The effect of Physiological concentrations of 17β-Estradiol and Progesterone on Fibrin network formation

by

Amcois Visagie

submitted in (partial) fulfilment of the requirements for the degree

Masters of Science

in the

Faculty of Health Sciences

Department of Physiology

University of Pretoria

October 2016

The effect of Physiological concentrations of 17β-Estradiol and Progesterone on Fibrin network formation

by

Amcois Visagie

Supervisor: Dr A.C Swanepoel

Department of Physiology, School of Medicine, Faculty of Health Sciences, University of Pretoria, South Africa

Degree: MSc

Abstract

17β-Estradiol (E_2) and progesterone (P4) have various important functions but the effect of these endogenous hormone concentrations on fibrin network formation has not been established. It is essential to understand natural hormone mechanisms since these hormones are still present in circulation while hormonal contraceptives, which are associated with increased risk of venous thromboembolism, are used. In this study the formation of a fibrin network is analysed when different physiological concentrations of E_2 and P4 is added to platelet poor plasma. Blood coagulation is critical for haemostasis but when the formation of a stable clot is influenced in such a way that hypercoagulation takes its course, it can have detrimental effects as it increases the risk of venous thrombosis. During blood coagulation fibrinogen is converted into fibrin in the presence of thrombin. The formation of a dense fibrin clot structure is quite an intense process and packaged in very specific ways. Both E_2 and P4 has the ability to shift the haemostatic balance to a hypercoagulable state and therefore viscoelastic studies, morphological analysis as well as

turbidimetry were used in this study to observe the possible changes in the fibrin network formation. Viscoelastic studies included thromboelastography (TEG) which gave insight to the properties of clot formation. Morphological studies included scanning electron microscopy (SEM) and atomic force microscopy (AFM) which delivered an investigation in fibrin network morphology, fibrin fiber diameter and surface roughness. Turbidimetry included further analysis of plasma fibrin clot formation and clot lysis time (CLT). Results showed that E_2 and P4 showed hypercoagulable viscoelastic properties with decreased fibrin diameter and surface roughness while increased occurrence of dense matted deposits (DMDs) were evident. Turbidimetry showed decreased CLT for E_2, but not P4. These results suggest in the presence of endogenous estrogen and progesterone, which is associated with hypercoagulability, the additional burden of synthetic hormones may result in a pro-thrombotic and hypercoagulable state in females with an inflammatory predisposition. It appears that both E_2 and P4, which are known for their anti- and pro-inflammatory action, may influence fibrin network formation on a molecular level. These results are of clinical importance when considering hormones as either a pathological agent or therapeutic intervention.

Keywords: 17β-estradiol, Progesterone, Fibrin, Fibrinogen, Thromboembolism, Visco-elasticity, Morphology, Turbidimetry

Declaration

I, Amcois Visagie, hereby declare that this research dissertation is my own and has not been presented for any degree at other Universities.

Signed at Pretoria on 11 October 2016

Signature

Department of Physiology, School of Medicine, Faculty of Health Sciences, University of Pretoria, South Africa

Abbreviations

Abbreviation	Description
α	Angle (degrees)
AFM	Atomic force microscopy
ANOVA	Analysis of variance
CLT	Clot lysis time
CRP	C-reactive protein
DHEA-s	Dehydro-epiandrosterone sulphate
DMDs	Dense matted deposits
DNA	Deoxyribonucleic Acid
E_1	Estrone
E_2	17β-estradiol
E_3	Estriol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERE	Estrogen response elements
ER-X	Estrogen receptor X
FII	Factor II / Prothrombin
FIIa	Activated factor II / Thrombin
FV	Factor V
FVII	Factor VII / Zymogen
FVIIa	Activated factor VII / Zymogen
FVIII	Factor VIII
FVIIIa	Activated factor VIII
FIX	Factor IX
FIXa	Activated factor IXa
FX	Factor X

FXa	Activated factor X
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor XII / Hageman factor
FXIIa	Activated factor XII / Activated hageman factor
FXIII	Factor XIII
FBG	Fibrinogen
FDP's	Fibrin degradation products
FEG SEM	Field Emission gun scanning electron microscopy
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
GPR30	Glycoprotein receptor 30
HMDS	Hexamethyldisilazane
IL-6	Interleukin 6
IL-8	Interleukin 8
IRMA	Intra-retinal microvascular abnormalities
K	Kinetics
LDL	Low density lipoprotein
MA	Maximum amplitude
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MRTG	Maximum rate of thrombus formation
OsO_4	Osmium tetraoxide
P4	Progesterone
PBS	Phosphate-buffered saline
PGR	Progesterone receptor
PGR-A	Progesterone receptor α
PGR-B	Progesterone receptor β
PPP	Platelet poor plasma
QNM	Quantitative Nanomechanical Property Mapping

R	Reaction time
RIA's	Radioimmunoassays
RMS	Root mean square
ROS	Reactive oxygen species
SANBS	South African National Blood Services
SEM	Scanning electron microscopy
SF	Serum Ferritin
SHBG	Sex hormone-binding globulin
TAFI	Thrombin activatable fibrinolysis inhibitor
TEG	Thromboelastography
TF	Tissue factor
TF	Transferrin
TMRTG	Time to maximum rate of thrombus formation
tPA	Tissue plasminogen activator
Trp	Tryptophan
TTG	Total thrombus generation
VTE	Thromboembolism
vWF	Von Willebrand factor

Acknowledgements

I would like to thank my supervisor, Doctor Albe Swanepoel, for giving me this amazing opportunity to make a contribution to the world of research. It was a great experience to be part of this research team and working on a project where the importance of the research is applicable in my own life as well. I am aware now that hormonal contraceptives could increase my risk for thrombosis when making important life decisions. Dr Swanepoel, thank you for all your hard work, effort and support during this study. Your guidance and motivation is highly appreciated and was needed during the tough times. You have set the example for a driven supervisor where the most important reason behind the research is emphasized and that is women's health. It was a privilege working under your supervision and I wish you all the best with your future research.

I would also like to thank my co-supervisor, Professor Etheresia Pretorius for all your guidance during this study. You are truly an inspiration, not only to me but also to every other upcoming researcher. Thank you for your continuous support and knowledge. I would also like to thank you for this opportunity for being part of such an amazing research team. All the best for your future research.

I want to express my gratitude to my co-workers in the lab for whom I have a very deep regard as well. Doctor Janette Bester, Doctor Mia van Rooy and Doctor Marnie Potgieter, thank you for supporting me throughout this entire study. You always believed in me and your motivation, guidance and friendliness carried me through the tough times. Thank you for being there and always putting a smile on my face. I have learnt so much from you all.

To all the staff at the Microscopy and Microanalysis Unit of the University of Pretoria, I would like to thank you for all your assistance and support during my study, especially assisting me on the electron microscope and enabling me to capture the best photo microns possible. Your patience and kindness are much appreciated. Miss Antoinette Buys, thank you for all your patience and assistance on the AFM as well as accommodating me in my needs that I had in the lab.

A great thanks to my friends in the lab, Sulette, Sthembisele and Priyaa. Thank you for being there for me to rely on. Your words of encouragement motivated me and always lifted my spirit.

A special thanks to all the male volunteers that participated in this study making it possible for me to complete this research project. I also want to thank Vermaak and Partners Pathologists for all the hormone and iron test results. Many thanks to Dr Catherina van Zijl from department of Urology, University of Pretoria, for providing me with equipment in the creation of the hormone stock dilutions.

On a personal level I would like to thank my family and friends for inspiring me and motivated me in finishing this project. Without your love and support I could not have done it. Thank you for always believing in me that I can achieve anything if I believe in it. Lastly but most importantly I am grateful and blessed by my Heavenly Father. Without His Spirit in me and my faith in Him I would've not been able to pull through and achieved the success I wanted to with this research project.

Table of Contents

List of figures

List of tables

Chapter 1: Introduction

Haemostasis is an extremely important process which is tightly regulated by mechanisms in place to prevent excessive blood loss. Platelets become activated and adhere to macromolecules in subendothelial tissues at the site of injury and then aggregate to form the primary haemostatic plug. Blood coagulation follows to form a fibrin network, sealing off a damaged vessel and preventing haemorrhage. Blood coagulation is a cascade of proteases that cleaves precursor enzymes to form active enzymes, forming and effective amplification system that culminates the formation of thrombin. Thrombin catalyses the conversion of soluble fibrinogen into insoluble fibrin to form a clot and regulates the system by providing stimulatory and inhibitory feedback (1). Platelets also stimulate the activation of plasma coagulation factors which results in the formation of a fibrin clot that reinforces the platelet aggregate. Wound healing occurs with the process fibrinolysis (2).

Any deficiency in components playing a key role in blood coagulation, where hormonal fluctuation is amongst these components, can lead to insufficient amounts of thrombin. High levels of thrombin results in altered clot formation. Continuous intravascular activation of coagulation and thrombin-mediated activation takes place and venous thrombosis or thromboembolism (VTE) occurs (3, 4).

Previous studies have shown that increased hormone concentrations such as 17β-estradiol (E_2) and progesterone (P4) could increase the risk of venous thrombosis by changing the structure of fibrin network formation during blood coagulation (5). However these studies have shown changes when both the hormones together influence the coagulation network simultaneously. The individual effects of E_2 and P4 on fibrin fiber network have not been established (6).

E_2 and P4 are steroid hormones with health benefits and important functions in the reproductive systems of both males and females. Previous studies suggested that the effects of E_2 and P4 may influence coagulation through its pro- and anti-inflammatory effects (7, 8).

The aim of this study was to compare platelet poor plasma (PPP) of healthy male participants in the presence and absence of different physiological concentrations of E_2 and P4. The effects of these hormones were investigated through the analysis of fibrin network formation, using viscoelastic, morphological and turbidimetric evaluation. Viscoelastic studies were conducted using thromboelastography (TEG) to evaluate the properties of clot formation. Morphological studies followed where scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to investigate the ultrastructural changes in the fibrin network and surface topography. Lastly turbidimetry gave an indication of plasma fibrin clot formation as well as clot lysis time. Statistical analysis was performed using one-way ANOVA with the measuring of viscoelastic parameters, fibrin fiber diameters, surface roughness and turbidimetric properties.

Chapter 2: Literature review

2.1 Chapter objective

In this chapter, a review regarding the literature supporting this study is explained.

2.2 Hormones and the menstrual cycle

The menstrual cycle is governed by hormonal fluctuations (9). E_2 and P4 are two of the most essential hormones in the menstrual cycle, each with critical functions. Two distinct cycles takes place during the menstrual cycle namely the ovarian cycle and the uterine cycle. Both these cycles can be divided in three different phases, known as menstruation, a proliferative phase and a secretary phase represented in figure 2-1. E_2 and P4 fluctuate in the ovarian cycle where estrogen is responsible for the growing and maturing of the uterine lining and assist in the maturation of the ovarian egg just before ovulation. As shown in figure 2-1, E_2 concentrations peak at ovulation where the egg is released and again during the secretary phase. As estriol (E_3) is the major estrogen form during pregnancy, effects of E_2 were investigated during the menstrual cycle where it has its critical role (10). P4 on the other hand is produced after ovulation by the corpus luteum but at lower concentrations than in pregnancy where it primarily assists in the maturation of the uterine lining. Therefore it is regarded that P4 is the hormone which dominates during pregnancy and it is for this reason that it is important to evaluate the effects of different concentrations of P4 during pregnancy rather than observing the effects of P4 concentrations present in contraceptives. When implantation does not take place, P4 levels decreases again and the lining of the uterus is shed in the menstruation phase (11).

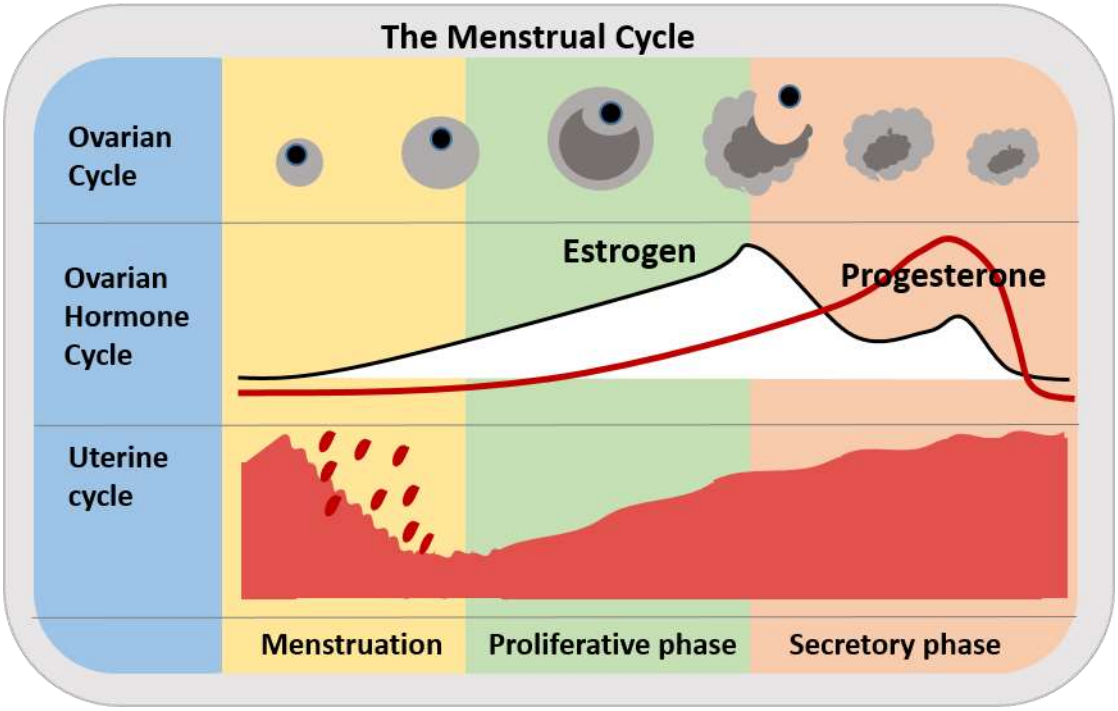


Figure 2-1: The normal menstrual cycle in females

The menstrual cycle includes the ovarian and the uterine cycle where changes occur in three different phases, menstruation, proliferation and secretary phase. Estrogen peaks in the menstrual cycle while P4 levels are high during pregnancy where the uterine lining needs to be maintained for implantation to occur. Adapted from Silverthorn, 2010 (11)

2.3 Steroid hormones

E_2 and P4 are lipophilic steroid hormones, not only important during the menstrual cycle and pregnancy but also critical for other functions in the reproductive systems in both males and females. E_2 together with estrone (E_1) and estriol (E_3) are the three major forms of estrogens (10).

2.3.1 The functions of E_2 and P4

E_2 assists primarily in females with the development of the secondary sex characteristics while P4 together with estrogen regulates the reproductive tract during the menstrual cycle as described above. As mentioned P4 is the main hormone that facilitates pregnancy (12).

In the male reproductive tract, these hormones also assist in critical functions. Although testosterone is the primary hormone, both E_2 and P4 are also involved in critical development and other functions. Both these hormones influences spermatogenesis, sertoli cell function, spermiogenesis and testosterone biosynthesis (12-15). These effects proves its regulatory effects as they assist in sperm production (9), regulate fluid reabsorption, maintain a differentiated epithelial morphology and affect proliferation and differentiation of gonocytes and spermatogonia (12-18). Estrogen also acts locally to regulate normal male gonodal development and is therefore critical for fertility in males (19).

Non-reproductive functions of E_2 includes blood coagulation where it increases platelet adhesiveness, improves lung function, maintains the skin and modulates immunity where it is primarily involve in inflammation (19). E_2 also provide protection in the brain against insult-induced neuronal damage (19). P4 is involved in neuro-behavioural expression associated with sexual responsiveness and in bone where it is able to prevent bone loss (20, 21).

2.3.2 The unique structures and receptors of E_2 and P4

These complex steroid hormones have unique structures which enable them to bind to their receptors and fulfil their functions. The aromatized C18 steroid structure of E_2 has two hydroxyl groups at 3-beta and 17-beta positions as shown in Figure 2-2 (a) being critical when it comes to the binding of E_2 to its receptor (9, 19, 22). P4 is a 21-carbon steroid, figure 2-2 (b), which is an important precursor molecule for the biosynthesis of steroids (21, 23).

P4 is transported in the blood by transcortin which is a corticosteroid-binding globulin and albumin, a plasma protein, where only 2% of P4 is present in blood in the free-unbound state. P4 has a half-life of almost 5 minutes and its degradation product is known as pregnanediol, which is formed in the liver (21).

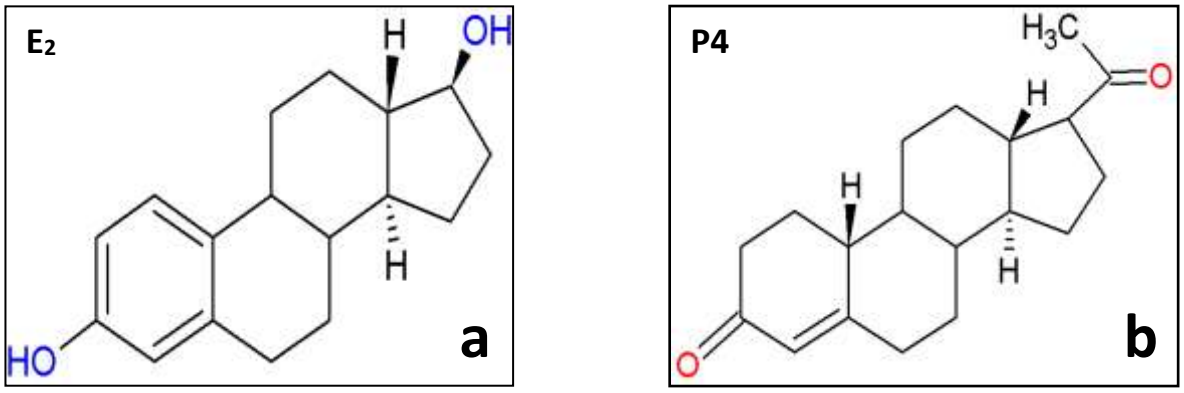


Figure 2-2: The unique structures of E_2 (a) and P4 (b)

The unique structure of E_2 showing the two hydroxyl groups at 3-beta and 17-beta positions (a) and the 21-carbon steroid, P4 (b). Adapted from Norman, A.W, 2004 (23)

Both E_2 and P4 have specific receptors to which they bind. A receptor must be able to recognise and bind the steroid hormone using the available free circulating concentration of the steroid. Plasma concentrations are usually 5-500 nM of steroids, while nuclear receptors are in the range of 0.05-50 nM, reflecting the strong, non-covalent binding of the hormone to its receptor. Sub-cellular localization of unoccupied steroid nuclear receptors varies with the specific steroid hormone, they can be present either in the cytoplasm attached to chaperones, in the nucleus or between the cytoplasm and the nucleus (23).

Steroid receptors are members of the nuclear receptor family and characterized by its unique ability to bind to its ligand within the cytoplasm, dimerize and enter the nucleus where it binds

to DNA to promote the transcription of target genes (24). Although the receptors of both E_2 and P4 belong to the same family of receptors, they each bind to their specific receptors.

Two nuclear receptors of E_2, estrogen receptor α (ERα) and β (ERβ) have been identified. Cell membrane receptors include GPR30 and ER-X (22). P4 binds to PGR (P4 receptor) (24). E_2 receptors are found throughout the male reproductive tract where ERα is localized in the efferent ductule epithelium where it is highly expressed. ERα is also localized in the sertoli and leydig cells of males. ERβ is found in the somatic cells or primary spermatocytes (18, 25). P4 receptor (PGR) is able to promote cellular responses independent of nuclear entrance and DNA binding. PGR has a high complexity and specificity as it functions via two distinct isoforms namely PGR-A and PGR-B (24).

2.3.3 The biosynthesis of E_2 and P4

The biosynthesis of E_2 and P4 is an intense biosynthetic process. E_2 is also regarded as the major product of the entire biosynthesis and the most potent estrogen during the premenopausal period (19). Estrogen is primarily produced in the ovaries, corpus luteum and placenta of females. In males, E_2 is produced in sizable quantities in the testes. Although early studies indicated that the primary source of E_2 in the immature male are the sertoli cells while the leydig cells are responsible for E_2 production in adult males, growing evidence shows that germ cells are also able to produce E_2. Therefore germ cells are regarded recently as the major source of this steroid hormone in the male reproductive tract (18). Other sources of small amounts of estrogen include non-gonadal organs such as the liver, heart, skin, muscle, bone and the brain in both genders (19). The major source of P4 in females is the granulosa-lutein cells of the corpus luteum, along with the placenta. P4 is only present in males at very small quantities and is mainly produced in the testes. In both genders, P4 is also produced from other sources including adrenal cortex (20, 21).

Both E_2 and P4 are produced from cholesterol. This biosynthetic process is represented by figure 2-3 which explains the synthesis of E_2 and P4 in the thecal and granulosa cells in the ovaries of females as well as the pathway from the brain to the ovaries and testes. Cholesterol is firstly converted into pregnenolone before it is converted to P4 in the thecal and granulosa cells. In the brain neurons and astrocytes expresses all the enzymes critical for the production of estrogen. Therefore pregnenolone is not only converted to P4 but also to dehydro-epiandrosterone sulphate (DHEA-s), which is converted to androstenedione. Androstenedione forms estrone in the ovaries and testosterone in the testes (22). From there, E_2 is synthesized from estrone via 17β-hydroxysteroid dehydrogenase, while the enzyme aromatase converts testosterone to E_2 (19, 22).

P4 in the thecal cells is converted to androgens as thecal cells are not able to produce estrogens. Androgens are released from thecal cells and transported across the basement membrane into the granulosa cells to be converted to E_2. This conversion can only proceed in the presence of aromatase, the same enzyme responsible for the conversion of testosterone into E_2. Therefore this is primarily responsible for the biosyntheses of E_2 in both males and females. Aromatase is highly expressed in the testes of males as well as the accessory glands

to maintain the required estrogen levels for normal spermatogenesis, sperm motility and sperm maturation (19, 22).

Estrogen deactivation can occur through metabolism where E_2 is converted to less active forms E_1 and E_3. Sulfation of E_2 also changes the structure so that it is unable to interact with estrogen receptors (26).

