with pre LMWH dose.



**Figure 3.8** Pearson's correlation was used to demonstrate the relationship between anti-Xa (iu/ml) and peak (nM) levels in women post caesarean section (CS1 n= 20). Blood samples were taken four hours post LMWH tinzaparin 4500iu/ml.



**Figure 3.9** Pearson's correlation was used to demonstrate the relationship between anti-Xa (iu/ml) and ETP (nM.min) levels in women post caesarean section (CS1 n= 20). Blood samples were taken four hours post LMWH tinzaparin 4500iu/ml.



**Figure 3.10** Pearson's correlation was used to demonstrate the relationship between anti-Xa (iu/ml) and TFPI (ng/ml) levels in women post caesarean section (CS1 n= 20). Blood samples were taken four hours post LMWH tinzaparin 4500iu/ml.

### 3.3 Discussion

LMWHs are widely used for thromboprophylaxis in pregnancy and have been shown to have an excellent safety profile in pregnancy [215]. However, the use of LMWH thromboprophylaxis in low risk women post elective CS is still controversial and largely based on invalidated risk stratification model. While it is widely agreed to commence LMWH thromboprophylaxis on women post emergency CS to avoid VTE (RCOG guideline 2009, CHEST 2008), there is no local or international consensus on the requirement or optimum timing of LMWH thromboprophylaxis for women post elective CS (RR 2.2 vs vaginal delivery). The American Society of Regional Anaesthesia (ASRA) recommends commencing LMWH prophylaxis between 4-6 hours post CS. In our centre, LMWH thromboprophylaxis is given to all patients post elective and emergency CS. This is commenced between 6-10 hours post surgery based on clinical preference and practice, not based on clinical characteristics of the patients. Thromboprophylaxis with LMWH aims to reduce or prevent excess thrombin generation, thereby reducing the risk of venous thrombosis. It is known that while LMWH exerts its effects mainly by anti-Xa activity, there is an increasing body of evidence suggesting the contribution of the anti-IIa and TFPI to the efficacy of LMWH especially in LMWHs such as tinzaparin, [280]. We did not determine the effects on fibrinolysis in this study. A previous study on women with moderate to high risk of VTE treated with tinzaparin during pregnancy reported that time since injection of tinzaparin did not affect markers of fibrinolysis such as plasmin- $\alpha_2$ -antiplasmin (PAP) or fibrin degradation product (FDP)[276].

The group of women undergoing elective caesarean delivery in this study were healthy women with no additional risk factors for VTE; hence their pre-delivery TAT levels were not elevated and were similar to those found in late pregnancy in previous studies [53, 281, 282]. However, six hours post delivery, thrombin formation as measured by TAT levels were significantly higher in post CS patients (CS1 and CS2 groups) compared to the vaginally delivered group (SVD). The rationale for the CS2 group was to provide values of TAT, ETP, Peak thrombin and TFPI at 10 hours post delivery without LMWH prophylaxis. TAT (thrombin generated) levels are significantly higher in CS2

compared to CS1 and SVD at this time point, suggesting that LMWH is effective in reducing the enhanced thrombin production in low risk women post elective CS. The increase in TAT level provides evidence of a more robust activation of the coagulation pathway *in vivo* following CS, and is comparable to the levels found in a previous study of women at moderate risk of VTE during pregnancy [282]. This enhanced activation and thrombin production may contribute to the risk of VTE seen post CS even in the absence of risk factors and provides a basis for LMWH prophylaxis in this group.

When LMWH prophylaxis is administered, this enhanced thrombin production is effectively reduced to levels found in the in SVD group. These findings suggest that LMWH thromboprophylaxis post elective CS, even in this healthy cohort, is justified to reduce thrombin generation. This observation is in contrast to a smaller study by *Boer et al* who observed no significant difference of coagulation activation (including TAT) between the post vaginal deliveries and post CS women. In addition treatment with LMWH, nadroparin had no effect on coagulation activation [283]. However, differences between the study time points and smaller sample numbers may explain the discrepancy with our study.

In our study, the anti-Xa 24 hour profile follows the predictable curve found in non pregnant patients. The mean peak anti-Xa level was 0.16 IU/ml (95% CI 0.11-0.2) found at 4 hours post LMWH with a gradual decrease reaching non-detectable levels at 18 and 24 hours dose. This is within the recommended prophylactic range and was in agreement with previous studies of LMWH prophylaxis and is unlikely to predispose haemorrhagic complications [281, 282, 284].

It is widely accepted the effects of LMWH on the haemostatic system are not fully reflected by anti-Xa levels. In this study, lower levels of TAT were found in the 24-hour period post LMWH dose compared with both pre delivery ( $P<0.001$ ) and pre-LMWH dose levels ( $P<0.0001$ ). In addition we found that TAT levels remained decreased even though anti-Xa levels were below accepted prophylactic range. This suggests that the women were still benefiting from an anticoagulant effect

of LMWH even when anti-Xa levels were negligible. This was supported by another study showing reduced TAT levels at anti-Xa levels below 0.1 IU/ml (prophylaxis)[282].

The thrombin generation test is a recently developed assay which measures the cleavage of a slow reacting substrate by thrombin over time. The resulting thrombin generation curves obtained provides a compound measure of a patient's total thrombin generation and degradation capacity and has been proposed as a useful marker of venous thrombosis risk. Increased peak thrombin production and ETP represent an intermediate phenotype for venous thrombosis and the test is also known to detect thrombophilia [285, 286]. Although CS is a risk factor for post partum venous thrombosis, we did not see an elevation in either peak or ETP which has been found in other at risk groups[287]. This may suggest that ETP and peak thrombin may not be a useful marker of VTE in an acute setting. ETP and peak thrombin production in the post CS group remained below levels found in the vaginal delivery group before LMWH prophylaxis. There was no recorded instance of primary or secondary post partum haemorrhage (blood loss of over 500mls) in any of the patient in this study. However, during CS there was more tissue trauma when compared to vaginal delivery, thus more coagulation factors are needed to maintain haemostasis. It is possible that thrombin and other coagulation factors *in-vivo* have been largely consumed during surgery, which in this study, is reflected by the higher TAT levels observed 6 hours post CS compared to pre-delivery. The consumption of coagulation factors during treatment would also explain the reduced ETP compared with patients undergoing vaginal delivery. There was a significant treatment effect of LMWH in decreasing ETP and peak thrombin levels at 4 hours post LMWH which coincided with peak anti-Xa levels post LMWH. This significant negative correlation reflects inhibitory activity of LMWH on thrombin generation. This is also shown in an *in-vitro* study using equivalent anti-Xa activity concentrations of several LMWH; tinzaparin was significantly more active than other LMWHs at inhibiting thrombin generation [288]. In the non pregnant population, ETP has been proposed as a more accurate method of assessing the anticoagulant activity of LMWH and as a possible monitoring tool. From our data, ETP and peak thrombin correlate closely with anti-Xa levels and would have the

most potential as markers of anticoagulant activity. However, large scale prospective studies are required to test the accuracy of these parameters in determining the efficacy of LMWH in a variety of clinical settings.

There is an accumulation of evidence that the release of potent TFPI from endothelial cell wall of patients treated with LMWH significantly contributes to the anticoagulant properties of LMWH[197, 280]. In the SVD group, TFPI levels were lower compared to both CS1 and CS2 groups prior to LMWH dose. Eighty percent of TFPI is membrane bound, hence plasma concentration may not reflect the TFPI availability in endothelial surface and it is possible that endothelial trauma post surgery caused TFPI increase [289]. In this study TFPI levels mirrored anti-Xa levels during the 24 hours following treatment in the CS1 group with peak TFPI levels coincided with peak anti-Xa levels. Treatment with LMWH significantly affected TFPI levels post delivery and shows significant positive correlation between TFPI and anti-Xa. In a comparative study, *Ellison et al* showed that three different LMWHs used post CS, generated peak anti-Xa levels similar to those expected in the non pregnant state [223]. At these doses, TAT levels were reduced and TFPI levels were increased; with tinzaparin showing the greatest effect on thrombin generation. This study supports this finding and underlines the important contribution of TFPI to antithrombotic effects of LMWH in particular tinzaparin.

In conclusion, our data shows that excess thrombin is produced *in-vivo* post elective CS compared with SVD and that LMWH thromboprophylaxis in this healthy cohort of patients effectively inhibits this production. This study provides the basis for thromboprophylaxis in this cohort of women. It is unlikely that there will be a randomised control trial in this cohort in the near future. Therefore, this study provides a pharmacokinetic rationale for LMWH thromboprophylaxis in low risk women post elective CS.

**Chapter 4 Result:  
Thrombin Generation as a  
Predictor of Venous  
Thrombosis in Pregnancy**

## 4.1 Background

In the developed world, VTE remains the leading cause of mortality and morbidity during pregnancy. Pregnant women who developed VTE may not have any risk factors. Hence there is a need for a biomarker with the ability to predict these 'at-risk' women. Increased ETP and peak thrombin have been correlated with risk of recurrent VTE. Measurements of CAT parameters in the presence and absence of thrombomodulin have an added value because they improve sensitivity of tests to the protein C pathway which are otherwise dampened in pregnancy [124]. Hence this assay has great potential as a screening tool for thrombosis in at risk populations [123]. The SCOPE (Screening for Pregnancy Endpoints) study is a prospective, multicentre cohort study of 'healthy' nulliparous women with the primary aim of developing screening tests to predict pre-eclampsia, small for gestational age infants and spontaneous preterm birth [277]. Using patients from the SCOPE study ([www.scopestudy.net](http://www.scopestudy.net)), women who developed VTE (confirmed by objective testing) in pregnancy or postpartum were identified and their thrombin generation profile measured from citrated blood samples taken at 15 weeks gestation, prior to the development of thrombosis. Thrombomodulin sensitivity ratios (n-TMs<sub>r</sub>) were also calculated (refer to section 2.4.2 in Methodology). The control group was matched for age, gestation, BMI and ethnicity.

A previous study of normal uncomplicated pregnant women observed a mean peak thrombin level of 500nM (SD100nM) [165]. From the SCOPE databank which has recruited in total of 5000 women, 20 women from the cohort have developed venous thrombosis. Based on these previous findings, it was estimated that for a comparison of means statistical test (probability of Type I error of 0.01, and a probability of Type II error of 0.1)  $n_1=11$  patients in the study group and  $n_2=33$  in the matched control group (matched for age, gestation, BMI, and ethnicity) were required to achieve statistical power of 82%. With  $n_1=20$  patients identified for the study group, sufficient statistical power was achieved to allow the study to be performed. This study investigated the CAT assay as a potential predictive tool of VTE in pregnancy.

## 4.2 Results

### Patients

Eighty-one women have been recruited from the SCOPE database (twenty patients in the VTE group, sixty-one patients in control group). Patients were matched for age, BMI and ethnicity. All patients recruited were primigravids who underwent singleton pregnancies with no personal or family history of VTE or known thrombophilia. Demographic profile of patients in this study was depicted in Table 4.1.

	VTE Group (n=20)	Control Group (n=61)	p-value
<b>Age</b>	26.8 (6.4)	27.5 (5.2)	NS
<b>Parity</b>	0	0	NS
<b>BMI</b>	25.9 (4.3)	25.8 (4.5)	NS
<b>Smoking status</b>			NS
<b>Yes</b>	6 (30%)	19 (31%)	NS
<b>No</b>	14 (70%)	43 (69%)	NS
<b>Ethnic Background</b>			
<b>Caucasian</b>	16 (80%)	50(81%)	NS
<b>African ancestry</b>	2 (10%)	5(8%)	NS
<b>Maori</b>	1 (5%)	4(6%)	NS
<b>Aboriginal</b>	1 (5%)	3(5%)	NS
<b>Mode of Delivery</b>			
<b>Unassisted Vaginal</b>	10 (50%)	27 (44%)	<0.05
<b>Operative Vaginal</b>	3 (15%)	23 (38%)	<0.05
<b>Prelabour CS</b>	4 (20%)	8 (13%)	<0.05
<b>CS in Labour</b>	3 (15%)	3 (5%)	<0.05

**Table 4.1 Demographic Profile.** Data presented as mean (SD) or percentage (%). NS denotes non-significant difference.

More than eighty percent of the women in the control group achieved vaginal delivery. Thirty five percent of the women who developed VTE in this study had caesarean delivery compared to only eighteen percent in the control group. Seventy percent of VTE in this study developed during antenatal period and the vast majority of these were DVTs in the third trimester. More than forty percent of women who developed antenatal VTE in this study did not have any known VTE risk

factors. Out of the thirty percent of women who developed postnatal VTE, sixty seven percent had caesarean delivery. All of the women who developed postnatal VTE in this study had at least one VTE risk factor. All women in this study delivered live healthy newborns (Table 4,2).

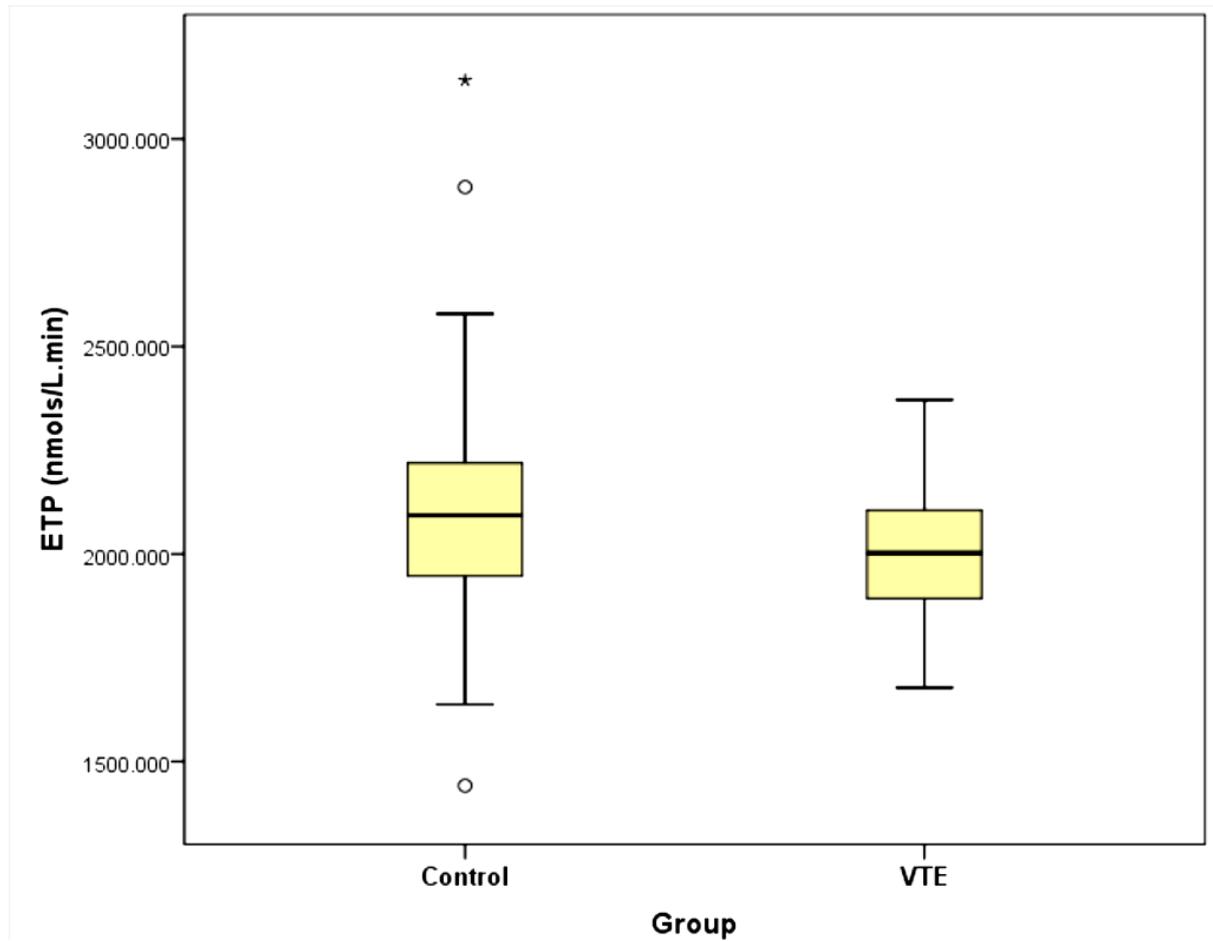
	<b>Antenatal VTE n=14</b>	<b>Postnatal VTE n=6</b>
<b>VTE</b>		
<b>Pulmonary Embolism</b>	2	2
<b>Proximal DVT</b>	2	0
<b>Distal DVT</b>	10	4
<b>VTE Risk Factors</b>		
<b>Anaemia</b>	2	0
<b>Smoking</b>	3	3
<b>APH</b>	2	NA
<b>Infection</b>	1	1
<b>Caesarean Delivery</b>	NA	4
<b>PPH (&gt; 1L)</b>	NA	1
<b>Known thrombophilia</b>	0	0
<b>Time of VTE event</b>		
<b>Second Trimester</b>	4	NA
<b>Third Trimester</b>	10	NA
<b>Post Natal</b>	NA	6
<b>Mode of Delivery</b>		
<b>Unassisted Vaginal</b>	9	1
<b>Operative Vaginal</b>	2	1
<b>Prelabour CS</b>	2	2
<b>CS in Labour</b>	1	2

**Table 4.2 Demographic Data of Women with VTE in Pregnancy.** Data represents n= number of women and NA = not applicable.

#### **4.2.1 Thrombin generation in Control and VTE Groups Prior to VTE Event (Figures 4.1 – 4.4)**

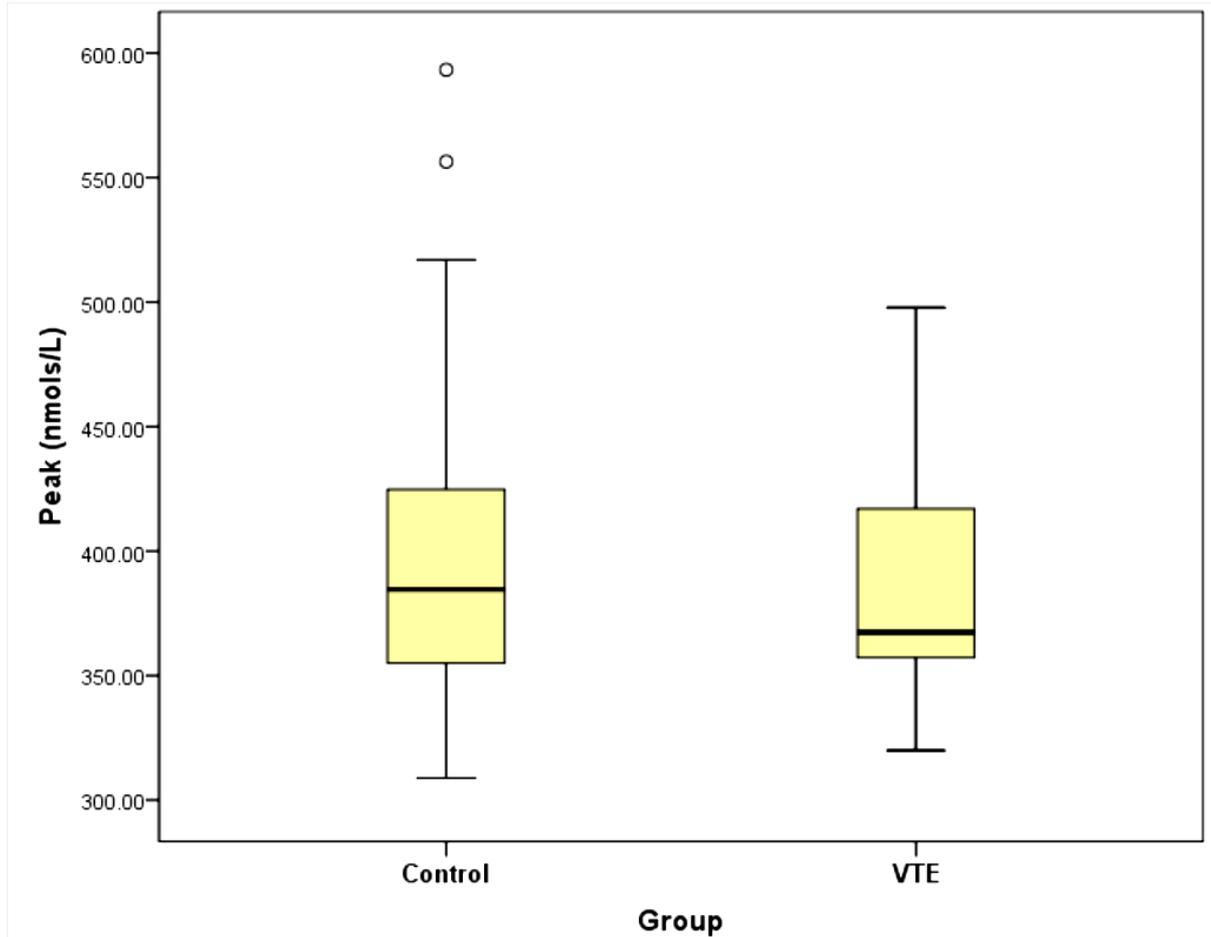
Figures 4.1- 4.4 showed ETP, peak, lagtime and time to peak for both control and VTE groups. There were no significant differences in all four parameters between the two groups.

i) ETP



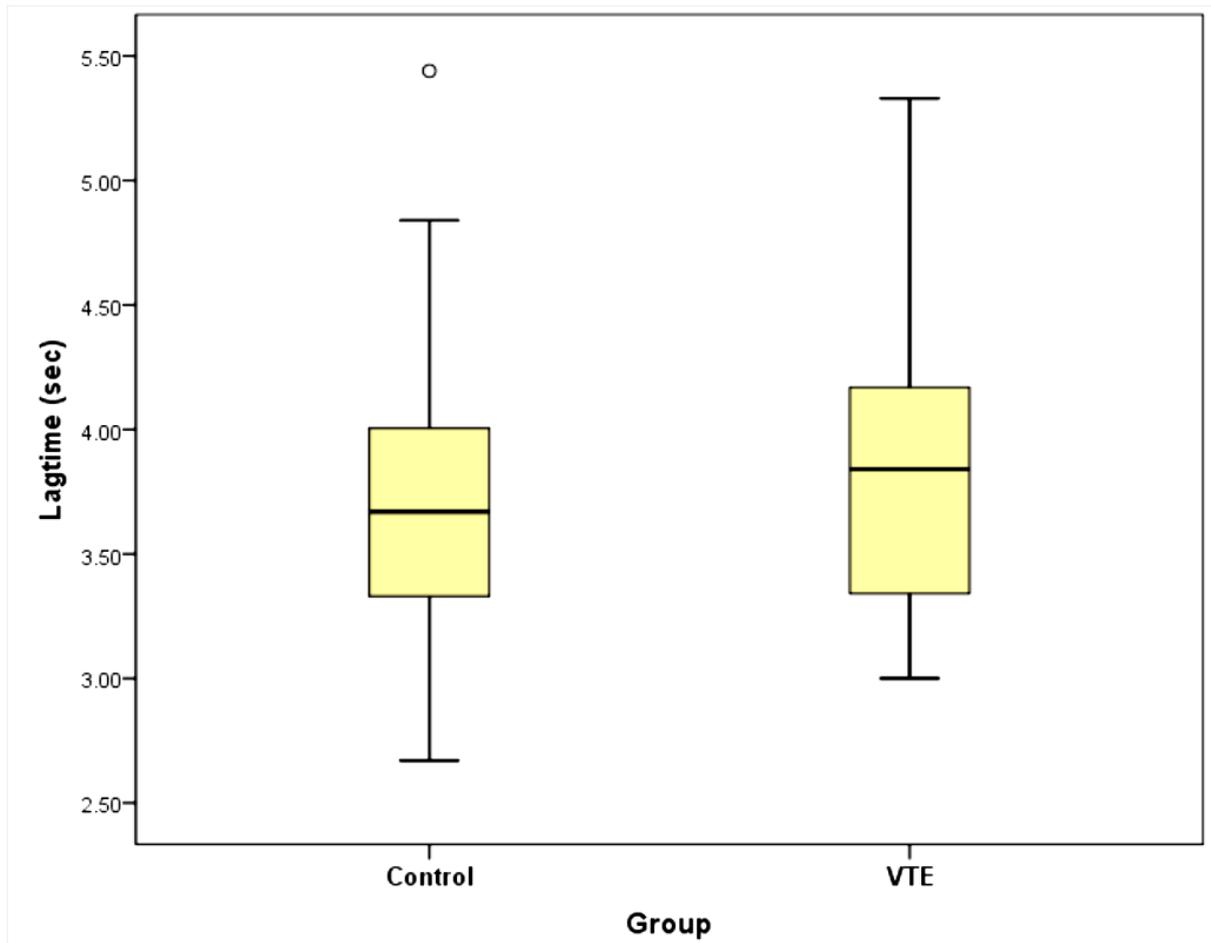
**Figure 4.1 ETP in 20 pregnant women with VTE and 61 healthy pregnant controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

ii) Peak



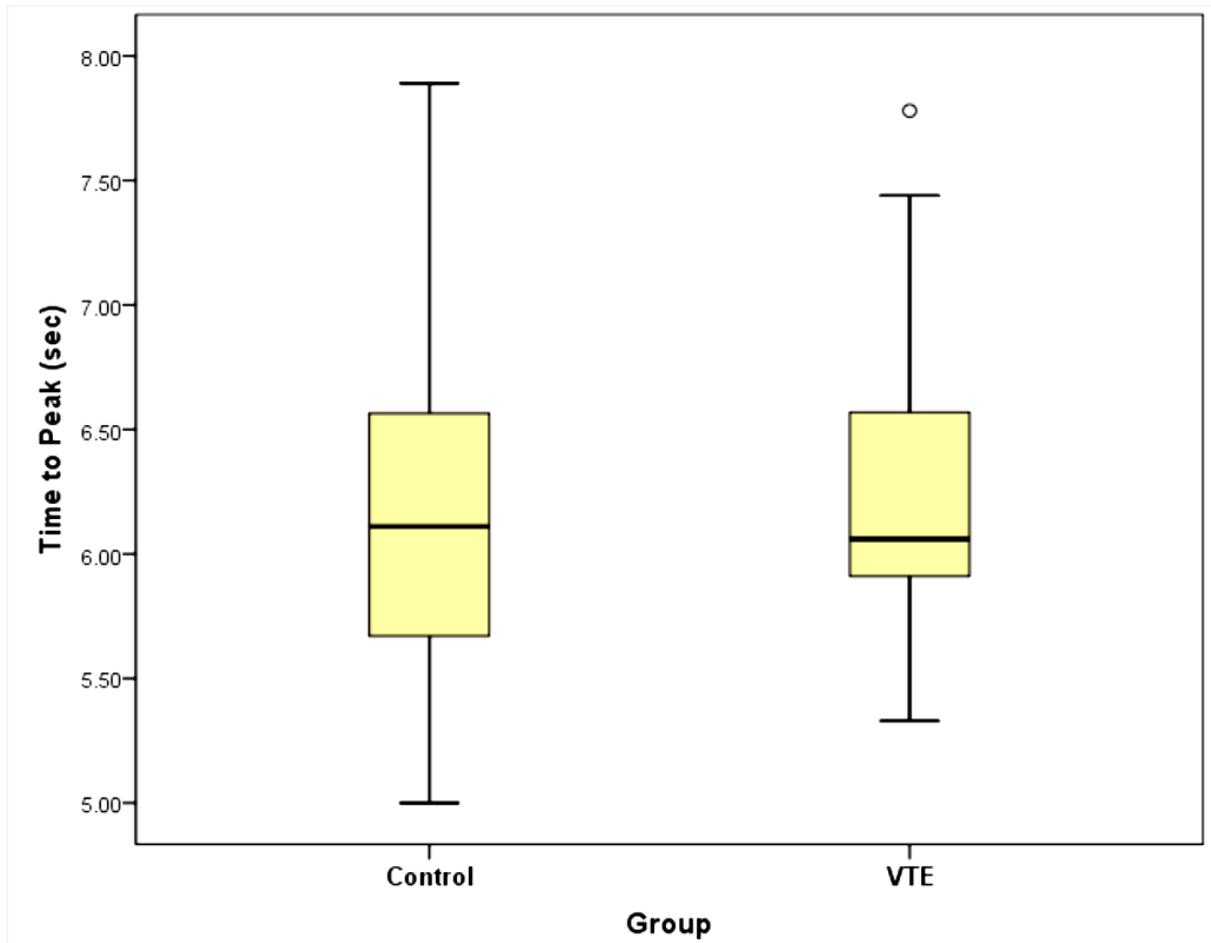
**Figure 4.2 Peak Thrombin in 20 pregnant patients with VTE and 61 controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