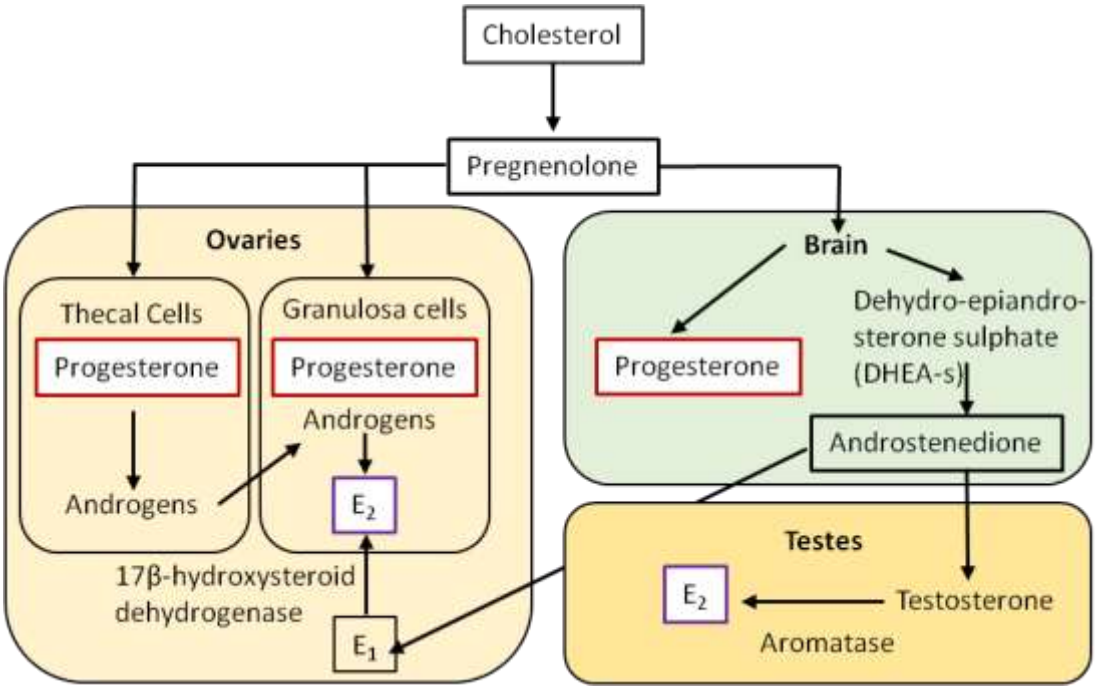


Figure 2-3: The biosynthetic process of E_2 and P4

Cholesterol is converted to pregnenolone to form P4. P4 is converted to androgens in the thecal cells where it is transported into the granulosa cells to form E_2. P4 in the brain is converted to androstenedione which is converted into estrone in the ovaries and testosterone in the testes. E_2 is formed from testosterone in males and estrone in females. Adapted from Ciu J, 2013 (22)

2.3.4 The mechanism of action of E_2 and P4

P4 and E_2 act through a ligand-dependent signalling cascade. They are able to diffuse through the plasma membrane as they are lipophilic. Reaching the nucleus, they encounter their receptors to which they bind with high affinity and specificity (27, 28). When these hormones bind to their receptors, they form a stable ligand-receptor complex and enter the nucleus where it binds to DNA to promote the transcription of target genes (24).

When E_2 binds to its receptor an estrogen receptor complex is formed, which binds to estrogen response element (ERE) sequences. This results in the recruitment of co-regulatory proteins and mRNA levels will be enhanced or decreased for the production of a specific protein to cause a physiological effect (28-30).

Although natural E_2 is known for many beneficial effects such as decreased risk for cardiovascular diseases observed in postmenopausal women (31) and osteoporosis (32), E_2 is capable of shifting the haemostatic balance to a hypercoagulable state in females, meaning that the coagulation process is up-regulated (5).

2.4 The blood coagulation system

There are various mechanisms in place favouring blood flow in a liquid phase in a healthy vasculature. It involves the flow of blood itself, diluting any activated or pro-thrombotic factors, the normal laminar flow of blood preventing contact of platelets to the endothelium and the expression of endothelial anti-platelet and anti-coagulant factors in excess of pro-coagulant factors (33). However, upon damage to the vasculature, other types of mechanisms should be in place to maintain stable haemostasis to protect against haemorrhage.

Blood coagulation or blood clotting is regarded as such a haemostatic mechanism where vascular walls interact with pro- and anti-coagulant factors in blood to prevent excessive blood loss and maintain blood fluidity and vascular integrity. Platelets and clotting factors are able to interact with each other to generate a protective haemostatic plug which reduces blood flow to the site of a vascular injury. The coagulation process follows which is dependent on many interactions including the assembly of even more platelets, along with the activation of circulating and tissue-bound coagulation factors to allow enzymatic conversions (33, 34). This process allows for the formation of a fibrin network which seals off a damaged vessel and prevents haemorrhage (2). A fibrinolytic system is in place for wound healing to occur where platelets aggregate and the fibrin clot is broken down, restoring perfusion through an injured vessel in which the protective clot has formed (35-40).

However the coagulation process mainly focuses on clot formation to prevent excessive blood loss and also removing clots to stabilize blood flow again, it is quite an intense process which involves various factors interacting with each other in order to maintain haemostasis. Some of these factors include platelets, thrombin, fibrinogen and fibrin (41).

Platelet activity is regarded as the initiation of blood coagulation. Damaged vasculature or trauma makes the subendothelial surface the primary target site for platelet action. Platelet localization and activation is dependent on various pro-coagulant factors such as Von Willebrand factor (vWF), thrombin, platelet receptors and vessel wall components such as collagen (42). When a platelet comes in contact with a break in the endothelium, either with damage to the blood vessels itself or tissue damage outside the blood vessel, it becomes activated. Upon platelet activation, the platelet changes shape, releases its granule contents

and gradually forms aggregates by adhering to each other. Platelet activation and localization sets the coagulation system in motion for the interactions between pro-coagulant factors (8, 20, 23, 43-45).

2.4.1 Models of coagulation

There are two proposed models for blood coagulation; the "cascade" or "waterfall" model and the cell-based model. A "cascade" or "waterfall" model involves an intrinsic and extrinsic pathway of coagulation that joins to form a classical or common pathway. In the cell-based model, the coagulation process is based on three basic phases namely the initiation phase, the amplification phase and the propagation phase.

2.4.1.1 The "cascade" or "waterfall" model

The "cascade" or "waterfall" model was first described in the 1960's (46, 47) and involves a series of steps where the activation of certain clotting factors results in the activation of other clotting factors, shown in figure 2-4. This model is described as the intrinsic and extrinsic pathways of coagulation which joins to form a classical or common pathway to culminate a thrombin burst and generate a fibrin clot (34, 41, 48).

The intrinsic pathway (figure 2-4) is activated by damage to the blood vessel itself. Platelets and factor VIII (FVIII) bound to von Willebrand factor, a plasma protein, leaves the vascular space and adhere to collagen and other matrix components at the site of injury. This binding to collagen activates platelets (42, 49). Von Willebrand factor (vWF) therefore assists in platelet adhesion by recruiting platelets to the site of injury where it promotes platelets to adhere to each other and to the walls of blood vessels. Various substances can trigger vWF secretion, including mediators of thrombosis and inflammation (49). Factor IXa diffuses through the surface of nearby activated platelets and binds to a specific platelet surface receptor (50). Factor IXa interacts with its cofactor, FVIIIa, and activate factor X directly on the platelet surface to provide a surface where phosphatidylserine is exposed. As soon as the membrane of a platelet becomes negatively charged, plasma protein FXII (Hageman factor) becomes activated (FXIIa). FXIIa accumulates and converts prekallikrein to kallikrein, which converts more FXII to FXIIa, in a positive feedback loop. Activated FXII cleaves FXI into activated FXI (FXIa), which in turn activates factor IX, another important protease. Activated

FIX cleaves FVIII into its activated form, which is stabilized by von Willebrand factor. FIXa, FVIIIa, Ca^{2+} and negatively charged phospholipids form a tenase tri-molecular complex which converts FX to FXa (34).

The expression of tissue factor (TF) in the extrinsic pathway, seems to be the main activator of coagulation currently (48,51, 52), which is up-regulated upon vascular injury, inflammation, plasmin and various other factors. Enhanced TF expression has also been identified due to oral contraceptives, shear stress, histamine, nicotine and estrogen (41).

When tissue factor is exposed, platelets aggregate and adhere to that site of injury and localize clotting reactions to the site of injury. An injury to the endothelium allows contact between FVII (zymogen) and tissue factor, for tissue factor to activate FVII into FVIIa, as tissue factor serving as a receptor for protein FVII (53). This binding of FVIIa to TF forms an enzyme complex, similar in function to the tenase complex in the intrinsic pathway, converting FX to its active form (FXa) and forms the common pathway from the intrinsic and extrinsic pathway, shown in figure 2-4. Activated factor X binds to cofactor V and is bound on membrane surfaces in the presence of calcium ions to generate the prothrombinase complex.

This complex is able to converge clotting signals coming from both the extrinsic and intrinsic pathways forming the common pathway. Activated factor X (FXa) has enzymatic activity converting the proenzyme prothrombin, to its enzyme form, thrombin. Thrombin (FIIa) is involved in the main coagulant function at the termination stage as it cleaves off fibrinogen (FBG) into fibrin monomers that cross link to produce insoluble blood clots. Tissue factor seems to be the main instigator of the coagulation process and rapid amplification of thrombin is the most important step in the development of a stable fibrin clot. The extrinsic pathway is thus complemented by the intrinsic pathway which ensures FIIa regeneration and further clot production as it activates FXIII, FXI, FVIII or FV which assures the propagation of blood coagulation (2, 34, 41).

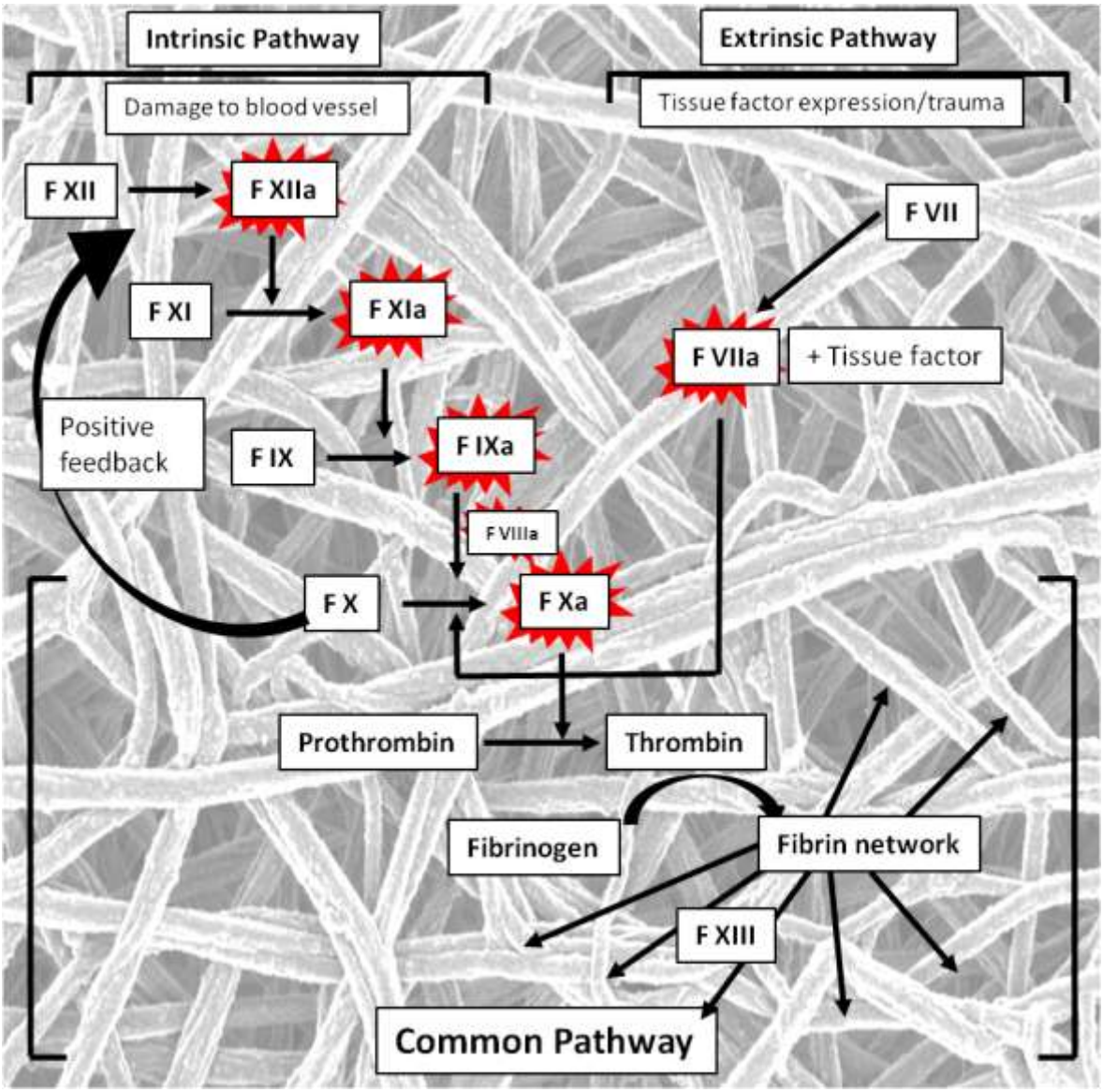


Figure 2-4: The intrinsic and extrinsic pathway involved in the blood coagulation process

In a final common pathway prothrombin (FII) is converted to thrombin (FIIa). Thrombin is essential to activate fibrin that cross-link that results in the formation of a stable fibrin clot. Transglutaminase enzyme factor XIIIa stabilizes the fibrin network for it to act as a scaffold for the binding of endothelial cells, leukocytes, platelets and plasma proteins to the fibrin clot. Adapted from Aleman M.M, 2014 (3)

2.4.1.2 The cell-based model

Three basic phases is regulated by cellular components *in vivo* namely; the initiation phase, the amplification phase and the propagation phase (54, 55). The initiation phase involves the exposure of tissue factor to blood and mainly entails the localization of the process on tissue factor expression cells and the amount of thrombin generated (9, 56). The duration of the initiation phase is dependent of TF:FVIIa concentration. The amplification phase entails the conversion from extrinsic to intrinsic thrombin generation (32, 57-59). Both the tenase complex and the prothrombinase complex is enhanced by their co-localization on the phospholipid membrane in the presence of calcium. The activated factors create a positive feedback loop to rapidly generate sufficient amounts of thrombin in order to form a stable clot. Thrombin accumulation further activates platelets. Finally the propagation phase relies on the recruitment of activated platelets at the site of injury, to provide localization of all the important components for the optimum generation of thrombin. A thrombin burst is observed which leads to the generation of fibrin from fibrinogen in order to produce a stable fibrin clot (11, 12, 33, 34, 60, 61).

2.4.2 The importance of thrombin and fibrinogen in the coagulation system

Activation factors V, VIII and XI up-regulates the coagulation system and thrombin is generated. Therefore thrombin is regarded as a strong pro-coagulant. Control of thrombin action allows for control for the whole blood coagulation system. Thrombin has various functions when it comes to the coagulation cascade. It not only converts fibrinogen into fibrin to create a fibrin fiber network, but also inhibit clot lysis by activating TAFI (Thrombin activatable fibrinolysis inhibitor) (1), which decreases the effectiveness of fibrinolysis (34, 60). Thrombin also regulates its own production by binding to a vascular endothelial cell protein called thrombomodulin. Thrombin is thus regarded as the central point in regulation of the coagulation process and plays an important role in haemostasis (1).

The most essential step in the coagulation process involves the conversion of fibrinogen into fibrin in the presence of thrombin. Fibrinogen is the most abundant protein present in the clear extracellular fluid component of blood known as plasma. The blood plasma together with the formed elements (blood cells and platelets) makes up whole blood (3, 29, 41).

Fibrinogen is a long structure made up of six paired polypeptide chains $(A\alpha\ B\beta\ \gamma)_2$. It has two domains; a central E domain and two D domains, one on either side, represented in figure 2-5. The cleavage of fibrinogen takes part in two steps. Both these steps are catalysed by thrombin. When fibrinogen is converted into fibrin, thrombin firstly cleaves fibrinogen at the E domain releasing fibrinopeptide A (FPA), and exposing a polymerisation site. This exposure allows for relatively weak association between this site and a D domain of another fibrin molecule. The molecules are then arranged in an end-to-middle, cross-linking fashion and fibrin polymerisation occurs. Secondly thrombin cleaves the E domain again releasing fibrinopeptide B (FPB) and exposes another fibrin polymerisation site. This site associates with another site on the D domains of other molecules, and the cross-linking is strengthened. The fibrinogen molecule is thus converted to a fibrin monomer. Thrombin then removes the Aα chain which eposes a new N-terminal sequence known as the A site. The A site permits the non-covalent association of the central E nodule with an exposed a pocket in the c-chain of the D nodule of another fibrinogen molecule. An ED association is observed which results in the formation of double stranded protofibrils. Removal of the N-terminal peptide of the β chains exposes the N-terminal sequence known as the B site. The B site interacts with an exposed β-pocket in the β chain of the D nodule of another molecule (62). This way fibrin monomers initiates fibrin polymerization (63), into oligomers that lengthen to form wide protofibrils. Protofibrils aggregates to form thick fibres, which branch along with lateral and longitudinal growth which results in a three-dimensional fibrin network as shown in figure 2-5 (62, 64). Thrombin also generates factor XIII, a transglutaminase which strengthens the fibrin matrix even more after the formation of the fibrin clot, which cross-links the fibrils, thus stabilizing the clot (1).

Having a closer look in fibrin formation or clot structure give insights into the mechanisms of many diseases associated with thrombotic complications (65-67). Fibrinogen concentration is directly related to the structure of a fibrin network. When fibrinogen levels are elevated, fibrin fiber network density increases, as well as clot stiffness and the resistance of the clot to fibrinolysis. The fibrin network is not only sensitive to the fibrinogen concentration, but also to the concentration of thrombin during fibrin polymerization. Various factors in blood plasma can change the ultrastructure of the fibrin network as fibrin fiber quality is dependent on plasma composition. Platelet rich clots contains platelet aggregates that are surrounded

by a dense meshwork of fibers that are thinner than fibres elsewhere in the clot, and many of them radiate out from the activated platelets, perhaps along the gradient of thrombin activity (68). Hormonal fluctuations are among these factors (3, 5). The fibrin network of platelet rich plasma is much denser than that of platelet poor (68).

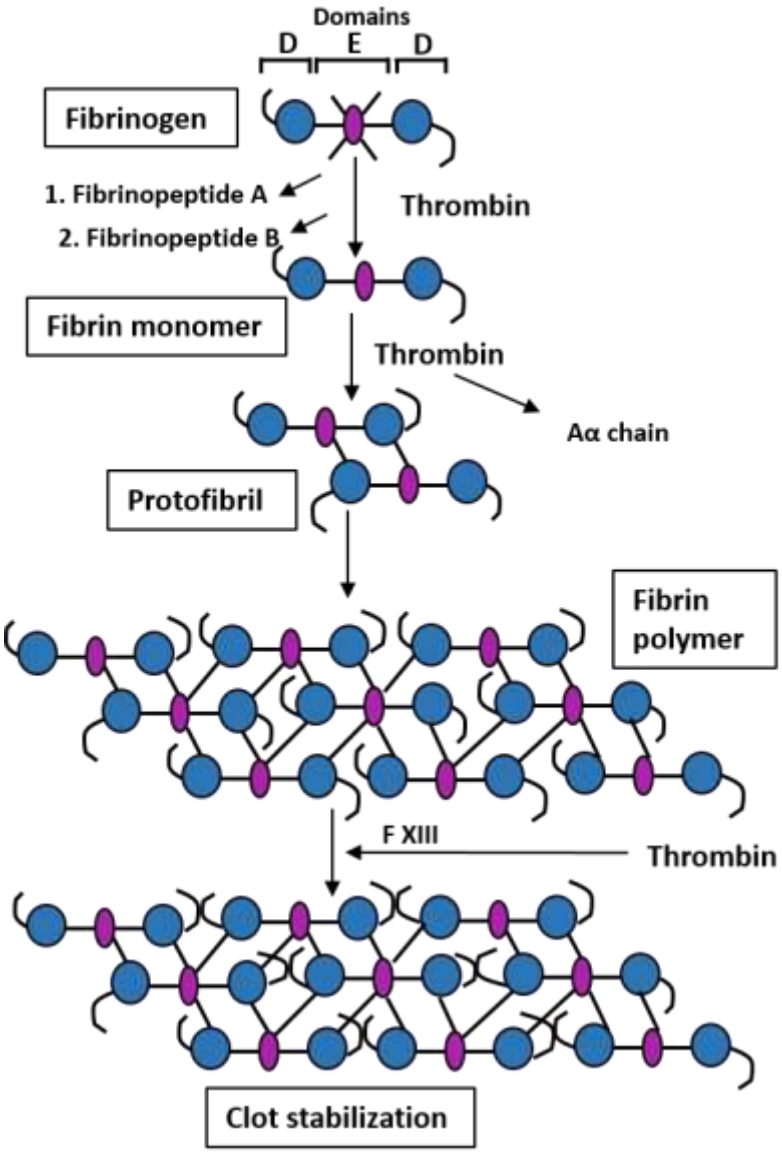


Figure 2-5: The process of fibrin polymerization

Fibrin polymerization where thrombin removes the two fibrinopeptides A and B from the central E nodule. Lateral aggregation and fibrin fiber formation occurs until fibrinolysis takes place which is facilitated by plasmin. Adapted from Weisel J.W, 2013 (63)

A fibrinolytic system, referred to as fibrinolysis, is in place to balance the coagulation process as healing occurs in order to restore normal perfusion of blood through the injured vessel in which the clot was formed (60). Fibrinolysis terminates the coagulation process by removing the clot formed and cellular proliferation repairs the injured vasculature. Circulating plasminogen is cleaved proteolytically to produce plasmin which interfere with fibrin polymerization and initiates the cleavage of fibrinogen or soluble fibrin. Plasmin is the principle mediator in fibrinolysis as it cleaves fibrin which results in the production of fibrin degradation products (FDP's) (33, 34). Elevated FDP's are usually associated with abnormal thrombotic states (33,34, 69-71).