iii) Lagtime



**Figure 4.3 Lagtime in 20 pregnant patients with VTE and 61 controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

iv) Time to Peak

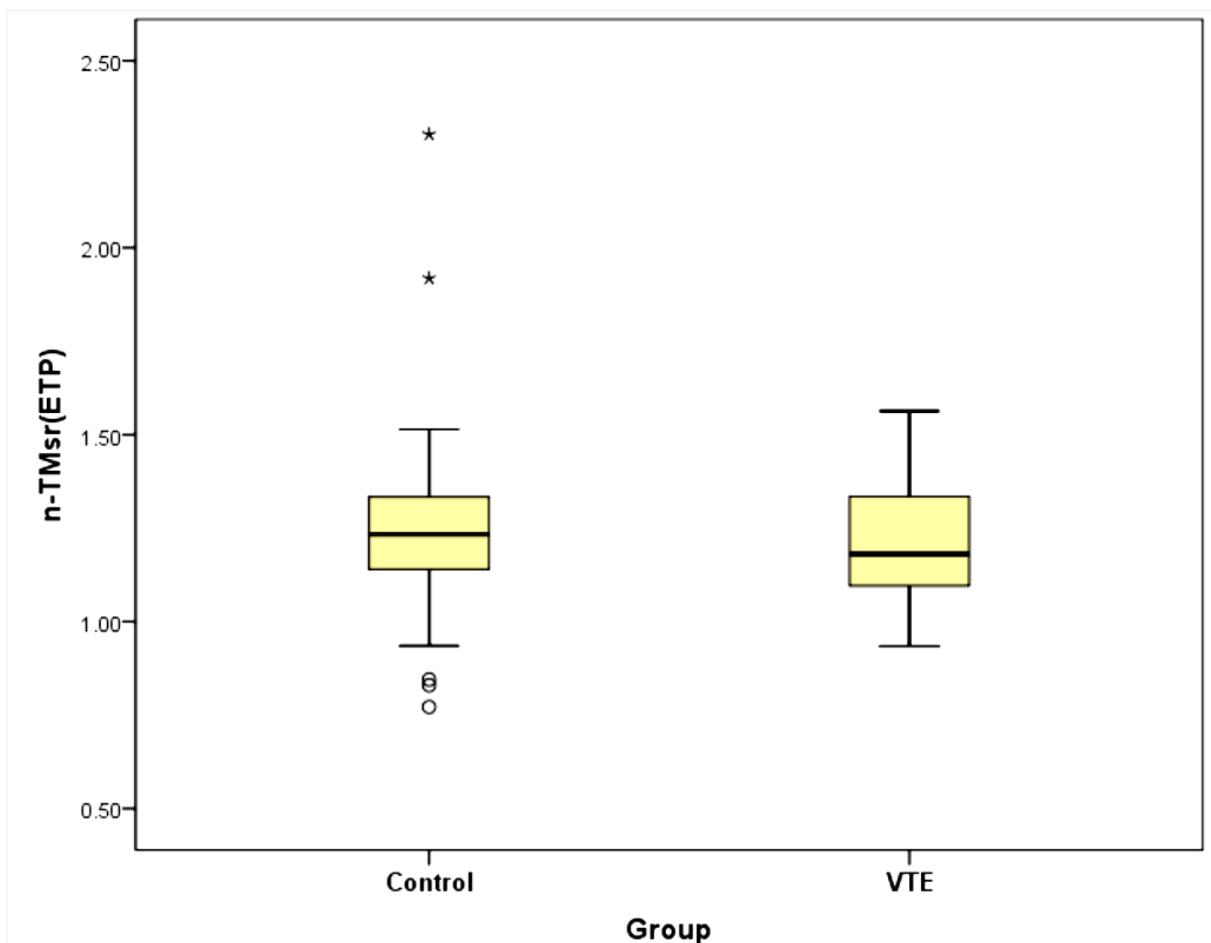


**Figure 4.4 Time to Peak in 20 pregnant patients with VTE and 61 controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

#### 4.2.2 n-TMsr in Control and VTE Group Prior to VTE Event (Figures 4.5 – 4.8)

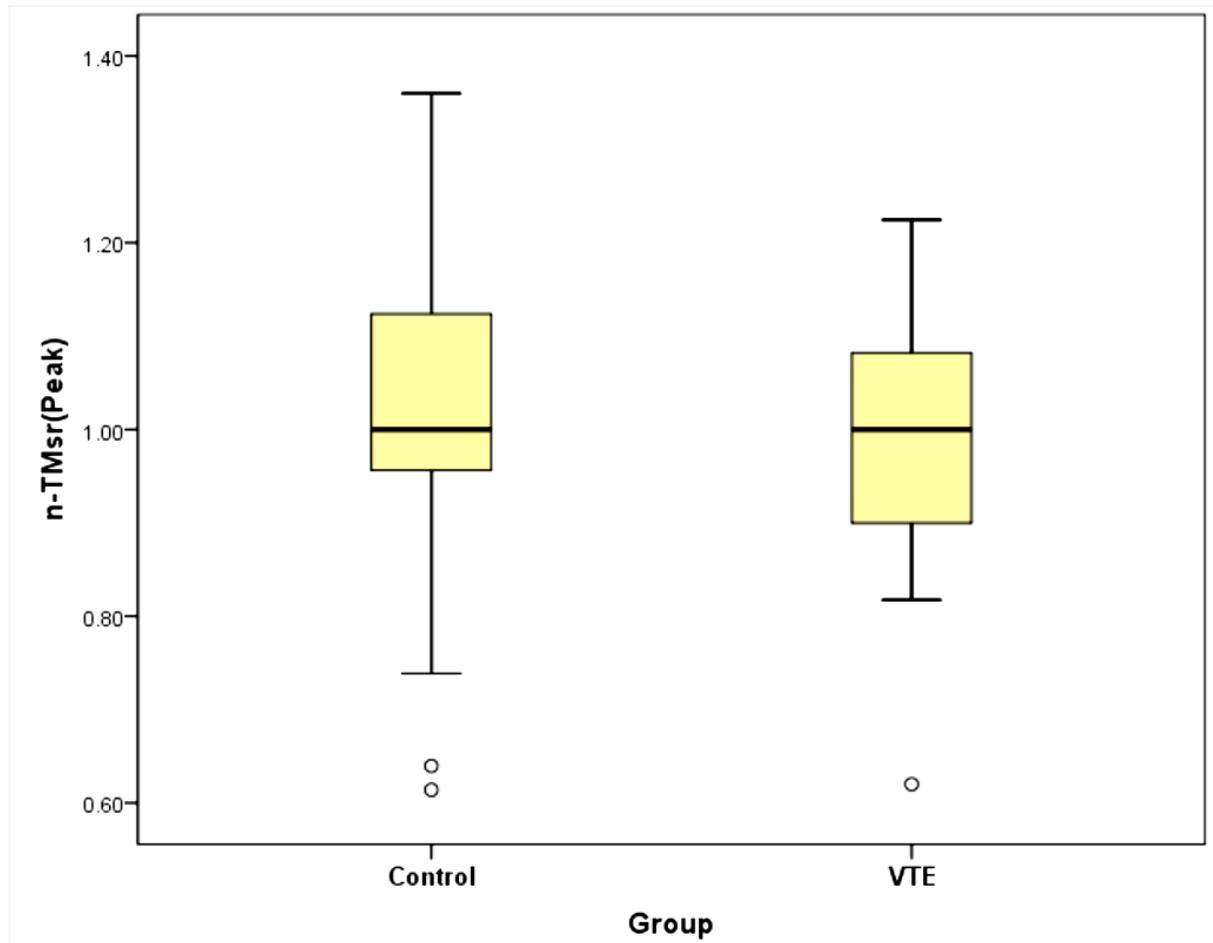
The ratio for each CAT parameter in presence and absence of thrombomodulin which was normalized against the same ratio determined in normal control plasma, n-TMsr, were calculated. Figures 4.5 to 4.8 showed n-TMs ratios of ETP, peak, lagtime and time to peak for both control and VTE groups. There were no significant differences in all four n-TMsr between control and VTE.

##### i) ETP



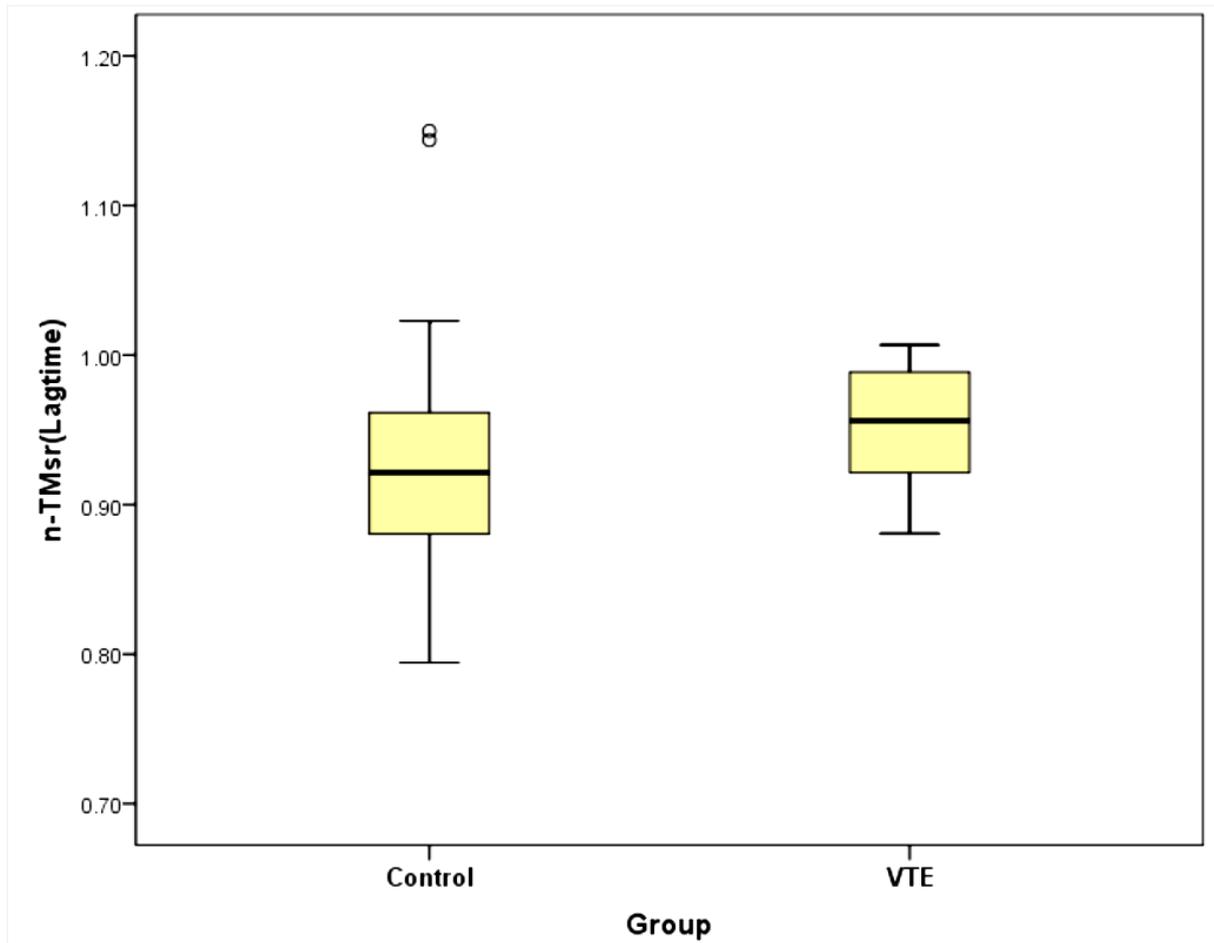
**Figure 4.5 Normalised thrombomodulin sensitivity ratio (ETP) in 20 pregnant women with VTE and 61 healthy pregnant controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

ii) Peak



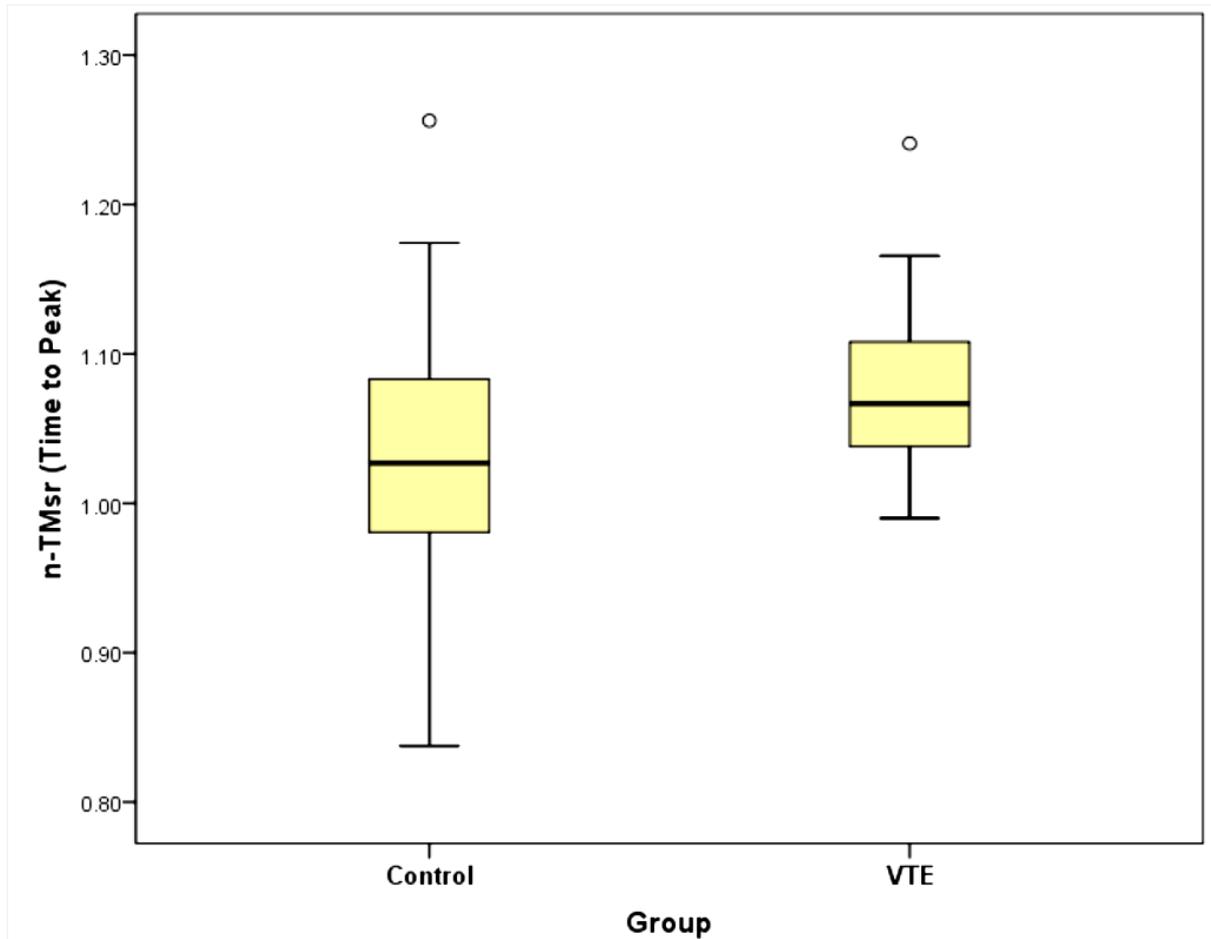
**Figure 4.6 Normalised thrombomodulin sensitivity ratio (peak) in 20 pregnant patients with VTE and 61 controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

iii) Lagtime



**Figure 4.7 Normalised thrombomodulin sensitivity ratio (lagtime) in 20 pregnant patients with VTE and 61 controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

iv) Time to Peak



**Figure 4.8 Normalised thrombomodulin sensitivity ratio (time to peak) in 20 pregnant patients with VTE and 61 controls.** All blood samples taken at 15 weeks gestation prior to any VTE event. The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> centile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).

### 4.3 Discussion

VTE in pregnancy is a known serious complication that can lead to serious morbidity and is still a leading cause of maternal mortality. Even though the availability of anticoagulants renders VTE as a potentially preventable disease, identification and stratification of pregnant women who will eventually develop VTE is still a challenge. Prophylaxis and management of VTE in pregnancy have a significant psychological and financial burden in recent times. Therefore, a predictive tool that is applicable in pregnancy would be extremely useful. Thrombin generation has been widely investigated in clinical studies for its predictive value for either first or recurrent VTE episodes.

In this study of pregnant primiparous women, there were no significant differences in any of the CAT parameters including ETP and peak thrombin, with or without thrombomodulin, between the sixty-one healthy control pregnant women and the twenty pregnant women who after blood sampling, developed VTE. This is in contrast of previous studies in non-pregnant subjects which reported higher ETP [119, 290] and peak [118] are predictive of future VTE occurrence or recurrence. Previous studies have reported coagulation activation from the first trimester of pregnancy [155, 164]; higher levels of thrombin generation found in this study were also previously reported in pregnant women by multiple authors [165, 291, 292]. In this study cohort, more women who developed VTE in pregnancy had caesarean delivery compared with pregnant women in the control group. Of the six women who developed post partum VTE, four had caesarean delivery. Equal number of women who underwent prelabour caesarean and caesarean in labour developed VTE.

Thrombin generation parameters by CAT assays evaluate a global haemostatic profile of patients at risk of VTE and measure the cumulative effect of prothrombotic tendencies [293, 294]. As CAT assay takes into account all pregnancy-related haemostatic modifications, its parameters are thought to be better indices than indirect thrombin generation markers such as prothrombin fragment 1.2, thrombin anti-thrombin complex and D-Dimers which only looked at ongoing coagulation activation. There was no correlation observed either between D-dimers or prothrombin

fragment 1,2 and ETP in a pregnancy study [292]. This is why the extensively studied D-Dimers, which is widely accepted as a useful tool to triage patients at-risk of VTE in non-pregnant population due to its high negative predictive value, is not as useful in pregnancy [295].

In pregnancy where the protein C pathway is diminished, measurements of thrombin generation parameters in the presence and absence of thrombomodulin have an added value because they improve sensitivity of tests to the protein C pathway. Thrombomodulin is a membrane bound protein and addition of thrombomodulin makes the assay sensitive to the APC pathway. Measurement of thrombin generation with and without thrombomodulin paints a more accurate picture of the global haemostatic system than thrombin generation with and without activated protein C [123, 287]. In this study, TF induced thrombin generation measured with CAT could not differentiate a prothrombotic state when the activated protein C system is boosted with thrombomodulin as calculated and analysed by n-TMsr. Indeed the ratio of endogenous thrombin potential determined in the presence and absence of thrombomodulin ( $nETP_{TMsr}$ ) in our pregnant study population is higher than that reported of that found in prothrombotic state such as in G20210A prothrombin gene mutation [123]. Judging from the elevated thrombin generation values, one would expect a higher rate of thrombosis in pregnancy. Yet, despite these high values of thrombin generation in pregnancy, the incidence of VTE remains relatively low. Different assay protocols and pre-analytical conditions may explain the differences reported between studies. It is possible that there are other mechanisms at play and it has been suggested that a *relative* increased in fibrinolysis may be a balancing act to reduce the incident of VTE in pregnancy [146]. A study observed a positive correlation between D-dimers (a marker of cross-linked fibrin turnover) and circulating fibrinogen levels[292]. The increased D-dimers level throughout pregnancy, may be an outcome of increased circulating fibrinogen [295].

It is possible that unlike in non-pregnant studies, the difference in ETP or peak thrombin between normal pregnant women and pregnant women who developed VTE were so small that our small study sample does not reach any significant difference. We chose a TM concentration designed

to inhibit 50% of peak thrombin (5pM TF). Using a different concentration of tissue factor (1pM TF) instead of 5pM TF may increase these difference [124]; however this technique does not reflect the physiological conditions *in-vivo*. Due to limited plasma availability it was not possible to perform serial assays with different tissue factor concentrations.

The vast majority of VTE in this study occurred either in late pregnancy or postpartum. None of the patients in this study had any personal or family history of thrombosis or known thrombophilia. The SCOPE study is not designed specifically to assess VTE in pregnancy; hence thrombophilia screening was not undertaken. Furthermore, the timing of samples collected (15 weeks gestation) might be too early to detect any changes in the CAT parameters relevant to a VTE event later in pregnancy or the post natal period. The timing of blood sampling may therefore exclude the women who developed VTE in the first trimester. It is possible that some of the women in the control group had thrombophilia, which could skew the results. Incidence of VTE in pregnancy is generally 1:1000. In this study, twenty women developed VTE in pregnancy out of a total of five thousand primigravids recruited in SCOPE; resulting in a four-fold higher VTE incidence (4:1000). This is in part, due to the SCOPE cohort containing a higher proportion of women who were overweight and of advanced age. Moreover, all women in this study are primigravids that would not have a previously challenged haemostatic system.

Recent studies have suggested that standardised pre-analytical conditions for the CAT assay need to be determined to ensure that the assay is a clinically useful predictive tests [296]. These include optimisation of sampling conditions, centrifugation and concentrations of TF used as stimulant. When this optimisation is achieved it may be possible that the CAT assay under different conditions than used in this study may have a role in the prediction of VTE in pregnancy.

**Chapter 5**

**Result: Antenatal  
Thromboprophylaxis in  
Morbidly Obese Women**

## 5.1 Background

In obese pregnant women, heparin prophylaxis has been recommended based on expert opinion [137], however no data exists on the anticoagulant effects of heparin in this group. The obese pregnant woman is particularly at risk in late pregnancy [137]. In the non pregnant patient, studies using anti-Xa as a marker of activity have shown that individualised weight based therapy generates anti-Xa levels in the required therapeutic range and the data did not support capping doses [231]. It is unknown whether this is the case in pregnancy. Similar studies in the obese pregnant woman (using anti-Xa and markers of thrombin generation) would provide important data leading to effective dosing strategies in this group.

The aim of this study was to investigate two different dosing regimens; fixed dose versus weight adjusted dose on the anticoagulant effects of the LMWH, tinzaparin used for thromboprophylaxis in obese pregnant women. Twenty morbidly obese pregnant women were invited to participate. At 30 weeks gestation, the patients were started on a fixed dose of Tinzaparin (4,500iu/day). Four hour post-dose levels of anti-Xa, CAT, TAT and TFPI were measured. The patients were crossed over to a weight adjusted dose (75iu/kg/day) and four-hour levels repeated. Patients continued on this dose (provided local anti-Xa levels are within the accepted range) and four hour post-dose levels were repeated every 2 weeks until delivery. Using peak anti-Xa levels as an endpoint, and based on previous studies in pregnant women, 75 iu/kg will give peak anti-Xa levels of  $0.25 \pm 0.10$  (SD) iu/ml [221]. In order to detect whether the fixed doses will yield anti-Xa levels lower than 0.2 iu/ml, a sample size of fourteen women in each group was required.

## 5.2 Results

### 5.2.1 Patients

Twenty patients in each group met the eligibility criteria and consented to take part in the study. Women in the normal BMI group acted as controls and were matched for age, parity and gestation with the obese group (Table 5.1). All women in this study were Caucasians enrolled at 30 weeks gestation and underwent singleton pregnancy. All patients had normal kidney and liver profile throughout this study. Average weight in the obese group was 138.9 ( $\pm$ 18.8) kg, corresponding to average BMI of 48.2 ( $\pm$ 22.9) kg/m<sup>2</sup>. Calculated creatinine clearance of women in the obese group were within normal pregnancy ranges (150-200ml/min) [297].

	<b>Obese Group</b>	<b>Normal BMI Group</b>	<b>p-value</b>
	<b>n=20</b>	<b>n=20</b>	
<b>Age (years)</b>	32.3 ( $\pm$ 4.7)	30.0 ( $\pm$ 5.4)	NS
<b>Parity</b>	1.2 ( $\pm$ 0.4)	1.0( $\pm$ 0.8)	NS
<b>Weight (kg)</b>	138.9( $\pm$ 18.8)	59.9 ( $\pm$ 7.3)	
<b>*BMI (kg/m<sup>2</sup>)</b>	48.2( $\pm$ 6.7)	22.9( $\pm$ 2.3)	
<b>Serum Creatinine (<math>\mu</math>mol/L)</b>	43.0( $\pm$ 2.3)	42.0( $\pm$ 3.4)	
<b>**Calculated Creatinine Clearance( ml/min)</b>	143.2 ( $\pm$ 16.7)		
<b>Mean Tinzaparin Dose (iu)</b>	10,420 ( $\pm$ 1031)		

**Table 5.1 Demographic Data of Study 3.** Values presented as mean ( $\pm$ SD). \*Body Mass Index (BMI). \*\*Creatinine clearance was calculated using adjusted body weight (ABW). Calculated creatinine clearance and tinzaparin dose only applicable to obese group.

There were no patients in the obese group who had anti-Xa levels outside the target range (0.2-0.5iu/ml) that was deemed harmful by the haematologist or obstetrician, hence, dose adjustment was not required. Two patients had anti-Xa levels between 0.11-0.14iu/ml at 32weeks but reached the target range on repeat testing at 34 weeks without dose adjustment. None of the

women on LMWH in this study developed VTE, abnormal bleeding or poor obstetric outcome. Two patients in the obese group reported discomfort bruising which resolved with rotating injection sites. All patients in this study delivered healthy live newborns. Twelve (60%) of the patients in the obese group had caesarean delivery compared with five in the normal weight group.