2.5 The crosstalk between blood coagulation, inflammation and steroid hormones

An extensive crosstalk exists between the blood coagulation system, inflammation and steroid hormones as displayed in figure 2-7, below (72). Activation of both inflammation and the coagulation system are not really associated in time, but they both have critical functions in host defence. Immune cells are important in the initiation of coagulation pathways, while inflammatory mediators are capable of altering haemostasis (72). At the site of inflammation, reactive oxygen species (ROS) are generated causing endothelial disruption and vascular injury (73) that will activate the coagulation system. Inflammation also increases fibrinogen and thrombin concentrations (74) resulting in the over-activation of the coagulation system that shifts the haemostatic balance toward a hypercoagulable state (75).

One of the major pathways that in which inflammation augments blood coagulation is the generation of thrombin which is mediated by tissue factor that is upregulated by monocytes, macrophages and endothelial cells (51). Thrombin exerts both pro- and-anti-inflammatory effects. Pro-inflammatory effects includes the production of pro-inflammatory and pro-coagulant factors including von Willebrand factor , growth factors and cytokines (IL-6 AND IL-8) (76, 77). Thrombin acts as a chemo attractant for neutrophils and monocytes, induces P-selectin expression in endothelial cells, assist in monocyte and neutrohpil chemotaxis (78, 79) and is involved in leukocyte adhesion molecule expression (80). Thrombin is also involved in IL-6 and IL-8 production by endothelial cells (81) and lymphocyte and monocyte activation

and proliferation (82, 83). All these reactions imitate an inflammatory response which triggers a coagulation response (77).

According to previous studies, the effects of E_2 and P4 may influence coagulation through its inflammatory effects. Both E_2 and P4 have pro- and anti-inflammatory effects (75). Estrogen receptors are regarded as modulator marker for inflammation and blood coagulation (31), as ER expression has been identified in cell types involved in inflammatory processes such as T cells, B cells, dentritic cells, monocytes and macrophages. Estrogen-mediated suppression of inflammation is mediated via ER-α and not ERβ (84).

Although natural endogenous E_2 is known for many beneficial effects such as decreased risk for cardiovascular diseases observed in postmenopausal women (31) and osteoporosis (32), E_2 is capable of shifting the haemostatic balance to a hypercoagulable state in females, meaning that the coagulation process is upregulated (5). E_2 also exerts its pro-thrombotic effects by increasing the levels of factor XIII, which is responsible for fibrin cross-linking, resulting in the formation of a thrombus that is more stable and resistant to fibrinolysis (85).

Recent studies have confirmed that a positive feedback loop for continues estrogens and prostaglandin E_2 production is established in pathologic tissue itself. As prostaglandin was found to be the most potent inducer of aromatase activity, a critical enzyme in the biosynthesis of estrogen, in endometriotic cells, estrogen upregulates prostaglandin E_2 formation by stimulating cyclo-oxygenase type 2 enzyme (8). Previous studies also confirmed that E_2 affects macrophage recruitment and activation (86) and decrease fibrinogen (87). Previous studies for P4 confirmed that prostaglandins and leukocytes was upregulated upon P4 withdrawal, indicating that P4 has a possible anti-inflammatory action (86). More recent studies suggested that P4 are also able to inhibit pro-inflammatory cytokine secretion (88).

2.6 Hypercoagulability

A deficiency in any of the important components contributing to the coagulation process or certain factors, such as hormonal fluctuations, can result in inadequate amounts of thrombin that allow timely fibrin clot formation which does not respond efficiently to vascular injury (34). When high levels of thrombin are produced, dense fibrin networks containing abnormal thin fibres are observed. This is regarded as a hypercoagulable state, where thrombus formation is observed (3). Continuous intravascular activation of coagulation and thrombin-mediated activation of fibrin deposition can result in venous thrombosis or thromboembolism (VTE) (3, 4).

Estrogen is known for many beneficial effects such as decreased risk for cardiovascular diseases observed in postmenopausal women (31) and osteoporosis (32), but it has been demonstrated that oral contraceptives and hormone replacement therapy are associated with thromboembolic events (31). Previous studies confirmed that synthetic estrogens are associated with this increased tendency to thrombosis in women, but natural induced estrogens did not produce a hypercoagulable state (7). More recent studies indicated changed plasma fibrinogen levels during the menstrual cycle in females, mainly due to the fluctuations in oestrogen levels, as an altered fibrin fiber assembly was observed. Estrogen is capable of shifting the haemostatic balance to a hypercoagulable state in females, meaning that the coagulation process is upregulated (5).

E_2 effects are mediated by both ERα and ERβ, displayed on circulation platelets (89). Estrogens are also capable of reducing venous flow by increasing venous dispensability, which can cause sufficient damage to initiate thrombus formation (7). E_2 also exerts its pro-thombotic effects by increasing the levels of factor XIII. Activated factor XIII catalyses the formation of cross-links between fibrin molecules, thus forming a more stable thrombus which is resistant to fibrinolysis. This confirms E_2's pro-thombotic effects (85).

It seems that the route of administration of estrogens has shown to have a major impact on the risk of venous thromboembolism. The action of estrogens are therefore strongly influenced by the dose, route and timing of the hormonal treatment alongside age, genetic and environmental factors (89).

According to the latest World contraceptive patterns report 13% of women of reproductive age use hormonal contraceptives (8.9% use oral contraceptives and 4.1% use injectable contraceptives) worldwide. In developed countries this number rises: the United States of America 16% (90), Europe 21% and in the United Kingdom it is a staggering 28% (91). Although it is arguably one of the most important medical breakthroughs of all times, hormonal contraceptives are associated with increased risk of VTE (6,44, 45, 92).

Since endogenous E_2 and P4 themselves may alter clot formation and therefore also augment the effect of synthetic hormonal contraceptives thus increase the risk of VTE, it is essential to investigate the effect of E_2 and P4 on clot formation.

2.7 Summary

Various factors in blood plasma can change the ultrastructure of the fibrin network as fibrin quality is dependent on plasma composition. Hormonal fluctuation are among these factors (3, 5). It is known that steroid hormones are able to change the fibrin network when coagulation occurs, but the individual effects or the specific concentrations where the ultrastructure is changed remains unknown. Blood coagulation is recognized as a host defence system that provides protection against threat and fatal haemorrhage (2, 33, 41, 60), but it is important to prevent a hypercoagulable state that could increase the risk of thrombosis. As the coagulation system and inflammation are linked and steroid hormones such as E_2 and P4 have both anti-and pro-inflammatory effects, it provides a possible mechanism to influence fibrin network.

Figure 2.6 provides a summary of the effects of E_2 and P4 on fibrin clot formation and the involvement of inflammation and coagulation factors.

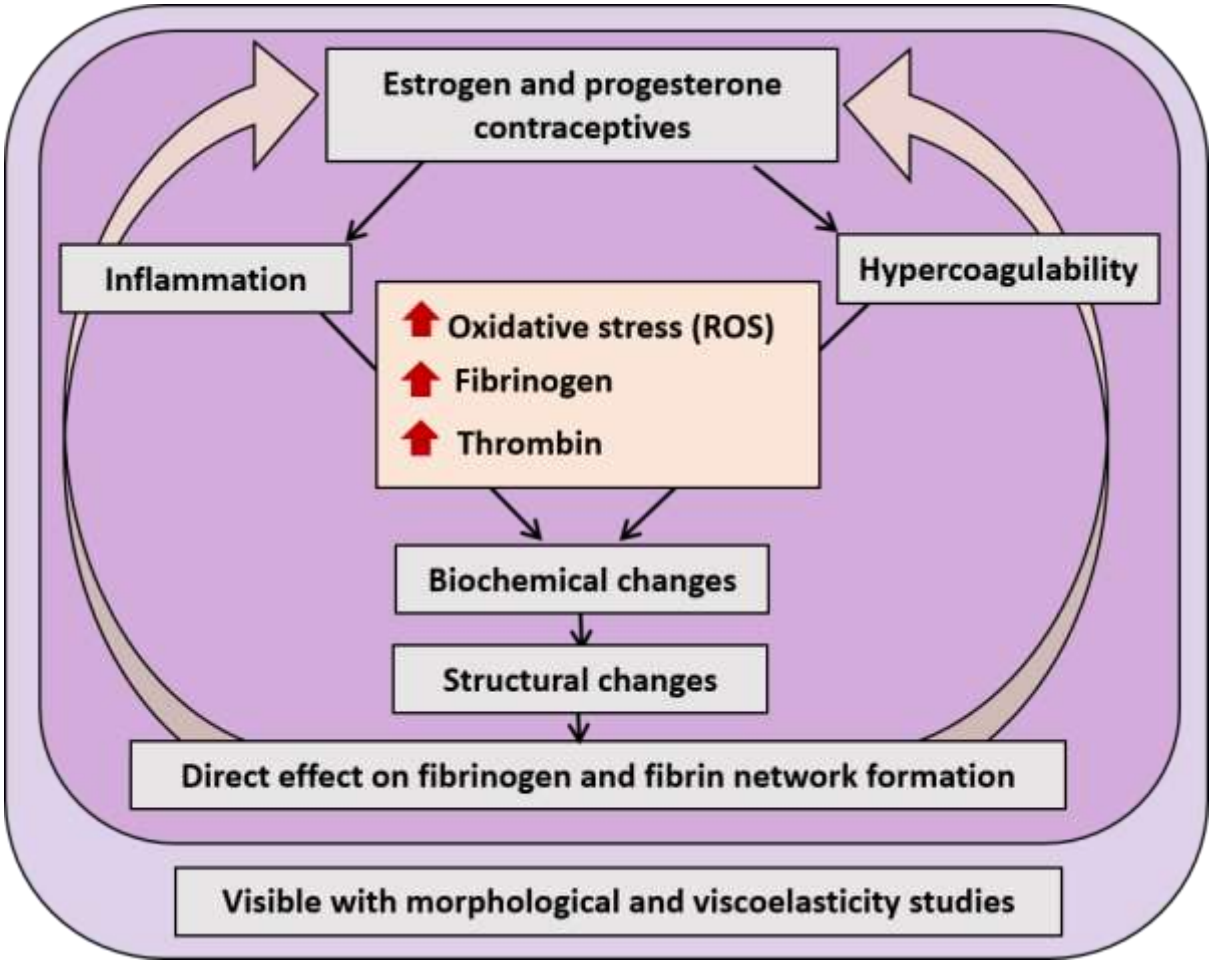


Figure 2-6: A summary of the interactions between coagulation and inflammation

Steroid hormones are able to shift haemostasis to a hypercoagulable state which related to biochemical and structural changes to fibrinogen and subsequent fibrin network formation which can be visualized with morphological and viscoelastic analysis.

Increased levels of E_2 and P4 can affect fibrinogen and thrombin directly causing ultrastructural changes when observing the morphology of fibrin networks. The binding of E_2 and P4 to the fibrin precursor, fibrinogen, before clot formation could lead to biochemical changes that result in ultrastructural changes. These alterations to the fibrin network is visible with morphological and viscoelastic studies. Therefore the possible pro-inflammatory properties of these hormones are able to trigger a pro-thrombotic state. This emphasizes the importance of establishing the specific effect of E_2 and P4 on clot formation.

2.8 Aim

To determine whether different physiological concentrations of E_2 and P4 have changing effects on the fibrin network formation of platelet poor plasma with regards to viscoelastic studies, morphological investigations and turbidimetry.

2.9 Objectives

Objective 1: To investigate viscoelastic properties of clot formation, using thromboelastography (TEG), before and after the addition of different physiological concentrations of E_2 and P4.

Objective 2: To establish morphological changes in the fibrin network before and after the addition of physiological concentrations of E_2 and P4 using scanning electron microscopy (SEM).

Objective 3: To observe surface topography of the fibrin networks prepared before and after the addition of the physiological concentrations of E_2 and P4 using atomic force microscopy (AFM).

Objective 4: To observe plasma clot formation and clot lysis time of clots before and after the addition of the physiological concentrations of E_2 and P4 using turbidimetry.

2.10 Hypothesis

H_0: No changes in clot formation or ultrastructure will be observed after the addition of physiological concentrations of E_2 and P4 when analysed by TEG, SEM, AFM and turbidimetry.

H_1: Changes in clot formation or ultrastructure will be observed after the addition of physiological concentrations of E_2 and P4 after analysed by TEG, SEM, AFM and turbidimetry.

Chapter 3: Materials and methods

3.1 Chapter objective

In this chapter the sample collection is explained along with the participant information to provide a clear overview on the recruitment of the participants. This chapter also provides detailed methods regarding the preparation of the platelet poor plasma (PPP) and the techniques carried out to evaluate the fibrin clots namely thromboelastography (TEG), scanning electron microscopy (SEM), atomic force microscopy (AFM) and turbidimetry. Figure 3-1 (below) shows the basic layout of this study.

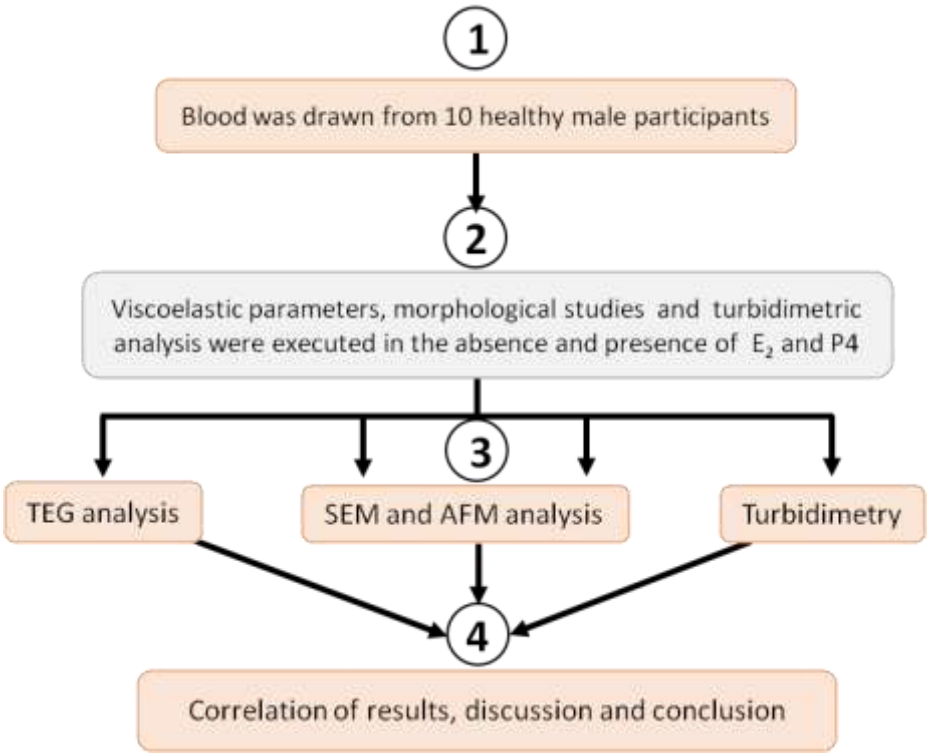


Figure 3-1: An overview summarizing the contents of this study

1. The healthy male participants' PPP was drawn by a qualified phlebotomist for laboratory analysis.

2 &3. Viscoelastic parameters (TEG), morphological studies (SEM and AFM) and turbidimetry were performed to investigate the properties of clot formation and changes in the fibrin network structure with analysis of fibrin diameter and surface roughness.

4. Results obtained were correlated and discussed

3.2 Participant information and sample collection

Blood samples of only male participants were obtained since E_2 and P4 occur at lower concentration and with less fluctuation in the plasma of males compared to that of females (93). In this study a total of 10 healthy male individuals between the age of 18 and 30 years were asked to participate freely. All male individuals were non-smokers, did not have a history of any type of thrombotic disease and were not using any chronic medication known to interfere with coagulation factors and/or platelet function. The participants did not use any aspirin or aspirin analogues within 48 hours before the blood samples were obtained. Ethical clearance was obtained and approved from the University of Pretoria Ethics Committee (reference 154/2014 and 66/2015). All participant information was handled anonymously.

The investigator discussed the project with each participant before blood was drawn and it was made clear that each participant was able to withdraw at any point of time. After the informed consent form was signed, blood was drawn by a qualified phlebotomist.

For viscoelastic studies and morphological analysis, blood samples were obtained via venepuncture in 5 mL citrate tubes which contained 0.5 mL of 3.8 % sodium citrate for 4.5 mL blood. Blood was also collected in 5 mL serum gel blood tubes that contain clot activator and gel for serum separation and taken to Vermaak and Partners Pathologists. These samples were collected to obtain iron profiles to assess the inflammatory status of each participant and hormone profiles, to ensure that endogenous hormones would not influence the results.

Iron profiles provide us with information regarding the presence or absence of inflammation. Hormone tests (E_2 and P4) are important to confirm normal hormone levels. Any abnormal levels of iron or hormones can interfere with results. Iron levels can quickly vary and therefore it is especially important to prevent an increase of iron levels as it can reach a point where it becomes toxic and can be harmful. Toxic iron concentrations have detrimental consequences on the vascular system and contribute to the progression of numerous pathological conditions that are associated with iron overload. Therefore high levels of iron will indicate the presence of inflammation which can cause alterations to the fibrin network (94, 95). Serum ferritin and transferrin (TF) saturation levels are good indicators that measures the iron levels as they are more accurate indicators of the iron levels than serum iron (96). Normal TF

levels are mostly higher in males than females because of the female menstrual cycle each month where iron is lost with menstrual flow.

The Serum Ferritin (SF) was measured by using the Bio-Rad Laboratories' QuantImune ferritin IRMA kit which is a single-incubation two-site immunoradiometric assay. In IRMA, which measures the most basic isoferritin, the highly purified I-labelled antibody to ferritin is the tracer and the ferritin antibodies are immobilized on polyacrylamide beads at the solid phase. Serum was mixed with the combined tracer/solid-phase antibody reagent, and the mixture was incubated. During incubation, the beads were diluted with saline, centrifuged and decanted. The level of I-labelled ferritin found in the pellets was measured by a gamma counter. There were a direct relationship between the radioactive levels of the pellets and the amount of endogenous ferritin in the serum, rather than the inverse relationship measured by most radioimmunoassays (RIA's). Serum Ferritin is the test of choice in the diagnosis of patients suffering from iron overload such as Haemochromatosis (97-99).

Hormone levels were tested at Vermaak and Partners Pathologists, using the E_2 ELISA test kit which specifically detects the estrogenic hormone E_2 in water and the P4 ELISA test kit which is developed for reliable and sensitive detection of P4 in serum samples. The E_2 ELISA test kit is based on a competitive reaction where enzyme-labelled standard E_2 competes with free E_2 in the sample for binding to a specific monoclonal antibody immobilised to the surface of the microtiter plate or tube. The amount of labelled E_2 bound to the plate is determined by addition of a non-coloured substrate which is converted into a coloured product. The colour intensity is measured at 450 nm and is inversely proportional to the amount of E_2 in the sample. The P4 ELISA test kit involves the addition of P4 enzyme reagent to the serum. After the addition of P4 biotin reagent, substrate and stop solution the absorbance is measured at 450 nm (100, 101). C-reactive protein (CRP) is known as a non-specific inflammatory marker and analysis is therefore important to ensure that iron levels are not affected by immunological states (102, 103).

The average iron and hormone profiles of the healthy participants are displayed in table 1. As compared to the reference values obtained from Kuhl, 2005 (104) all the healthy male participants had their iron panels and hormone profiles within the normal ranges.

Table 1: Summary of the participant information, iron and hormone profiles with the references ranges

Variables	Healthy individuals (n = 10)	Reference ranges Males	Reference ranges Females
Age (years)	23 (±2.26)		
Gender	Males		
Iron profiles			
Iron (µmol. L^{-1})	14.30 (± 4.42)	11.60 - 31.30	
Transferrin (g.L^{-1})	2.45 (± 0.19)	2.20 - 3.70	
Saturation (%)	22.50 (± 7.37)	20 – 50	
Serum Ferritin (ng.mL^{-1})	96.00 (± 49.72)	20 – 250	
Hormone profiles			
E_2 (pmol. L^{-1})	57.00 (± 24.79)	28 – 156	46 - 1828
P4 (nmol. L^{-1})	1.67 (± 0.79)	0.7 – 4.3	0.60 - 86
E_3 (ng.mL^{-1})	<0.07	<0.07	<0.08
Testosterone (nmol. L^{-1})	17.23 (± 5.60)	8.64 – 29	0.29 – 1.67
SHBG (nmol. L^{-1})	36.04 (± 11.92)	14.50 – 48.40	26.10 – 110

Inflammatory status were normal of all the male participants and the endogenous hormone levels were normal indicating that this endogenous hormone concentrations could not influence the results with regards to viscoelastic studies, morphology analysis and turbidimetry.

3.3 Sample preparation

After the blood samples were collected in the citrate blood tubes from the healthy male participants, the citrated blood was centrifuged for 15 min at 1250*g*. The supernatant plasma was transferred to Eppendorf tubes and centrifuged for a second time at 1250*g* for another 10 minutes to obtain platelet poor plasma (PPP) and frozen at -80°C for at least 48 hours.

The frozen PPP samples were thawed before analysis commenced. The PPP was incubated at 37°C for 15 minutes with different concentrations of both E_2 and P4 separately to attain the final concentrations as indicated in table 2.

Table 2: Final concentrations of platelet poor plasma (PPP) of E_2 and P4

	E_2 final concentration in PPP ($pg.mL^{-1}$) in the menstrual cycle	**P 4 final concentration in PPP ($ng.mL^{-1}$) in pregnancy**
Control (no addition of hormone)	0	0
Lowest physiological concentration of hormone	60	60
Highest physiological concentration of hormone	300	300

After the incubation period each PPP-hormone mixture was used for viscoelastic studies, morphology analysis and turbidimetry.

3.4 Laboratory analysis

3.4.1 Methodology for viscoelastic studies using thromboelastography

Thromboelastography (TEG) was employed to investigate the effect of E_2 and P4 on the viscoelasticity of fibrin clot formation. Thromboelastography (TEG) shows the kinetics of clot formation and growth as well as the strength and stability of the formed clot (105) by yielding a graphical and numerical output relating to the cumulative effects of various plasma and cellular elements of all phases of the coagulation and fibrinolysis (106). By measuring the viscoelastic changes that occur during the haemostatic process, TEG provides a real-time functional evaluation of the coagulation cascade beginning with the initial platelet-fibrin interaction, through platelet aggregation, clot strengthening and fibrin cross-linkage and eventually clot lysis (106, 107).

The different PPP-hormone mixtures (concentrations indicated in Table 2) were analysed by adding 340µL of each PPP-hormone mixture into a TEG cup, where after 20µL $CaCl_2$ was added to the PPP-hormone mixture to reverse the effect of the sodium citrate. Samples were then placed in a Thromboelastograph 5000 Hemostasis Analyser System to be analysed.

3.4.2 Methodology for morphological studies: Scanning electron and atomic force microscopy

SEM and AFM were used to investigate the effect of E_2 and P4 on fibrin network surface morphology with regards to structural analysis and surface roughness.

The different PPP-hormone mixtures (concentrations as displayed in Table 2) were used to prepare the fibrin clots for both SEM and AFM.

The different PPP-hormone mixtures (10µl) were individually mixed with thrombin (5µl) on round glass coverslips in order to make the plasma smears. Control samples was made with 10µL pure PPP (without any added hormone) and 5µL thrombin only while experimental smears were made 10 µL PPP exposed to different concentrations of either E_2 or P4 along with 5 µL thrombin. The addition of thrombin creates the fibrin network as explained in the literature review. Thrombin was provided by the South African National Blood Services

(SANBS). The glass coverslips with the different PPP-hormone and thrombin mixtures were immediately placed on filter paper dampened with 0.075 M sodium potassium phosphate buffer solution (pH = 7.4) inside an airtight container to ensure a humid environment. The enclosed samples were incubated at 30°C for 5 minutes and then washed in buffer solution for 20 minutes using a plate shaker to remove any trapped blood proteins within the fibrin network. Samples subsequently underwent a primary fixation step with 4% formaldehyde for 30 minutes followed by three rinsing changes with buffer solution (PBS) for 5 minutes each. A secondary fixation step with 1% osmium tetraoxide (OsO_4) was done for 15 minutes to ensure the preservation of membrane phospholipids. Three more rinsing steps with PBS followed as mentioned before. Procedures were concluded with a series of dehydration steps of different ethanol concentrations (30 %, 50%, 70%, 90% and three times 100%) for 5 minutes each. Thereafter samples underwent hexamethyldisilazane (HMDS) submersion for 30 minute and lastly air-drying of samples in a flow hood (5, 108-110).

3.4.2.1 Scanning electron microscopy (SEM)

For SEM preparation, the glass coverslips were mounted on an aluminium platform with carbon tape and coated with carbon before samples were examined with a Zeiss Ultra plus FEG SEM (located in the Microscopy and Microanalysis laboratory of the University of Pretoria, Pretoria, South Africa) at 1 kV with InLens capabilities to analyse the morphology of the fibrin network formations.

Micrographs were firstly taken to observe the morphology of the fibrin network formations in the absence and presence of physiological concentrations of E_2 and P4 separately. Thereafter the fibrin fiber diameters were measured using ImageJ Software.

3.4.2.2 Atomic force microscopy (AFM)

AFM sample preparation did not include any mounting or coating with carbon. The samples on the glass coverslips were placed directly in the AFM Dimension Icon with ScanAsyst, Bruker, USA for analysis of surface roughness using the PeakForce QNM (Quantitative Nanomechanical Property Mapping) imaging mode. This technique is almost similar to the standard tapping mode of scanning probe microscopy, where the probe and the sample is brought together, but conversely to tapping mode where the oscillation amplitude is kept

constant. This mode operates by controlling the maximum force applied by the probe to the sample (111).

A 1 µm by 1 µm scan was performed on each sample with a peak force of 6 µN. Offline software (NanoScope Analysis version R3, Bruker, USA) was used to process all images and measure the surface roughness.

3.5 Sample preparation for Turbidimetric assays

Frozen PPP samples were thawed before analyses commenced. Samples were exposed to different concentrations of each hormone as described above. For turbidimetric analysis more concentrations were tested than for viscoelastic and morphological analysis to give a more detailed account of these hormones 'effect on clot formation and also clot lysis time. Plasma fibrinolytic potential of tissue factor induced clots, lysed by exogenous tissue plasminogen activator (tPA) (112-114) were determined for control plasma and plasma with different concentrations of E_2 and P4 displayed in table 3. Clot formation and lysis were measured at 405nm and 37°C until clots were lysed. Final concentrations in clots were: 700 x diluted tissue factor (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany), 17 mmol/l $CaCl_2$, 60 ng/mL tPA (Boehringer Ingelheim, Germany) and 10 µmol/L phospholipid vesicles (Rossix, Mölndal, Sweden).

Table 3: Different concentrations of E_2 and P used for turbidimetric analysis

E_2	30 $pg.mL^{-1}$	60 $pg.mL^{-1}$	140 $pg.mL^{-1}$	220 $pg.mL^{-1}$	300 $pg.mL^{-1}$	1 $ng.mL^{-1}$
P4	30 $ng.mL^{-1}$	60 $ng.mL^{-1}$	140 $ng.mL^{-1}$	220 $ng.mL^{-1}$	300 $ng.mL^{-1}$	

3.6 Statistical analysis

The seven parameters measured with TEG are explained in table 4. For the different concentrations of each hormone all parameter values were obtained for statistical analysis.

Table 4: Summary of thrombo-elastic parameters

	Parameters	Description
R	Reaction time (minutes)	Time of latency from start of test to initial fibrin formation i.e. initiation time
K	Kinetics (minutes)	Time taken to achieve a certain level of clot strength i.e. amplification
α	Angle (degrees)	The angle measures the speed at which fibrin build up and cross linking takes place, hence assesses the rate of clot formation; i.e. thrombin burst
MA	Maximum amplitude (mm)	Maximum strength/stiffness of clot. Represents the ultimate strength of the fibrin clot, i.e. overall stability of the clot
MRTG	Maximum rate of thrombus formation ($Dyn.cm^{-2}.s^{-1}$)	The maximum velocity of clot growth
TMRTG	Time to maximum rate of thrombus formation (minutes)	The time interval observed before the maximum speed of the clot growth
TTG	Total thrombus generation ($Dyn.s^{-1}$)	The clot strength: the amount of total resistance generated during clot formation. This is the total area under the velocity curve during clot growth representing the amount of clot strength generated during clot growth

SEM micrographs were taken at 40 000x machine magnification and used to measure fibrin strand thickness. Image J (http://rsb.info.nih.gov/ij/download.html) was used to measure a

total of 1000 fibrin fibres (100 strands per individual) for the control as well as different concentrations of each hormone.

Surface roughness was measured with the use of AFM. De Oliveira et al provide a comprehensive overview of AFM (115). We employed AFM in tapping mode to investigate surface roughness of individual fibrin fibers. As the cantilever tap on the sample while it scans over the specimen it intermittently makes contact with the surface of the fibrin fiber and surface texture is then conveyed. Roughness (also referred to as being uneven, irregular or coarse) describes closely spaced irregularities. It is quantified by the vertical spacing of a real surface form its ideal form. Roughness relates to large spacing compared to the control surface while smoothness relates to small spacing compared to the control surface. RMS (root mean square) was then used as statistical measure (115). AFM software was used to evaluate the surface roughness of 150 areas on individual fibrin fibers (3 points on 5 randomly selected fibrin fibers of each participant) for the controls and different concentrations of each hormone.

Turbidimetric parameters measured include: Lag time (the time it takes for the coagulation cascade to be activated and fibrin fibers to grow enough for lateral aggregation), slope (the rate of lateral aggregation), maximum absorbance (an indication of fibre thickness) and clot lysis time (CLT), which is the midpoint of maximum turbidity to clear, minus clot formation time-midpoint of clear to maximum turbidity (116).

Graphpad Prism 5 was employed to perform one-way ANOVA for all statistical analysis with a p value of ≤ 0.005 considered significant.

Chapter 4: Results

4.1 Chapter objectives

In this chapter the effect of different concentrations of E_2 and P4 will be discussed with regards to

- The viscoelastic properties of fibrin clots
- The morphological effect on fibrin fiber networks and individual fibrin fibres
- The surface roughness of individual fibrin fibres
- Turbidimetric analysis

4.2 Influence of E2 on PPP clot formation

As described in chapter 3, different physiological concentrations of E_2 as found throughout the menstrual cycle were added to the PPP of healthy male participants before clot formation, fibrin network morphology, individual fibrin fiber diameter, surface topography and turbidimetric parameters were analysed.

4.2.1 The viscoelastic properties of fibrin clots

Thromboelastography (TEG) shows the kinetics of clot formation and growth as well as the strength and stability of the formed clot (105) by yielding a graphical and numerical output relating to the cumulative effects of various plasma and cellular elements of all phases of coagulation and fibrinolysis (106) as explained in chapter 3. The 7 parameters were analysed and compared in the absence and presence of E_2.

Table 5: Summary of the effects of different concentrations of E_2 on viscoelastic properties of clot formation

Viscoelastic parameters					
	Control	**Lowest physiological concentration**	**Highest physiological concentration**	**F-value [F=3,36]**	**P-value**
R (minutes)	8.40 (± 2.56)	8.75 (± 2.66)	***4.85 (± 1.81)***	**7.373**	**0.0006**
K (minutes)	5.30 (± 2.78)	2.70 (± 3.11)	***1.85 (± 1.09)***	**3.651**	**0.0214**
Angle (degrees)	66.50 (± 8.96)	65.80 (± 6.85)	73.20 (± 8.26)	**2.942**	**0.0460**
MA (mm)	21.70 (± 3.44)	27.93 (± 3.86)	***31.33 (± 5.45)***	**6.402**	**0.0014**
MRTG ($Dyn.cm^2.s^{-1}$)	4.55 (± 1.93)	4.31 (± 1.29)	***7.17 (± 4.51)***	**7.388**	**0.0006**
TMRTG (minutes)	9.63 (± 2.72)	10.34 (± 3.10)	***6.02 (± 2.18)***	**6.109**	**0.0018**
TTG ($Dyn.s^{-1}$)	138.87 (±30.40)	193.98 (±36.02)	***227.55 (±66.58)***	**5.917**	**0.0022**

An analysis of variance (ANOVA) showed that all viscoelastic parameters were significantly influenced by E_2 (refer to table 5 for mean ±s standards deviation of each concentration evaluated for E_2, F-values and p-values). Dunnett post hoc criterion for significance was performed to compare the effects of the different concentrations of E_2 to the control group (significance indicated with blue, bold and italic in table 5).

This viscoelasticity study showed that only the highest concentration of E_2 at $300pg.mL^{-1}$ significantly influenced almost all the thrombo-elastic parameters analysed, while the lowest physiological concentration at $60pg.mL^{-1}$ had no significant effect on these parameters. E_2 at a concentration of $300pg.mL^{-1}$ significantly decreased the initiation time (R), amplification (K) and time interval before maximum velocity of clot growth is observed (TMRTG), while it significantly increased the overall stability of the clot (MA), maximum velocity of clot growth (MRTG) and clot strength (TTG). However, it did not have a significant effect on the thrombin burst (α, angle). This specific trend of decreases and increases in the specific parameters is

indicative of hypercoagulability, specifically at a concentration at 300pg.mL^{-1}, which produces hypercoagulable fibrin clots while 60pg.mL^{-1} produced fibrin clot formations similar to that of the controls.

4.2.2 The morphological effect on fibrin networks and individual fibers

SEM was used to investigate the effect of E_2 on fibrin network surface morphology with regards to structural analysis and fibrin diameter investigations.

Using scanning electron microscopy (SEM) provides us with information on surface topography, crystalline structure, chemical position and electrical behaviour. Alternative advantages of SEM analysis provides for a large field depth so most of the specimen surface is simultaneously in focus independent of the roughness of the surface and higher magnification settings for topography analysis. Large specimens can be analysed with SEM as it is does not destroy the specimen and has a short specimen preparation time (117).

It is of great importance to study the morphology of fibrin network formation under different circumstances as a comparative picture can be obtained. Scanning electron microscopy can also guide us to different parameters which can be analysed such as fibrin thickness or fibrin fibre diameter. Detailed descriptions can be obtained from this morphological technique including features like granules or dense matted deposits (DMDs).

After SEM micrographs were taken, the micrographs were carefully studied and are shown below in figure 4-1.

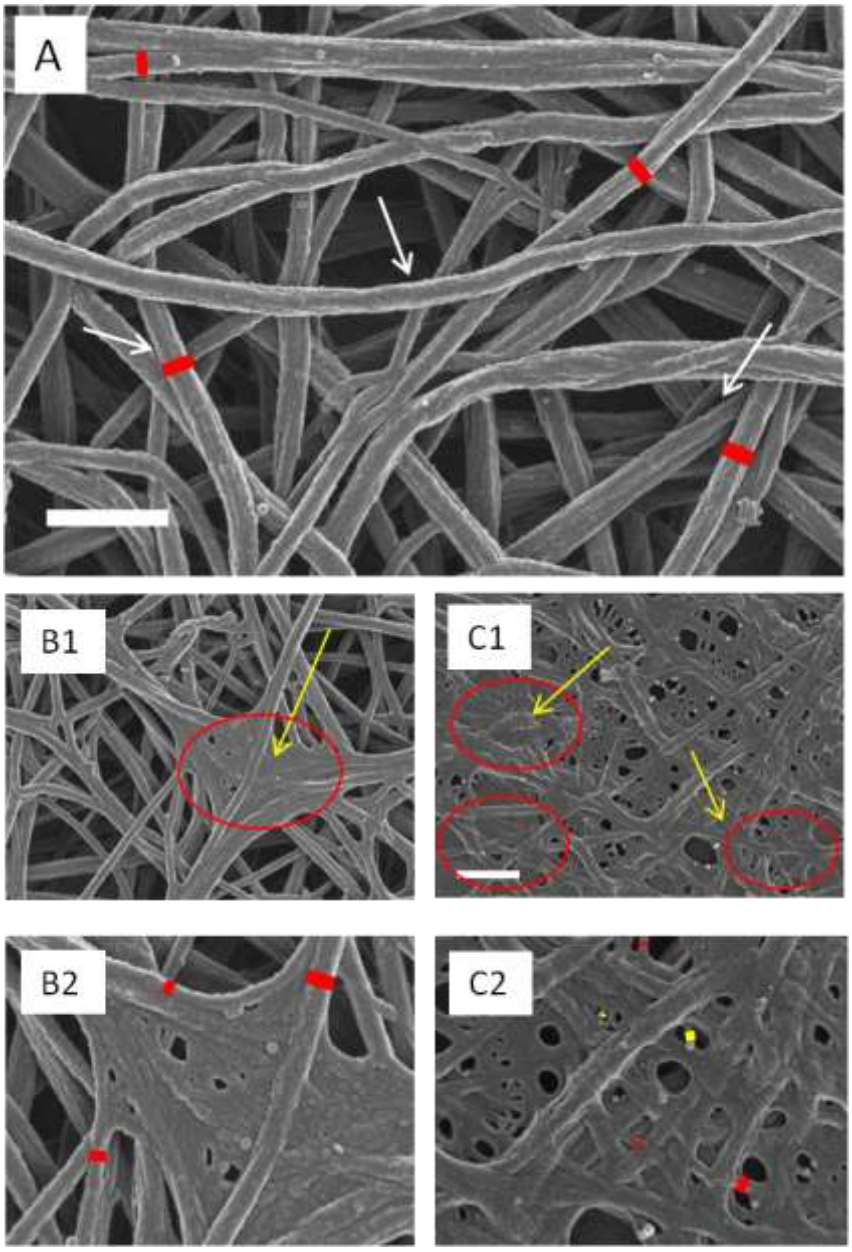


Figure 4-1: SEM micrographs representing the morphological features of the fibrin fiber networks of E_2

A representation of a control sample (A), E_2 at a concentration of 60 pg.mL^{-1} machine magnification of 40 000x (B1) and 100 000x (B2), and E2 at concentration of 300 pg.mL^{-1} at machine magnification of 40 000x (C1) and 100 000x nm (C2). Scale bars represent 1 µm (A, B1 & C1) and 100nm (B2 & C2). White arrows represent normal fibrin fibers, while yellow arrows show DMDs. Red and yellow bars are indicative of individual fibrin fibers where the yellow bars display the smaller fibrin fibers.

Figure 4-1 (A) shows fibrin strands that are evenly dispersed and mainly composed of thick fibrin fibers which are typical without any addition of hormones. B1-C2 shows the effect of the different concentrations of E_2 and at different machine magnifications; an accumulation of thin fibers are seen which forms DMDs which are present at both concentrations although it is most prevalent with the highest physiological concentration (300 $pg.mL^{-1}$ final concentration) where it covers most of the surface (C1). These DMDs give the fibrin network a sticky appearance.

The fibrin diameters were measured and analysed through Image J from the micrographs taken with SEM. Results obtained were analysed with ANOVA, along with a Dunnett post hoc criterion for significance, (table 6 shows the mean, standard deviation, F and p-values. Significance indicated with blue, bold and italic).

Table 6: Changes in fibrin fiber diameter for different concentrations of E_2

Morphological analysis: fibrin diameter					
	Control	**Lowest physiological concentration**	**Highest physiological concentration**	**F-value [F=3,36]**	**P-value**
Fibrin diameter (nm)	125.97 (±48.84)	***113.02 (±46.12)***	***108.34 (±45.75)***	**42.11**	**<0.0001**

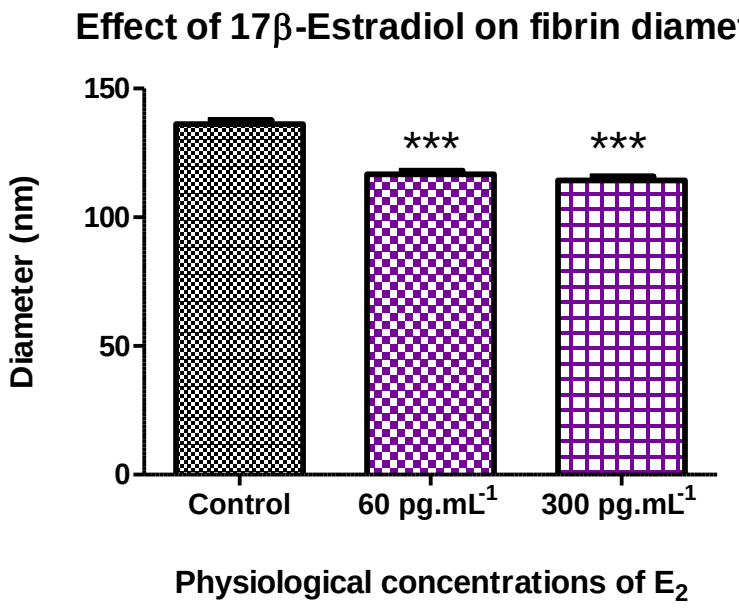


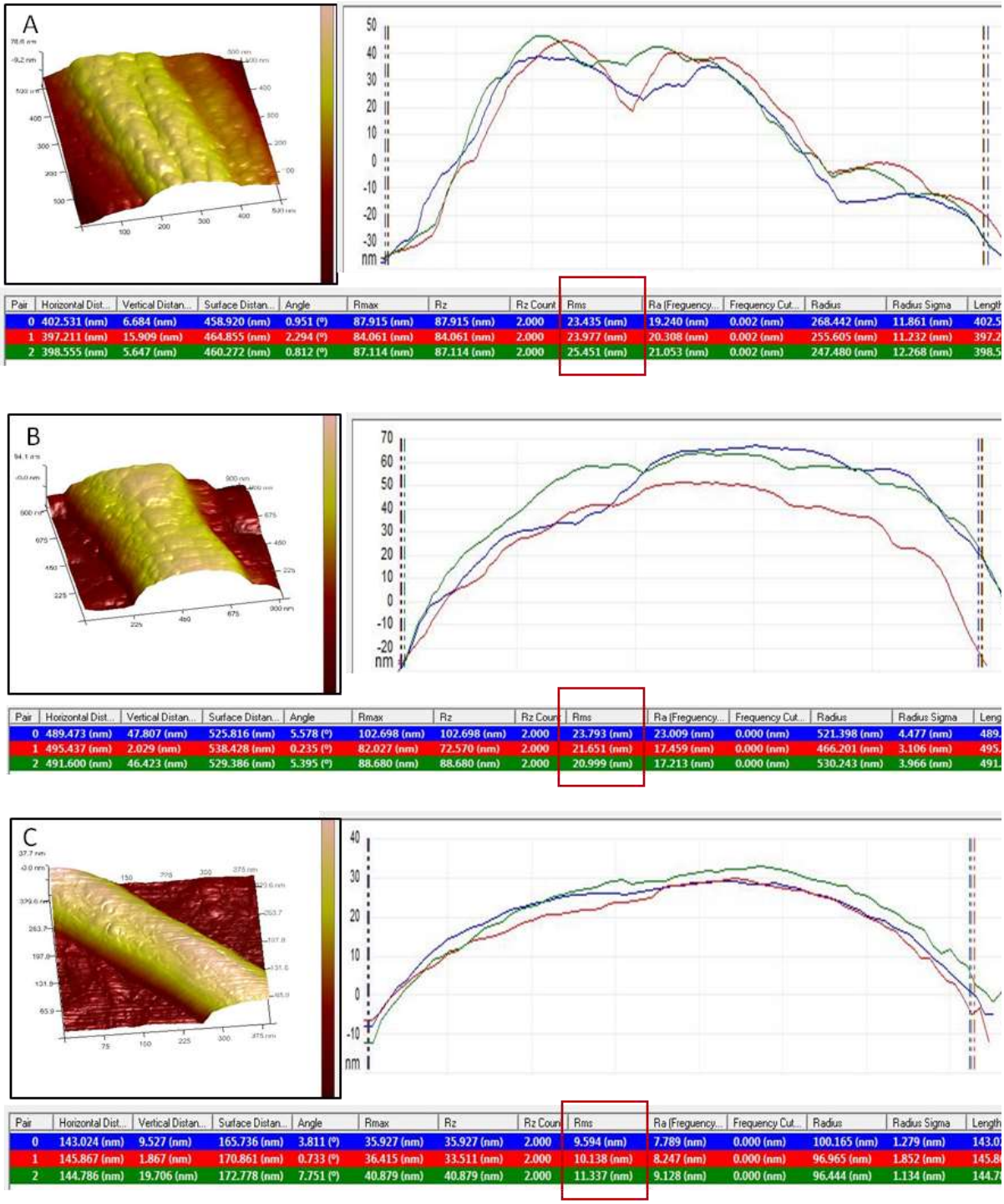
Figure 4-2: Graph displaying the changes in fibrin fiber diameter when the different physiological concentrations of E_2 were added to the PPP

All concentrations of E_2 significantly decreased the diameters of individual fibrin fibers compared to the fibrin fibers formed without addition of hormones as shown in table 6 and figure 4-2.