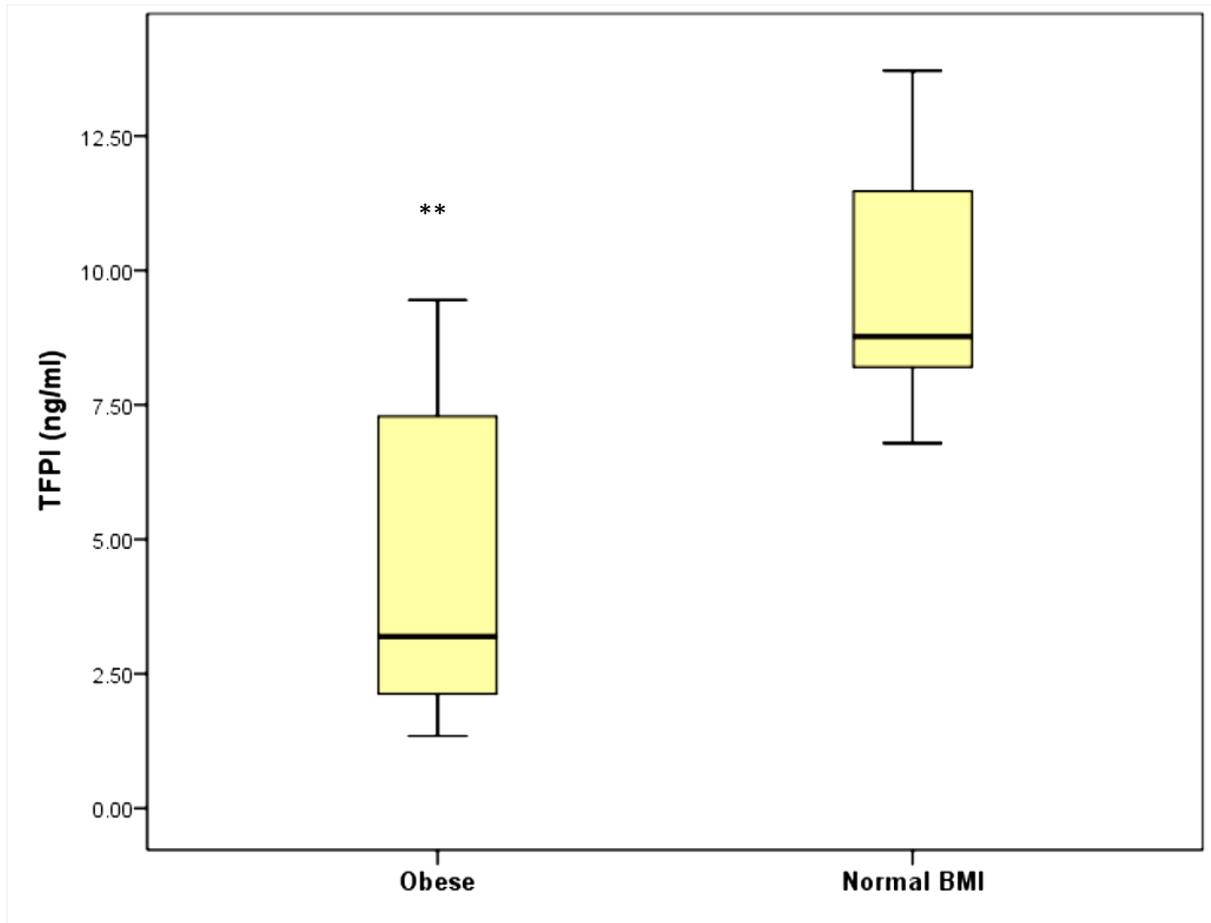
### **5.2.2 Coagulation Parameters at 30 Weeks Gestation in the Morbidly Obese and Normal Weight Pregnant Women**

#### **i) TFPI and TAT (Figures 5.1 and 5.2)**

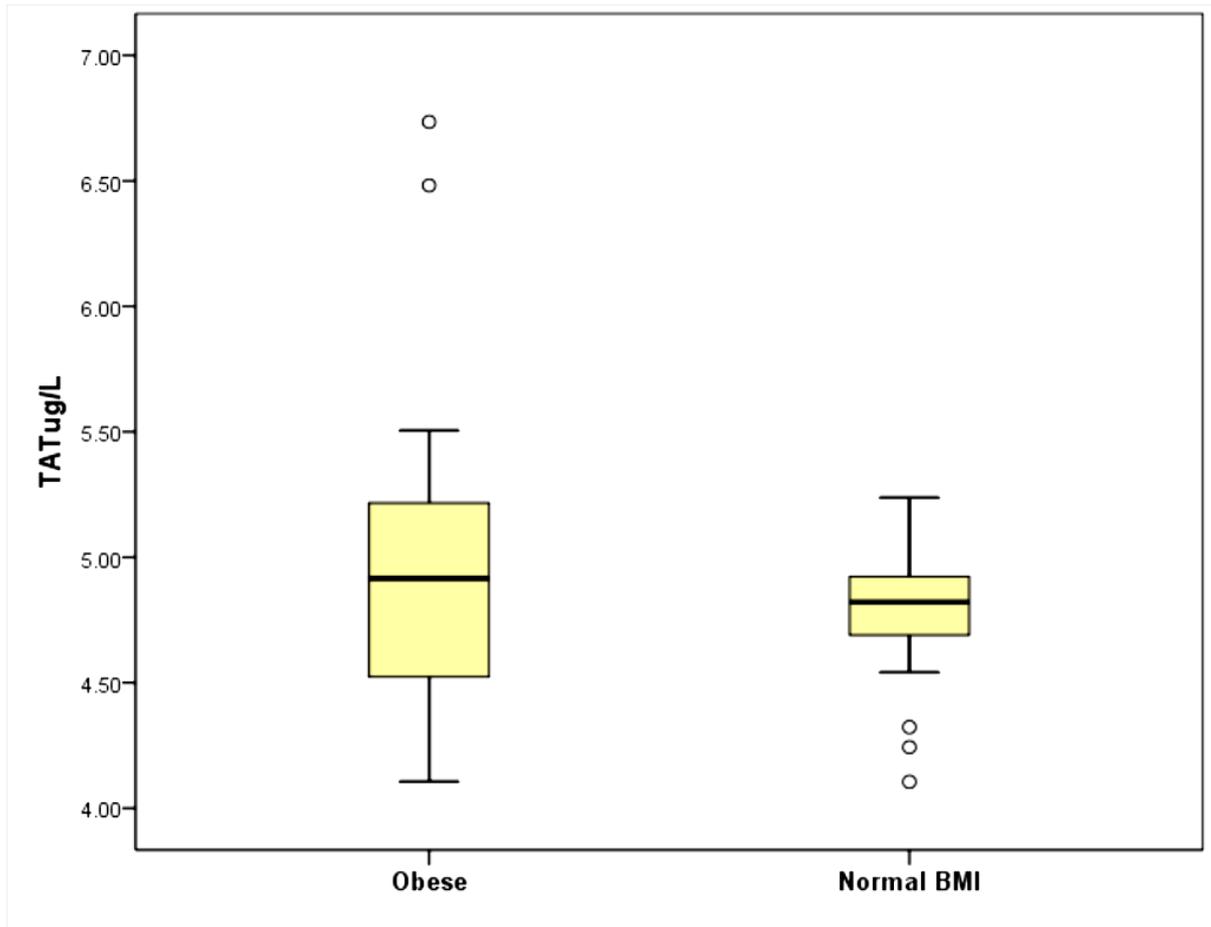
TFPI and TAT levels were measured in the obese group (prior to LMWH administration) and compared with those in a group of control women of normal weight. TFPI levels in the obese group were significantly lower than control group at 30 weeks gestation ( $p < 0.001$ ). TAT levels in obese group were higher than control group at 30 weeks but this did not reach statistical significance ( $p > 0.05$ ).

#### **ii) CAT Parameters (Figures 5.3-5.6)**

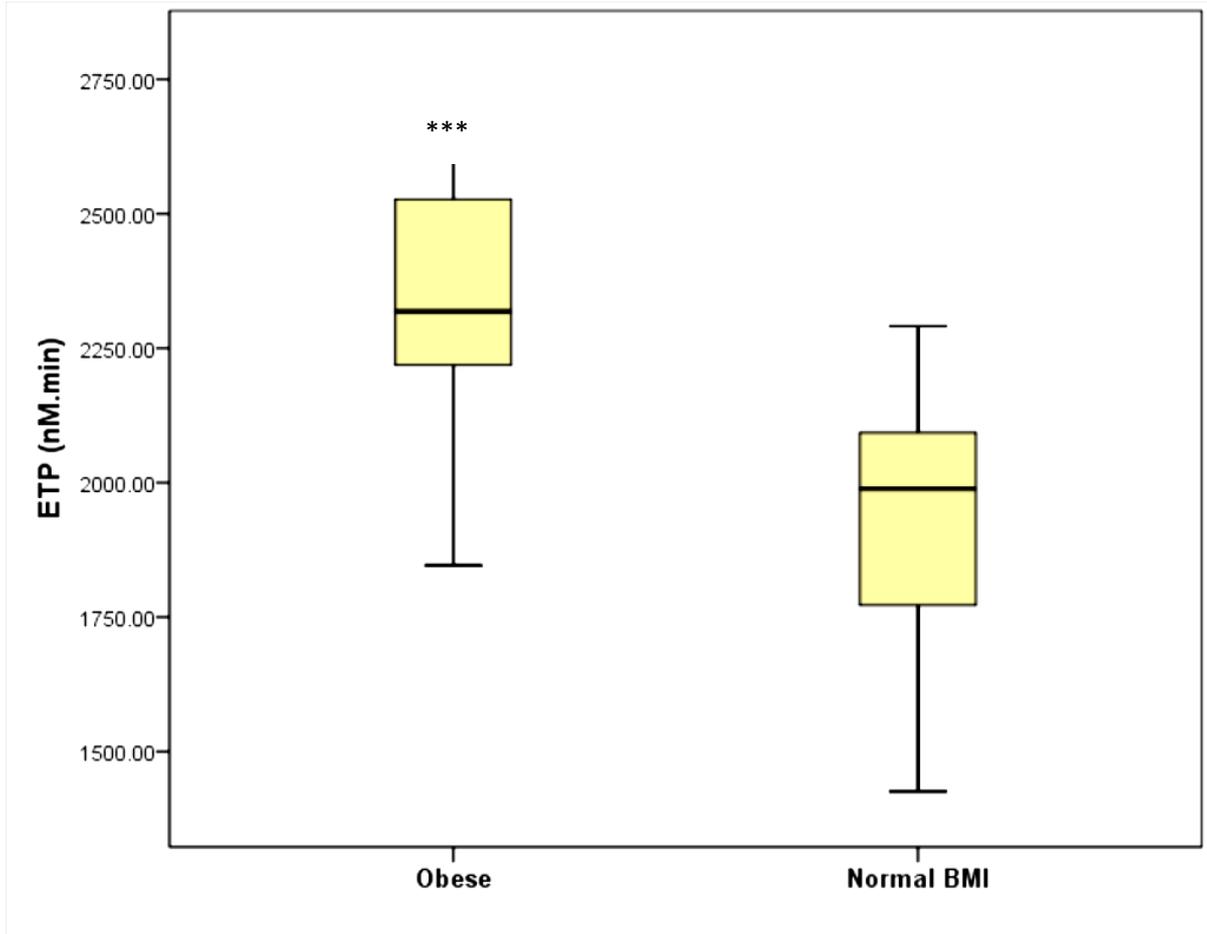
Thrombin potential was measured in the obese group (prior to LMWH administration) and compared with those in a group of control women of normal weight. Mean ETP and Peak thrombin levels in obese group were significantly higher compared with control group at 30 weeks ( $p < 0.0001$ ;  $p < 0.001$  respectively). Similarly lag time and time to peak was significantly shorter in the obese group obese group (prior to LMWH administration) compared with that in a group of control women of normal weight ( $p < 0.001$ ).



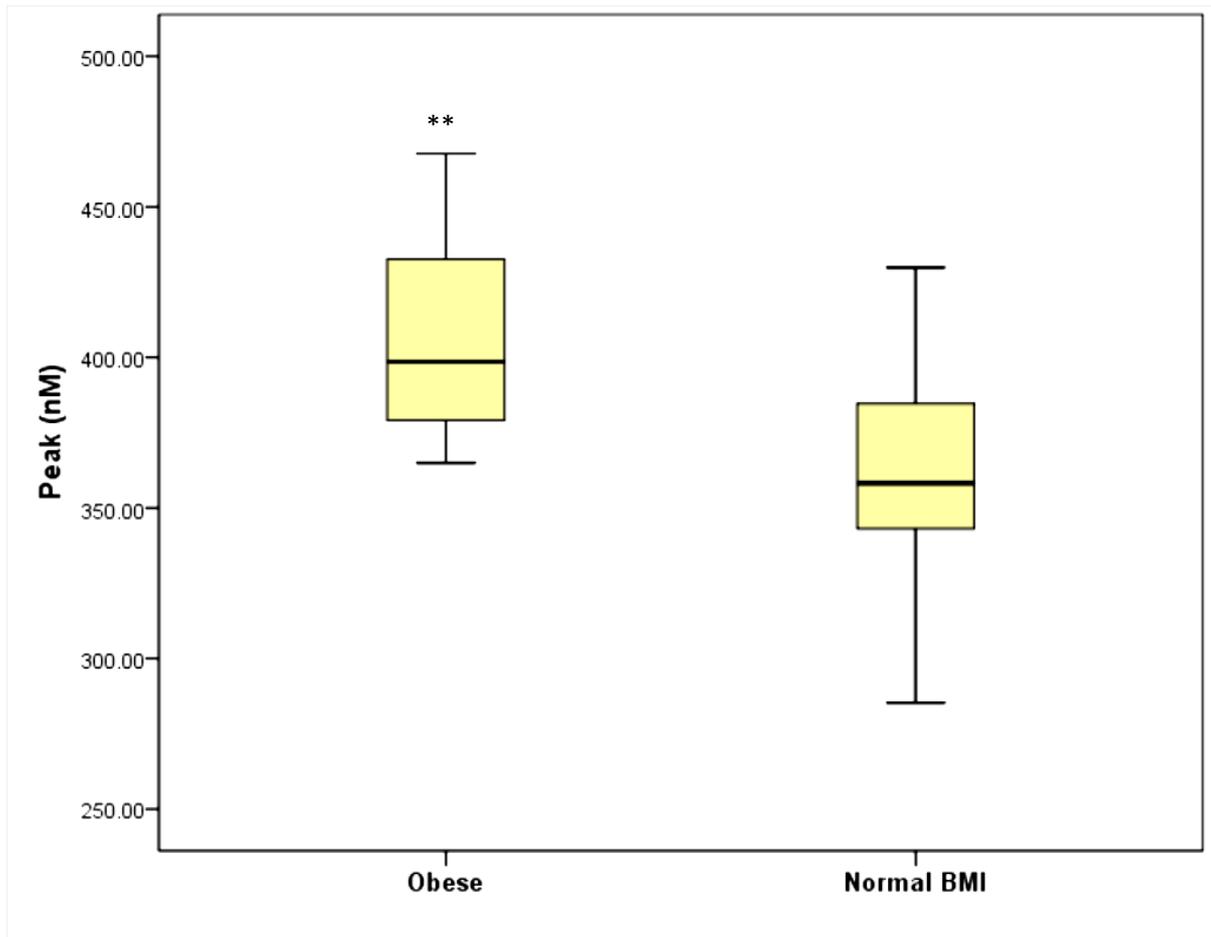
**Figure 5.1 TFPI in Morbidly Obese and Normal Weight Pregnant Women at 30 Weeks Gestation.** TFPI levels were measured at 30 weeks in the obese group (prior to LMWH administration) (n=20) and compared with those in a group of control women of normal weight (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\* (p<0.001).



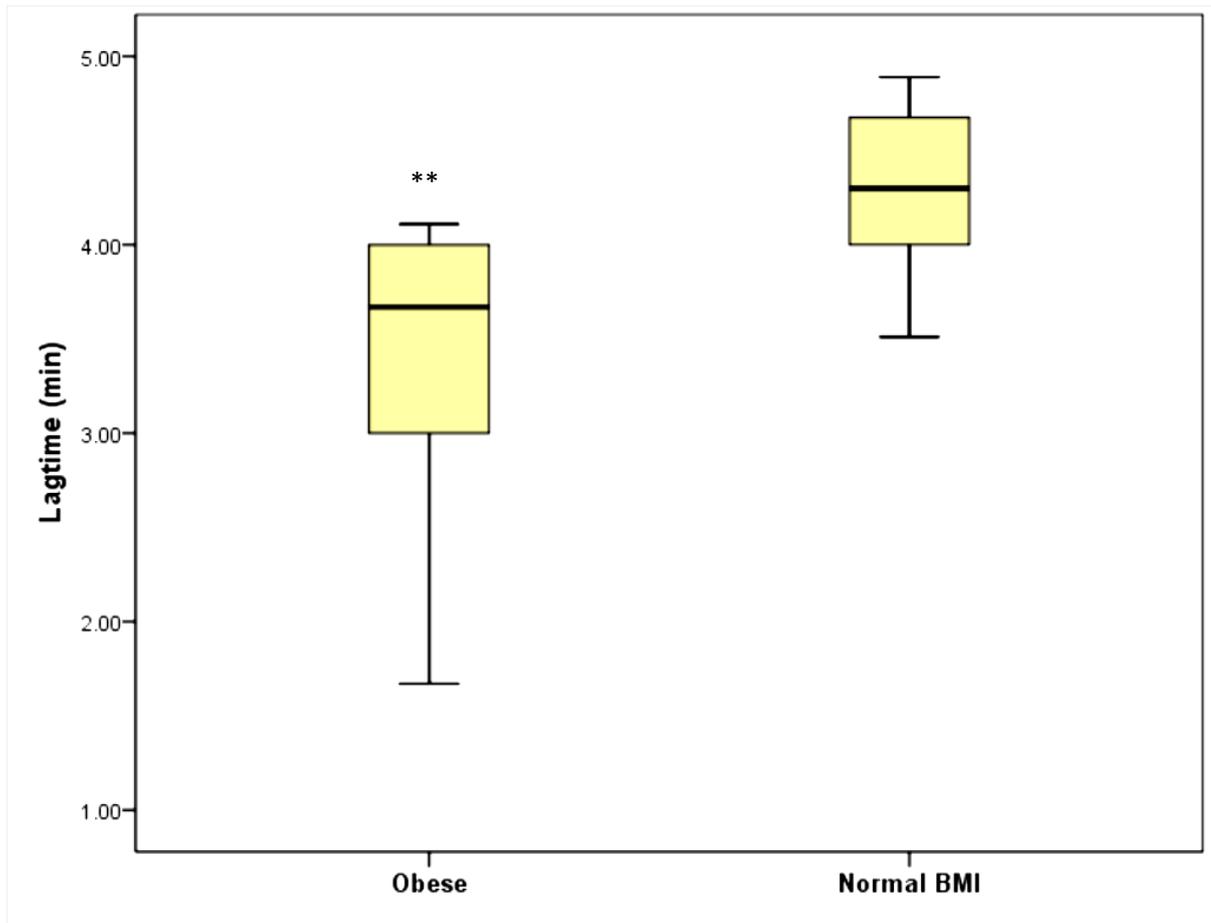
**Figure 5.2 . TAT in Morbidly Obese and Normal weight Pregnant women at 30 Weeks Gestation.** TAT levels were measured at 30 weeks in the obese group (prior to LMWH administration) (n=20) and compared with those in a group of control women of normal weight (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles).



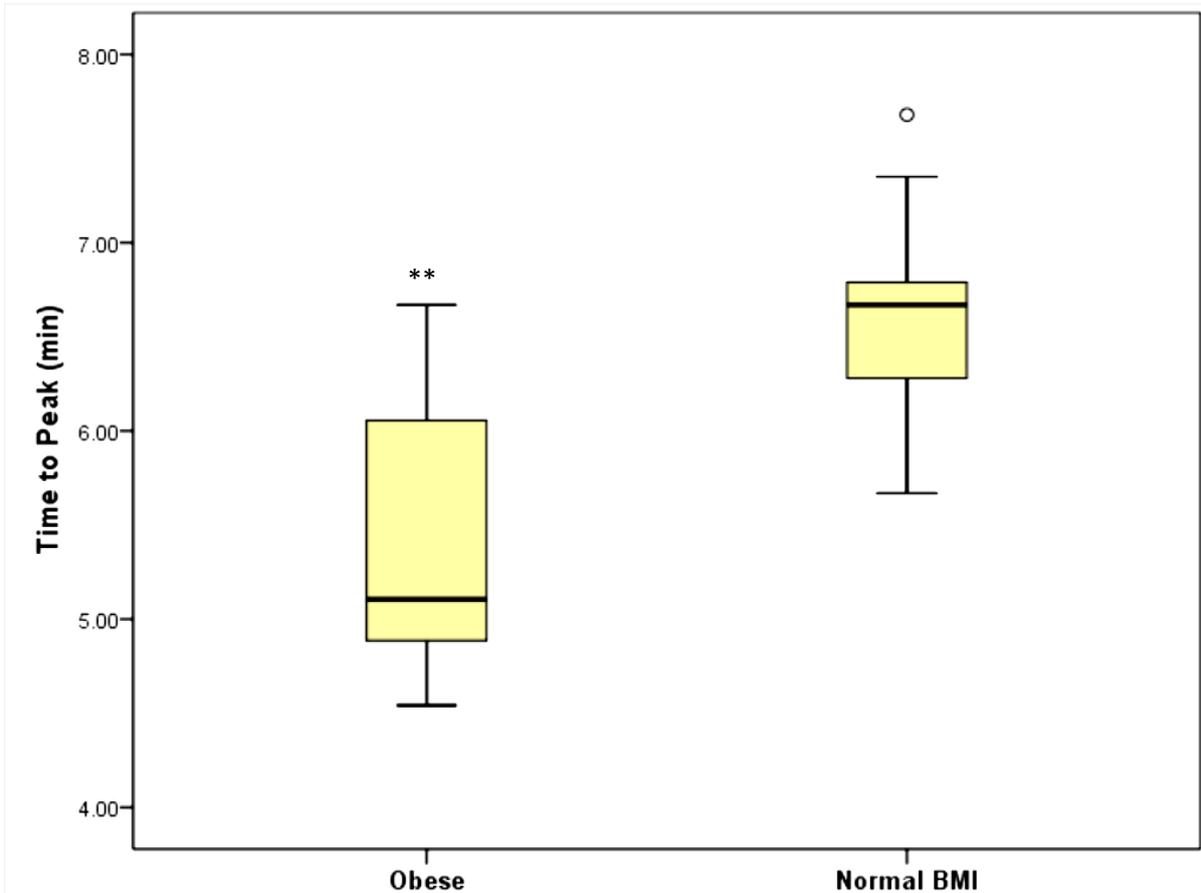
**Figure 5.3 ETP in Morbidly Obese and Normal Weight Pregnant Women at 30Weeks Gestation.** ETP levels were measured at 30 weeks in the obese group (prior to LMWH administration) (n=20) and compared with those in a group of control women of normal weight (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\*\* (p<0.0001).



**Figure 5.4 Peak in Morbidly Obese and Normal Weight Pregnant Women at 30 Weeks Gestation.** Peak thrombin levels were measured at 30 weeks in the obese group (prior to LMWH administration) (n=20) and compared with those in a group of control women of normal weight (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\* (p<0.001).



**Figure 5.5 Lagtime in Morbidly Obese and Normal Weight Pregnant Women at 30 Weeks Gestation.** Lagtime were measured at 30 weeks in the obese group (prior to LMWH administration) (n=20) and compared with those in a group of control women of normal weight (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\* (p<0.001).



**Figure 5.6 Time to Peak in Morbidly Obese and Normal Weight Pregnant Women at 30 Weeks Gestation.** Time to peak were measured at 30 weeks in the obese group (prior to LMWH administration) (n=20) and compared with those in a group of control women of normal weight (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\* (p<0.001).

### **5.2.3 Effects of LMWH on TFPI in the Morbidly Obese Pregnant Women in Third Trimester**

**(Figures 5.7 and 5.8)**

Within the obese group, paired t-test showed that TFPI levels were increased significantly post standard LMWH dose 4500iu tinzaparin ( $p < 0.001$ ) and weight adjusted dose ( $p < 0.0001$ ) (Figure 5.7) compared with pre dose levels. Venous blood taken post standard LMWH dose (4500iu tinzaparin) yielded a significantly lower TFPI level compared with post weight-adjusted dose (75iu/kg tinzaparin) ( $p < 0.0001$ ). Repeated ANOVA showed there was a significant effect of LMWH prophylaxis on TFPI levels ( $p < 0.0001$ ) (Figure 5.8). Post hoc test showed that TFPI was significantly increased at 32 ( $p < 0.0001$ ), 34 ( $p < 0.0001$ ) and 36 ( $p < 0.0001$ ) weeks in the obese group compared with baseline at 30 weeks gestation. There were no significant changes in TFPI levels in the normal BMI control group throughout gestation.

### **5.2.4 Effects of LMWH on TAT in the Morbidly Obese Pregnant Women in Third Trimester**

**(Figures 5.9 and 5.10)**

In the obese group, TAT levels pre-LMWH dose were significantly reduced compared with levels post standard LMWH dose 4500iu tinzaparin ( $p < 0.01$ ) (Figure 5.9) and levels found post weight-adjusted LMWH dose, 75iu/kg tinzaparin ( $p < 0.001$ ). Venous blood taken post standard LMWH dose 4500iu tinzaparin showed significantly higher TAT levels compared with post weight-adjusted dose (75iu/kg tinzaparin) ( $p < 0.05$ ). Repeated ANOVA showed there was a significant effect of LMWH treatment on TAT, ( $p = 0.005$ ) (Figure 5.10). Post hoc tests showed TAT levels at 32 weeks were significantly lower in the obese group ( $p < 0.001$ ) compared with pre-dose (30 weeks) levels. The decreased levels at 34 and 36 weeks gestation were not significantly different from pre-dose levels. TAT levels increased significantly in the normal BMI group with

gestation, this reached significance at 32 ( $p<0.0001$ ), 34 ( $p<0.0001$ ) and 36 weeks ( $p<0.0001$ ) compared with levels at the 30 weeks gestation.

### **5.2.5 Effects of LMWH on CAT Parameters in the Morbidly Obese Pregnant Women in Third Trimester**

#### **i) ETP (Figure 5.11 and 5.12)**

Within the obese group, paired t-Test showed that there was no significant difference between ETP levels pre-LMWH dose and post standard LMWH dose 4500iu tinzaparin (Figure 5.11). However, following weight-adjusted LMWH dose, 75iu/kg tinzaparin, ETP levels were significantly lower compared with pre-dose and post standard LMWH dose 4500iu tinzaparin levels, ( $p<0.0001$ ). Repeated ANOVA showed there was a significant LMWH treatment effect on ETP, ( $p<0.0001$ ) (Figure 5.12). Post hoc test showed significantly lower levels were observed at 32 ( $p<0.0001$ ), 34 ( $p<0.0001$ ) and 36 weeks ( $p<0.0001$ ) compared with 30 wks (pre dose levels) in the obese group. There were no significant changes in ETP levels in the normal BMI control group.

#### **ii) Peak (Figure 5.13 and 5.14)**

Paired t-Test showed that peak thrombin levels pre-LMWH dose were significantly higher compared with post standard LMWH dose( 4500iu tinzaparin) in the obese group ( $p<0.0001$ ) and post weight-adjusted LMWH dose (75iu/kg tinzaparin) ( $p<0.0001$ ) (Figure 5.13). Post standard LMWH dose (4500iu tinzaparin), peak thrombin levels were also significantly higher compared with post weight-adjusted dose (75iu/kg tinzaparin) ( $p<0.0001$ ). Repeated ANOVA showed there was a significant LMWH prophylaxis effect on peak thrombin ( $p<0.0001$ ) (Figure 5.14). Post hoc tests showed a significant decrease in peak thrombin at 32 ( $p<0.0001$ ), 34 ( $p<0.0001$ ) and 36 weeks ( $p<0.0001$ ) compared with pre-dose (30wks) levels in the obese group. There was no significant change in peak thrombin levels in the normal BMI control group.

### **iii) Lagtime Figure (5.15 and 5.16)**

In the obese group, paired t-Test showed that lagtime pre-LMWH dose was significantly shorter compared with post standard LMWH dose (4500iu tinzaparin) ( $p < 0.0001$ ) and post weight-adjusted LMWH dose (75iu/kg tinzaparin) ( $p < 0.0001$ ) (Figure 5.15). Longer lag times were found post weight-adjusted dose (75iu/kg tinzaparin) compared with post standard LMWH dose (4500iu tinzaparin), ( $p < 0.0001$ ). Repeated ANOVA showed there was a significant LMWH prophylaxis effect on lagtime ( $p < 0.0001$ ) (Figure 5.16). Lag times were significantly longer at 32 ( $p < 0.001$ ), 34 ( $p < 0.001$ ) and 36 weeks ( $p < 0.001$ ) compared with 30 wks (pre dose) in the obese group. There was no significant change in lagtime in the normal BMI control group.

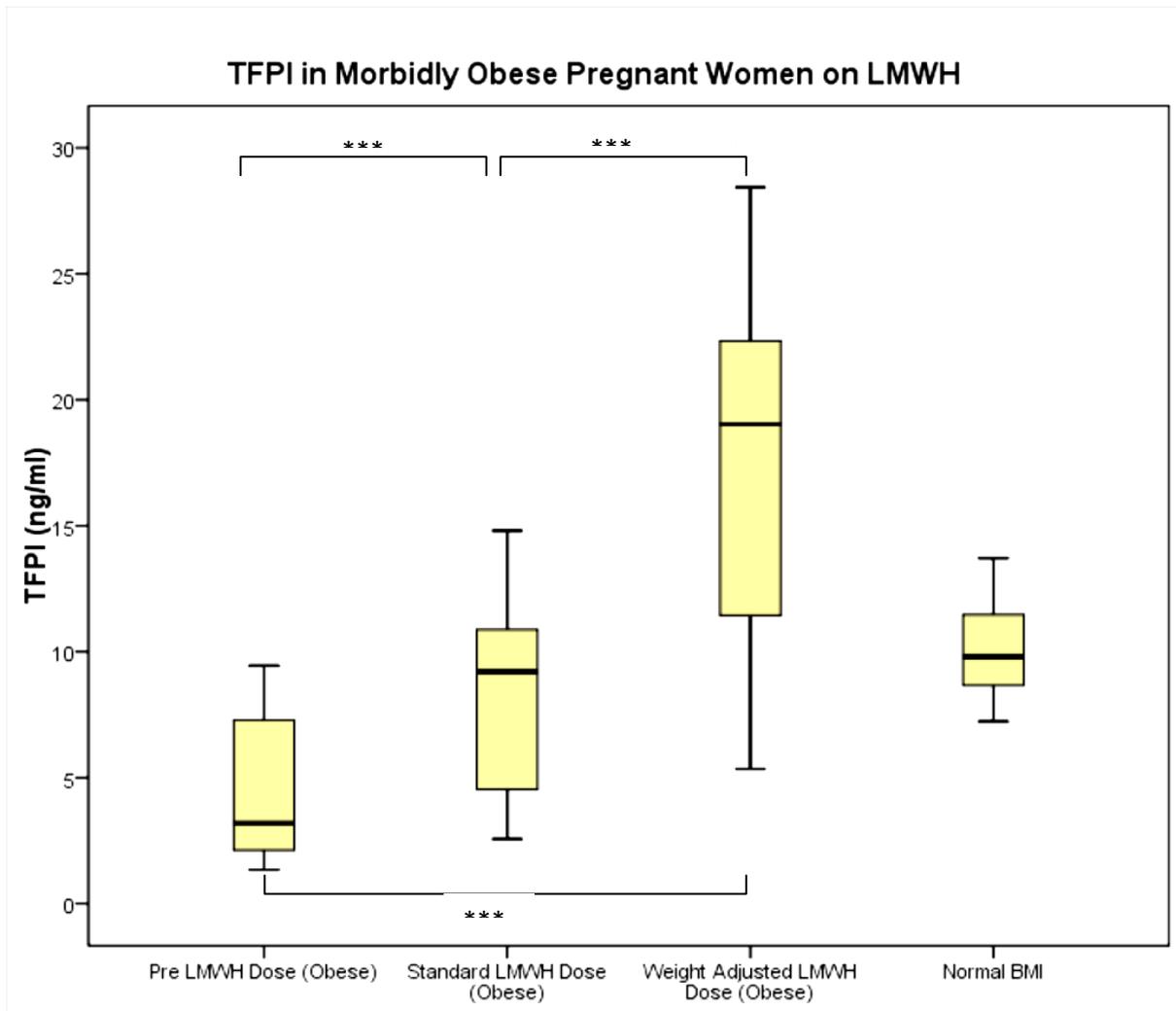
### **iv) Time to Peak (Figure 5.17 and 5.18)**

Within the obese group, paired t-Test showed that time to Peak pre-LMWH dose was significantly shorter compared with post standard LMWH dose (4500iu tinzaparin) ( $p < 0.0001$ ) and post weight-adjusted LMWH dose (75iu/kg tinzaparin) ( $p < 0.0001$ ) (Figure 5.17). Post standard LMWH dose (4500iu tinzaparin), time to peak was significantly shorter compared with post weight-adjusted dose (75iu/kg tinzaparin) ( $p < 0.0001$ ). Repeated ANOVA showed there was a significant LMWH prophylaxis effect on time to peak ( $p < 0.0001$ ) (Figure 5.18). Post hoc test showed that time to peak was longer at 32 ( $p < 0.001$ ), 34 ( $p < 0.001$ ) and 36 weeks ( $p < 0.001$ ) compared with pre LMWH dose (30 wks) in the obese group. There was no significant change in time to peak levels in the normal BMI control group.

### 5.2.6 Effects of LMWH on Anti Xa in the Morbidly Obese Pregnant Women in Third Trimester

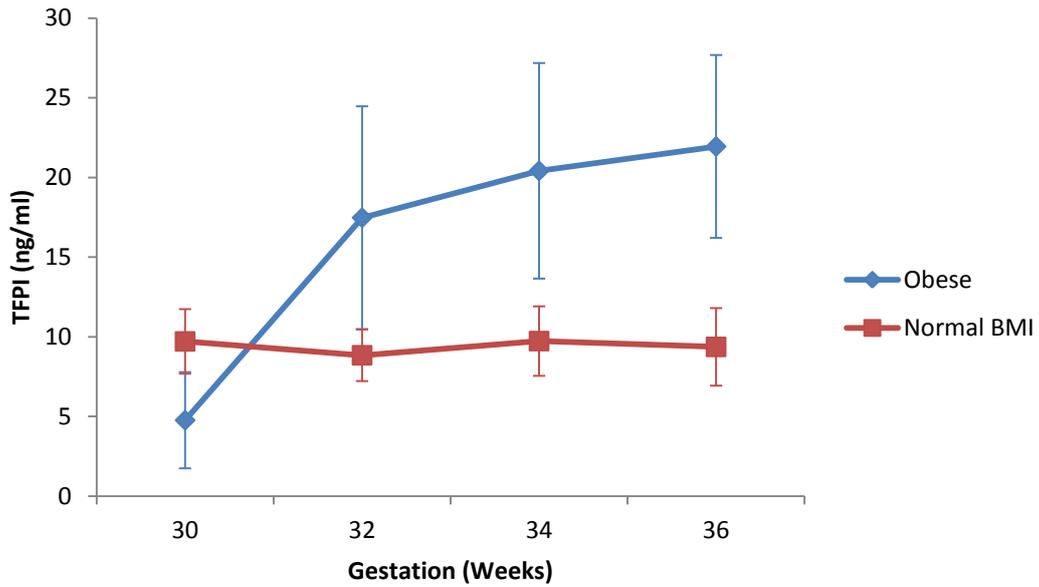
(Figure 5.19 and 5.20)

Venous blood taken post standard LMWH dose (4500iu tinzaparin) yielded significantly lower anti-Xa levels compared with levels found in women post weight-adjusted dose (75iu/kg tinzaparin) ( $p < 0.0001$ ) (Figure 5.19). Pre-LMWH administration, peak anti-Xa levels were generally lower than limit of detection of the assay (0.05 iu/ml). Repeated ANOVA showed there was no significant effect of gestation on anti-Xa levels (Figure 5.20).

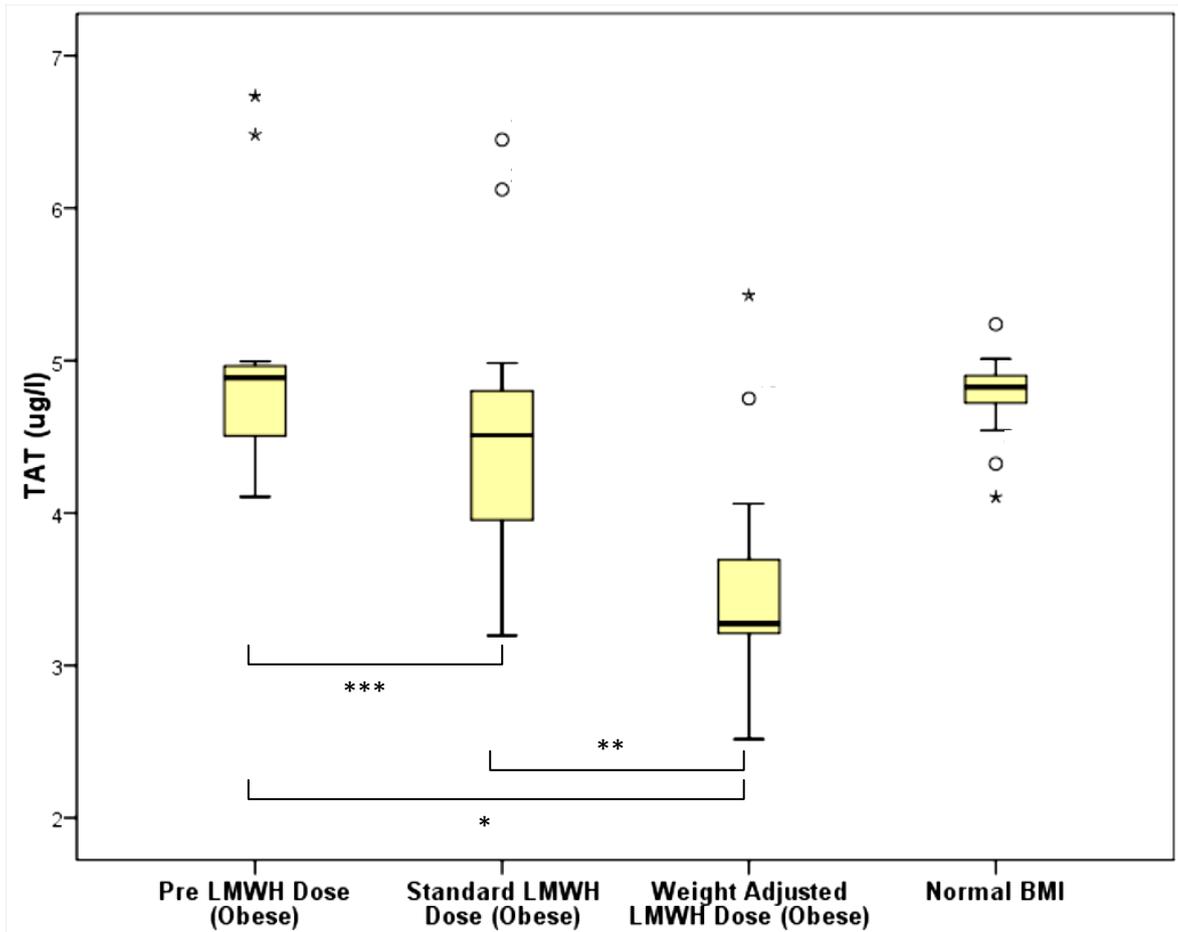


**Figure 5.7 TFPI in Morbidly Obese Pregnant Women on LMWH at 30-32weeks gestation**

In the Obese group TFPI was measured at 30 weeks prior to LMWH administration (Pre LMWH Dose) and four hours post LMWH (4500iu tinzaparin) (Standard LMWH Dose) at 32 weeks (n=20). After a 48-hour washout period, the Obese group was changed to a weight adjusted dose at 32 weeks and TFPI was determined four hours post LMWH (75iu/kg tinzaparin) (n=20). Normal BMI group was (not on LMWH) included as reference point (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\*\* (p<0.0001).

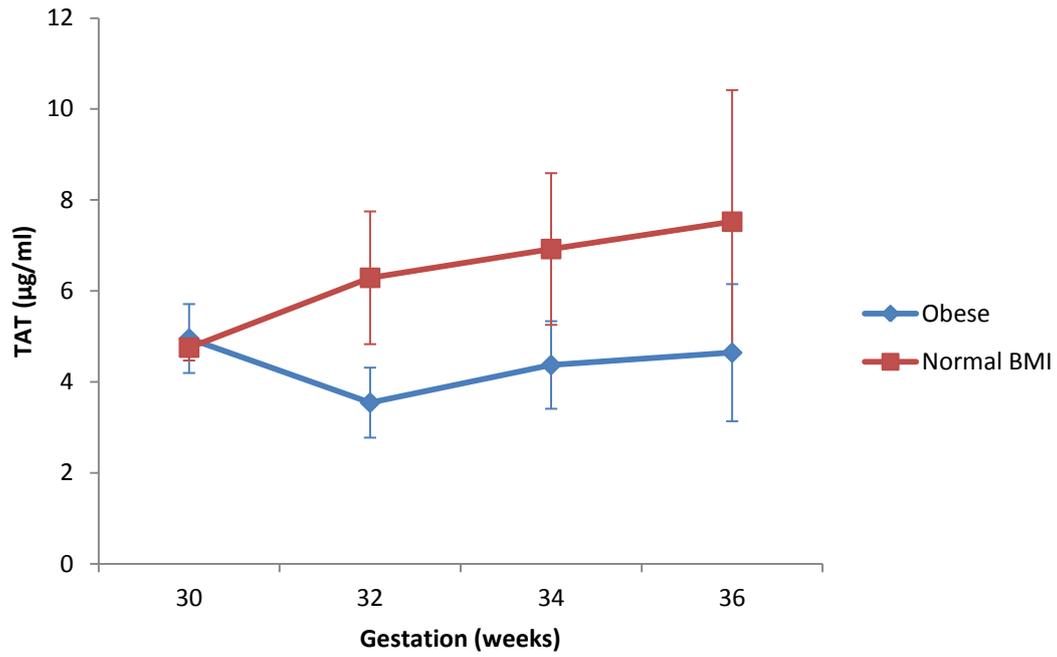


**Figure 5.8 TFPI in Morbidly Obese Pregnant Women on Weight Adjusted LMWH in Third Trimester.** TFPI was measured from 30 to 36 weeks gestation. Obese group was morbidly obese pregnant women on weight adjusted dose LMWH (75iu/kg tinzaparin) from 32 weeks onwards. Samples taken at 30 wks were pre-LMWH dose. Control group was normal weight pregnant women without LMWH prophylaxis. Data represented as mean +/- SD.

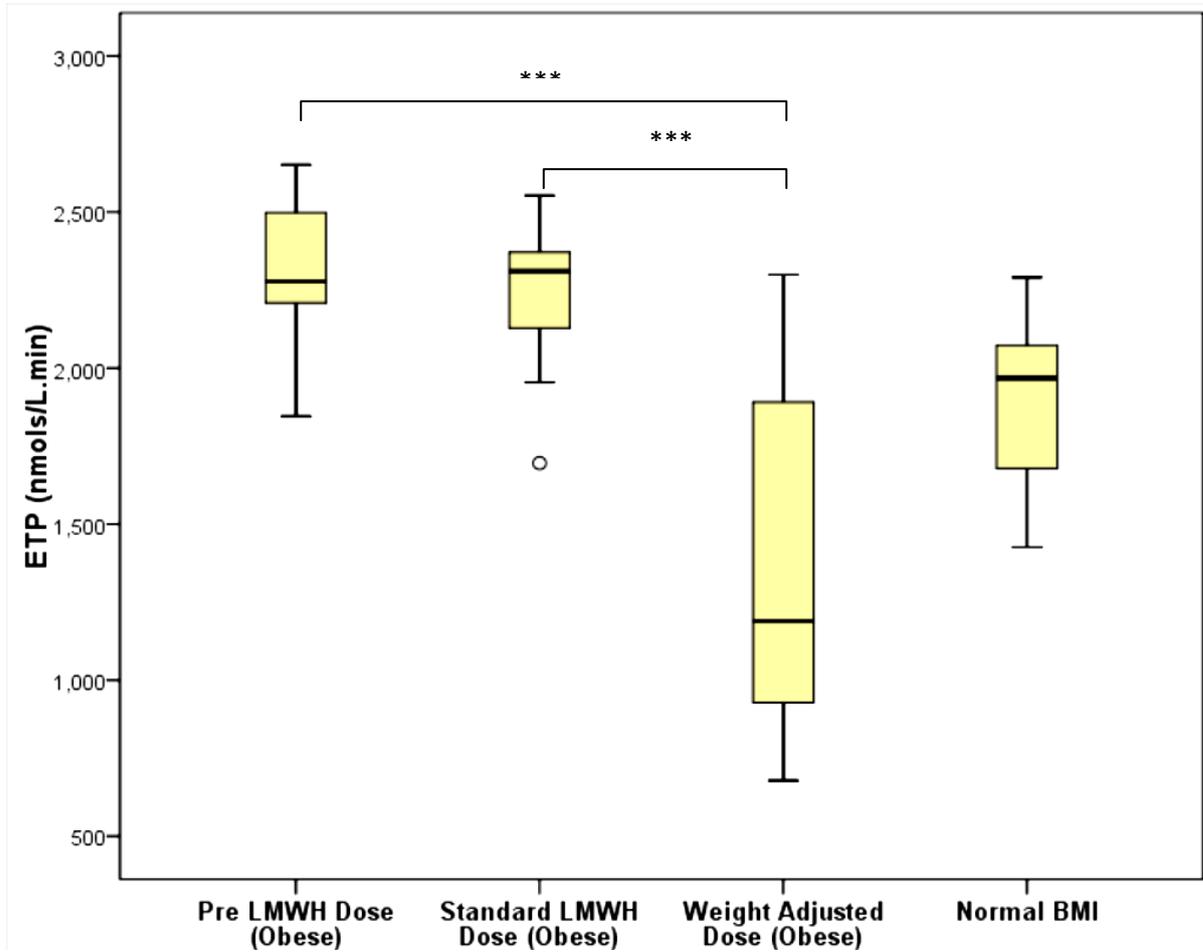


**Figure 5.9 TAT in Morbidly Obese Pregnant Women on LMWH at 30-32 Weeks Gestation.**

In the Obese group TAT was measured at 30 weeks prior to LMWH administration (Pre LMWH Dose) and four hours post LMWH (4500iu tinzaparin) (Standard LMWH Dose) at 32 weeks (n=20). After a 48-hour washout period, the Obese group was changed to a weight adjusted dose at 32 weeks and TAT was determined four hours post LMWH (75iu/kg tinzaparin) (n=20). Normal BMI group was (not on LMWH) included as reference point (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*, \*\* and \*\*\* denotes significant findings (p<0.05).

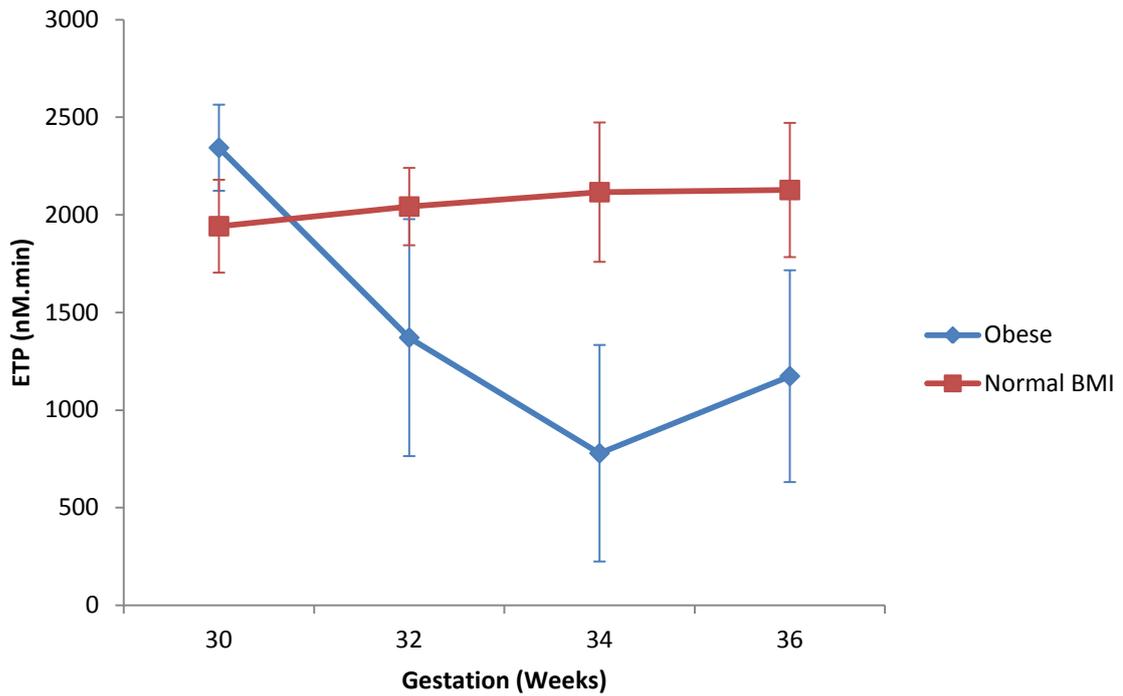


**Figure 5.10 TAT in Morbidly Obese Pregnant Women on Weight Adjusted LMWH in Third Trimester.** TAT was measured from 30 to 36 weeks gestation. Obese group was morbidly obese pregnant women on weight adjusted dose LMWH (75iu/kg tinzaparin) from 32 weeks onwards. Samples taken at 30 wks were pre-LMWH dose. Control group was normal weight pregnant women without LMWH prophylaxis. Data is represented as mean +/- SD.

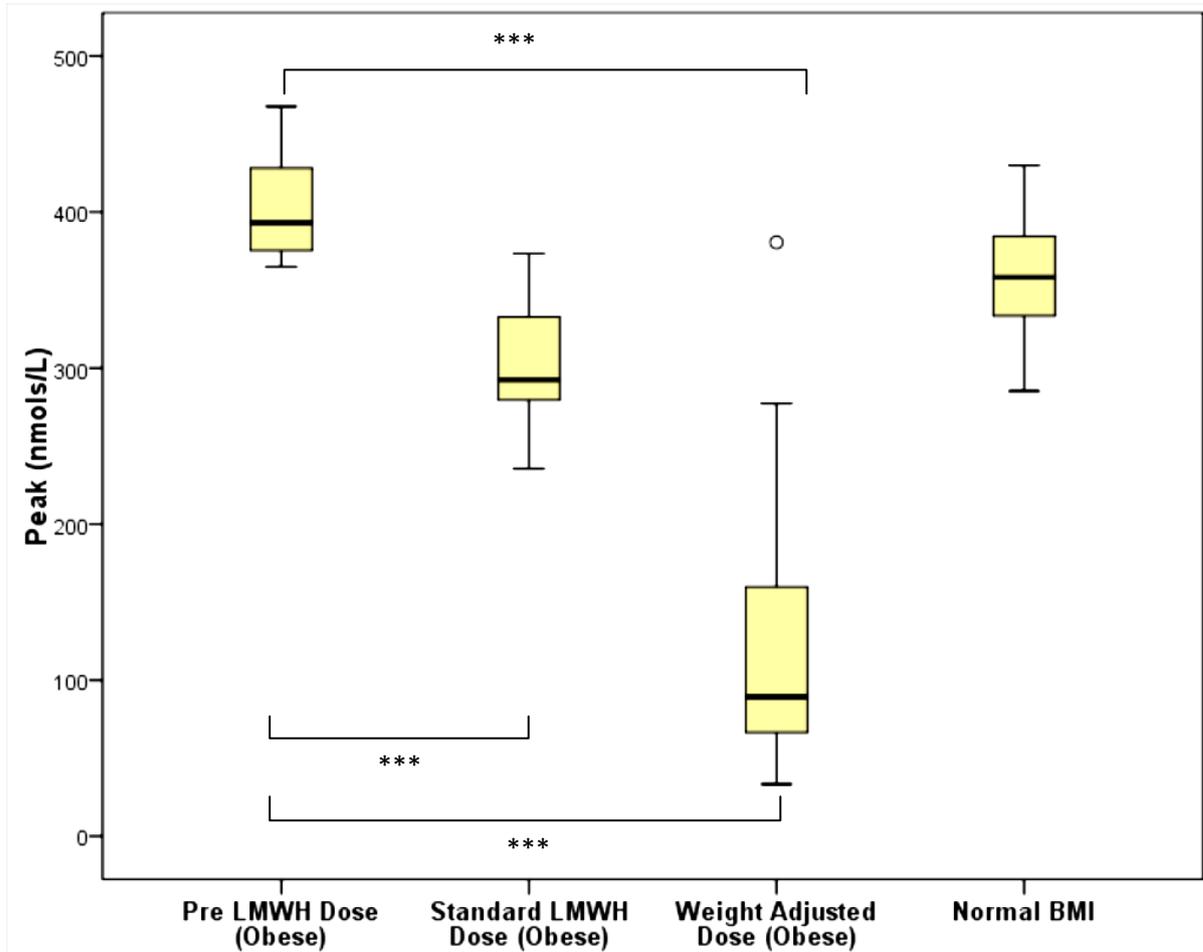


**Figure 5.11 ETP in Morbidly Obese Pregnant Women on LMWH at 30-32 Weeks Gestation.**

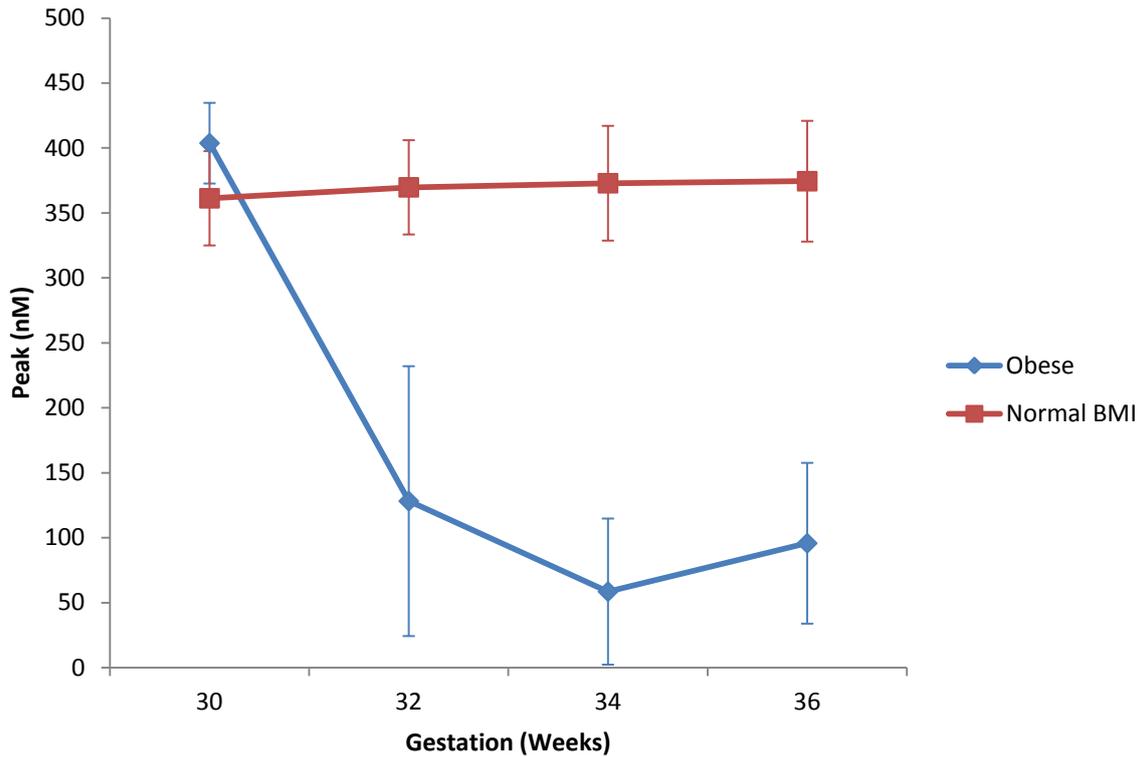
In the Obese group ETP was measured at 30 weeks prior to LMWH administration (Pre LMWH Dose) and four hours post LMWH (4500iu tinzaparin) (Standard LMWH Dose) at 32 weeks (n=20). After a 48-hour washout period, the Obese group was changed to a weight adjusted dose at 32 weeks and ETP was determined four hours post LMWH (75iu/kg tinzaparin) (n=20). Normal BMI group was not on LMWH included as reference point (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\*\* (p<0.0001).