4.2.3 The surface roughness of individual fibrin fibers

The effects of the different concentrations of E_2 on the surface roughness of individual fibrin fibers are displayed in figure 4-3 (below).

As previously mentioned, a 1 µm by 1 µm scan was performed on each sample with a peak force of 6 µN. Figure 4-3 represents images from the Offline software (NanoScope Analysis version R3, Bruker, USA) which was used to process all images and measure the surface roughness.



Pair	Horizontal Dist...	Vertical Distan...	Surface Distan...	Angle	Rmax	Rz	Rz Count	Rms	Ra (Frequency...	Frequency Cut...	Radius	Radius Sigma	Length
0	402.531 (nm)	6.684 (nm)	458.920 (nm)	0.951 (°)	87.915 (nm)	87.915 (nm)	2.000	23.435 (nm)	19.240 (nm)	0.002 (nm)	268.442 (nm)	11.861 (nm)	402.5
1	397.211 (nm)	15.909 (nm)	464.855 (nm)	2.294 (°)	84.061 (nm)	84.061 (nm)	2.000	23.977 (nm)	20.308 (nm)	0.002 (nm)	255.605 (nm)	11.232 (nm)	397.2
2	398.555 (nm)	5.647 (nm)	460.272 (nm)	0.812 (°)	87.114 (nm)	87.114 (nm)	2.000	25.451 (nm)	21.053 (nm)	0.002 (nm)	247.480 (nm)	12.268 (nm)	398.5

Pair	Horizontal Dist...	Vertical Distan...	Surface Distan...	Angle	Rmax	Rz	Rz Count	Rms	Ra (Frequency...	Frequency Cut...	Radius	Radius Sigma	Leng
0	489.473 (nm)	47.807 (nm)	525.816 (nm)	5.578 (°)	102.698 (nm)	102.698 (nm)	2.000	23.793 (nm)	23.009 (nm)	0.000 (nm)	521.398 (nm)	4.477 (nm)	489.
1	495.437 (nm)	2.029 (nm)	538.428 (nm)	0.235 (°)	82.027 (nm)	72.570 (nm)	2.000	21.651 (nm)	17.459 (nm)	0.000 (nm)	466.201 (nm)	3.106 (nm)	495.
2	491.600 (nm)	46.423 (nm)	529.386 (nm)	5.395 (°)	88.680 (nm)	88.680 (nm)	2.000	20.999 (nm)	17.213 (nm)	0.000 (nm)	530.243 (nm)	3.966 (nm)	491.

Pair	Horizontal Dist...	Vertical Distan...	Surface Distan...	Angle	Rmax	Rz	Rz Count	Rms	Ra (Frequency...	Frequency Cut...	Radius	Radius Sigma	Length
0	143.024 (nm)	9.527 (nm)	165.736 (nm)	3.811 (°)	35.927 (nm)	35.927 (nm)	2.000	9.594 (nm)	7.789 (nm)	0.000 (nm)	100.165 (nm)	1.279 (nm)	143.0
1	145.867 (nm)	1.867 (nm)	170.861 (nm)	0.733 (°)	36.415 (nm)	33.511 (nm)	2.000	10.138 (nm)	8.247 (nm)	0.000 (nm)	96.965 (nm)	1.852 (nm)	145.8
2	144.786 (nm)	19.706 (nm)	172.778 (nm)	7.751 (°)	40.879 (nm)	40.879 (nm)	2.000	11.337 (nm)	9.128 (nm)	0.000 (nm)	96.444 (nm)	1.134 (nm)	144.7

Figure 4-3: Surface roughness of individual fibrin fibers after the addition of different concentrations of E_2

A control fibrin fiber (A), a fiber exposed to E_2 at a concentration of 60 pg.mL^{-1}(B) and 300 pg.mL^{-}(C). Figures A-C display the 3-dimentional structure, the 2-dimentional curve when a fiber is dissected along with the study of the root mean square (RMS) set values obtained, enclosed by the red block.

The control fibrin fiber showed a rougher surface then those at the different concentrations of E_2. It seems that the higher the concentration of E_2, the smoother the surface topography becomes.

Level of significance is shown in table 7 as the RMS along with the display of a graph representing surface topography (Figure 4-4).

Table 7: Statistical analysis of surface roughness after the addition of different physiological concentrations of E_2

Morphological analysis: surface roughness					
	Control	**Lowest physiological concentration**	**Highest physiological concentration**	**F-value [F=3,36]**	**P-value**
Surface roughness (nm)	21.07 (± 2.63)	17.95 (± 10.29)	***15.17 (± 9.36)***	**2.678**	**0.0463**

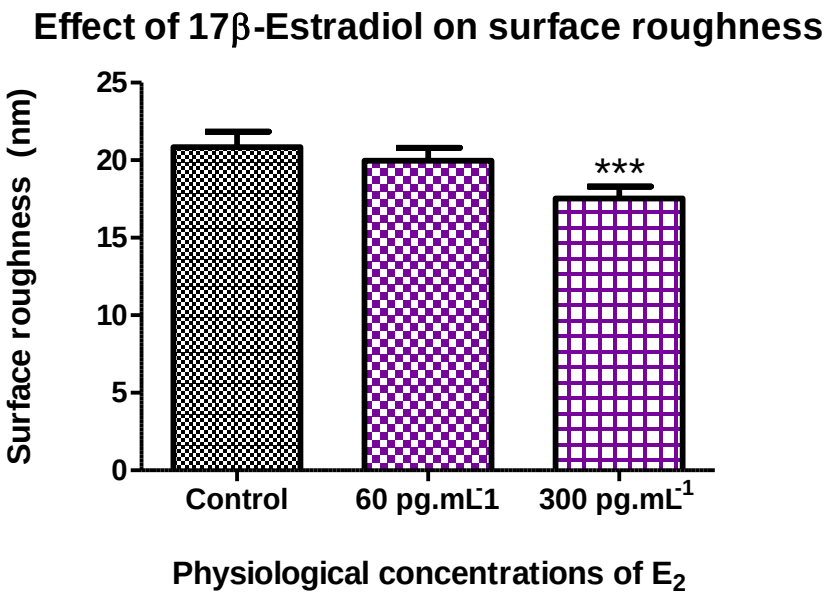


Figure 4-4: Graph displaying the effect of E_2 at different physiological concentrations on fibrin fiber surface roughness

AFM analysis revealed that the lowest physiological concentration of E_2 did not influence the roughness of the fibrin strands significantly, although the dissected fiber showed decreased roughness. The highest physiological concentration of the hormone however significantly decreased the roughness of the fibrin fibers. Thus E_2 only at a concentration of 300pg.mL^{-1} had a statistically significant effect on fibrin fiber roughness. At this specific concentration the fibrin fibers become smoother compared to fibrin fibers with no hormones added.

4.2.4 Turbidimetric analysis for E_2

Table 8 provides a summary of the results obtained by turbidimetry, where the lag time, slope, maximum absorbance and clot lysis time were determined for the different concentrations of E_2.

Table 8: Summary of the effects of different concentrations of E_2 on turbidimetric parameters indicated as mean ± standard deviation

Turbidimetric parameters				
	Lag time (minutes)	**Slope ($x10^{-3}$ au/s)**	**Maximum absorbance**	**CLT (minutes)**
Control	4.852 (±0.532)	6.348 (±2.016)	0.496 (±0.136)	71.050 (±3.736)
30pg.mL^{-1}	5.343 (±0.670)	5.806 (±1.248)	0.439 (±0.096)	67.380 (±3.857)
60pg.mL^{-1}	5.096 (±0.600)	5.108 (±0.647)	0.444 (±0.103)	67.830 (±3.904)
140pg.mL^{-1}	5.081 (±0.566)	5.283 (±0.692)	0.444 (±0.112)	***66.370 (±2.903)***
220pg.mL^{-1}	5.139 (±0.639)	5.582 (±1.38)	0.448 (±0.111)	***66.520 (±3.677)***
300pg.mL^{-1}	5.208 (±0.444)	5.341 (±0.577)	0.449 (±0.112)	***66.060 (±4.010)***
1ng.mL^{-1}	5.100 (±0.600)	5.265 (±0.700)	0.445 (±0.124)	66.640 (±4.568)
F-value [F = 6;63]	**0.662**	**1.381**	**0.297**	**2.007**

ANOVA p-value	**0.681**	**0.236**	**0.936**	**0.078**

An ANOVA showed no statistically significant overall change to turbidimetric analysis, however Dunnett post hoc criterion for significance indicated that only at concentrations 140, 220 and 300pg.mL^{-1} of 17β-estradiol significantly decreased the CLT (significance indicated with blue, bold and italic in Table 8).

All concentrations of 17β-estradiol tested increased the lag time and decreased the slope, but this was not statistically significant. The maximum absorbance was also decreased for all concentrations of 17β-estradiol and although not statistically significant correlates with the significant decrease in fibrin fiber diameter as visualised with SEM.

This indicates that the activation of the coagulation cascade and rate of lateral aggregation are slower (although not statistically significant) in the presence of 17β-estradiol compared to the control samples. Decreased fiber thickness confirms the decreased fibrin diameters visualised with SEM and measured with ImageJ. The overall decreased clot lysis time, with statistical significance for the 140-300 pg.mL^{-1} indicate that fibrin clots exposed to different concentrations of 17β-estradiol cause the clots to lyse faster than that of the control fibrin clots.

4.3 Influence of P4 on PPP clot formation

As described in chapter 3, different physiological concentrations of P4 were added to the PPP of healthy male participants before clot formation, fibrin network morphology and individual fibrin fiber diameter and surface topography were analysed.

4.3.1 The viscoelastic properties of fibrin clots

Table 9 provides a summary of the effects of the different concentrations of P4 on the viscoelastic properties of fibrin clot formation. The same viscoelastic parameters analysed for E_2 were also investigated for P4 using TEG.

Table 9: Summary of the effects of different concentrations of P4 on viscoelastic properties of clot formation

Viscoelastic studies					
	Control	**Lowest physiological concentration**	**Highest physiological concentration**	**F-value [F=3,36]**	**P-value**
R (minutes)	8.40 (± 2.56)	7.25 (± 2.14)	7.05 (± 1.29)	**1.211**	**0.0006**
K (minutes)	5.30 (± 2.78)	***2.60 (± 0.96)***	***2.25 (± 0.99)***	**6.065**	**0.0214**
Angle (degrees)	66.50 (± 8.96)	65.35 (± 6.73)	66.60 (± 5.07)	**0.9516**	**0.0460**
MA (mm)	21.70 (± 3.44)	29.45 (± 4.45)	***29.45 (±7.22)***	**2.595**	**0.0014**
MRTG ($Dyn.cm^{-}.s^{-1}$)	4.55 (± 1.93)	6.36 (± 2.81)	***6.15 (± 2.56)***	**2.838**	**0.0006**
TMRTG (Minutes)	9.63 (± 2.72)	9.56 (± 2.26)	9.29 (± 1.48)	**0.7013**	**0.0018**
TTG ($Dyn.s^{-1}$)	138.87 (±30.40)	197.10 (±45.13)	***212.87 (±75.57)***	**2.546**	**0.0022**

Referring to table 9, P4 did not influence fibrin clot formation in the same manner as E_2. It was mainly the K (amplification), MA (maximum velocity), MRTG (maximum velocity of clot growth) and TTG (clot strength) values that were influenced by different concentrations of

P4. The amplification (K) was significantly decreased by both concentrations of P4, while the overall stability (MA), maximum velocity of clot growth (MRTG) and clot strength (TTG) was significantly increased by the highest physiological concentration of 300ng.mL^{-1}.

4.3.2 The morphological effect on fibrin networks and individual fibers

SEM was used to investigate the effect of P4 on fibrin network surface morphology with regards to structural analysis and fibrin diameter investigations. All SEM samples were prepared in the exact same manner for both E_2 and P4.

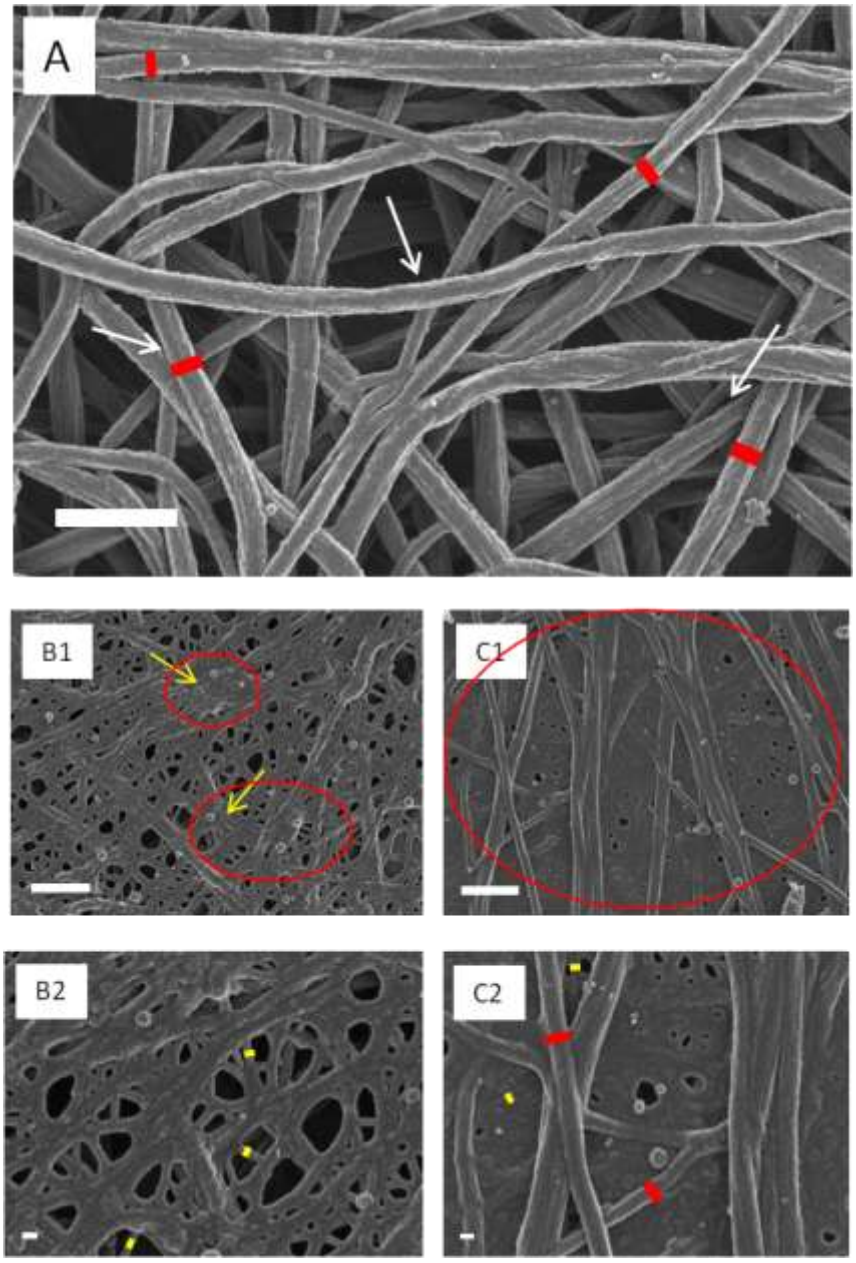


Figure 4-5: SEM micrographs representing the morphological features of the fibrin fiber networks of P4

A representation of a control sample (A), P4 at a concentration of 60 ng.mL^{-1} of machine magnification of 40 000x (B1) and 100 000x (B2), and P4 at a concentration of 300 ng.mL^{-1} at machine magnification 40 000x (C1) and 100 000x (C2). Scale bars represent 1 µm (A, B1 & C1) and 100nm (B2 & C2). White arrows represent normal fibrin fibers while yellow arrows show DMDs. Red and yellow bars are indicative of different fibrin diameters where the yellow bars display the smaller fibrin fibers.

Figure 4-5 shows the effect of different concentrations of P4 on fibrin network morphology. Like E_2 DMDs are present for all concentrations but appear more closely packed for the lowest and highest physiological concentrations compared to E_2. When compared to only P4 the fibrin fiber network for the highest concentration of 300 ng.mL^{-1} appears denser than that of the lowest concentration at 60 ng.mL^{-1}.

The fibrin diameters were also measured for P4 and analysed through Image J from micrographs taken with SEM. Results obtained were analysed with ANOVA along with a Dunnett post hoc criterion for significance, (Table 10 shows the mean, standard deviation, F and p-values. Significance indicated with blue, bold and italic).

Table 10: changes in fibrin fiber diameter after addition of different concentrations of P4

Morphological studies: Fibrin diameter					
	Control	**Lowest physiological concentration**	**Highest physiological concentration**	**F-value [F=3,36]**	**P-value**
Fibrin diameter (nm)	125.97 (±48.84)	***111.26 (±58.28)***	***111.79 (±53.10)***	**37.90**	**<0.0001**

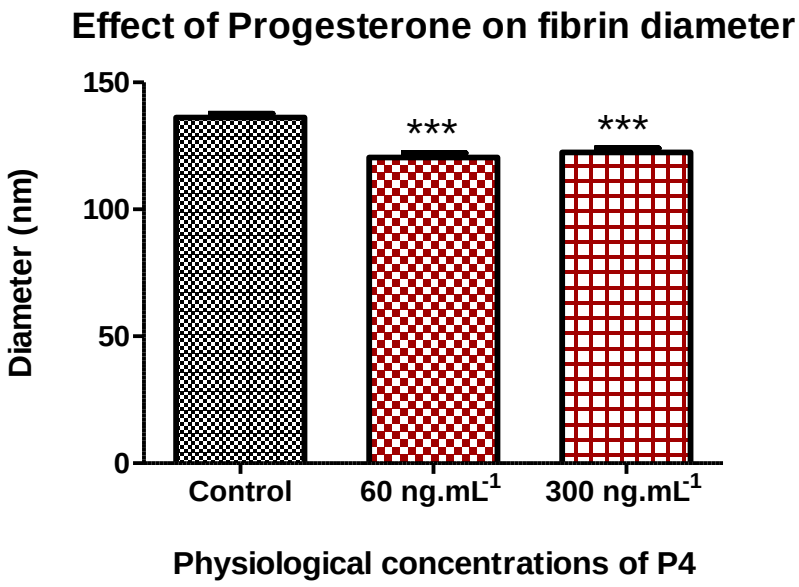
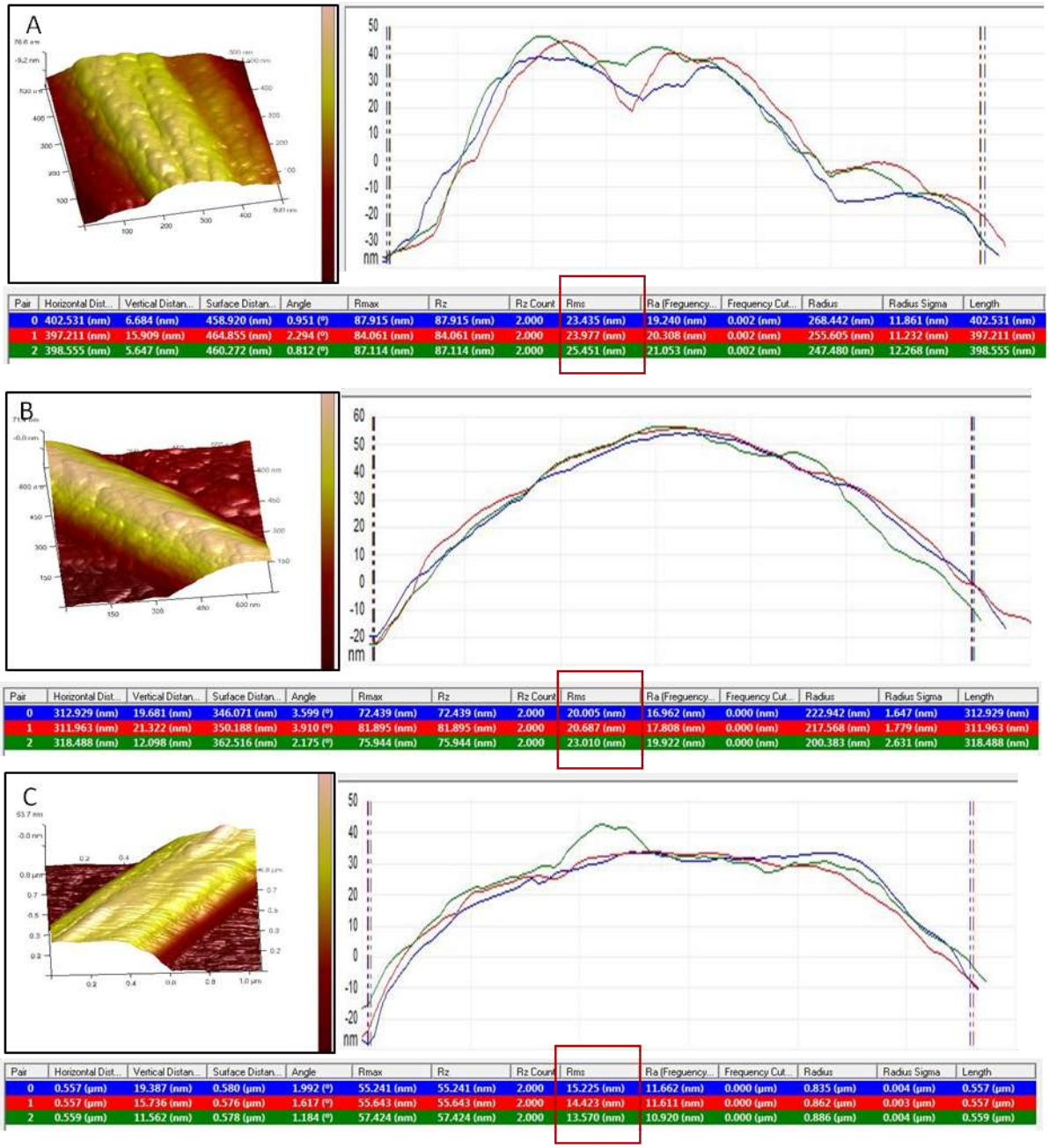


Figure 4-6: Changes in fibrin fiber diameter after addition of different physiological concentrations of P4 to PPP

Both concentrations of P4 significantly decreased the fibrin diameters of the individual fibers compared to the fibrin fibers formed without the addition of any of these hormones. (Figure 4-6 and Table 10, above)

4.3.3 The surface roughness of individual fibres

AFM was used to analyse surface roughness for P4. Figure 4-7 represents images from the Offline software (NanoScope Analysis version R3, Bruker, USA) which was used to process all images and measure the surface roughness.