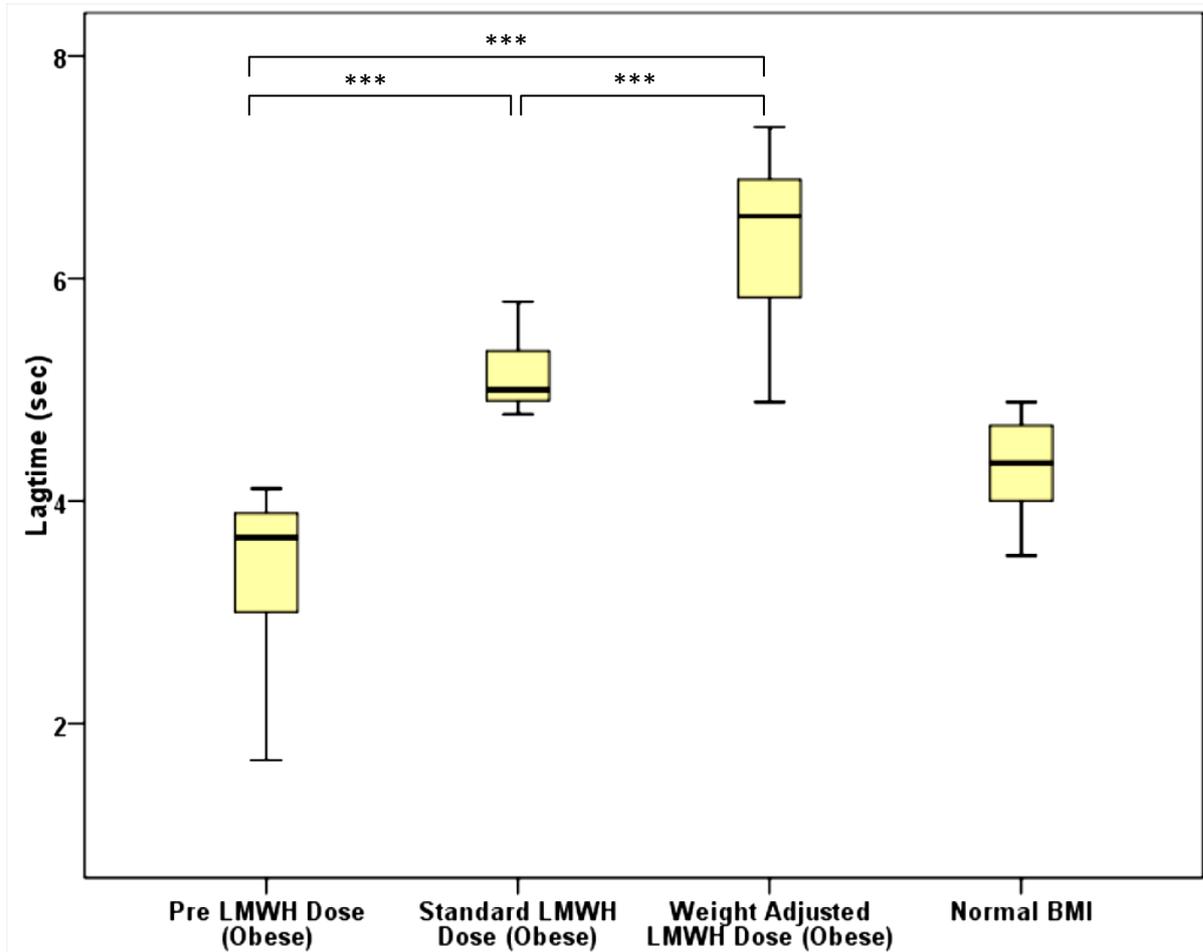
**Figure 5.12 ETP in Morbidly Obese Pregnant Women on Weight Adjusted LMWH in Third Trimester.** ETP was measured from 30 to 36 weeks gestation. Obese group was morbidly obese pregnant women on weight adjusted dose LMWH (75iu/kg tinzaparin) from 32 weeks onwards. Samples taken at 30 wks were pre-LMWH dose. Control group was normal weight pregnant women without LMWH prophylaxis. Data represented as mean +/- SD.



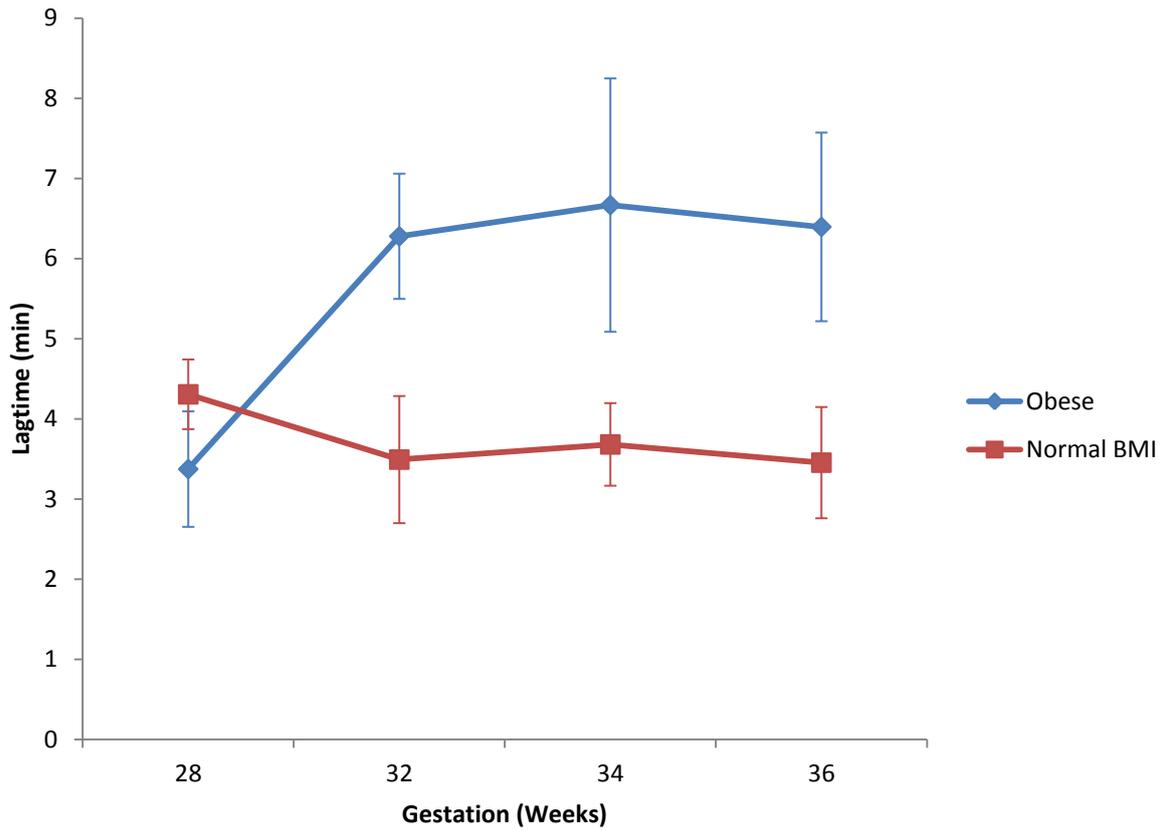
**Figure 5.13 Peak thrombin in Morbidly Obese Pregnant Women on LMWH at 30-32 Weeks Gestation.** In the Obese group peak thrombin was measured at 30 weeks prior to LMWH administration (Pre LMWH Dose) and four hours post LMWH (4500iu tinzaparin) (Standard LMWH Dose) at 32 weeks (n=20). After a 48-hour washout period, the Obese group was changed to a weight adjusted dose at 32 weeks and peak thrombin was determined four hours post LMWH (75iu/kg tinzaparin) (n=20). Normal BMI group was not on LMWH included as reference point (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\*\* (p<0.0001).



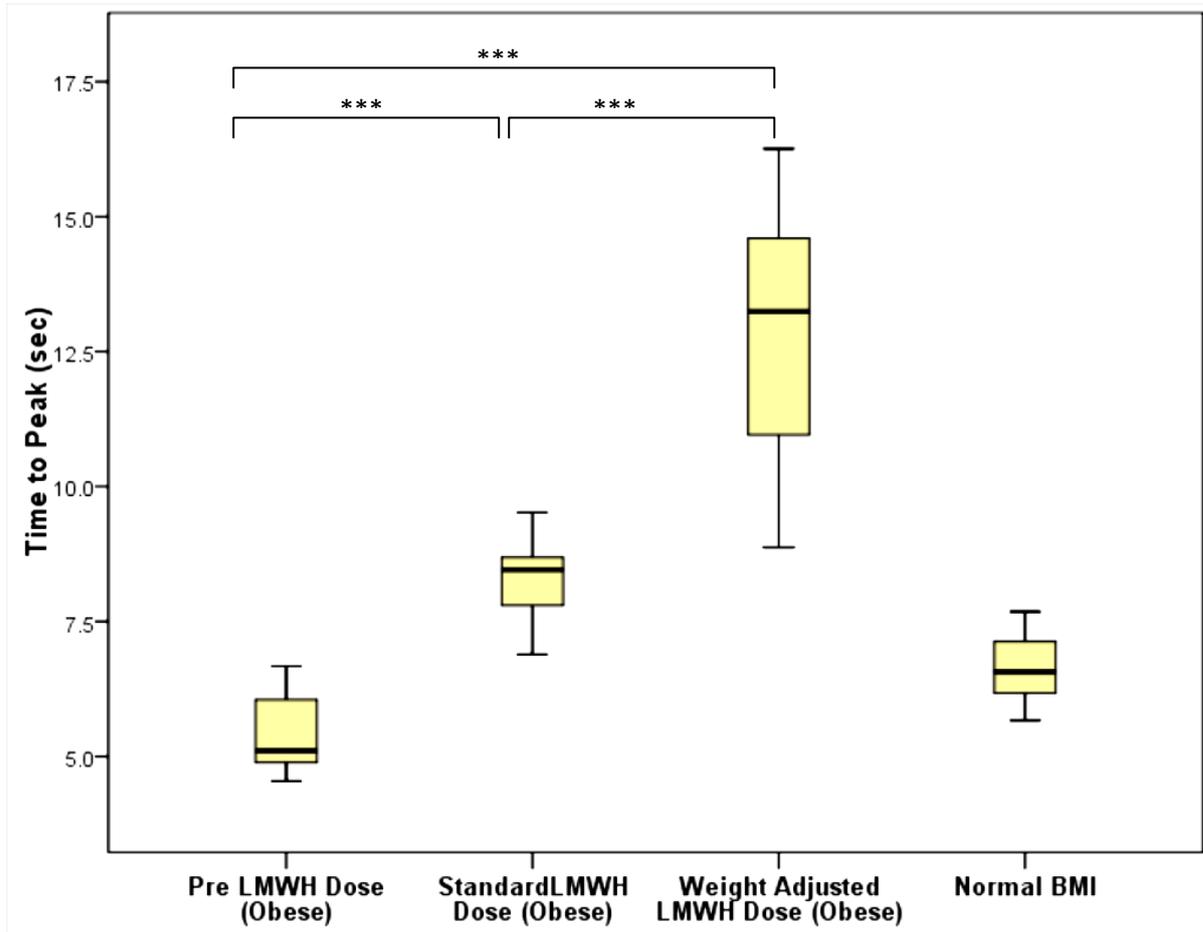
**Figure 5.14 Peak Thrombin in Morbidly Obese Pregnant Women on Weight Adjusted LMWH in Third Trimester.** Peak thrombin was measured from 30 to 36 weeks gestation. Obese group was morbidly obese pregnant women on weight adjusted dose LMWH (75iu/kg tinzaparin) from 32 weeks onwards. Samples taken at 30 wks were pre-LMWH dose. Control group was normal weight pregnant women without LMWH prophylaxis. Data is represented as mean +/- SD.



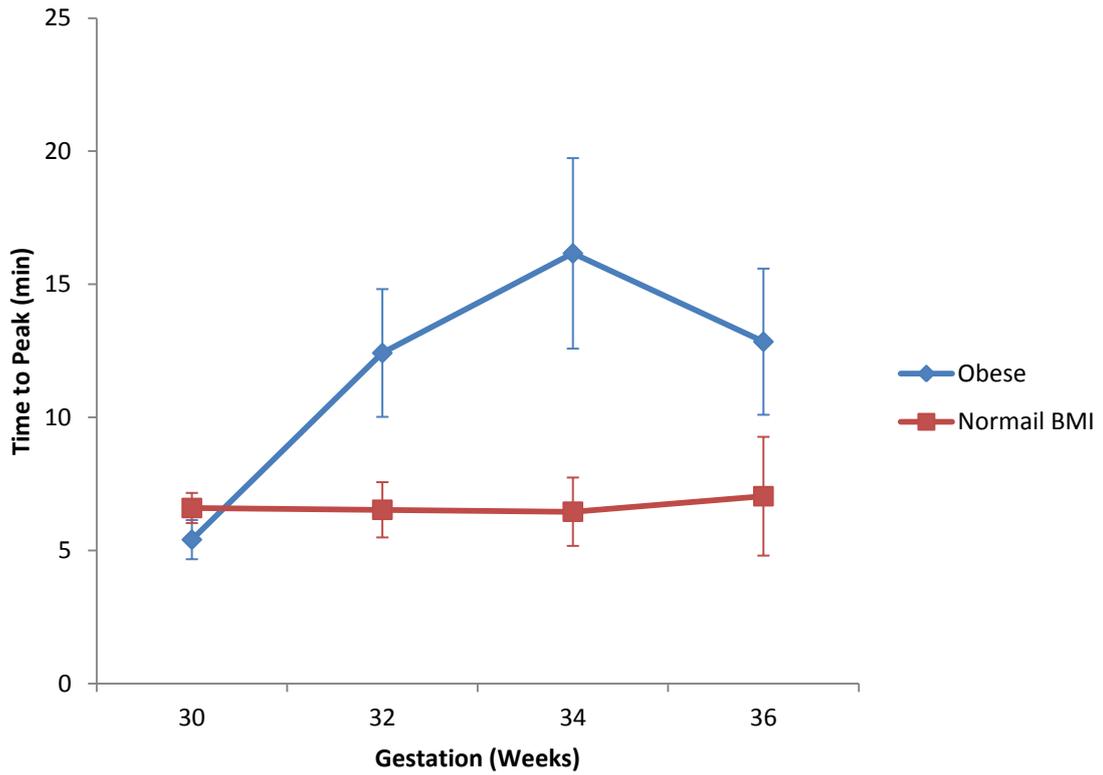
**Figure 5.15 Lagtime in Morbidly Obese Pregnant Women on LMWH at 30- 32 Weeks Gestation**  
 In the Obese group lagtime was measured at 30 weeks prior to LMWH administration (Pre LMWH Dose) and four hours post LMWH (4500iu tinzaparin) (Standard LMWH Dose) at 32 weeks (n=20). After a 48-hour washout period, the Obese group was changed to a weight adjusted dose at 32 weeks and lagtime was determined four hours post LMWH (75iu/kg tinzaparin) (n=20). Normal BMI group was not on LMWH included as reference point (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\*\* (p<0.0001).



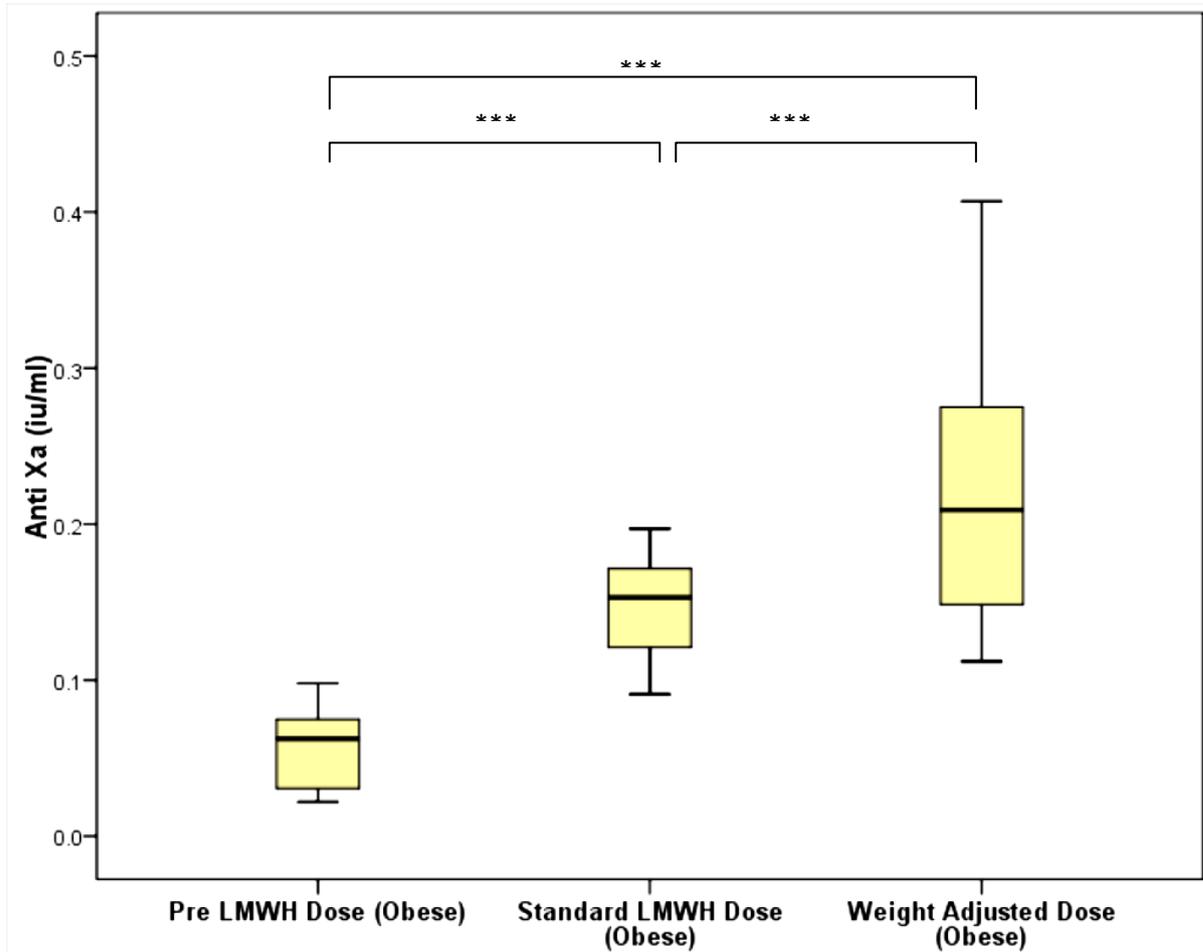
**Figure 5.16 Lagtime in Morbidly Obese Pregnant Women on Weight Adjusted LMWH in Third Trimester.** Lagtime was measured from 30 to 36 weeks gestation. Obese group was morbidly obese pregnant women on weight adjusted dose LMWH (75iu/kg tinzaparin) from 32 weeks onwards. Samples taken at 30 wks were pre-LMWH dose. Control group was normal weight pregnant women without LMWH prophylaxis. Data represented as mean +/- SD.



**Figure 5.17 Time to Peak thrombin in Morbidly Obese Pregnant Women on LMWH at 30-32 Weeks Gestation** In the Obese group time to peak was measured at 30 weeks prior to LMWH administration (Pre LMWH Dose and four hours post LMWH (4500iu tinzaparin) (Standard LMWH Dose) at 32 weeks (n=20). After a 48-hour washout period, the Obese group was changed to a weight adjusted dose at 32 weeks and time to peak was determined four hours post LMWH (75iu/kg tinzaparin) (n=20). Normal BMI group was not on LMWH included as reference point (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\*\* (p<0.0001).

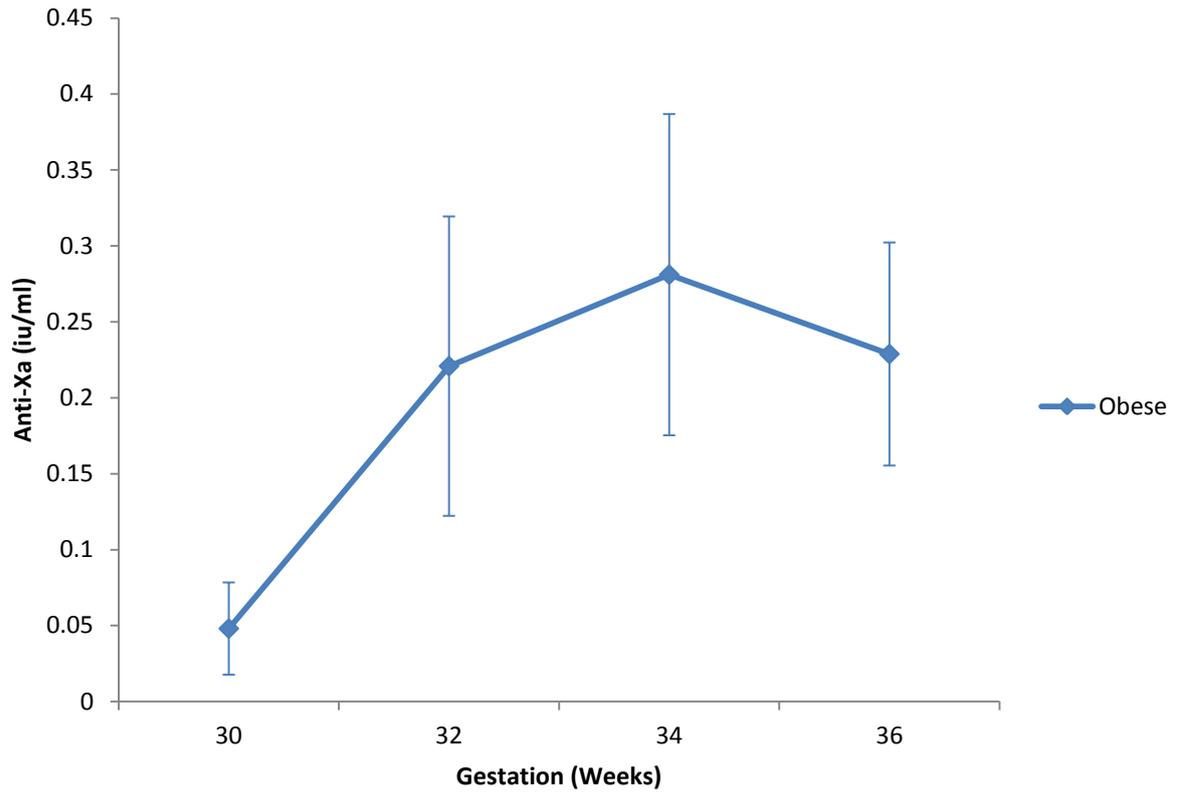


**Figure 5.18 Time to Peak in Morbidly Obese Pregnant Women on Weight Adjusted LMWH in Third Trimester.** Time to peak was measured from 30 to 36 weeks gestation. Obese group was morbidly obese pregnant women on weight adjusted dose LMWH (75iu/kg tinzaparin). Control group was normal weight pregnant women without LMWH prophylaxis. Data is represented as mean +/-SD.



**Figure 5.19 Anti-Xa in Morbidly Obese Pregnant Women on LMWH at 30-32 Weeks Gestation.**

In the Obese group anti-Xa was measured at 30 weeks prior to LMWH administration (Pre LMWH Dose) and four hours post LMWH (4500iu tinzaparin) (Standard LMWH Dose) at 32 weeks (n=20). After a 48-hour washout period, the Obese group was changed to a weight adjusted dose at 32 weeks and anti-Xa was determined four hours post LMWH (75iu/kg tinzaparin) (n=20). Normal BMI group was not on LMWH included as reference point (n=20). The boundary of each box closest to zero indicates 25<sup>th</sup> percentile, the boundary furthest from zero indicated 75<sup>th</sup> percentile. The line within each box represents the median. Error bars represent the 90<sup>th</sup> and 10<sup>th</sup> percentiles. Samples outside of these parameters are represented as outliers (open circles). \*\*\* (p<0.0001).



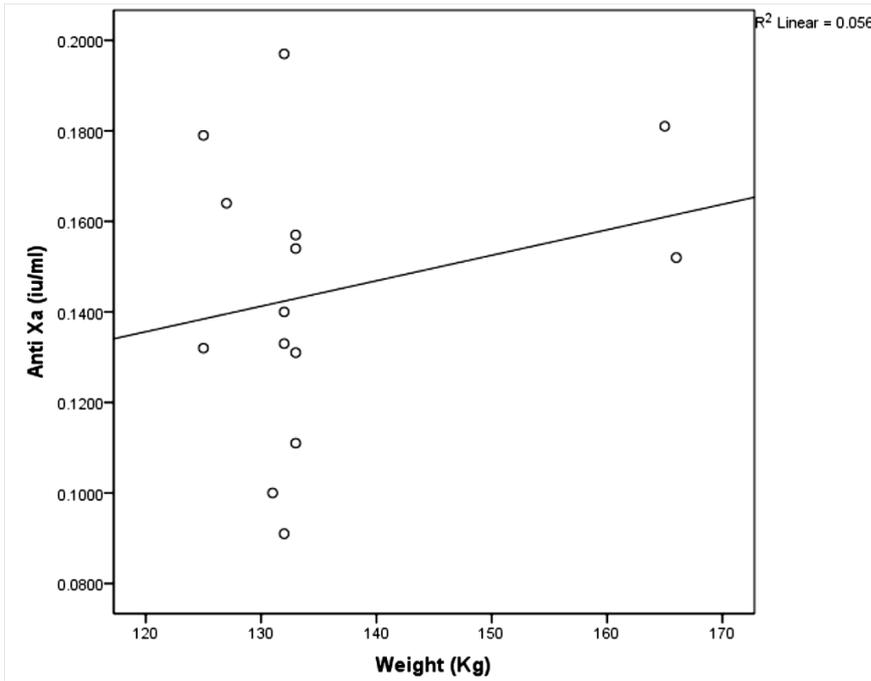
**Figure 5.20 Anti-Xa in Morbidly Obese Pregnant Women on Weight Adjusted LMWH in Third Trimester.** Anti-Xa was measured prior to LMWH at 30 weeks and at four hours post LMWH administration from 32 to 36 weeks gestation in morbidly obese pregnant women on weight adjusted dose LMWH (75iu/kg tinzaparin). Data represented as mean +/- SD.