Pair	Horizontal Dist...	Vertical Distan...	Surface Distan...	Angle	Rmax	Rz	Rz Count	Rms	Ra (Frequency...	Frequency Cut...	Radius	Radius Sigma	Length
0	402.531 (nm)	6.684 (nm)	458.920 (nm)	0.951 (°)	87.915 (nm)	87.915 (nm)	2.000	23.435 (nm)	19.240 (nm)	0.002 (nm)	268.442 (nm)	11.861 (nm)	402.531 (nm)
1	397.211 (nm)	15.909 (nm)	464.855 (nm)	2.294 (°)	84.061 (nm)	84.061 (nm)	2.000	23.977 (nm)	20.308 (nm)	0.002 (nm)	255.605 (nm)	11.232 (nm)	397.211 (nm)
2	398.555 (nm)	5.647 (nm)	460.272 (nm)	0.812 (°)	87.114 (nm)	87.114 (nm)	2.000	25.451 (nm)	21.053 (nm)	0.002 (nm)	247.480 (nm)	12.268 (nm)	398.555 (nm)

Pair	Horizontal Dist...	Vertical Distan...	Surface Distan...	Angle	Rmax	Rz	Rz Count	Rms	Ra (Frequency...	Frequency Cut...	Radius	Radius Sigma	Length
0	312.929 (nm)	19.681 (nm)	346.071 (nm)	3.599 (°)	72.439 (nm)	72.439 (nm)	2.000	20.005 (nm)	16.962 (nm)	0.000 (nm)	222.942 (nm)	1.647 (nm)	312.929 (nm)
1	311.963 (nm)	21.322 (nm)	350.188 (nm)	3.910 (°)	81.895 (nm)	81.895 (nm)	2.000	20.687 (nm)	17.808 (nm)	0.000 (nm)	217.568 (nm)	1.779 (nm)	311.963 (nm)
2	318.488 (nm)	12.098 (nm)	362.516 (nm)	2.175 (°)	75.944 (nm)	75.944 (nm)	2.000	23.010 (nm)	19.922 (nm)	0.000 (nm)	200.383 (nm)	2.631 (nm)	318.488 (nm)

Pair	Horizontal Dist...	Vertical Distan...	Surface Distan...	Angle	Rmax	Rz	Rz Count	Rms	Ra (Frequency...	Frequency Cut...	Radius	Radius Sigma	Length
0	0.557 (µm)	19.387 (nm)	0.580 (µm)	1.992 (°)	55.241 (nm)	55.241 (nm)	2.000	15.225 (nm)	11.662 (nm)	0.000 (µm)	0.835 (µm)	0.004 (µm)	0.557 (µm)
1	0.557 (µm)	15.736 (nm)	0.576 (µm)	1.617 (°)	55.643 (nm)	55.643 (nm)	2.000	14.423 (nm)	11.611 (nm)	0.000 (µm)	0.862 (µm)	0.003 (µm)	0.557 (µm)
2	0.559 (µm)	11.562 (nm)	0.578 (µm)	1.184 (°)	57.424 (nm)	57.424 (nm)	2.000	13.570 (nm)	10.920 (nm)	0.000 (µm)	0.886 (µm)	0.004 (µm)	0.559 (µm)

Figure 4-7: Changes in surface roughness of fibrin fibers after addition of physiological concentrations of P4

A control fiber (A), a fiber exposed to P4 at a concentration of 60 ng.mL^{-1}(B) and 300 ng.mL^{-1} (C). Figures A-C display the 3-dimentional structure, the 2-dimentional curve when a fiber is dissected along with the study of the root mean square (RMS) set values obtained, enclosed by the red block

The control fibrin fiber showed a rougher surface then those at the different concentrations of P4. It seems that the higher the concentration of P4, the smoother the surface topography becomes.

Table 11 summarises the effects of the different concentrations of P4 on the surface roughness of individual fibrin fibers, while figure 4-8 shows the study of root mean square.

Table 11: Statistical analysis of surface roughness after addition of different physiological concentrations of P4

Morphological studies: Surface roughness					
	Control	**Lowest physiological concentration**	**Highest physiological concentration**	**F-value [F=3,36]**	**P-value**
Surface roughness (nm)	21.07 (±12.63)	18.77 (± 8.16)	***14.40 (± 6.78)***	**13.94**	**<0.0001**

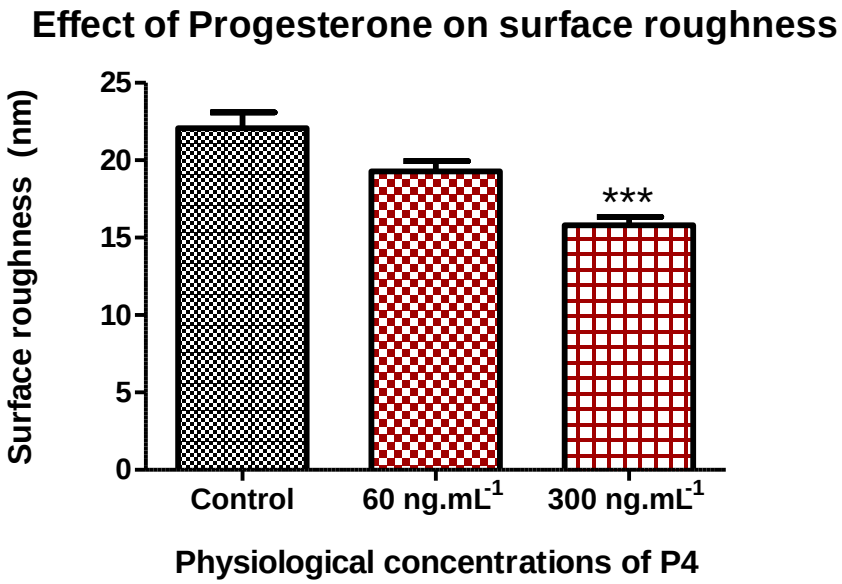


Figure 4-8: Surface roughness of individual fibrin fibers after addition of different concentrations of P4

AFM analysis showed that the lowest physiological concentration of P4 did not have a statistically significant influence on the fibrin strand roughness. It was the highest physiological concentration of this hormone that followed the trend of the highest physiological concentration of E_2 by significantly decreasing the roughness of the fibrin fibers.

4.3.4 Turbidimetric analysis for P4

Table 12 provides a summary of the results obtained by turbidimetry, where the lag time, slope, maximum absorbance and clot lysis time were determined for the different concentrations of P4.

Table 12: Summary of the effects of different concentrations of P4 on turbidimetric parameters indicated as mean ± standard deviation

Turbidimetric parameters				
	Lag time (minutes)	**Slope (x10^{-3} au/s)**	**Maximum absorbance**	**CLT (minutes)**
Control	4.852 (±0.532)	6.348 (±2.016)	0.496 (±0.136)	71.050 (±3.736)
30ng.mL^{-1}	5.013(±0.496)	5.478 (±1.637)	0.444 (±0.121)	68.360 (±6.508)
60ng.mL^{-1}	5.127 (±0.557)	5.298 (±0.920)	0.458 (±0.130)	67.270 (±3.661)
140ng.mL^{-1}	5.064 (±0.593)	5.196 (±0.695)	0.457 (±0.134)	68.820 (±3.915)
220ng.mL^{-1}	5.061 (±0.552)	5.703 (±1.771)	0.462 (±0.134)	70.140 (±5.738)
300ng.mL^{-1}	5.262 (±0.631)	5.724 (±1.885)	0.459 (±0.133)	68.790 (±4.994)
F-value [F = 6;63]	**0.573**	**0.692**	**0.176**	**0.753**
ANOVA p-value	**0.721**	**0.632**	**0.971**	**0.588**

Progesterone followed the same trend as that of 17β-estradiol by increasing the lag time as well as decreasing the slope, maximum absorbance and CLT, but not to a statistically significant effect.

This indicates that Progesterone like 17β-estradiol decreased the activation time of the coagulation cascade, the rate of lateral aggregation, fiber thickness and clot lysis time, none of which was shown to be statistically significant.

4.4 Concluding remarks

Viscoelasticity studies showed that only the highest concentration of E_2 at 300pg.mL^{-1} significantly influenced almost all the thrombo-elastic parameter analysed, while the lowest physiological concentration at 60pg.mL^{-1} had no effect on these parameters. P4 did not influence fibrin clot formation in the same manner as E_2. It was mainly the K (amplification), MA (maximum velocity), MRTG (maximum velocity of clot growth) and TTG (clot strength) values that were influenced by different concentrations of P4. This specific trend of decreases and increases in the specific parameters is indicative of hypercoagulability, specifically at the highest concentration of both E_2 and P4, which produces hypercoagulable fibrin clots while E_2 at 60pg.mL^{-1} produced fibrin clot formations similar to that of the controls.

From SEM analysis it seems that P4 at the lowest physiological concentration produced patches of DMDs dispersed over thin fibrin fibers. E_2 at the highest physiological concentration had a similar effect on the morphology of the fibrin network as P4 at the lowest physiological concentration where DMDs are prominent with holes still visible between very thin fibrin fibers. P4 are the highest physiological concentration formed thick masses of fibrin with very few small holes in between the thin fibrin fibers.

Fibrin fiber diameter was decreased for both hormone concentrations at all the concentrations compared to that of the control fibers with no hormones added.

Surface topography was decreased as hormone concentration increased for both E_2 and P4. Thus a smoother surface was observed at the highest concentrations of both hormones.

Turbidimetry indicated that P4 like E_2 decreased the activation time of the coagulation cascade, the rate of lateral aggregation, fiber thickness and clot lysis time, none of which was shown to be statistically significant.

Chapter 5: Discussion

The individual haematological effects of endogenous hormones, specifically E_2 and P4 in the two most important life cycles of a woman, namely the menstrual cycle and pregnancy (90, 91). It is also critical to determine the individual effect of these endogenous steroid hormones on coagulation since they are present in circulation when additional synthetic hormones are introduced into the body in the form of hormonal contraception, which may have thrombotic implications (9).

Thromboelastography revealed the viscoelastic properties of E_2 and P4 with regards to several parameters as described in chapter 3. A standard curve (Figure 5-1) is displayed below for normal viscoelastic properties, in other words a control sample. This also represents a clear indication of how the mentioned parameters either increase or decrease and whether it is linked to a hypo- or hypercoagulable state.

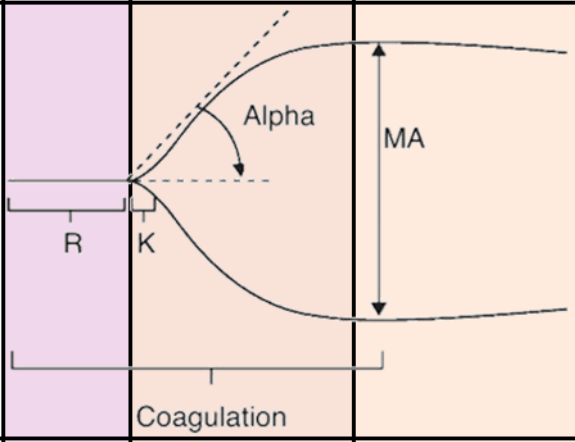
Parameter	Clot time	Clot rate	Maximum clot strength
Haemostatic activity	Thrombin generation Fibrin formation	Platelets Fibrin cross-linking	Maximum platelet-fibrinogen interactions
Haemostatic component	Coagulation pathways	Coagulation pathway	Platelets (± 80%) Fibrinogen (± 20%)
			
Hypo-coagulable	↑ R (min)	↑ K (min) ↓ α (deg)	↓ MA (mm)
Hyper-coagulable	↓ R (min)	↓ K (min) ↑ α (deg)	↑ MA (mm)

Figure 5-1: Viscoelastic parameters indicating hypo- and hypercoagulability

Increased R time (R), kinetics (K) and decreased maximum amplitude (MA) indicates hypo-coagulation while decreased R time, kinetics and increased amplitude indicates a hypercoagulable state

When plotting E_2 and P4 onto the same graph and comparing it to the standard curve of normal plasma fibrin formation, we are able to see that the rate of the clot formation after E_2 addition was influenced while that for P4 was not (Figure 5-2). Initiation time for clot formation after addition of E_2 at the highest physiological concentration was shorter than that for P4 and the control. Decreased kinetics and time interval observed before the maximum speed of the fibrin clot growth was decreased relating to more rapid clot formation compared to that of controls. Not only was clot formation faster, the overall stability and strength of the clot was increased. As compared to E_2 at the highest physiological concentration P4 at its highest physiological concentration did not affect the initiation time of clot formation. All concentrations tested increased clot amplification, while P4 at its highest physiological concentration increased the overall stability and strength of the clot with P4 addition was not as stable and strong as that of E_2 at the highest physiological concentration.

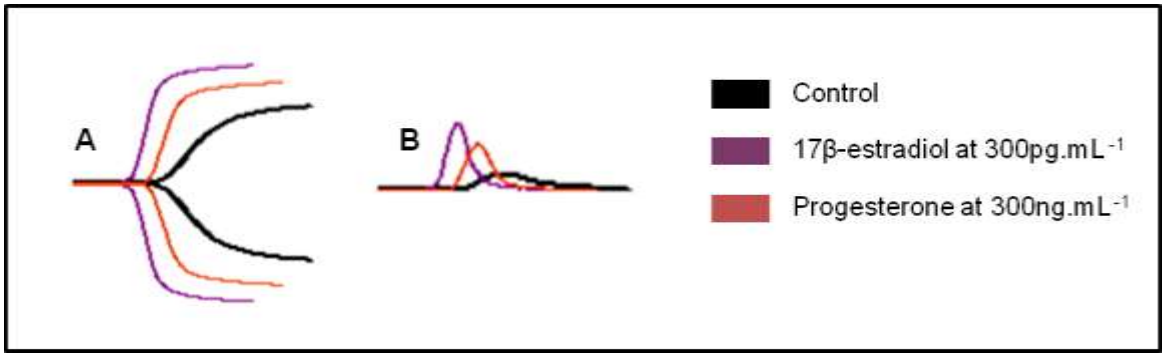


Figure 5-2: Representative viscoelastic traces of normal viscoelastic properties (black), E_2 (purple) and P4 (red)

A = Traditional TEG waveforms, B = Clot growth velocity curves

Referring to Figure 5-3, clot velocity is displayed with regards to TMRTG and MRTG. Again normal, hyper- and hypocoagulation profiles are indicated. As shown in chapter 4 for the viscoelastic parameters (Table 5 and 9), both E_2 and P4 increased the overall velocity of clot growth.

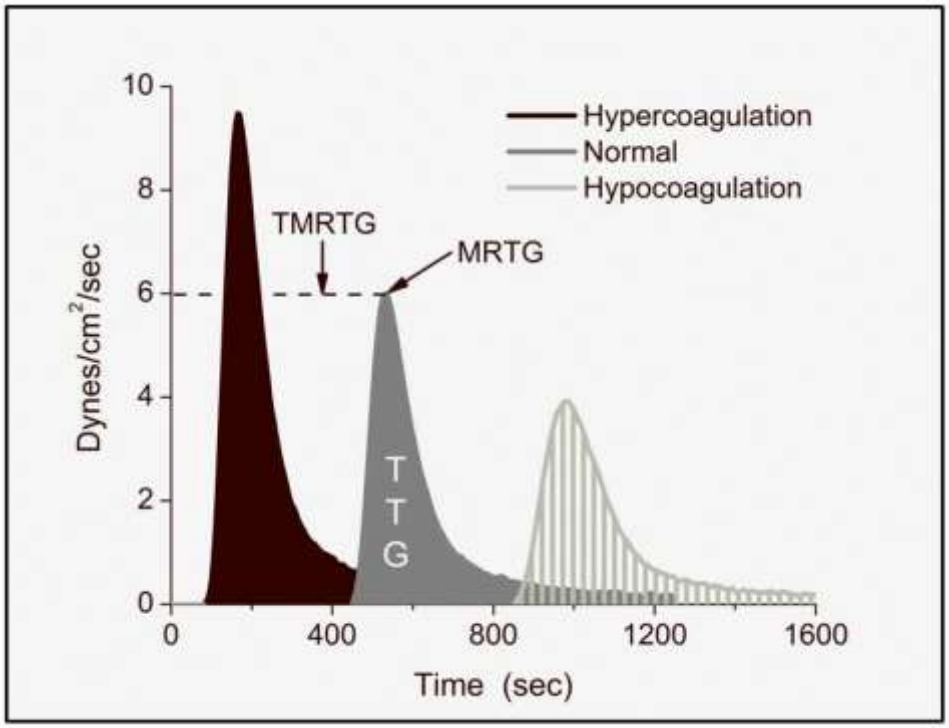


Figure 5-3: Representative clot velocity curve indicating the difference between normal, hyper- and hypocoagulation

Therefore E_2 at its highest physiological concentrations resulted in more rapid formation of strong, thick fibrin clots. P4 increased clot amplification overall but at a concentration of 300ng.mL^{-1}, formation rate of fibrin clots was increased even more and was also stronger and denser. These viscoelastic alterations were confirmed with SEM. Piechocka et al (118) recently showed that the hierarchical architecture of the fibrin fibers are reflected by the strain-stiffening response of fibrin networks. Duval et al (119) has suggested a connection between clot structure and viscoelastic properties via fibre tautness.

Both E_2 and P4 decreased the fibrin fiber diameter while forming a more closely-packed arrangement known as DMDs. Fibrin network architecture is closely associated with fibrinolysis. Although individual thinner fibers lyse more easily (120) a dense network of thin fibrin fibers are more resilient to lysis than thick fibers arranged in a more dispersed configuration, regardless of the quantity of the lytic agent (121). Since both hormones decrease the fibrin fiber diameter which in turn possibly decreases the local enhancement and acceleration of fibrinolysis (121), we could deduce that this might increase the risk of thrombosis.

Fibrin formation or clot structure gives insights into the mechanism of many diseases associated with thrombotic complications (65-67). Fibrinogen concentration is directly related to the structure of a fibrin network. When fibrinogen levels are elevated, fibrin network density increases, as well as clot stiffness and the resistance of the clot to fibrinolysis. The fibrin network is not only sensitive to the fibrinogen concentration, but also to the concentration of thrombin during fibrin polymerization. Therefore elevated fibrinogen (122, 123) and thrombin levels (124) are associated with denser fibrin clot structure and reduced susceptibility to lysis. Plasma fibrinogen is susceptible to oxidative stress (125), and fibrinogen exposed to reagents present during oxidative stress resulted in decreased fibrin fiber diameter (126). Inflammation further increases fibrinogen and thrombin concentrations (74) resulting in the over-activation of the coagulation system that shifts the haemostatic balance toward a hypercoagulable state (75).

Given that samples were collected at a single time point, we can assume that the fibrinogen concentration remained stable for all samples. And since the viscoelastic studies showed that the hormones did not influence the thrombin burst (α, angle) and the same thrombin

concentration and volume was used throughout it seems that change in fibrin diameter may have been brought on by oxidative stress induced by the hormones. Although E_2 is more commonly known to attenuate oxidative stress (127) it has recently been shown that chronic E_2 exposure induces oxidative stress in the hypothalamus (128) and even in organisms that do not possess estrogen receptors like *Eisenia fetida* (129).

Santanam et al has shown that physiological concentrations of estradiol do not inhibit the oxidation of LDL by copper. LDL samples isolated from pre- and postmenopausal females as well as females during different phase of their menstrual cycle oxidized at the same rate although the plasma estradiol levels differed significantly. Only LDL samples from females receiving estradiol concentrations above 2000 $pg.ml^{-1}$ were resistant to oxidation by copper. They concluded that physiological concentrations of estradiol is unlikely to act as an antioxidant and that it might rather induce myeloperoxidase (MPO) and become a pro-oxidant (130).

It is interesting that, although both hormones at all concentrations decreased the fibrin diameter and resulted in the formation of DMDs, the fibrin fiber roughness was decreased by all concentrations of both hormones. The reason behind the fact that AFM results did not follow the same pattern as that for thromboelastic and morphological studies, it is important to take a closer look at the way fibrin strands bind to each other and the different ways they could be arranged.

Fibrinogen (Figure 5-4), is a large centrosymmetric glycoprotein (131, 132), which contains three pairs of polypeptide chains (Aα, Bβ and γ polypeptides) curved into a central E-region with two distal D-regions (133, 134). Each fibrinogen monomer contains a total of 72 Trp residues (11 on each α-chain, 14 on each β-chain and 11 on each γ-chain (http://www.ncbi.nlm.nih.gov/sites/entrez) which are primarily situated inside the hydrophobic core of the fibrinogen protein.