### **5.2.7 Correlation between Anti-Xa and Total Body Weight in Morbidly Obese Pregnant Women on LMWH (Figure 5.21 and 5.22)**

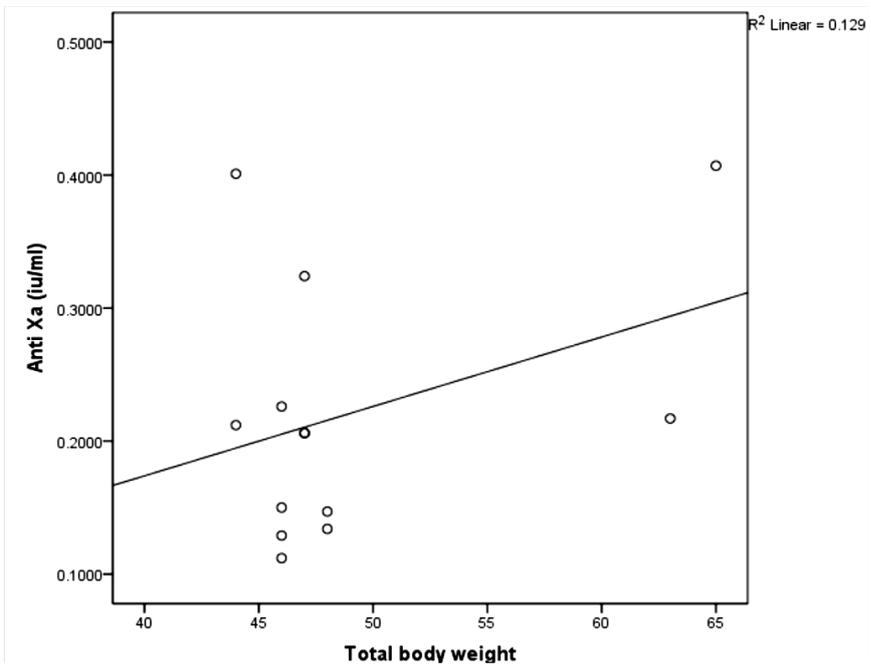
Using Pearson's correlation coefficient, peak anti-Xa levels did not significantly correlate with total body weight at standard dose 4,500iu tinzaparin ( $r=0.236$ ) ( $p>0.05$ ) (Figure 5.21) or weight adjusted dose 75iu/kg tinzaparin ( $r=0.359$ ) ( $p>0.05$ ) (Figure 5.22).

### **5.2.8 Correlation between ETP and Total Body Weight in Morbidly Obese Pregnant Women on LMWH (Figure 5.23 and 5.24)**

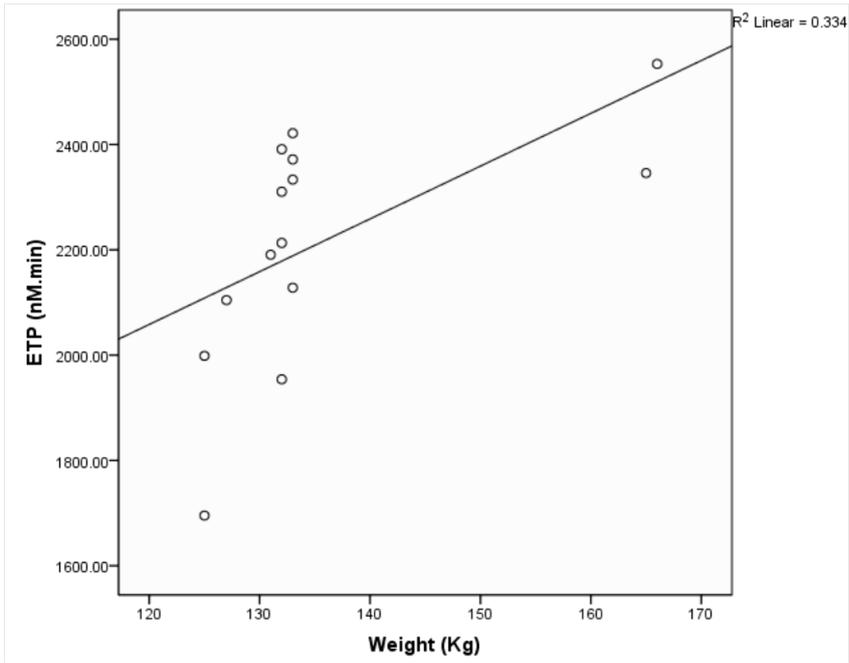
ETP levels had a significant positive correlation with total body weight at standard dose 4,500iu tinzaparin ( $r=0.578$ ) ( $p<0.05$ ) (Figure 5.23). At weight adjusted dose 75iu/kg tinzaparin, ETP levels demonstrated a weak negative correlation ( $r= -0.430$ ) but does not reach significance ( $p=0.059$ )(Figure 5.24).



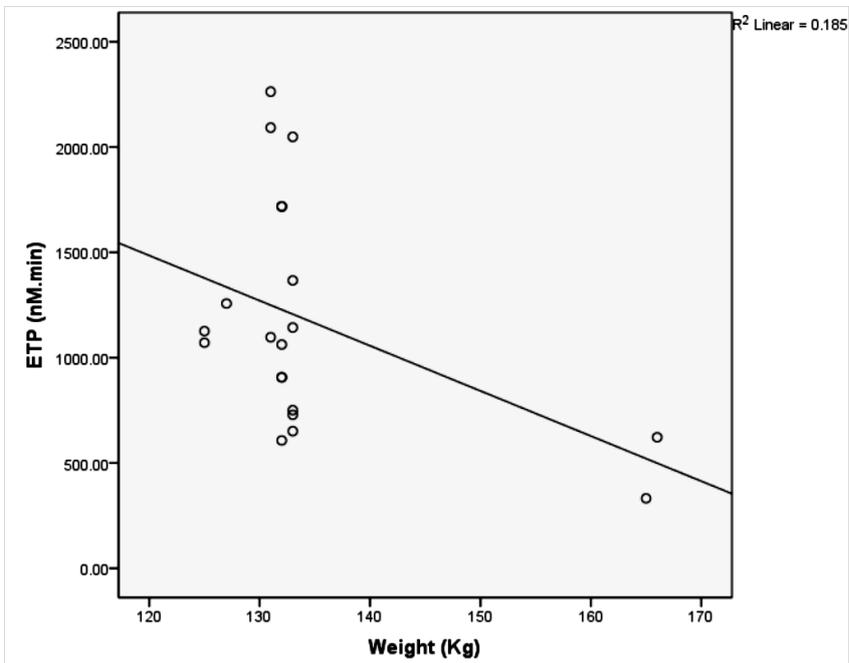
**Figure 5.21 Peak Anti-Xa Levels in Morbidly Obese Women in Third Trimester on Fixed dose LMWH Prophylaxis in Third Trimester.** There was no correlation between peak anti-Xa levels and total body weight in morbidly obese pregnant women on standard dose 4500iu tinzaparin.



**Figure 5.22 Peak Anti-Xa Levels in Morbidly Obese Women in Third Trimester on Weight Adjusted Dose LMWH Prophylaxis in Third Trimester.** There was no correlation between peak anti-Xa levels and total body weight (kg) in morbidly obese pregnant women on weight adjusted dose 75iu/kg tinzaparin ( $p < 0.01$ ).



**Figure 5.23 ETP Levels in Morbidly Obese Women in Third Trimester on Fixed dose LMWH Prophylaxis in Third Trimester.** There was a weak positive correlation between ETP levels and total body weight in morbidly obese pregnant women on standard dose 4500iu tinzaparin ( $p < 0.05$ ).



**Figure 5.24 ETP Levels in Morbidly Obese Women in Third Trimester on Weight Adjusted Dose LMWH Prophylaxis in Third Trimester.** There was no correlation between ETP levels and total body weight in morbidly obese pregnant women on weight adjusted dose 75iu/kg tinzaparin.

### 5.3 Discussion

Currently, it is not known whether the risk of VTE increases with increasing obesity or whether morbid obesity as a single risk factor warrants thromboprophylaxis. It is widely accepted that pregnancy results in increased coagulation activity and reduction in anticoagulant and fibrinolytic response [164]. This is more evident in obesity whereby adipose tissue secretes plasminogen activator inhibitor 1 (PAI-1), further impairing fibrinolysis [298]. Haemostatic studies have shown that increased levels of circulating TF in morbidly obese patients were reduced after bariatric surgery and weight loss [299, 300]. Additionally, evidence from murine studies showed up-regulation of TF gene expression in obesity [301, 302]. In this study, we showed that the haemostatic parameters reflect a significant activation of the coagulation system in the morbidly obese pregnant women compared with normal weight pregnant women in late pregnancy. TFPI levels of normal weight pregnant women in late pregnancy in this study is comparable to levels we reported previously [303]. The reduction in TFPI levels found in obese group may suggest that the reduction in natural anticoagulant activity in pregnancy is exacerbated by obesity. TAT and prothrombin F-1.2 are markers of thrombin already formed *in vivo*. We did not find a difference in TAT levels between obese and normal group in this study. This is in contrast to a previous study in non-pregnant population which showed a higher F1.2 levels in the morbidly obese [300]. Our findings also differ from animal study which reported significantly higher levels of TAT complex in obese mice [304]. TAT levels at 30 weeks in our morbidly obese pregnant women are comparable to that found in pregnant women at moderate risk of VTE at similar time point [276]. Yoneda et al also reported a higher TAT levels in women at high risk of thrombosis than that found in morbidly obese pregnant women in this study at the similar gestation [276]. This suggests that *in vivo* thrombin formation in morbidly obese pregnant women is as substantial as in pregnant women at moderate risk of thrombosis (including some with thrombophilia).

However, at the same time point, the 'potential thrombin formation', as measured by ETP and peak thrombin is higher in morbidly obese group. This supports the premise that obese pregnant

women have more readily activated coagulation system than normal weight pregnant women [305]. At around 30 weeks, our values of ETP and Peak thrombin in normal BMI pregnant women in this study is comparable to values reported at late pregnancy (30-34weeks gestation) in a previous study looking at tissue factor-dependent thrombin generation across gestation in normal weight pregnant women [193]. We have no basis of comparison in pregnancy for other CAT parameters lagtime and time to peak. However, in non-pregnant morbidly obese patients, lagtime is found to be shorter when compared to patients with normal weight [299]. This is replicated in our study of pregnant women. Lagtime reflects the initiation phase of thrombin generation [306]. Shorter lagtime and time to peak in this obese group is consistent with the hypercoagulability associated with obesity. Collectively, they showed an obvious haemostatic disturbance due to ongoing thrombin activation that could potentially lead to thrombosis.

TFPI levels were increased by nearly 2-fold after standard LMWH dose (tinzaparin 4,500iu/kg) and over 3-fold after weight adjusted-dose (tinzaparin 75iu/kg). This higher TFPI release with increased LMWH exposure may suggest that TFPI release post LMWH may be invoked in a dose dependant fashion. Our findings are comparable to data from non-pregnant obese subjects where increased levels of TFPI (3-fold above basal) post tinzaparin at 75iu/kg were demonstrated in obese and in normal healthy subjects. Their data suggest a normal responsiveness of vascular endothelial cells and other cellular compartments to tinzaparin with regard to the pharmacodynamic profiles of plasma TFPI in obese subjects [307].

Previous reports have shown that TAT levels were reduced after acute LMWH administration in healthy pregnant women [303]. More importantly, reports have shown that in women at high risk of VTE during pregnancy, higher dose of chronic LMWH exposure in pregnant women at high risk of VTE reduced *in-vivo* thrombin formation as measured by TAT [276]. Our finding in the morbidly obese pregnant women on LMWH is consistent with these reports. LMWH, as a factor-Xa inhibitor, displays a delaying effect on thrombin generation (lagtime and time to peak) as well as reducing the

total amount of thrombin formed (peak and ETP) [308]. Similar effects of LMWH on decreasing the quantitative parameters of CAT (ETP and Peak thrombin) were seen outside pregnancy [306]. We showed similar results in our study. In the morbidly obese pregnant women, potential to clot as shown by ETP levels were significantly lower in weight-adjusted dose compared with pre-dose LMWH, but does not seem to portray the same lowering effect at standard dose LMWH. More importantly, in the group of morbidly obese pregnant women a 41% v 5% reduction of ETP and 68% v 26% reduction of peak thrombin from baseline levels after weight adjusted dose v standard dose LMWH, corresponding to mean anti-Xa levels of 0.23 v 0.15 iu/ml respectively. It is noteworthy that previous *in-vitro* pharmacokinetic studies done on LMWH showed that tinzaparin inhibited ETP and peak thrombin by a targeted 50% at anti-Xa concentration of over 0.2iu/ml [288]. Another *in-vitro* study on enoxaparin reported 40% and 60% reduction of ETP correlated with anti-Xa of 0.39 and 0.59iu/ml respectively [309]. This is followed up by an *in-vivo* study also using enoxaparin which reported a decrease of 55% of ETP is associated with anti-Xa of 0.5iu/ml [306]. These anti-Xa levels were within the recommended prophylactic range, unlikely to predispose haemorrhagic complications and agreeable with previous studies on pregnant women [221, 276, 284]. None of the women in this study experience any abnormal bleeding.

In normal weight pregnant women, serial data in this study found levels of TFPI, ETP, peak thrombin, lagtime and time to peak does not vary in the third trimester of pregnancy. This is not surprising as other authors have found that ETP and peak thrombin increased in first trimester compared to pre-pregnancy levels, before stabilising in the third trimester [165, 193, 292]. Prior to LMWH dosing, the results of the CAT assay suggest that morbidly obese women are more hypercoagulable than women of normal weight. It is unclear whether further increases occur in the last trimester as pregnancy advances since we do not have a group of untreated obese women. However, it would seem likely that the increase in thrombin potential is at least maintained until delivery. Our results show that tinzaparin effectively inhibits the potential for thrombin formation throughout the last trimester without the need for dose adjustment.

In accordance with this, the anti-Xa levels did not significantly vary from 32 to 36 weeks gestation in the morbidly obese women who were on weight adjusted LMWH. This finding was quite reassuring as it suggested that in morbidly obese pregnant women on weight based LMWH dose, there was no effect of gestation on anti-Xa in the third trimester. This might indicate that in this special group of women, anti-Xa monitoring might not be necessary once the target anti-Xa level was achieved (provided there were no acute events during the pregnancy that could upset haemostasis). Within the obese group women who received weight adjusted LMWH prophylaxis, the ETP and peak thrombin levels were reduced; lagtime and time to peak lengthened; and TFPI increased with gestation compared to pre-dose. It is possible that the treatment effect of LMWH is under-estimated by TAT as in contrast to other haemostatic parameters in this study, TAT levels were increased with gestation in normal weight pregnant women. In the obese group, standard dose LMWH prophylaxis reduced TAT levels and increased TFPI to levels similar to normal weight pregnant women. However, ETP levels remained unchanged in the obese group at this dose. Weight adjusted LMWH dose produces a greater effect on TAT, TFPI and all CAT parameters. The TAT assay measured the degree of thrombin generated at a given time. On the other hand, ETP from the CAT assay, is an activity assay which represents a woman's potential to generate thrombin should the coagulation activation event transpires [292]. This may explain why unlike ETP, TAT levels in this study were not able to demonstrate the hypercoagulable state of the morbidly obese parturient prior to LMWH initiation. ETP, peak thrombin, lagtime and time to peak are more global haemostatic indices; they communally demonstrated in this study that weight-based dosing is probably more appropriate in obese pregnant women. Hence, ETP may likely be a better tool for monitoring and measuring LMWH efficacy [296, 310].

Clinical studies in the non-pregnant obese reported that fixed-dose LMWH prophylaxis resulted in high VTE rates [311] and a negative correlation with peak anti-Xa [312]. This study found

that peak anti-Xa levels had no correlation with total body weight when LMWH was administered at both fixed dose or weight adjusted dose. Correlation between weight adjusted dose peak anti-Xa levels and total body weight in the morbidly obese non-pregnant population were previously reported using LMWH 0.5mg/kg enoxaparin [229]. The result from this study replicated the findings of Rondina et. al. [229] However, it is difficult to directly compare these results due to different use of LMWH and anti-Xa assay. It is of concern that ETP levels showed a positive correlation with total body weight when LMWH tinzaparin was administered at standard 4,500iu dose in obese pregnant women. This indicates that thrombin generation is still increased with patient weight when LMWH was given at fixed dose. It also suggested that standard LMWH dose did not confer adequate anti-coagulation in the morbidly obese women in third trimester. Additionally, when weight-adjusted dose LMWH was administered, ETP showed no correlation with total body weight. These results indicated that LMWH at weight-adjusted dose seems to be better at curbing the possible excess thrombin formation in these women.

A previous pharmacokinetic study in pregnancy reported peak LMWH concentration is lower and the half-life is shorter in pregnancy compared with non-pregnant population [196]. This is further complicated by obesity where there is increase in volume distribution and drug clearance [313]. Despite a non-linear relationship between total body weight and blood volume, evidence suggested that weight-based dosing results in acceptable range in peak anti-Xa levels [227, 229]. Moreover, available data evaluating LMWH in the non-pregnant obese population does not support capping of doses and that more aggressive prophylactic LMWH doses may be needed [231]. There is evidence suggesting that there is a gradient risk for VTE with increasing levels of obesity [305]. On the other hand, a retrospective subgroup analysis of non-pregnant obese women recruited in a large VTE prophylaxis trial did not demonstrate a difference in LMWH efficacy compared with non-obese with LMWH dose modification [314]. Hence, at time of writing, guidelines refrained from specifying dosage recommendation in pregnant obese patients [216] or if dose modification is recommended, this is not based on high level evidence [217].

Findings in this study suggested that in morbidly obese pregnant women, coagulation activation and thrombin generation is substantially increased. ETP is sensitive to the anticoagulant effects of LMWH at different dosages and is a potential tool for monitoring LMWH in the morbidly obese. No women in this study suffered from abnormal bleeding, thrombotic event or poor obstetric outcome. Although this study is too small to establish efficacy and safety, the prothrombotic state in pregnant morbidly obese women was substantially attenuated by weight adjusted LMWH doses without abnormal bleeding. Since there is no indication that the obesity epidemic is faltering and more women in this group is entering pregnancy, more large studies are needed to prove dosage efficacy and safety in obese pregnant women.

# **Chapter 6**

**Result:**

## **Effects of LMWH on Uteroplacental Haemostasis**

## 6.1 Background

The haemostatic system within the uteroplacental circulation faces distinctly different physiological challenges as pregnancy proceeds. Uteroplacental haemostasis is tightly regulated during antenatal period. Blood fluidity must be maintained through the low resistance haemochorial system while simultaneously the haemostatic system must be primed for delivery when the significant blood flow into the uteroplacental circulation must be staunched over a matter of seconds [53]. Perturbation of haemostasis in the uteroplacental compartment has been proposed as an explanation in placenta mediated pregnancy complications in thrombophilic women. LMWH have been widely used despite the scientific uncertainty, for the prevention of placenta mediated pregnancy complications including pre-eclampsia, fetal growth restriction and pregnancy loss. The focus of research in this area has been on clinical trials and there is a dearth of data on the effect of LMWH within the uteroplacental unit.

The hypothesis of this study is that LMWH may be effective in altering local thrombin production in the uteroplacental compartment. Eight thrombophilic women (who had been on antenatal LMWH prophylaxis) and fifteen healthy women undergoing planned CS for obstetric indications were recruited. The LMWH group were thrombophilic pregnant women who were on antenatal LMWH prophylaxis. The control group were healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. During elective CS, after delivery of the baby but prior to delivery of placenta, venous blood was taken simultaneously, from maternal antecubital fossa, maternal uterine vein and fetal umbilical cord vein. Placental biopsies were taken from all patients as previously described [278]. Real-time PCR and ELISA were used to quantify mRNA and protein expression of TFPI, TFPI<sub>2</sub> and TF in placental tissue. In order to investigate whether changes in the expression of placental coagulation proteins are translated into altered procoagulant activity TFPI, TAT and CAT assays were measured. Independent T-test was performed to compare means between VTE group and normal control group.

The aim of the study was to investigate coagulation gene and protein expression in placentas from patients treated with LMWH and determine the effect of any changes in expression on in vivo hypercoagulability. This data will increase our understanding of the effects of LMWH at a local level.

### **Data Analysis**

Data were first assessed for normal distribution. mRNA fold change results were expressed as the ratio of target gene cDNA to the internal control using the  $2^{-\Delta\Delta CT}$  method [279]. The means were compared using independent t-test. Data is presented using column charts with error bars. In all circumstances, a p-value of <0.05 was considered statistically significant.

## **6.2 Results**

### **6.2.1 Patients**

Patients in both groups were matched for age, parity, BMI and gestation. All women included underwent elective caesarean delivery for obstetric indications. None of the women in this study experienced a thrombotic event, abnormal bleeding, long-term adverse effects of LMWH administration or required additional surgical procedures. Demographic data of each group is depicted in Table 6.1.

<b>Mean</b>	<b>Control n=15</b>	<b>LMWH n=8</b>	<b>p-value</b>
<b>Age</b>	35.9(4.2)	36.2(3.9)	NS
<b>Parity</b>	1.7(0.8)	1.8(0.7)	NS
<b>BMI</b>	25.4(2.3)	26(2.2)	NS
<b>Gestation</b>	39.1(0.5)	38.8(0.6)	NS

**Table 6.1 Demographic Data Study 4.** Values presented as mean(SD). NS-not significant.

<b>Patient</b>	<b>Indication for LMWH Prophylaxis</b>
<b>Patient 1</b>	Protein S deficiency and history of pulmonary embolism outside pregnancy.
<b>Patient 2</b>	Anti phospholipid syndrome. History of four consecutive early pregnancy lost.
<b>Patient 3</b>	Combined Factor V Leiden Heterozygote and Prothrombin Gene Mutation and history of pulmonary embolism outside pregnancy.
<b>Patient 4</b>	Anti phospholipid syndrome. History of two mid-trimester and one early pregnancy lost.
<b>Patient 5</b>	Anti phospholipid syndrome. History of three consecutive early pregnancy lost
<b>Patient 6</b>	Factor V Leiden Heterozygote with history of pulmonary embolism outside pregnancy.
<b>Patient 7</b>	Anti phospholipid syndrome
<b>Patient 8</b>	Factor V Leiden Heterozygote with history of DVT in pregnancy.

**Table 6.2 Indication for LMWH Prophylaxis in the LMWH Group**

Within the LMWH group, four women had heritable thrombophilia and four had confirmed antiphospholipid syndrome (Table 6.2). All women on LMWH in this study were commenced on tinzaparin 4500iu/day at confirmation of pregnancy. The four women with anti phospholipid syndrome had confirmed antibodies and were also on aspirin 75mg/day, which was stopped at 36 weeks gestation. Last administration of LMWH in this group is at least 12 hours prior to surgery.

### **6.2.2 Effect of LMWH on Thrombin Generation in the Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing Caesarean Section Compared with Controls.**

#### **i) ETP (Figure 6.1)**

ETP levels were significantly higher in peripheral and uterine circulation compared to cord circulation ( $p < 0.0001$ ) in both control and LMWH groups. LMWH treatment significantly reduced ( $p < 0.05$ ) ETP levels in the uterine circulation compared with the untreated control group. No significant differences were found between the groups in the peripheral or cord circulation.

**ii) Peak Thrombin (Figure 6.2)**

Peak thrombin is higher in peripheral and uterine circulation compared with cord circulation ( $p < 0.0001$ ) in both the control and LMWH groups. There were no significant differences in peak thrombin between LMWH and control group within the peripheral, uterine circulation and cord circulation

**iii) Lagtime (Figure 6.3)**

Lagtime is longer in uterine circulation compared with peripheral circulation ( $p < 0.05$ ) in the control group but not in the LMWH group. Lagtime time in the cord circulation were shorter compared to peripheral and uterine circulations in both control and LMWH groups (all  $p < 0.001$ ). There was no significant difference in lagtime within the cord circulation between the two groups.

**iv) Time to Peak (Figure 6.4)**

There were no significant differences in time to peak between the control and LMWH groups in the peripheral, uterine or cord circulations. Time to peak is longer within the uterine circulation compared to peripheral and cord circulations in both groups ( $p < 0.05$ ).

**6.2.3 Effect of LMWH on Thrombin antithrombin (TAT) in Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing Caesarean Section Compared with Controls (Figure 6.5).**

TAT levels within uterine vein are significantly higher compared to maternal peripheral circulation in both the control group ( $p < 0.0001$ ) and LMWH group ( $p < 0.02$ ). In the LMWH group, TAT was reduced

compared with controls in the uterine vein samples ( $p < 0.001$ ) but not in the peripheral or cord circulation.

#### **6.2.4 Placental TFPI Gene and Antigen Expression (Figure 6.6)**

Placental TFPI mRNA expression was down-regulated 2-3 fold in the LMWH group compared with placentae from the control group ( $p < 0.05$ ). Protein expression of TFPI antigen as measured by ELISA was also decreased in LMWH group compared with the control group.

#### **6.2.5 Effect of LMWH Tissue Factor Pathway Inhibitor antigen (TFPI) in Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women Compared with Controls (Figure 6.7)**

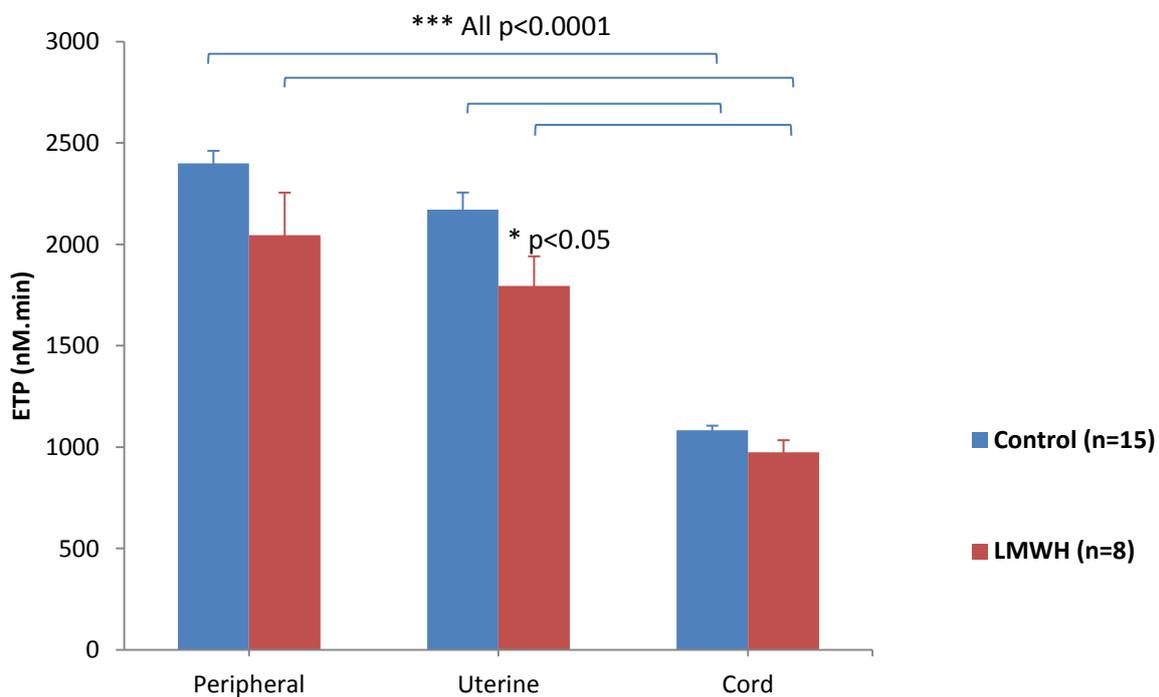
TFPI within uterine circulation is reduced significantly in the LMWH group compared with control ( $p < 0.02$ ). In the control group, TFPI levels are higher in uterine circulation compared to peripheral ( $p < 0.05$ ) and cord ( $p < 0.001$ ) circulations. There is no significant difference between TFPI levels in peripheral and uterine circulation in the LMWH group. Cord TFPI levels are lower compared to peripheral and uterine circulations in both groups ( $p < 0.05$ ). There are no significant changes in TFPI between the two groups within the peripheral and cord circulations.