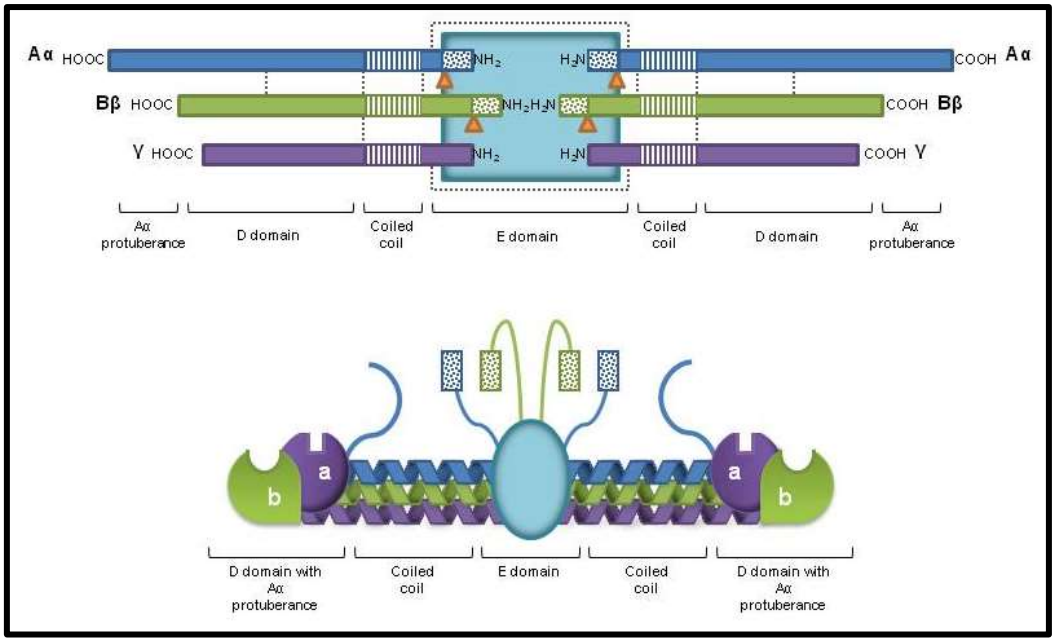


Figure 5-4: Fibrinogen structure

The top diagram indicates the polypeptide organisation of fibrinogen with the three chains (α,β and γ) while the bottom diagram depicts domain organisation (D domain and E domain) and binding sites of fibrinogen (135, 136)

E_2 has been shown to bring about conformational changes to the fibrinogen molecule. It directly interacts with at least one high affinity site in the fibrinogen macromolecule resulting in conformational changes to the protein and subsequent exposure of more tryptophan (Trp) residues (137). Alterations of Trp residues, in the D domain results in increased polymerization while complete loss of the polymerization activity of fibrinogen is associated with modifications to Trp residues in the E domain (138). Since the ultrastructural and viscoelastic studies showed hypercoagulable clot formation, which is associated with increased polymerization, at the highest physiological concentration of E_2 we can hypothesise that this specific concentration of E_2 may alter Trp residues in the D domain.

Fibrinogen orientation is also influenced by fibrinogen concentration (139, 140) as well as the surface charge (141). At high fibrinogen concentrations and negatively charged surfaces the molecules tend to stand on the surface in an end-on fashion rather than in a side-on

manner (139-141) (Figure 5-5). Hydrophobicity has a direct correlation to the degree of conformational alterations (142, 143). Clark et al attributed these variances to altered binding mechanisms and post-adsorption conformational changes (144). Crosslinking of the Aα and γ constituent chains in fibrinogen can also occur in different formations as indicated in Figure 5-5 (145). When fibrinogen concentrations decreases, a side-on configuration can occur where the D domains line up sideways and form a fibrin network formation horizontally (a) When fibrinogen concentrations increase, an end-on configuration is observed with the D domains rotated, forming a vertical arrangement of fibrin fibers (b).

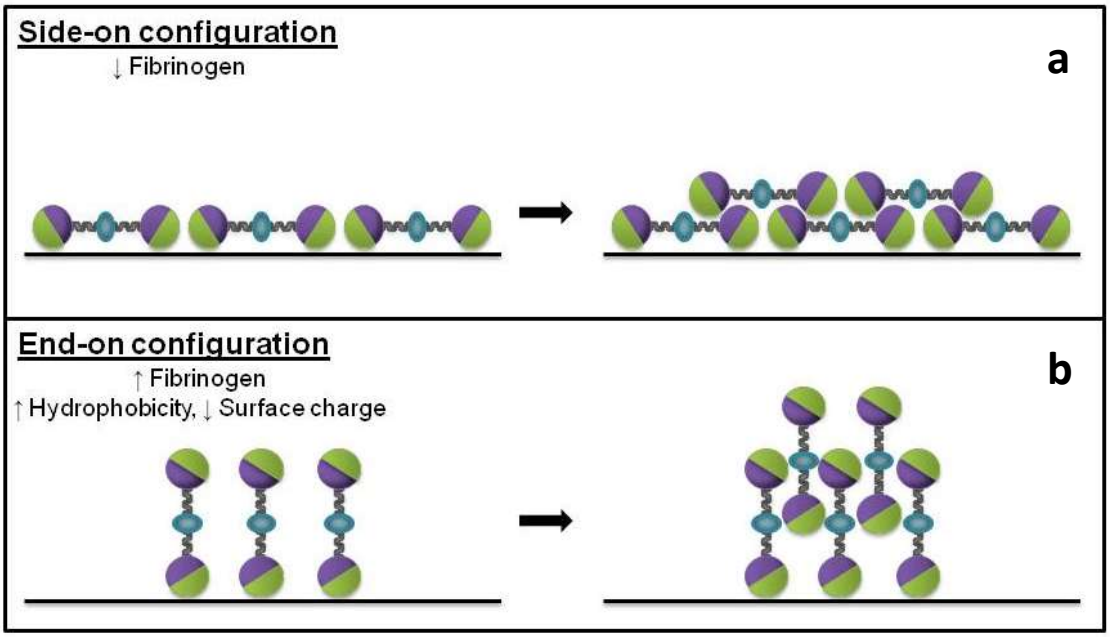


Figure 5-5: Two possible configurations of fibrinogen packaging (139-143)

Two other configurations are also known (Figure 5-6) namely angled end-to-end (a) and angled end-to-centre (b). AFM results of this study provides some insight with regards to the internal packaging of fibrinogen within fibrin fibers and the specific influence that E_2 and P4 have on these structural arrangements.

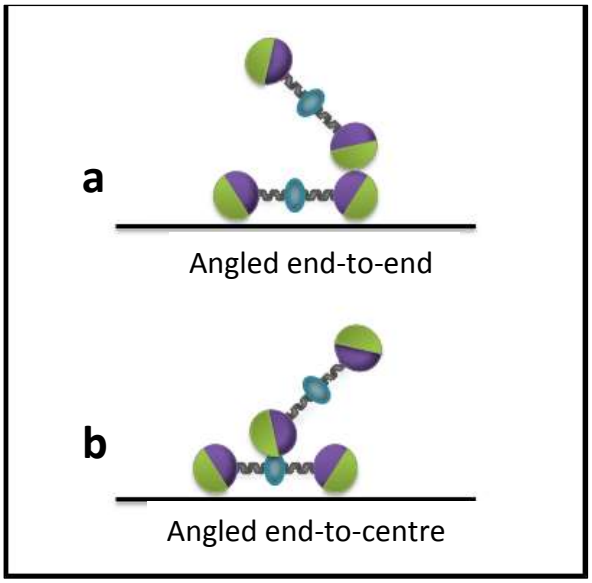


Figure 5-6: Two different angled configurations fibrinogen molecules showing (a) angled end-to-end (b) and angled end-to-centre (145)

For control samples the surface roughness had a higher RMS value with a rough surface appearance. This could possibly be a result of a side-on configuration with the fibrin structure lined up horizontally giving the surface a rougher surface. With both E_2 and P4, the surface roughness became smoother with the RMS value decreased. This could possibly indicate an end-on configuration where the D domains line up vertically and appears smoother.

With regards to turbidimetric analysis, the only parameter that was significantly influenced was CLT after the addition of 300 $pg.mL^{-1}$. Clot lysis time was decreased, meaning that the clot lysed faster.

Although the viscoelastic, morphological and turbidimetric results indicate a hypercoagulable state associated with E_2 and P4 addition to PPP it should be noted that not all females will suffer a thrombotic event. It may be that females predisposed to inflammatory or haematological conditions have a greater risk of thrombosis during their periods associated with increased hormonal concentrations, for example during pregnancy. Females using synthetic hormones therapies, for example combined oral contraceptives, can also be at risk for thrombosis since synthetic estrogens and/or progestins now join E_2 and P4 which are already present in circulation. It is possible that the synthetic estrogens and progestins may alter the fibrin network packaging in the same manner as that of natural endogenous hormones.

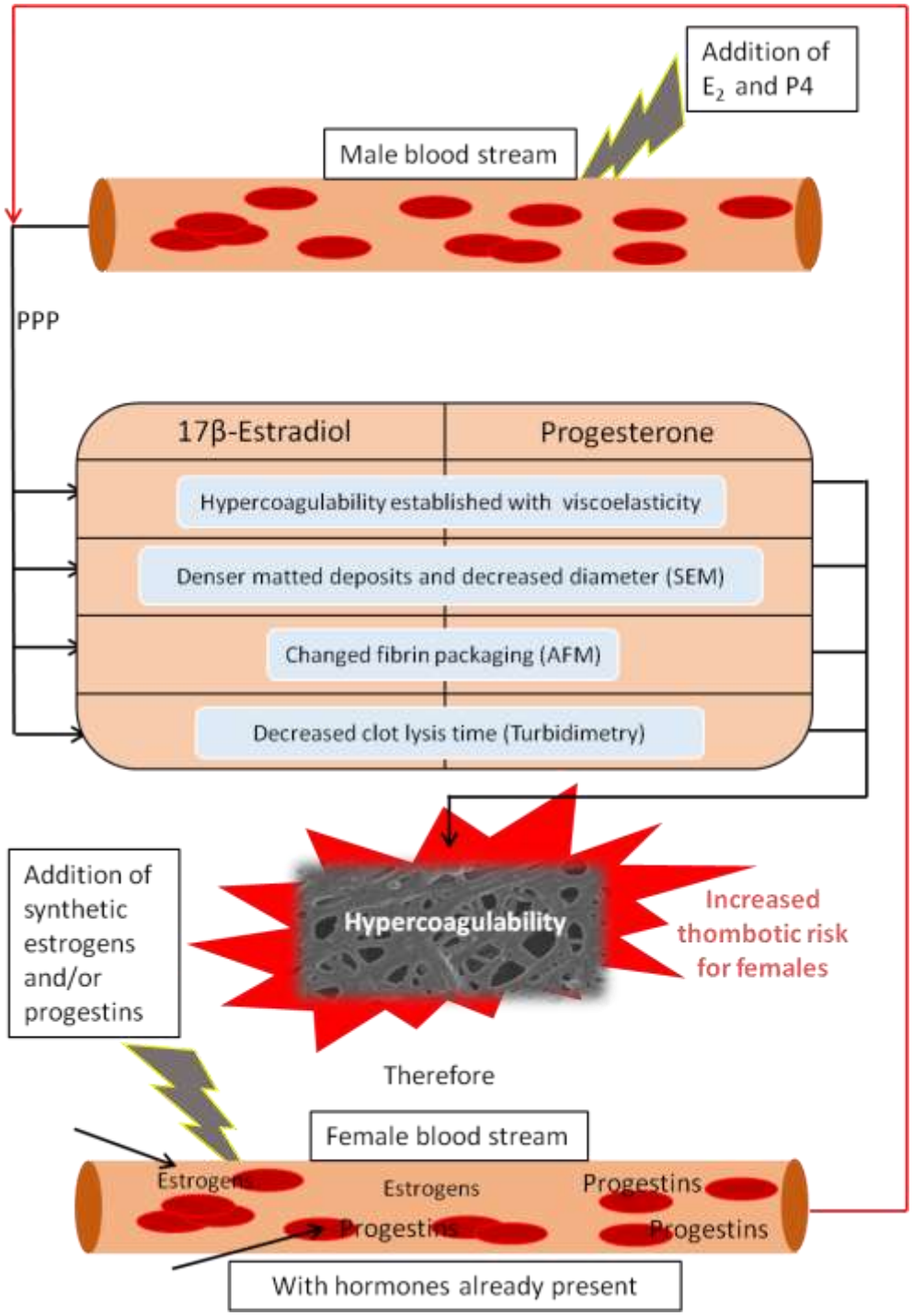


Figure 5-7: A summary diagram representing the clinical relevance of this study

With the addition of E_2 and P4 to male plasma, both hormones indicated 'n hypercoagulable state with regards to viscoelasticity, morphology and turbidimetry. Adding additional estrogens and progestins to female plasma where hormones are already present in the bloodstream, with inflammatory pre-disposition can result in increased thrombotic risk.

Chapter 6: Conclusion

From the viscoelastic, morphological studies and turbidimetric observations, it is clear that fibrin packaging and coagulation properties are influenced by E_2 and P4, especially at the highest physiological concentrations of these hormones.

Viscoelastic studies showed that the highest physiological concentrations of both E_2 and P4 resulted in hypercoagulable fibrin plasma clots. Morphological analysis with SEM showed DMD formation and decreased fibrin diameter in the presence of E_2 and P4. Although decreased fibrin diameter is associated with hypo-fibrinolysis, turbidimetric analysis revealed that both hormones did not significantly decrease CLT, but E_2 in fact decreased lysis time at specific concentrations. AFM analysis showed that surface topography was significantly influenced by the highest physiological concentrations of both hormones, indicating that these hormones possibly influence fibrinogen packaging.

Table 13 shows concluding remarks with regards to the existing knowledge, the new knowledge and suggested future research endeavours to further clarify the biochemical interactions of these two hormones on fibrinogen packaging, and determining the dose dependent effect of these hormones by testing the effect of lower doses of these hormones.

Table 13: Past knowledge, current findings and future work on E_2 and P4

Past findings What did we know about E_2 and P4 from literature?	**Present findings** What knowledge did this paper add concerning the effect of E_2 and P4 on plasma fibrin clot formation?	**Future prospectives** What needs to be done in fuure studies?
• Cardioprotective role (31, 146) • Prothrombotic (85) • Pro and anti-inflammatory properties (75)	• ↑ Hypercoagulability • ↓ Fibrin diameter • ↑ DMDs • ↓F ibrin fiber surface roughness • Unchanged or ↓ CLT	• Determine possible ROS generation during fibrin packaging after exposure to hormones • Determine specific orientation and conformation of altered fibrinogen packaging after exposure to hormones • Determine the specific Trp residues that are exposed by E_2 and how it influences fibrinogen packaging • Determine whether P4 also influence Trp residues (similar to E_2)

Not all females will suffer a thrombotic event. Our results suggest that the additional burden of synthetic hormonal load, together with the presence of endogenous E_2 and P4, may result in a prothrombotic and hypercoagulable state in females with an inflammatory predisposition. Metabolic syndrome, including obestity and glucose intollerance and or diabetes, together with lifestyle choices like smoking, may burden the hematological health

of the individual since all these confounding factors are known to already increase thrombotic risk and inflammation.

From a historical clinical perspective, the relative hypercoagulability associated with E_2 and P4 has caused both adverse and adventagous outcomes in a variety of settings. Pathological thrombosis in the setting of oral contraception (147), assisted reproductive technology (148), or postmenopausal hormone replacement (149) have been well documented. However, female gender has been found to bestow better outcomes after trauma, with associated superior post traumatic fibrinogen concentrations (150). In fact, the therapeutic administration of estrogen in the setting of orthotopic liver transplantation resulted in decreased transfusion requirements for fresh-frozen plasma, red blood cells, and platelets (151). While some works have implicated roles for decreased tissue factor pathway inhibitor concentrations (102), activated protein C resistance (102), and elevated heparanase procoagulant activity (152) as contributors to hormone associated hypercoagulability, our previous work (put in references of your choice) and the viscoelastic data of the present manuscript **implicate** a major role of hormone-enhanced fibrinogen as a **potential** source of thrombophilia. In sum, our findings are clinically relevant when considering hormones as either pathological agent or therapeutic intervention as will be assessed in future investigation.

References

1. Hongbao M YJ, Shen C,. Thrombin. Nature and Science. 2008;6(2):90-3.
2. Riddel J.P ABE, Miaxkowski C, Lillicrap D.P. Theories of blood coagulation. Journal of pediatric oncology nursing. 2007;24(3):123-31.
3. Aleman M.M WBL, Byrnes J.R, Wolberg A.S. Fibrinogen and red blood cells in venous thrombosis. Thrombosis research. 2014;133:38-40.
4. Pruissen D.M.O SAJC, Rosendaal F.R, Van der Graaf Y, Algra A. . Coagulation factor XIII gene variation, oral contraceptives, and the risk of ischemic stroke. Blood Journal. 2008;111(3):1282-6.
5. Swanepoel A.C LBG, Swart P.J, Abdool Z, Pretorius E. . Estrogen causes ultrastructural changes of fibrin networks during the menstrual cycle: A qualitative investigation. Microsc Res Tech. 2014;77:594-601.
6. Plu-Bureau G. Combined oral contraceptives and risk of venous thromboembolism: there is higher risk in new generations compared to second generations, but paradoxically not in norgestimate-containing-pills. Evidence Based Medicine. 2015;20(5):189-.
7. Notelovitz M GHBW. The effect of natural oestrogens on coagulation. S Afr Med J. 1975;49(101):101-5.
8. Vigano P RE, Pagliardini L, Somigliana E, Candiani M, Vercellini P,. Progesterone resistance, aromatase, and inflammation: the important relationships between hormones and inflammation. Curr Obstet Gynecol Rep. 2012;1:146-52.
9. Cui X WEE, Chumanevich A. P, Chumanevich A.A, Poudyal D, Subramanian, V, et al, . The Induction of microRNA-16 in Colon Cancer Cells by Protein Arginine Deiminase Inhibition Causes a p53-Dependent Cell Cycle Arrest. PLoS One. 2013;8(1):e53791. Epub 2013/01/12.
10. Wingeier M. LM-G, P., Zimmermann R, Ehlert U. Is salivary estriol detectable in very early pregnancy? The Journal of Maternal-Fetal & Neonatal Medicine. 2016:1-5.
11. Silverthorn. Human Physiology - an intergrated approach. 2010;6 th edision:San Francisco: Pearson Ed.
12. Sauber K EDP, Meizel S, . Human sperm plasma membrane progesterone receptor(s) and the acrosome reaction. Biol Reprod. 1996;54:993-1001.
13. El-Hefnaway T MPR, Luconi M, Baldi E, Slotte JP, Huhtaniemi I, . Progesterone action in a murine Leydig tumor cell line (mLTC-1), possibly through a nonclassical receptor type. Endocrinology. 2000;141:247-55.
14. Schwarzenbach H MPR, Stocco D.M, Chakrabarti G, Mukhopadhyay A.K,. Stimulatory effect of progesterone on the expression of steroidogenic acute regulatory protein in MA-10 Leydig cells. Biol Reprod. 2003;68:1054-63.
15. Oettel M MAK. Progesterone: the forgotten hormone in men? Aging Male. 2004;7:236-57.
16. O'Donnell L RKM, Jones M.E, Simpson E.R, . Estrogen and spermatogenesis. Endocr Rev. 2001;22:289-318.
17. Hess R.A CK. The role of estrogen in testis and the male reproductive tract: a review and species comparison. Anim Reprod. 2004;1(5-30).
18. Hess R.A. Estrogen in the adult male reproductive tract: A review. Reproductive Biology and Endocrinology. 2003;1(52):1-14.
19. Jackson T LWLW, Trusselle M.N, Chander S.K, Purohit A, Reed M.J, et al. Dual aromatase-sulfatase inhibitors based on the anastrozole template: synthesis, in vitro SAR, molecular modeling and in vivo activity. Org Biomol Chem. 2007(5):2940-52.
20. Diagniostics B. ADVIA Centaur Progesterone product insert, Revision F.
21. Al-Asmak M. Review: Reproductive functions of progesterone. Middle East Fertility Society Journal. 2007;12(3):147-52.

22. Ciu J SY, Li R,. Estrogen synthesis and signaling pathways during aging: from periphery to brain. Trends in molecular medicine. 2013;19(3):197-209.
23. Norman A.W MMT, Norman D.P.G,. Steroid-hormone rapid actions, membrane receptors and a conformation ensemble model. Nature reviews: Drug discovery. 2004;3(27-41).
24. Wetendorf M DFJ. Progesterone receptor signaling in the initiation of pregnancy and preservation of a healthy uterus. Int J Dev Biol. 2014;58:95-106.
25. HAN Y FHL, Sandlow J.I, Haines C.J, . Comparing expression of progesterone and estrogen receptors in testicular tissue from men with obstructive and nonobstructive azoospermia. Journal of Andrology. 2009;30(2):127-33.
26. Jackson T LWLW, Trusselle M.N, Chander S.K, Purohit A, Reed M.J, et al. Dual aromatase-sulfatase inhibitors based on the anastrozole template: synthesis, in vitro SAR, molecular modeling and in vivo activity. . Org Biomol Chem. 2007;5 2940- 52.
27. Anderson E. The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. Breast cancer Res. 2002;4:197-201.
28. Deroo B.J KKS. Estrogen receptor and human disease. J Clin Invest. 2006;116(3):561-70.
29. Heldring N PA, Andersson S, Maatthews J, Cheng G, Hartman J. et al. Estrogen receptors: How they signal and what are their targets? . Physiological reviews. 2007;87:905-31.
30. Weatherman R.V. Untangling the estrogen receptor. Nature Chemical Biology. 2006;2:175-6.
31. Lemini C FY, Avila M.E, Jaimez R. Contrasting effects of estradiol and 17B - aminoestrogens on blood clotting time in rats and mice. European journal of pharmacology. 2005;510:229-33.
32. Riggs B.L. The mechanisms of estrogen regulation of bone resorption. JCI. 2000;10:1203-4.
33. Campbell J.E B-ZKE, Butenas S, Mann K.G. Cellular regulation of blood coagulation: a model for venous stasis. Blood. 2010;116(26):6082-91.
34. Adams R.L.C BRJ. Review article: coagulation cascade and therapeutics update: relevance to nephrology. Part 1: overview of coagulation, thrombophilias and history of anticoagulants. Nephrology. 2009;14:462-70.
35. Loscalzo J. S. A. I. Thrombosis and hemorrhage (3rd ed.). . Philadelphia: Lippincott Williams & Wilkins. 2003.
36. Niinuma T, Suzuki H, Nojima M, Nosho K, Yamamoto H, Takamaru H, et al. Upregulation of miR-196a and HOTAIR drive malignant character in gastrointestinal stromal tumors. Cancer Res. 2012;72(5):1126-36. Epub 2012/01/20.
37. Hoffbrand V. A CD, Tuddenham E. Postgraduate haematology (5th ed.) Malden, MA: Blackwell. 2005.
38. Lefkowitz J.B. Coagulation Pathway and Physiology. In: Hemostasis Physiology. . JB Lippincott Co, Philadelphia, PA 2006:3-12.
39. Hoffman Y, Bublik DR, Pilpel Y, Oren M. miR-661 downregulates both Mdm2 and Mdm4 to activate p53. Cell Death Differ. 2014;21(2):302-9. Epub 2013/10/22.
40. Naldini A CDH, Pucci A, Pasquali A, Carraro F,. Thrombin regulates the expression of proangiogenic cytokines via proteolytic activation of protease-activated receptor-1. Gen Pharmacol. 2000;35(5):255-9.
41. Chu A.J. Tissue factor, blood coagulation, and beyond: An overview. International Journal of Inflammation. 2011;10(4061):1-31.
42. Vinik A.I ET, Sun Park T, Nolan R, Pittenger G.L,. Platelet dysfunction in type 2 diabetes. Diabetes care. 2001;24(8):1476-85.
43. Zeerleder S HCE, Wuillemin W.A, . Disseminated intravascular coagulation in sepsis,. Chest. 2005;128(4):2864-75.
44. Vinogradova Y, Coupland C, Hippisley-Cox J. Use of combined oral contraceptives and risk of venous thromboembolism: nested case-control studies using the QResearch and CPRD databases. bmj. 2015;350:h2135.