#### **6.2.6 Placental TFPI<sub>2</sub> Gene Expression (Figure 6.8)**

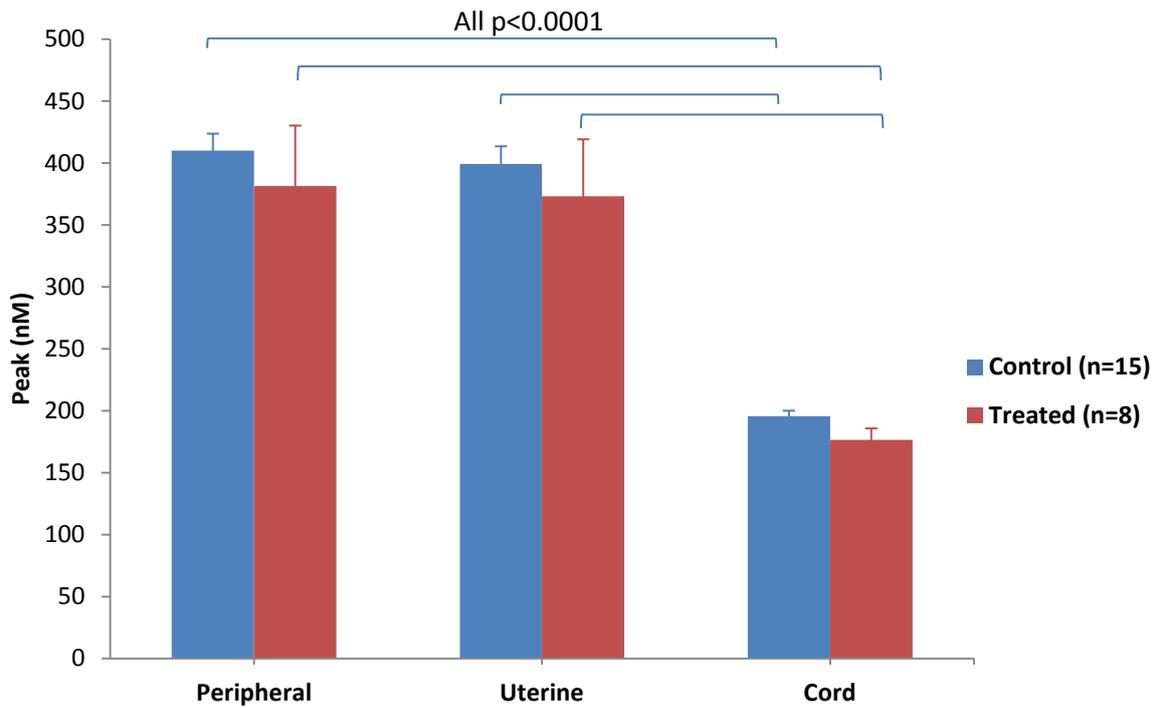
A more marked down-regulation of placental TFPI<sub>2</sub> isoform was found following LMWH treatment. A 6 fold down regulation of TFPI<sub>2</sub> mRNA expression was found in the LMWH group ( $p < 0.05$ ) compared to control group.

### 6.2.7 Placental Tissue Factor Gene and Antigen Expression (Figure 6.9)

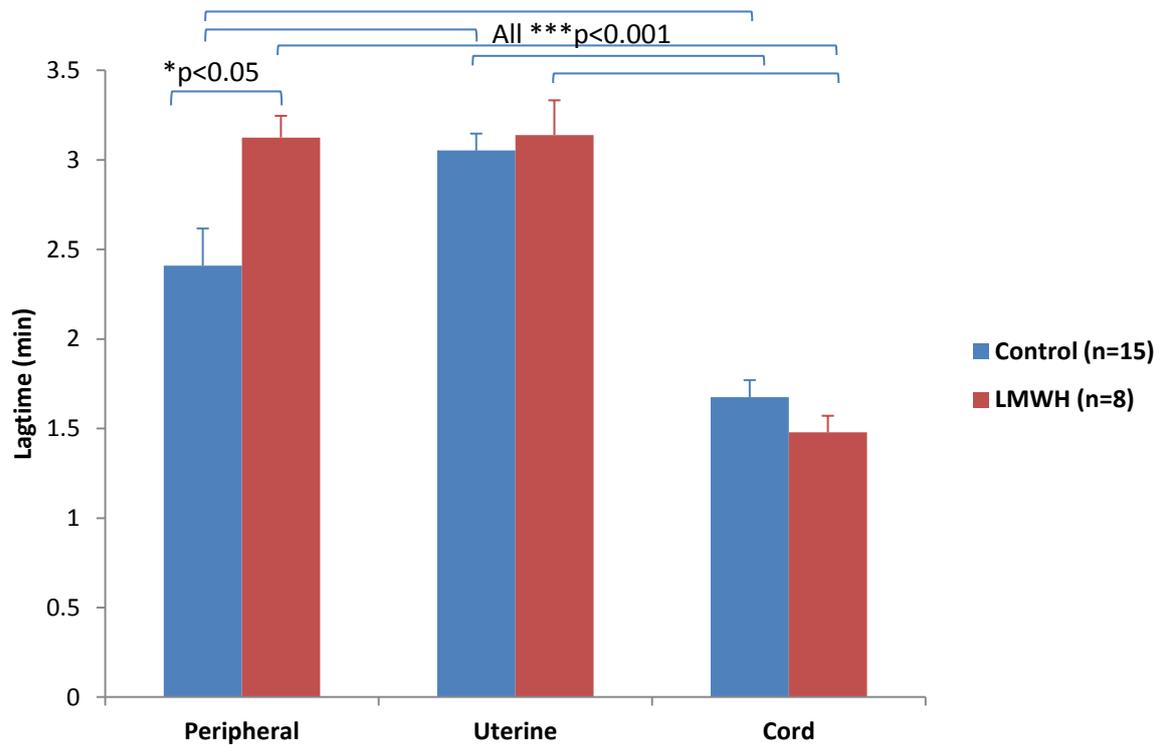
Placental tissue factor mRNA gene expression and placental tissue factor antigen in the LMWH group was similar in the LMWH group and the control groups.



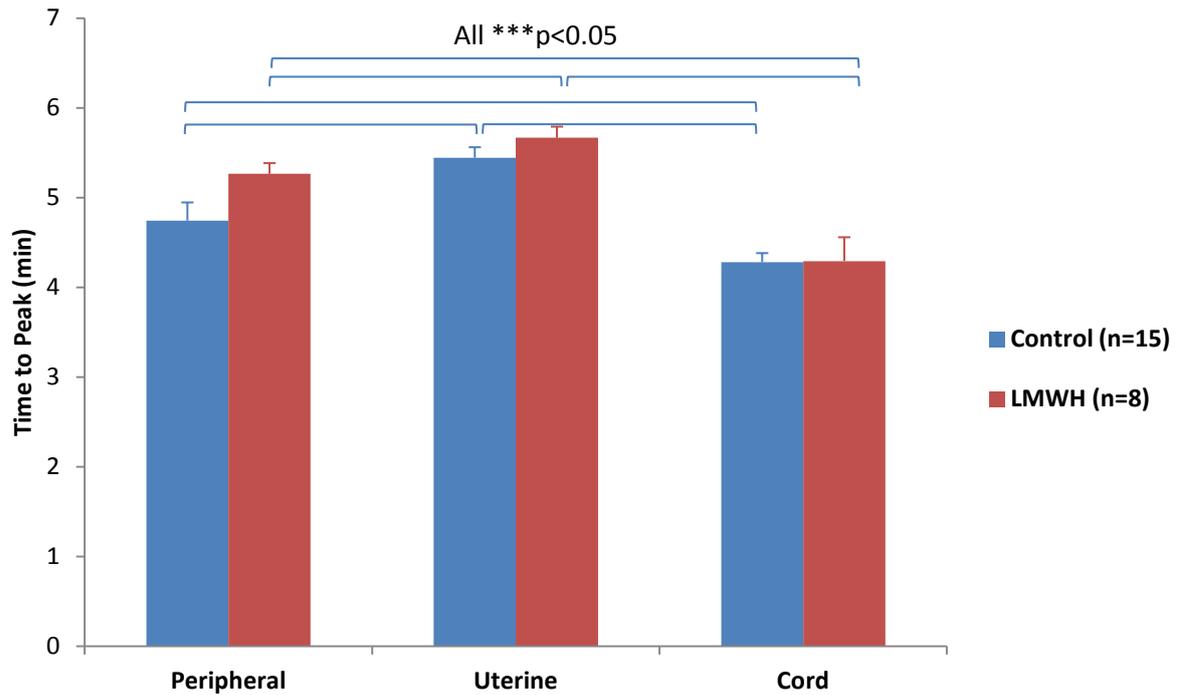
**Figure 6.1 Effect of LMWH on Thrombin Generation (ETP) in the Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing caesarean section compared with controls.** LMWH group are thrombophilic pregnant women who were on antenatal LMWH prophylaxis (n=8). Control group (n=15) are healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. Values represent mean and SD. \* =  $p < 0.05$  and \*\*\* =  $p < 0.0001$  following student's *t*-test.



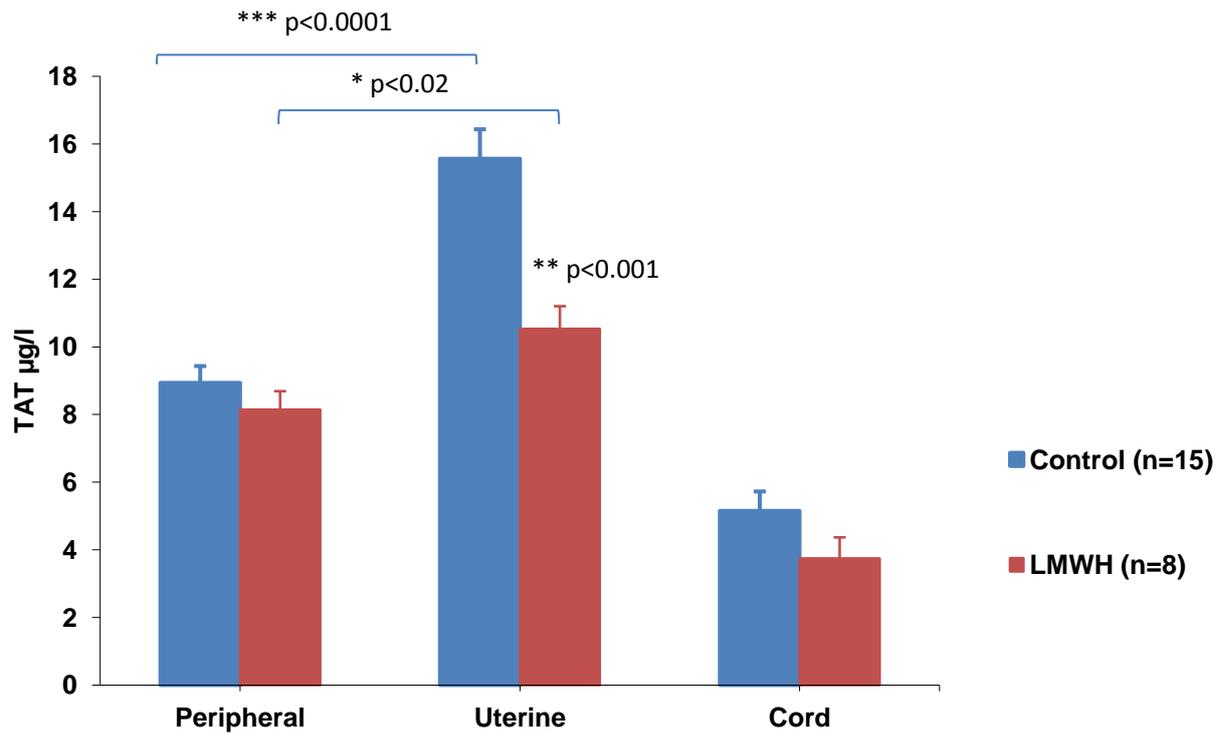
**Figure 6.2 Effect of LMWH on Thrombin Generation (Peak Thrombin) in the Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing caesarean section compared with controls.** LMWH group are thrombophilic pregnant women who were on antenatal LMWH prophylaxis (n=8). Control group (n=15) are healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. Values represent mean and SD. \*\*\* =  $p < 0.0001$  following student's *t*-test.



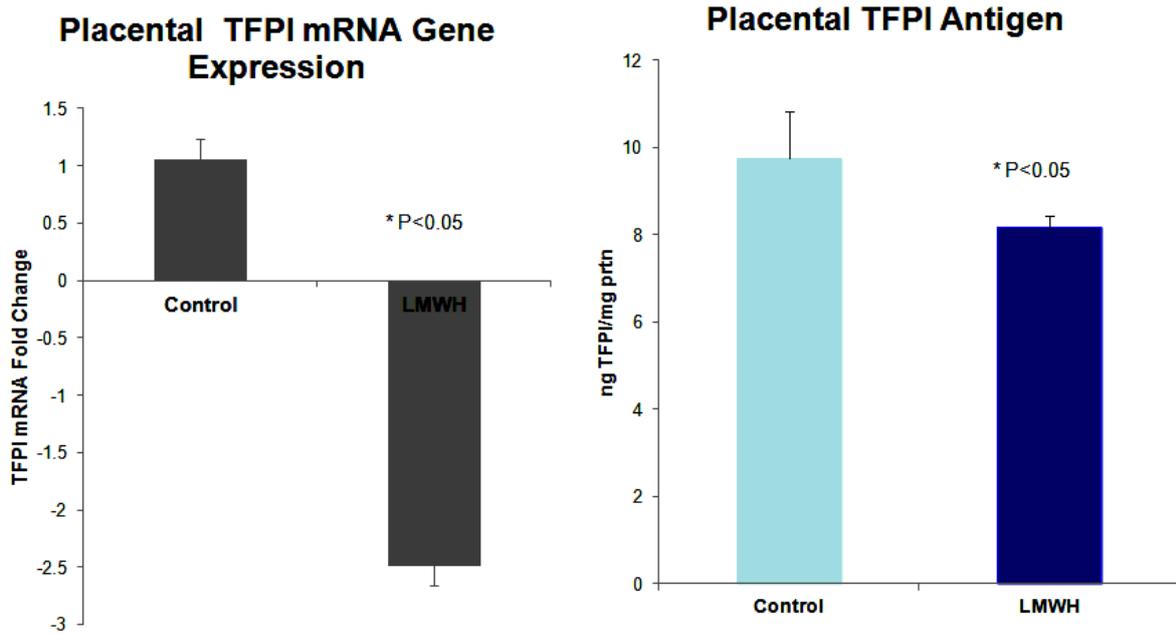
**Figure 6.3 Effect of LMWH on Thrombin Generation (Lagtime) in the Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing caesarean section compared with controls.** LMWH group are thrombophilic pregnant women who were on antenatal LMWH prophylaxis (n=8). Control group (n=15) are healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. Values represent mean and SD. \* =  $p < 0.05$ , \*\*\* =  $p < 0.001$  following student's *t*-test.



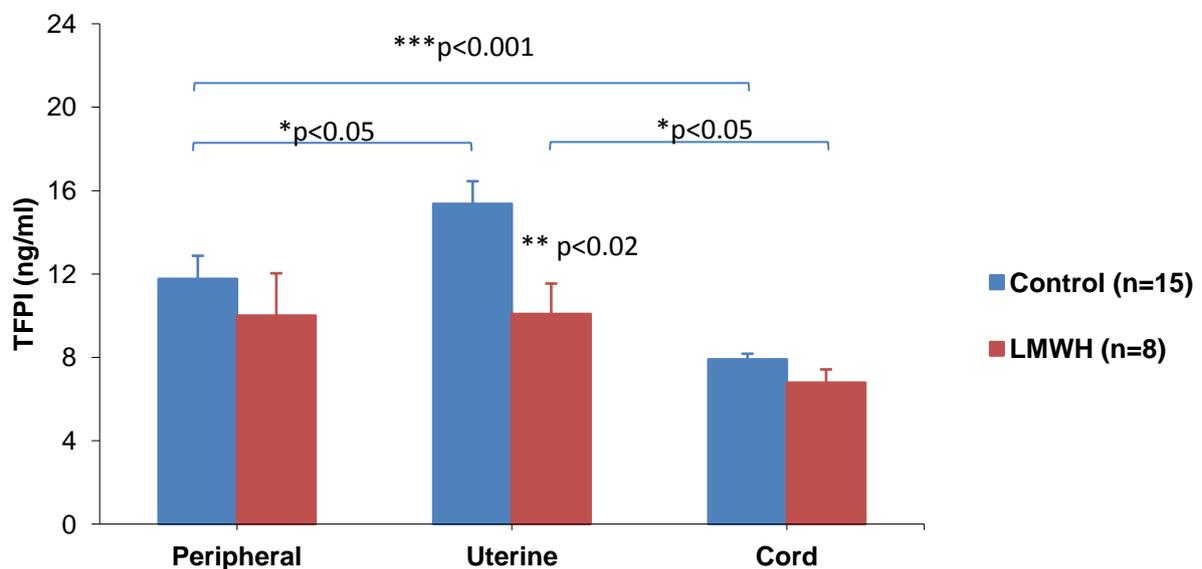
**Figure 6.4 Effect of LMWH on Thrombin Generation (Time to Peak Thrombin) in the Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing caesarean section compared with controls.** LMWH group are thrombophilic pregnant women who were on antenatal LMWH prophylaxis (n=8). Control group (n=15) are healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. Values represent mean and SD. \*\*\* =  $p < 0.05$  following student's *t*-test.



**Figure 6.5 Effect of LMWH on Thrombin antithrombin (TAT) in the Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing caesarean section compared with controls.** LMWH group are thrombophilic pregnant women who were on antenatal LMWH prophylaxis (n=8). Control group (n=15) are healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. Values represent mean and SD. \* =  $p < 0.02$ , \*\* =  $p < 0.001$  and \*\*\* =  $p < 0.0001$  following student's *t* -test.



**Figure 6.6 TFPI Gene and Antigen Expression in Placenta from Thrombophilic Pregnant Women on LMWH Prophylaxis (n=8) Compared with Control Healthy Pregnant Women (n=15).** mRNA fold change calculated using  $2^{-\Delta\Delta Ct}$ . TFPI antigen expressed as ng TFPI/mg of extracted protein. Results expressed as mean and SD. \* denotes  $p < 0.05$



**Figure 6.7 Effect of LMWH on Tissue Factor Pathway inhibitor (TFPI) in the Peripheral, Uterine and Umbilical Cord Venous Circulation of Thrombophilic Pregnant Women undergoing caesarean section compared with controls.** LMWH group are thrombophilic pregnant women who were on antenatal LMWH prophylaxis (n=8). Control group (n=15) are healthy pregnant women matched for age, BMI, parity, gestation and ethnicity. Values represent mean and SD. \* =  $p < 0.05$ , \*\* =  $p < 0.02$  and \*\*\* =  $p < 0.001$  following student's *t*-test.

## Placental TFPI<sub>2</sub> mRNA Gene Expression

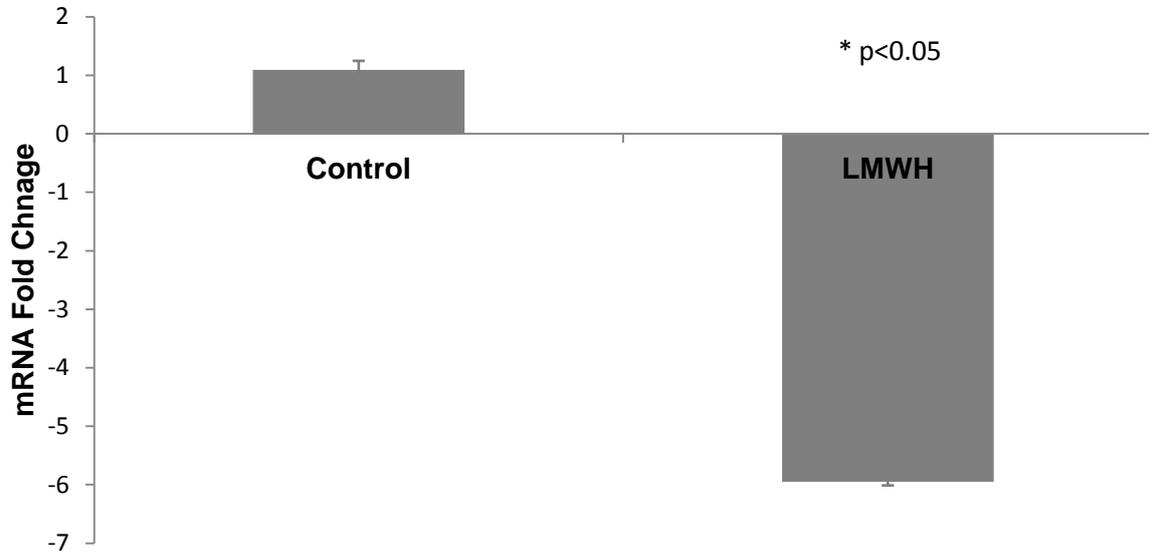


Figure 6.8 TFPI<sub>2</sub> Gene Expression in Placenta from Thrombophilic Pregnant Women on LMWH Prophylaxis (n=8) Compared with Control Healthy Pregnant Women (n=15). mRNA fold change calculated using  $2^{-\Delta\Delta ct}$ . Results expressed as mean and SD. \* denotes  $p < 0.05$

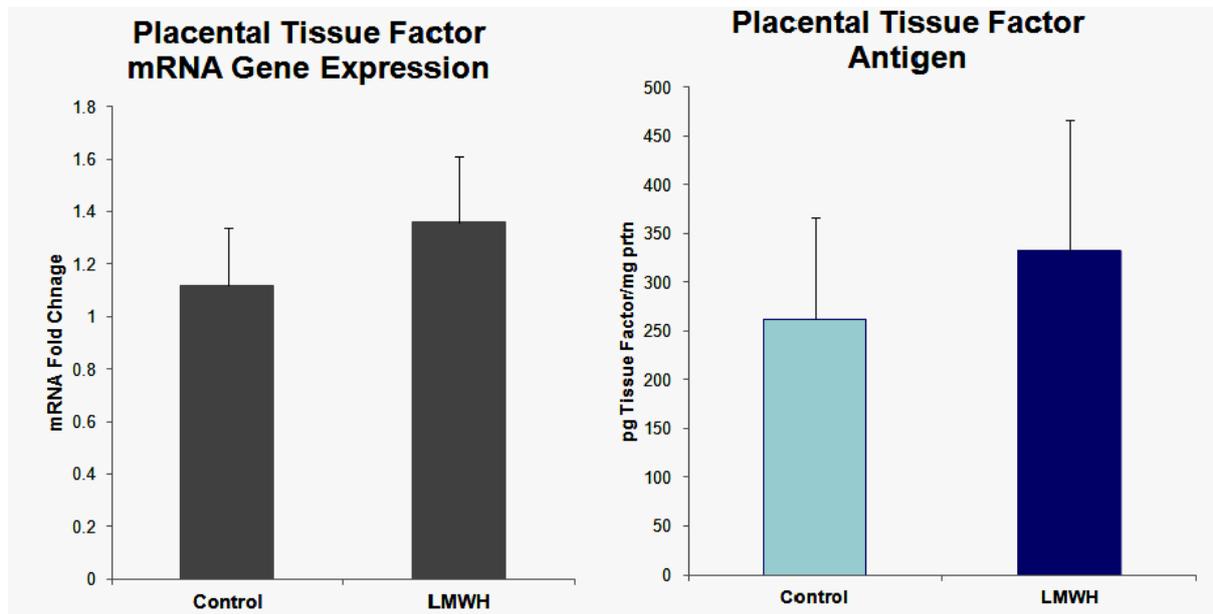


Figure 6.9 Tissue Factor Gene and Antigen Expression in Placenta from Thrombophilic Pregnant Women on LMWH Prophylaxis (n=8) Compared with Control Healthy Pregnant Women (n=15). mRNA fold change calculated using  $2^{-\Delta\Delta ct}$ . Tissue factor antigen expressed as ng tissue factor/mg of extracted protein. Results expressed as mean and SD. \* denotes  $p < 0.05$

### 6.3 Discussion

The results of this study showed there are different haemostatic profiles between the peripheral, uteroplacental and cord circulation. The CAT assay parameter such as ETP and peak thrombin provides a compound measure of the patients total thrombin generation. In contrast, TAT levels reflect the ongoing *in-vivo* production of thrombin. Peripheral ETP levels found in the control group of this study are comparable to previously reported studies in the late pregnancy [165, 193, 303]. In non-pregnant studies, ETP [123] and peak thrombin [267] levels have been reported to be increased in thrombophilic individuals compared to normal controls. In this study, we do not see a quantitative difference in ETP or peak thrombin within the peripheral circulation between the control and thrombophilic women on LMWH. This might be due to the chronic exposure to LMWH in the peripheral circulation.

ETP and peak thrombin were reported to increase in non-pregnant thrombophilic individuals who are susceptible to thrombosis [109, 123, 267]. Segers et al found that CAT assay is sensitive to genetic variation in haemostatic genes, rendering the assay a promising tool to identify genetic risk factors to thrombosis. Studies in pregnancy found that thrombin generation measured by ETP and peak thrombin were increased in women who developed pre-eclampsia compared to normotensive pregnant women [315]. Furthermore, three months after delivery, ETP and peak thrombin were reported to be higher in women with history of pre-eclampsia compared with normal pregnant controls [316]. This makes the CAT assay, specifically ETP and peak thrombin, a potentially useful tool in screening for thrombophilia and placenta mediated pregnancy complications. ETP levels within the uteroplacental unit are reduced in thrombophilic women exposed to LMWH compared to the healthy control group. This is an encouraging finding as it potentially reflects that LMWH may reduce thrombin generation within the uteroplacental circulation that otherwise is likely to be increased in thrombophilic women. Within the cord circulation, ETP and peak thrombin levels are reduced in both groups compared to peripheral

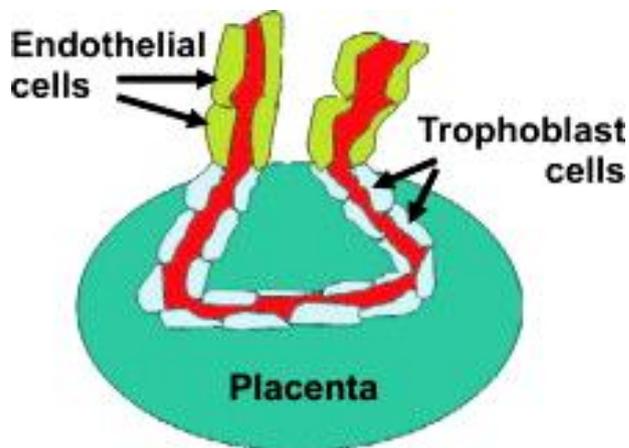
and uteroplacental levels. This could be explained by the immature development of the fetal coagulation system.