45. Suchon P, Al Frouh F, Henneuse A, Ibrahim M, Brunet D, Barthet M-C, et al. Risk factors for venous thromboembolism in women under combined oral contraceptive. Thrombosis and haemostasis. 2015.
46. MacFarlane R.G. An enzyme cascade in the blood clotting mechanism and its function as a biochemical amplifier. Nature. 1964;202(498-499).
47. Davie E.W RSI. Waterfall sequence for intrinsic blood clotting. Science. 1964;145(1310-1312).
48. Anastasiou G GA, Merkouri E, Politou M, Travlou A,. Thrombomodulin as a regulator of the anticoagulant pathway: implication in the development of thrombosis. Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis. 2012;23:1-10.
49. Hassan I SA, Ahmad F,. Structure and function of von Willebrand factorL the protein that is deficient and/or abnormal in inherited von Willebrand disease. Blood coagulation and fibrinolysis. 2012;23:11-22.
50. Rawala-Sheikh R ASS, Monroe D.M, et al,. Role of gamma-carboxyglutamic acid residues in the binding of factor IXa to platelets and in factor-X activation. Blood. 1992;79:398-405.
51. Mackman N. The many faces of tissue factor. Journal of Thrombosis and Haemostasis. 2009;7(1):136-9.
52. Bungay S. Modelling the effect of amplification pathway factors on thrombin generation: A comparison of hemophilias. . TransfusApher Sci 2008;38(41-47).
53. Hoffman M MDM. Coagulation 2006: A modern view of hemostasis. . Hematol Oncol Clin North Am. 2007;21(1-11).
54. Vinogradov AE. Systemic factors dominate mammal protein evolution. Proc Biol Sci. 2010. Epub 2010/01/08.
55. Mann K.G B-ZK, Orfeo T, Butenas S, . Models of blood coagulation. Blood Cells Mol Dis 2006;36(108-117).
56. Bach R.R. Tissue factor encryption. . Arterioscler Thromb Vasc Biol 2006;26:456-61.
57. Monroe D.M HM. What does it take to make the perfect clot? . Arterioscler Thromb Vasc Biol 2006;26(41-18).
58. O'Donnell JM, Kalichira A, Bi J, Lewandowski ED. In vivo, cardiac-specific knockdown of a target protein, malic enzyme-1, in rat via adenoviral delivery of DNA for non-native miRNA. Current gene therapy. 2012;12(6):454-62. Epub 2012/09/15.
59. Orfeo T BN, Nesheim M.E et al, . The factor V activation paradox. J Biol Chem 2004;279(19580-19591).
60. Versteeg H.H HJWM, Levi M, Reitsma P.H. New fundamentals in haemostasis. Physiol Rev. 2013;93:327-58.
61. Hoffman M MDM. Coagulation 2006: a modern review of hemostasis. Hematol Oncol Clin North Am. 2007;21(1).
62. Wolberg A. Thrombin generation and fibrin clot structure Blood reviews. 2007;21:131-42.
63. Weisel J.W LRI. Mechanisms of fibrin polymerization and clinical implications. Blood. 2013;121(10):1712-9.
64. Bhattacharjee P BD. Chapter 1: An Insight into the Abnormal Fibrin Clots — Its Pathophysiological Roles. Fibrinolysis and Thrombolysis2014.
65. Undas A ARAS. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. . Arteriosclerosis, Thrombosis and Vascular Biology 2011;31(12):e88-e99.
66. Monroe D HM, Roberts H. R, . Transmission of a procoagulant signal from tissue factor-bearing cell to platelets. Blood Coagulation Fibrinolysis, . 1996;74:459-64.
67. Wolberg A.S MDM, Roberts H.R, Hoffman M, . Elevated prothrombin results in clots with an altered fiber structure: a possible mechanism of the increased thrombotic risk. . Blood. 2003;101:3008-13.
68. Weisel J.W LRI. Mechanisms of fibrin polymerization and clinical implications. BLOOD. 2012;121(10):1712-9.

69. Luchtman-Jones L BJ. The current status of coagulation. Annals of Medicine,. 1995;27(1):47-52.
70. Marlar R.A KAJ, Griffin J.H, . An alternative extrinsic pathway of human blood coagulation. Blood. 1982;60(1353-1358).
71. Undas A NT, Cieoela-Dul M, Sadowski J, . Abnormal plasma fibrin clot characteristics are associated with worse clinical outcome in patients with peripheral arterial disease and thrombangiitis obliterans. . Atherosclerosis 2011;215(2):481-6.
72. Opal S.M. Phylogenetic and functional relationships between coagulation and the innate immune response. Critical care medicine. 2000;28:77-80.
73. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxidants & redox signaling. 2014;20(7):1126-67. Epub 2013/09/03.
74. Xu J, Lupu F, Esmon C. Inflammation, innate immunity and blood coagulation. Hamostaseologie. 2010;30(1):5.
75. Okamoto T, Tanigami H, Suzuki K, Shimaoka M. Thrombomodulin: a bifunctional modulator of inflammation and coagulation in sepsis. Critical care research and practice. 2012;2012.
76. Vergnolle N WJL, Bunnett N.W, Hollenberg M.D,. Protease-activated receptors in inflammation, neuronal signaling and pain. Trends in pharmacological sciences. 2001;22(3):146-52.
77. Okamoto T TH, Suzuki K, Shimaoka M, . Thrombomodulin: A bifunctional modulator of inflammation and coagulation in Sepsis. Critical care research and practice. 2012:1-10.
78. Bizios R LL, Fenton J.W, Malik A.B,. Thrombininduced chemotaxis and aggregation of neutrophils,. Journal of Cellular Physiology. 1986;128(3):485-90.
79. McEver R.P. Leukocyte-endothelial cell interactions. Current Opinion in Cell Biology. 1992;4(5):840-9.
80. Popovic M SK, Dobutovic B, Syrovets T, Simmet T, Isenovic E.R,. Thrombin and vascular inflammation. Molecular and Cellular Biochemistry. 2012;359(1-2):301-13.
81. Johnson K CY, DeGroot E, Samuels I, Creasey A, Aarden L, . Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation. Journal of Immunology. 1998;160(10):5130-5.
82. Naldini A CDH, Bocci V et al, . Thrombin enhances T cell proliferative responses and cytokine production. Cellular immunology. 1993;147(2):367-77.
83. Naldini A CDH, Pucci A, Carraro F, . Human alpha-thrombin stimulates proliferation of interferon-gamma differentiated, growth-arrested U937 cells, overcoming differentiation-related changes in expression of p21CIP1/WAF1 and cyclin D1. Journal of Cellular Physiology. 2002;191(3):290-397.
84. Dulos J VP, van Doorn C, Hofstra C.L, Veening-Griffioen D, de Graaf J, . Supression of the inflammatory response in experimental arthritis is mediated via estrogen receptor a but not estrogen receptor b. Arthritis Research and therapy. 2010;12:1-12.
85. Reiner A.P HR, Vos H.L, Ariens R.A.S, Lemaitre R.N, Smith N.L, et.al. Genetic variants of coagulation factor XIII, postmenopausal estrogen therapy, and risk of nonfatal myocardial infarction. Blood Journal. 2003;120(1):25-30.
86. Critchley H.O.D JRL, Lea R.G, Drudy T.A, Kelly R.W, Williams A.R.W, . Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. Journal of clinical endocrinology and metabolism. 1999;84(1):240-8.
87. Stork S VSC, Angerer P,. The effect of 17b-estradiol on endothelial and inflammatory markers in postmenopausal women: a randomized, controlled trial. Atherosclerosis. 2002;165:301-7.
88. Tait A.S BCL, Sternberg E.M, . The role of glucocorticoids and progestins in inflammatory, autoimmune and infectious disease. Journal of leukocyte biology. 2008;84(924-931).
89. Valera M GM, Gourdy P, Lenfant F, Cabou C, Toutain C.E. et.al. Chronic estradiol treatment reduces platelet responses and protects mice from thromboembolism through the hematopoietic estrogen receptor alpha. Blood Journal. 2012;120(8):1703-12.

90. Daniels K, Daugherty J, Jones J. Current contraceptive status among women aged 15–44: United States, 2011–2013. NCHS data brief. 2014;173:1-8.
91. Affairs DoEaS. World contraceptive pattern. http://www.un.org/en/development/desa/population/publications/family/contraceptive-wallchart-2013.shtml2013 [cited 2015 13 November].
92. Jick SS. Fresh evidence confirms links between newer contraceptive pills and higher risk of venous thromboembolism. BMJ. 2015;350:h2422.
93. Moro L RS, Piranda D, Pietrapiana D, Lova P, Bertoni A et al, . Nongenomic effects of 17B-estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor B and Src kinase. Blood. 2005;105(1):115-21.
94. Lipinski B, Pretorius, E. Novel pathway of iron-induced blood coagulation: implications for diabetes mellitus and its complications. 2012.
95. Lipinski B, Pretorius, E., Oberholzer, H.M., Van der Spuy, W.J. Interaction of fibrin with red blood cells: the role of iron. Ultrastructural Patology. 2012;36:79-84.
96. Pretorius E, Kell, D.B. Diagnostic morphology: biophysical indicators for iron-driven inflammatory disease. Intergrative Biology. 2014.
97. Adams BD, Furneaux H, White BA. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein expression in breast cancer cell lines. Mol Endocrinol. 2007;21(5):1132-47. Epub 2007/02/22.
98. Powell LW, George, D.K., Mcdonnell, S.M., Kowdley, K.V. . Dagnosis of Hemochromatosis Annals of Internal Medicine. 1998;129:925-32.
99. Finch CA, Stray, S., Huebers, H.A., Bellotti, V., Lipschitz, D.A., Cook, J.D., et. al. Plasma ferritin determination as a diagnostic tool. Western Journal of Medicine. 1986;145:657.
100. Abraham G.E. The application of natural steroid radioimmunoassay to gynecologic endocrinology,. Radioassay Systems in Clinical Endocrinology,. 1981:475-529.
101. Bauman J. Basal body temperature: unreliable method of ovulation detection. Fertility Sterility. 1981;36:729-33.
102. Johnson KC, Aragaki AK, Jackson R, Reiner A, Sandset PM, Rosing J, et al. Tissue Factor Pathway Inhibitor, Activated Protein C Resistance, and Risk of Coronary Heart Disease Due To Combined Estrogen Plus Progestin Therapy. Arteriosclerosis, thrombosis, and vascular biology. 2015:ATVBAHA. 115.306905.
103. Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS, Benz CC. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J Biol Chem. 2007;282(2):1479-86.
104. Kuhl H. Pharmacology of estrogens and progestogens: influence of different routes of administration. CLIMACTERIC. 2005;8(1):3-63.
105. De Wolf A. Thromboelstography. 2012.
106. Subramanian A, Albert V, Agrawal D, Saxena R, Pandey R.M, . Evaluation of the Utility of Thromboelastography in a Tertiary Trauma Care Centre. ISRN Hematology. 2014:1-6.
107. Mallett S.V CDJ. Thrombelastography. Br J Anaesth. 1992;69(307-313).
108. Pretorius E VWA, Oberholzer H. M, Auer R. E. J,. Comparative Scanning Electron Microscopy of Platelets and Fibrin Networks of Human and Differents Animals. Int J Morphol. 2009;27(1):69-76.
109. Pretorius E Oj, Oberholzer H.M, van der Spuy W.J, . Scanning electron microscopy investigation of fibrin networks after thermal injury. Onderstepoort Journal of Veterinary Research. 2011;78(1):1-4.
110. Oosthuizen M.A PE, Oberholzer H.M, van der Spuy W.J, . Ultrastructural Analyses of Platelets and Fibrin Networks in BALB/c Mice after Inhalation of Spherical and Rod-Shaped Titanium Nanoparticles. Int J Morphol. 2010;28(4):1263-71.
111. Dufrene YF, Martinez-Martin,D., Medalsy I, Alsteens D, Muller D.J,. Multiparametric imagaing of biological systems by force-distance curve-based AFM. . Nature methods. 2013;10:847-54.

112. Lisman T dGPG, Meijers J.C, Rosendaal F.R, . Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. Blood. 2005;105:1102-5.
113. Talens S, Malfliet J.J., Rudez, G., Spronk, H.M., Janssen, N.A., Meijer, P., et al. Biological variation in tPA-induced plasma clot lysis time. Thromb Haemost. 2012;7108:640-6.
114. De Lange Z, Pieters, M., Jerling, J.C., Kruger, A., Rijken, D.C. Plasma clot lysis time and its association with cardiovascular risk factors in black Africans. PloS One. 2012(7):e48881.
115. De Oliveira R, Albuquerque D, Leite F, Yamaji F, Cruz T. Measurement of the nanoscale roughness by atomic force microscopy: basic principles and applications: INTECH Open Access Publisher; 2012.
116. Kotze RC, Ariens, R.A., de Lange, Z., Pieters, M. CVD risk factors are related to plasma fibrin clot properties independent of total and or gamma' fibrinogen concentration. Thromb Res. 2014;134:963-9.
117. Vernon-Parry K.D. Scanning electron microscopy : an introduction. Analysis. 2000;13(4):41-4.
118. Piechocka IK, Jansen KA, Broedersz CP, Kurniawan NA, MacKintosh FC, Koenderink GH. Multi-scale strain-stiffening of semiflexible bundle networks. Soft matter. 2016.
119. Duval C, Allan P, Connell SD, Ridger VC, Philippou H, Ariens RA. Roles of fibrin alpha- and gamma-chain specific cross-linking by FXIIIa in fibrin structure and function. Thromb Haemost. 2014;111(5):842-50. Epub 2014/01/17.
120. Bucay I, O'Brien III ET, Wulfe SD, Superfine R, Wolberg AS, Falvo MR, et al. Physical Determinants of Fibrinolysis in Single Fibrin Fibers. PloS one. 2015;10(2):e0116350.
121. Collet J, Park D, Lesty C, Soria J, Soria C, Montalescot G, et al. Influence of Fibrin Network Conformation and Fibrin Fiber Diameter on Fibrinolysis Speed Dynamic and Structural Approaches by Confocal Microscopy. Arteriosclerosis, thrombosis, and vascular biology. 2000;20(5):1354-61.
122. Weisel JW, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. Biophysical journal. 1992;63(1):111.
123. Scott EM, Ariëns RAS, Grant PJ. Genetic and Environmental Determinants of Fibrin Structure and Function: Relevance to Clinical Disease. Arteriosclerosis, Thrombosis, and Vascular Biology. 2004;24(9):1558-66.
124. Wolberg AS, Monroe DM, Roberts HR, Hoffman M. Elevated prothrombin results in clots with an altered fiber structure: a possible mechanism of the increased thrombotic risk. Blood. 2003;101(8):3008-13.
125. Shacter E, Williams JA, Lim M, Levine RL. Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. Free Radical Biology and Medicine. 1994;17(5):429-37.
126. Štikarová J, Kotlín R, Riedel T, Suttnar J, Pimková K, Chrastinová L, et al. The effect of reagents mimicking oxidative stress on fibrinogen function. The Scientific World Journal. 2013;2013.
127. Arias-Loza P-A, Muehlfelder M, Pelzer T. Estrogen and estrogen receptors in cardiovascular oxidative stress. Pflügers Archiv-European Journal of Physiology. 2013;465(5):739-46.
128. MohanKumar SM, Kasturi BS, Shin AC, Balasubramanian P, Gilbreath ET, Subramanian M, et al. Chronic estradiol exposure induces oxidative stress in the hypothalamus to decrease hypothalamic dopamine and cause hyperprolactinemia. Am J Physiol Regul Integr Comp Physiol. 2011;300(3):R693-9. Epub 2010/12/24.
129. Heger Z, Michalek P, Guran R, Havelkova B, Kominkova M, Cernei N, et al. Exposure to 17beta-Oestradiol Induces Oxidative Stress in the Non-Oestrogen Receptor Invertebrate Species Eisenia fetida. PLoS One. 2015;10(12):e0145426. Epub 2015/12/24.
130. Santanam N, Shern-Brewer R, McClatchey R, Castellano PZ, Murphy AA, Voelkel S, et al. Estradiol as an antioxidant: incompatible with its physiological concentrations and function. Journal of lipid research. 1998;39(11):2111-8.
131. Neeves K, Illing D, Diamond S. Thrombin flux and wall shear rate regulate fibrin fiber deposition state during polymerization under flow. Biophysical Journal. 2010;98(7):1344-52.

132. Ferri F, Greco M, Arcovito G, De Spirito M, Rocco M. Structure of fibrin gels studied by elastic light scattering techniques: dependence of fractal dimension, gel crossover length, fiber diameter, and fiber density on monomer concentration. Physical Review E. 2002;66(1):011913.
133. Yeromonahos C, Polack B, Caton F. Nanostructure of the fibrin clot. Biophysical Journal. 2010;99(7):2018-27.
134. Yang Z, Kollman JM, Pandi L, Doolittle RF. Crystal structure of native chicken fibrinogen at 2.7 Å resolution. Biochemistry. 2001;40(42):12515-23.
135. Undas A, Ariëns RAS. Fibrin Clot Structure and Function: A Role in the Pathophysiology of Arterial and Venous Thromboembolic Diseases. Arteriosclerosis, Thrombosis, and Vascular Biology. 2011;31(12):e88-e99.
136. Mosesson M. Fibrinogen and fibrin structure and functions. Journal of Thrombosis and Haemostasis. 2005;3(8):1894-904.
137. Gonçalves S, Santos NC, Martins-Silva J, Saldanha C. Fluorescence spectroscopy evaluation of fibrinogen–β-estradiol binding. Journal of Photochemistry and Photobiology B: Biology. 2007;86(2):170-6.
138. Ishida Y, Takiuchi H, Matsushima A, Inada Y. Functional consequences of tryptophan modification in human fibrinogen. Biochimica et Biophysica Acta (BBA) - Protein Structure. 1978;536(1):70-7.
139. Dyr JE, Tichý I, Jiroušková M, Tobiška P, Slavık R, Homola J, et al. Molecular arrangement of adsorbed fibrinogen molecules characterized by specific monoclonal antibodies and a surface plasmon resonance sensor. Sensors and Actuators B: Chemical. 1998;51(1):268-72.
140. Riedel T, Suttnar J, Brynda E, Houska M, Medved L, Dyr JE. Fibrinopeptides A and B release in the process of surface fibrin formation. Blood. 2011;117(5):1700-6.
141. Bratek-Skicki A, Żeliszewska P, Adamczyk Z, Cieśla M. Human fibrinogen monolayers on latex particles: role of ionic strength. Langmuir. 2013;29(11):3700-10.
142. Lu DR, Park K. Effect of surface hydrophobicity on the conformational changes of adsorbed fibrinogen. Journal of colloid and interface science. 1991;144(1):271-81.
143. Khot G, Kuforiji F, Wright R, Roach P, editors. Dynamic Assessment of Fibrinogen Adsorption and Secondary Structure Perturbation. Conference Papers in Science; 2014: Hindawi Publishing Corporation.
144. Clarke ML, Wang J, Chen Z. Conformational changes of fibrinogen after adsorption. The Journal of Physical Chemistry B. 2005;109(46):22027-35.
145. Murthy SP, Wilson JH, Lukas TJ, Veklich Y, Weisel JW, Lorand L. Transglutaminase-catalyzed crosslinking of the Aα and γ constituent chains in fibrinogen. Proceedings of the National Academy of Sciences. 2000;97(1):44-8.
146. Lee T-M, Su S-F, Tsai C-C, Lee Y-T, Tsai C-H. Cardioprotective Effects of 17β-Estradiol Produced by Activation of Mitochondrial ATP-Sensitive K+Channels in Canine Hearts. Journal of Molecular and Cellular Cardiology. 2000;32(7):1147-58.
147. Castelli WP. Cardiovascular disease: pathogenesis, epidemiology, and risk among users of oral contraceptives who smoke. American journal of obstetrics and gynecology. 1999;180(6):S349-S56.
148. Vloeberghs V, Peeraer K, Pexsters A, D'Hooghe T. Ovarian hyperstimulation syndrome and complications of ART. Best Practice & Research Clinical Obstetrics & Gynaecology. 2009;23(5):691-709.
149. Canonico M. Hormone therapy and risk of venous thromboembolism among postmenopausal women. Maturitas. 2015;82(3):304-7.
150. Gee AC, Sawai RS, Differding J, Muller P, Underwood S, Schreiber MA. The influence of sex hormones on coagulation and inflammation in the trauma patient. Shock. 2008;29(3):334-41.
151. Frenette L, Cox J, McArdle P, Eckhoff D, Bynon S. Conjugated estrogen reduces transfusion and coagulation factor requirements in orthotopic liver transplantation. Anesthesia & Analgesia. 1998;86(6):1183-6.

152. Matan M, Axelman E, Brenner B, Nadir Y. Heparanase procoagulant activity is elevated in women using oral contraceptives. Human Reproduction. 2013;28(9):2372-80.

Addendum 1: Published article

Swanepoel AC, Visagie A, de Lange Z, Emmerson O, Nielsen VG, Pretorius E. The clinical relevance of altered fibrinogen packaging in the presence of 17beta-estradiol and progesterone. Thombosis Research. 2016; 146 : 23-34. Impact factor = 2.32.