Peripheral plasma TAT levels found in this study were comparable to previous studies in late pregnancy either in healthy women [53] or women with moderate VTE risk (including thrombophilia) who were exposed to chronic LMWH administration [276]. In this study, TAT levels within the uteroplacental circulation are higher than in peripheral circulation in normal pregnant women. This finding is similar to a previous report which found increased uterine TAT levels in normal pregnant women at delivery compared to peripheral circulation [53]. Increased TAT levels were reported in non-pregnant thrombophilic individuals [107, 109, 317, 318]. Therefore, even though this study lacks a control group of thrombophilic women without LMWH exposure, it is reasonable to expect a higher TAT levels in this group of women. We do not find any difference in TAT levels between our control and thrombophilic women on LMWH. This may be due to the timing of sampling. Samples were taken during caesarean delivery which was 24 hours from the last LMWH injection. Taking this into account, peripheral TAT levels found in this LMWH group are similar to that found in pregnant women with thrombotic state also chronically exposed to LMWH at 24 hours post LMWH injection [276]. Within, the uteroplacental circulation, it is reassuring that TAT levels are reduced in thrombophilic women exposed to LMWH in this study. This suggests that LMWH prophylaxis in this condition may reduce the possibility of uteroplacental thrombosis and prevent placenta mediated complications.

We found no difference in peripheral plasma levels of TFPI between the control and LMWH groups. This is possibly due to the fact that blood sampling was done 24 hours post LMWH and not 4 hours post LMWH injection where TFPI release is at its peak before it returns to pre-dose levels [47, 307]. Indeed, the peripheral TFPI levels found in the control and LMWH group in this study is comparable to that found at pre-delivery and 24-hours post LMWH injection respectively [303]. With the fall of ongoing thrombin generation as shown by decreased ETP and

TAT levels within the uteroplacental circulation, it is logical to expect TFPI levels to be increased, especially with exposure to LMWH. This does not seem to be the case in this study. However, as 80% of TFPI are membrane bound, plasma TFPI concentration may not reflect the availability of TFPI in vascular endothelium [289]. Levels of TFPI within uterine circulation were reduced significantly in the thrombophilic women on LMWH compared with control group in this study. This finding corresponded to expression of TFPI in placentae from the LMWH group at both the mRNA and protein level. This result is comparable to another study which also reported lower TFPI placental antigen expression in thrombophilic women treated with LMWH compared with normal pregnant women [319]. However, Aharon et al uses glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as their housekeeping gene while in this study, 18s was used (18s has been shown to be more inert in hormone influenced tissues such as progesterone). This may be why their reported placental TFPI mRNA expression in LMWH treated thrombophilic women is in contrast to this study. The result of this study is in contrast to report showing that TFPI-mRNA expression is up-regulated in endothelial cell line following heparin treatment *in vitro* [320]. TFPI<sub>2</sub> gene expression was also down-regulated in the LMWH group of our study. In this study, human Quantikine TFPI immunoassay was used where no significant cross reactivity or interference observed with human TFPI<sub>2</sub>. These results suggest that LMWH is having an effect (either direct or indirect) on the transcription of the TFPI<sub>1</sub> and TFPI<sub>2</sub> gene. Chronic exposure to LMWH may result in downregulation of TFPI mRNA gene expression. Different mechanism may be involved by which TFPI gene is regulated. A cell culture study demonstrated that LMWH (tinzaparin) and UFH at pharmacological doses, significantly decreased lipopolysaccharide induced production of inflammatory cytokines, as well as nuclear factor-kappa B (NFκB) translocation in human monocytes [321]. Hochart et al also showed that heparins did not have similar effect on unstimulated monocytes. This indicated that a significant anti-inflammatory effects of heparin on monocyte mediated immune response and inhibition of NFκB may be one of the mechanisms the anti-inflammatory effects is exerted [321].

A series of ground breaking murine studies demonstrated that the haemostatic system plays an essential role in normal placental development. Human and rodents share the unique 'haemochorial' placenta not found elsewhere in the body in which, the wall of maternal blood vessels is breached and maternal blood bathes placental trophoblast cells [322] (Figure 6.10). Trophoblast cells mimic the ability of endothelial cells in regulating local haemostasis by expressing a group of thrombo-regulatory genes such as thrombomodulin, endothelial protein C receptor (EPCR) and TFPI. Studies on knock-out mice showed that absence of thrombomodulin expression on trophoblast cells leads to inadequate placentation and early embryonic lethality[180]. This embryonic lethality was shown to be dependant on tissue factor expression on trophoblast cells and thrombin generation. These genes are potential risk modifiers of placenta mediated pregnancy complication in mothers with thrombophilia.



**Figure 6.10** Maternal blood directly bathes trophoblast cells of the hemochorial placenta [322]

In this study no difference of placental TF mRNA and antigen expression were seen between the control and thrombophilic LMWH group. This result is similar to previously reported by other authors [319]. In human decidual cell culture studies, TF protein expression was reported to be

enhanced by more than two-fold in the tissues mimicking thrombotic pregnant state (with presence of progestin and thrombin) [323] while on the other hand, LMWH has been reported to result in downregulation of TF gene expression [324, 325]. With no difference in TF expression but down-regulation of TFPI expression, the increased in TF/TFPI ratio seen in this study suggested a more profound haemostatic activation at the placental level in the thrombophilic women compared to normal pregnant controls. Manipulation of thrombin generation within uteroplacental circulation by way of modifying haemostatic gene expression such as TF and TFPI may reverse the risk of placenta mediated pregnancy complication in mothers with thrombophilia.

In this study, placental biopsies were randomly taken from the trophoblast side of the placentae which may have affected the results. However, haemostatic gene expression including TF, TFPI and TFPI<sub>2</sub> were documented to be not significantly different in various areas of placentae [272]. Other author has reported that in normal pregnancy, local haemostasis at the placental trophoblast is characterised by increased TF and low TFPI mRNA expression [47, 164].

The timing of blood sampling in this study is vital for interpretation of the results. Although there would be some degree of haemostatic activation during delivery, it is re-assuring that the peripheral levels of ETP, Peak, TAT and TFPI within the control group is comparable to that previously reported, in the same late gestation prior to delivery [291, 303]. This indicates that the peripheral levels reported in this study reflects the haemostatic profile in the late pregnancy and not the acute effects of delivery. Comparing results for the uteroplacental circulation is challenging as haemostatic markers within the uteroplacental circulation are very rarely reported. At present time, to this author's knowledge, there is no documented data on ETP or peak thrombin measured within the uterine vein. This study suggested that coagulation is more profoundly activated in the uteroplacental circulation in both normal and thrombophilic women

than in systemic circulation. Higgins et al showed that TAT was significantly increased in uterine vein in normal and pre-eclamptic women [53]. While Boyle et al in 1992 found no difference in levels of fibrin degradation product (D-Dimer) within the systemic and uterine circulation, she also reported a higher fibrinolytic potential (measured by t-PA and u-PA) in the uterine vein, which possibly counter-balanced the D-Dimer levels [326]. All haemostatic markers within the cord circulation were far reduced when compared to levels in peripheral and uterine vein. This is not unexpected as the fetal coagulation system is yet to be fully developed. No changes between control and LMWH group in the cord vein are not surprising as LMWH does not cross the placental barrier hence, has negligible effect on the fetal circulation.

LMWH is used widely for the prevention of placenta mediated pregnancy complications (pregnancy loss, fetal growth restriction, pre-eclampsia and abruption) both in thrombophilic and non thrombophilic patients [273] although reports are mixed as to whether LMWH is truly beneficial in preventing these complications. Despite these uncertainties the excellent safety profile of LMWH outweighs the possible devastating outcome of fetal loss. This study provides novel data on the haemostatic effects of LMWH on the uteroplacental unit. LMWH may be effective in reducing *in-vivo* thrombin production in the uteroplacental circulation of thrombophilic women while maintaining the capacity for the haemostatic activation required as the placenta separates. These effects need to be explored further and more data on the precise mechanisms involved are required to maximise potential benefits of this therapeutic intervention.

# **Chapter 7**

## **General Discussion**

## 7.1 General Discussion and Conclusion

VTE remains a leading cause of maternal mortality and morbidity. The rate of maternal death caused by VTE had plateaued until the most recent triennial report when it has fallen. Due to the low absolute risk of VTE (<1 per 1000), there are very few randomised trials to guide management choices. Clinical guidelines rely significantly on expert opinion. Evidence is frequently based on data from the non-pregnant population which has been extrapolated to pregnant women.

The first controlled trial for treatment of VTE was carried out in 1960 [327]. LMWH has replaced UFH in the last two decades and they have transformed VTE therapy [201, 328]. Compared with UFH, LMWHs show a more predictive dose response curve, do not require regular monitoring and have a significantly better side effect profile in terms of heparin induced thrombocytopenia or osteoporosis. LMWH are the anticoagulant of choice for thromboprophylaxis in pregnancy and there is now a considerable experience of their use in pregnancy. They have been utilised for treatment of venous thrombosis, arterial and VTE thromboprophylaxis and prevention of fetal loss in placental dysfunction in thrombophilic women [196]. There is now good data demonstrating the safety of LMWH in pregnancy.

In post CS women, peak thrombin generation and TAT showed that the LMWH tinzaparin was successful in maintaining levels comparable to that of women delivered vaginally. The results showed that tinzaparin produced a predictable 24 hour profile of anti-Xa activity with peak levels at 4 hours dropping sharply at 10 -24 hours to levels at or below the detection limit of the assay. In contrast, thrombin production (as measured by ETP) was reduced at both 4 and 10 hours gradually declining at 18-24 hours post treatment. TAT remains suppressed within 24 hours post tinzaparin administration despite declining of anti-Xa levels. This study showed that although anti-Xa activity was reduced 10 hours post tinzaparin, ETP and TAT was still inhibited. As a RCT in this cohort is unlikely in the near future, this study provided a pharmacokinetic rationale for LMWH thromboprophylaxis in low risk women post elective CS.

Thrombin generation parameters as measured by CAT assays evaluate a global haemostatic profile of patients at risk of VTE and measure the cumulative effect of prothrombotic tendencies [293, 294]. As the CAT assay takes into account all pregnancy-related haemostatic modifications, its parameters are thought to be better indices than *in vivo* thrombin generation markers such as prothrombin fragment 1.2, thrombin anti-thrombin complex and D-Dimers which looked at ongoing coagulation activation. However, in the study using the SCOPE data and biobank, there were no significant differences of any CAT parameters in venous blood taken prior to a thrombotic event, between pregnant women who eventually developed VTE and controls. It is possible that the timing of samples collected (15 weeks gestation) is too early for any haemostatic changes that later led to thrombosis in the third trimester or postpartum, to be apparent in the CAT parameters. Lack of standardisation of assays and no standard bench mark for pregnancy levels makes direct comparison to previous predictive study difficult. Additionally, in this study, twenty women developed VTE in pregnancy out of a total of five thousand primigravids recruited in SCOPE; resulting in a four-fold higher VTE incidence (4:1000). This is in part, due to fact that the SCOPE cohort was more overweight and of advanced age. Moreover, all women in this study were primigravids that would not have a previously challenged haemostatic system.

The study on morbidly obese pregnant women showed that this group of women were far more prothrombotic than normal weight pregnant women. Prior to LMWH prophylaxis, TFPI levels in the obese group at 30 weeks were significantly lower and ETP and peak thrombin levels in obese group were significantly higher compared with controls. Within the obese group, there was no significant difference between ETP levels before and after fixed LMWH dose. However, ETP levels were significantly lower post weight-adjusted dose (75iu/kg tinzaparin) compared with post fixed dose (4500iu/day). There was a significant effect of LMWH on TFPI levels. ETP correlated positively with total body weight at fixed dose but not at weight adjusted dose. Although this study is too small to establish efficacy and safety, the prothrombotic state of the morbidly obese pregnant women was substantially attenuated by weight adjusted LMWH doses without abnormal bleeding.

Uteroplacental haemostasis was also studied. TAT levels within uterine vein were significantly higher compared to maternal peripheral circulation in both the control group and LMWH group. In the LMWH group, TAT is reduced compared with controls in the uterine vein. ETP and TFPI within uterine circulation were reduced significantly in the LMWH group. Down-regulation of placental TFPI and TFPI<sub>2</sub> mRNA expression was also found. Placental TF mRNA expression in LMWH group showed a non significant increase compared to control and this is replicated in placental TF antigen expression. However, this thesis reported a novel set of data, which included thrombin generation from the CAT assay, on the haemostatic effects of LMWH within the uteroplacental circulation.

Increasing CS rates and an increasingly obese population mean that care of these at-risk groups of pregnant women is increasingly important as they impose a significant adverse effect on perinatal outcome and health economy. The results of this study showed an increasing role of LMWH in reducing thrombotic disease risk. However, the downside as in many pharmacological interventions is increased cost. There is also potential abnormal bleeding, subcutaneous bruising and irritation. A highly sensitive, specific and cheap predictive test is still needed but evidence from this thesis did not suggest that CAT assay in its present form can be utilised as a tool for predicting VTE in pregnancy. Evidence has emerged suggesting that thrombophilic women may be at higher risk of developing placenta-mediated pregnancy complications. Evaluation of the use of LMWH in placental mediated pregnancy complications in thrombophilic women are currently focused on time-consuming but critically needed clinical trials. Data from this thesis adds knowledge in understanding how LMWH works within the uteroplacental unit. While LMWH is safe and effective for treating and preventing thrombosis in pregnancy, current evidence is not robust enough to establish causal association between inherited thrombophilia and placenta mediated pregnancy complications. Hence, in addition to clinical trials, more mechanistic studies are needed to have a better grasp of the pathogenesis of the disease and to justify the use of LMWH for this indication.

## 7.2 Recommendations for Future Research

The author acknowledges limitations in this thesis. In the caesarean section study, an ideal control group would include low risk women post elective CS without any LMWH thromboprophylaxis. However, this study was done within the context of clinical practice in our unit (CUMH) whereby, all women post CS (elective CS and emergency CS) would receive LMWH thromboprophylaxis hence the inclusion of such a control group was not feasible. This study would be strengthened if all time points are present for CS2 group. In the predictive study, plasma was limited hence only one concentration of TF was used, varying the assay conditions may have yielded a different result. However, additional analysis with assays requiring minimal amount of plasma such as D-Dimer and P selectin would be possible and the data obtained may be predictive of VTE. Predictive work within a group of pregnant women with higher risk of VTE; such as analysing the pre-thrombotic levels of CAT parameters, D-Dimer and P selectin in women with previous history of personal VTE or thrombophilia, would be an interesting area to venture in the future. In the third study (Study 3), a control group consisting of morbidly obese pregnant women without LMWH thromboprophylaxis would have been ideal to discern effect of obesity on haemostatic parameters as pregnancy progresses in third trimester. Moving forward, this would be an interesting point to investigate. In Study 4, the LMWH treated thrombophilic group represents a convenience sample; a less heterogeneous set of thrombophilia and having a group of thrombophilic women without LMWH prophylaxis would be ideal. At this present time, pending results from randomised control trials, it is extremely difficult to impart on this group of women to discontinue LMWH prophylaxis, especially when they achieved successful pregnancy outcome in the past while on LMWH therapy.



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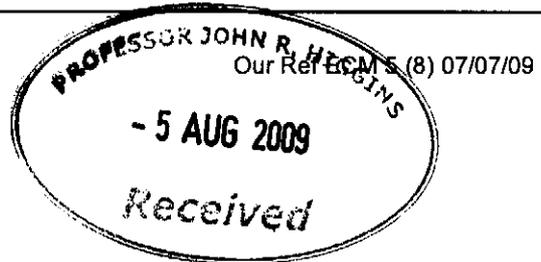
Appendix A  
COISTE EITICE UM THAIGHDE CLINICIÚIL  
**Clinical Research Ethics Committee**

Lancaster Hall,  
6 Little Hanover Street,  
Cork,  
Ireland.

Coláiste na hOllscoile Corcaigh, Éire  
**University College Cork, Ireland**

8<sup>th</sup> July 2009

Professor John Higgins  
Professor in Obstetrics & Gynaecology  
Anu Research Centre  
5<sup>th</sup> Floor  
Cork University Maternity Hospital  
Wilton  
Cork



**Re: Heparin in human pregnancy**

Dear Professor Higgins

The Clinical Research Ethics Committee of the Cork Teaching Hospitals reviewed your correspondence at its recent meeting held on 7<sup>th</sup> July 2009.

Full approval is granted by the Committee to carry out the above study at:

- Cork University Maternity Hospital.

The Committee approved the following documents:

- Application Form
- Study Protocol
- Patient Information/Consent Form Groups 1-5 version 1 dated 18<sup>th</sup> June 2009.

We note that the co-investigators involved in this trial will be:

- Dr Siti Khadijah Ismail
- Dr Shanthi Muttukrishna.

The following Committee Members attended the above meeting.

Dr Michael Hyland – (Chairman)  
Dr Mike O'Connor  
Dr Eugene Dempsey  
Dr Deirdre O'Hanlon (by phone)

Kieran Doran  
Kathryn Neville  
Dr Dan McKenna (by phone)

Yours sincerely

Dr Michael Hyland  
Chairman  
Clinical Research Ethics Committee  
of the Cork Teaching Hospitals

COISTE EITICE UM THAIGHDE CLINIÚIL  
**Clinical Research Ethics Committee**

Lancaster Hall,  
 6 Little Hanover Street,  
 Cork,  
 Ireland.



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Coláiste na hOllscoile Corcaigh, Éire  
**University College Cork, Ireland**



Our Ref ECM 3 (II) 06/10/09

17<sup>th</sup> September 2009

Professor John Higgins  
 Professor in Obstetrics & Gynaecology  
 Anu Research Centre  
 5<sup>th</sup> Floor  
 Cork University Maternity Hospital  
 Wilton  
 Cork

**Re: CKY18: Heparin in human pregnancy**

Dear Professor Higgins

The Chairman approved the following:

- Amendment Application Form
- Revised Study Protocol
- Revised Consent Forms version 2 dated 12<sup>th</sup> August 2009.

Yours sincerely

Dr Michael Hyland  
 Chairman  
 Clinical Research Ethics Committee  
 of the Cork Teaching Hospitals

*The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational medicinal products. The Committee is fully compliant with the Regulations as they relate to Ethics Committees and the conditions and principles of Good Clinical Practice.*

## Clinical Research Ethics Committee Of The Cork Teaching Hospitals

### CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL

#### **Section A**

Protocol Number: CKZ 47

Patient Name: \_\_\_\_\_

Title of Protocol: Thrombosis and Human Pregnancy (Group 2: Post caesarean section)

Doctor(s) Directing Research: Professor John Higgins  
Dr Siti Khadijah Ismail

Phone: 021-4205020  
021-4205038

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

#### **Section B**

##### I. NATURE AND DURATION OF PROCEDURE(S):

- i) Blood clotting (venous thromboembolism) remains the leading cause of ill health or even death to mothers. Most of them underwent caesarean section. In this group of patients, further research is required to determine the correct dose of blood thinner called tinzaparin, a low molecular weight heparin (LMWH).
- ii) Blood samples will be taken pre-treatment and at 4,10, 18 and 24 hours post tinzaparin treatment
- iii) Thrombophilia screening will be done before treatment and six weeks postpartum to identify and rule out patients with thrombophilia.
- iv) At delivery a small sample of after birth (placental biopsies) will be taken for investigation. These samples will be anonymous and stored in the laboratory until analysis is carried out. You have the choice to participate in this study and your clinical care will not be compromised by your decision. You can decide to discontinue your participation in this study at any time.

##### II. POTENTIAL RISKS AND BENEFITS:

Your participation and results of this research could inform us the correct dose of treatment to prevent blood clotting (venous thromboembolism) in women undergoing caesarean section. It will enable us to develop new therapies that could better outcome of similar patients in the future.

##### III. POSSIBLE ALTERNATIVES:

Your participation is voluntary.

#### **Section C**

#### AGREEMENT TO CONSENT

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

## Appendix C

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor: \_\_\_\_\_

\_\_\_\_\_  
Signature of Subject,

Witness: \_\_\_\_\_

Date: \_\_\_\_\_ Time: \_\_\_\_\_ AM  
(Circle) PM

*Consent Form Version 2 – dated 12/08/2009*



## Clinical Research Ethics Committee Of The Cork Teaching Hospitals

### CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL

#### **Section A**

Protocol Number: CKZ 47

Patient Name: \_\_\_\_\_

Title of Protocol: Thrombosis and Human Pregnancy (Group 2: Normal Vaginal Delivery)

Doctor(s) Directing Research: Professor John Higgins

Phone: 021-4205020

Dr Siti Khadijah Ismail

021-4205038

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

#### **Section B**

##### I. NATURE AND DURATION OF PROCEDURE(S):

- i) Blood clotting (venous thromboembolism) remains the leading cause of ill health or even death to mothers. Further research is required to determine the correct dose of blood thinner called tinzaparin, a low molecular weight heparin (LMWH).
- ii) Blood samples will be taken at four different time points within 24 hours post delivery.
- iii) These samples will be anonymous and stored in the laboratory until analysis is carried out. You have the choice to participate in this study and your clinical care will not be compromised by your decision. You can decide to discontinue your participation in this study at any time.

##### II. POTENTIAL RISKS AND BENEFITS:

Your participation and results of this research could inform us the correct dose of treatment to prevent blood clotting (venous thromboembolism) in women undergoing caesarean section. It will enable us to develop new therapies that could better outcome of similar patients in the future.

##### III. POSSIBLE ALTERNATIVES:

Your participation is voluntary.

#### **Section C**

#### AGREEMENT TO CONSENT

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor: \_\_\_\_\_

\_\_\_\_\_  
Signature of Subject,

Witness: \_\_\_\_\_

Date: \_\_\_\_\_ Time: \_\_\_\_\_ AM  
(Circle) PM

## **Appendix D**

*Consent Form Version 2 – dated 12/08/2009*

**Clinical Research Ethics Committee Of The Cork Teaching Hospitals**

**CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL**

**Section A**

Protocol Number: CKZ 47

Patient Name: \_\_\_\_\_

Title of Protocol: Thrombosis and Human Pregnancy (Group 1: Overweight pregnant Women)

Doctor(s) Directing Research: Professor John Higgins  
Dr Siti Khadijah Ismail

Phone: 021-4205020

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

**Section B**

**I. NATURE AND DURATION OF PROCEDURE(S):**

Blood clotting (venous thromboembolism) remains the leading cause of ill health or even death to mothers even while on blood thinning medication. Most of them are overweight. In this group of patients, further research is required to determine the correct dose of blood thinner, low molecular weight heparin (LMWH).

You will be started on LMWH tinzaparin 4500units injection daily from 32 weeks pregnancy. After 48 hours, blood sample (10mls- 2 tablespoon) will be taken four hours after LMWH injection, by which your injection may be corrected. Another blood sample will be taken 1 week after this corrected dose. You will continue on this corrected dose until delivery. Blood sample will be taken every two weeks.

These samples will be anonymous and stored in the laboratory until analysis is carried out. You have the choice to participate in this study and your clinical care will not be compromised by your decision. You can decide to discontinue your participation in this study at any time.

**II. POTENTIAL RISKS AND BENEFITS:**

Your participation and results of this research could inform us the correct dose of treatment to prevent blood clotting (venous thromboembolism) in overweight pregnant women. It will enable us to develop new therapies that could better outcome of similar patients in the future.

**III. POSSIBLE ALTERNATIVES:**

Your participation is voluntary.

---

**Section C**

**AGREEMENT TO CONSENT**

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

**Appendix E**

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor: \_\_\_\_\_

\_\_\_\_\_  
Signature of Patient

Witness: \_\_\_\_\_

Date: \_\_\_\_\_ Time: \_\_\_\_\_ AM  
(Circle) PM

**Clinical Research Ethics Committee Of The Cork Teaching Hospitals**

**CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL**

**Section A**

Protocol Number: CKY 47

Patient Name: \_\_\_\_\_

Title of Protocol: Thrombosis and Human Pregnancy (Normal Weight Pregnant Women-Control)

Doctor(s) Directing Research: Professor John Higgins

Phone: 021-4205020

Dr Siti Khadijah Ismail

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

**Section B**

**I. NATURE AND DURATION OF PROCEDURE(S):**

Blood clotting (venous thromboembolism) remains the leading cause of ill health or even death to mothers. Most of them are overweight or underwent caesarean section. In this group of patients, further research is required to determine the correct dose of low molecular weight heparin (LMWH). Healthy pregnant women will act as controls. Blood samples (20mls or 2-3 tablespoons) will be taken at 30, 32,34 and 36 weeks pregnancy. These samples will be anonymous and stored in the laboratory until analysis is carried out. You have the choice to participate in this study and your clinical care will not be compromised by your decision. You can decide to discontinue your participation in this study at any time.

**II. POTENTIAL RISKS AND BENEFITS:**

Your participation and results of this research could inform us the correct dose of treatment to prevent blood clotting (venous thromboembolism) in overweight pregnant women. It will enable us to develop new therapies that could better outcome of similar patients in the future.

**III. POSSIBLE ALTERNATIVES:**

Your participation is voluntary.

**Section C**

**AGREEMENT TO CONSENT**

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor: \_\_\_\_\_

\_\_\_\_\_  
Signature of Subject,

Witness: \_\_\_\_\_

Date: \_\_\_\_\_ Time: \_\_\_\_\_ AM  
(Circle) PM



**Clinical Research Ethics Committee Of The Cork Teaching Hospitals**

**CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL**

**Section A**

Protocol Number: CKZ 47

Patient Name: \_\_\_\_\_

Title of Protocol: Prediction and Prevention of Venous Thromboembolism in Pregnancy (Group 4: uterine circulation)

Doctor(s) Directing Research: Professor John Higgins  
Dr Siti Khadijah Ismail

Phone: 021-4205020  
021-4205038

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

**Section B**

**I. NATURE AND DURATION OF PROCEDURE(S):**

- i) Blood clotting (venous thromboembolism) remains the leading cause of ill health or even death to mothers. Most of them underwent caesarean section. In this group of patients, further research is required to determine the effects of blood thinner called tinzaparin, a low molecular weight heparin (LMWH). Before delivery of placenta, blood sample (15mls- 2 tablespoon) will be taken from your arm. At the same time, during the surgery, 10mls of uterine venous blood and. 5mls of cord blood and placental biopsy will also be taken.
- ii) These samples will be anonymous and stored in the laboratory until analysis is carried out. You have the choice to participate in this study and your clinical care will not be compromised by your decision. You can decide to discontinue your participation in this study at any time.

**II. POTENTIAL RISKS AND BENEFITS:**

Your participation and results of this research could inform us the correct dose of treatment to prevent blood clotting (venous thromboembolism) in women undergoing caesarean section. It will enable us to develop new therapies that could better outcome of similar patients in the future.

**III. POSSIBLE ALTERNATIVES:**

Your participation is voluntary.

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**Section C**

**AGREEMENT TO CONSENT**

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

## Appendix G

Doctor: \_\_\_\_\_

\_\_\_\_\_  
Signature of Subject,

Witness: \_\_\_\_\_

Date: \_\_\_\_\_ Time: \_\_\_\_\_ AM  
(Circle) PM