The clinical relevance of altered fibrinogen packaging in the presence of 17β-estradiol and progesterone

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Running heading: 17β-estradiol and progesterone alter fibrinogen.

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Highlights

- The effect of 17β-estradiol and progesterone on fibrin network formation is unknown.
- SEM, AFM and TEG provided insight regarding morphology, surface roughness and viscoelasticity.
- 17β-estradiol and progesterone are shown to induce a prothrombotic state.
- The clinical importance when considering hormones as therapeutic intervention.

Abstract

Background: The effect of endogenous hormone concentrations, specifically 17β-estradiol and progesterone, on fibrin network formation has not been established. *Objectives:* 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. *Methods:* Due to the fact that these hormones are known to increase hypercoagulability and the prothrombotic state scanning electron microscopy (SEM), atomic force microscopy (AFM), thromboelastography (TEG) and turbidimetry were employed to investigate the morphology, surface roughness, viscoelastic properties and formation and lysis of fibrin. *Results:* 17β-estradiol and progesterone showed hypercoagulable viscoelastic properties and decreased the diameter and surface roughness of fibrin while increasing dense matted deposit occurrence. Our results suggest that the additional burden of hormonal load, together with the presence of endogenous estrogen and progesterone, may result in a prothrombotic and hypercoagulable state in females with an inflammatory predisposition. *Conclusion:* Our results are of clinical importance when considering hormones as either pathological agent or therapeutic intervention as will be assessed in future investigation.

Keywords: 17β-estradiol, Fibrinogen, Fibrin, Progesterone, Thrombophilia

Introduction

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% [1], Europe 21% and in the United Kingdom it is a staggering 28% [2]. Although it is arguably one of the most important medical breakthroughs of all times, hormonal contraceptives are associated with increased risk of venous thromboembolism (VTE) [3-6].

The menstrual cycle is governed by hormonal fluctuations. 17β-estradiol and progesterone are lipophilic steroid hormones that are critical for normal reproductive functions. 17β-estradiol assists in the development of the secondary sex characteristics while progesterone regulates the reproductive tract during the menstrual cycle as it induces ovulation, facilitates implantation and maintains early pregnancy in females [7].

Although natural estrogen is known for many beneficial effects such as decreased risk for cardiovascular diseases observed in postmenopausal women [8] and osteoporosis [9], estrogen is capable of shifting the haemostatic balance to a hypercoagulable state in females, meaning that the coagulation process is upregulated [10]. Estrogen also exerts its prothrombotic effects by increasing the levels of factor XIII, responsible for fibrin cross-linking, resulting in the formation of a thrombus that is more stable and resistant to fibrinolysis [11].

An extensive crosstalk is observed between the coagulation system and inflammation [12]. At the site of inflammation, reactive oxygen species (ROS) are generated causing endothelial disruption and vascular injury [13] that will activate the coagulation system. Inflammation also increases fibrinogen and thrombin concentrations [14] resulting in the over-activation of the coagulation system that shifts the haemostatic balance toward a hypercoagulable state. Estrogen and progesterone both have pro- and anti-inflammatory effects [15]. Increased levels of estrogen and progesterone can affect fibrinogen and thrombin directly causing ultrastructural changes when observing the morphology of fibrin networks.

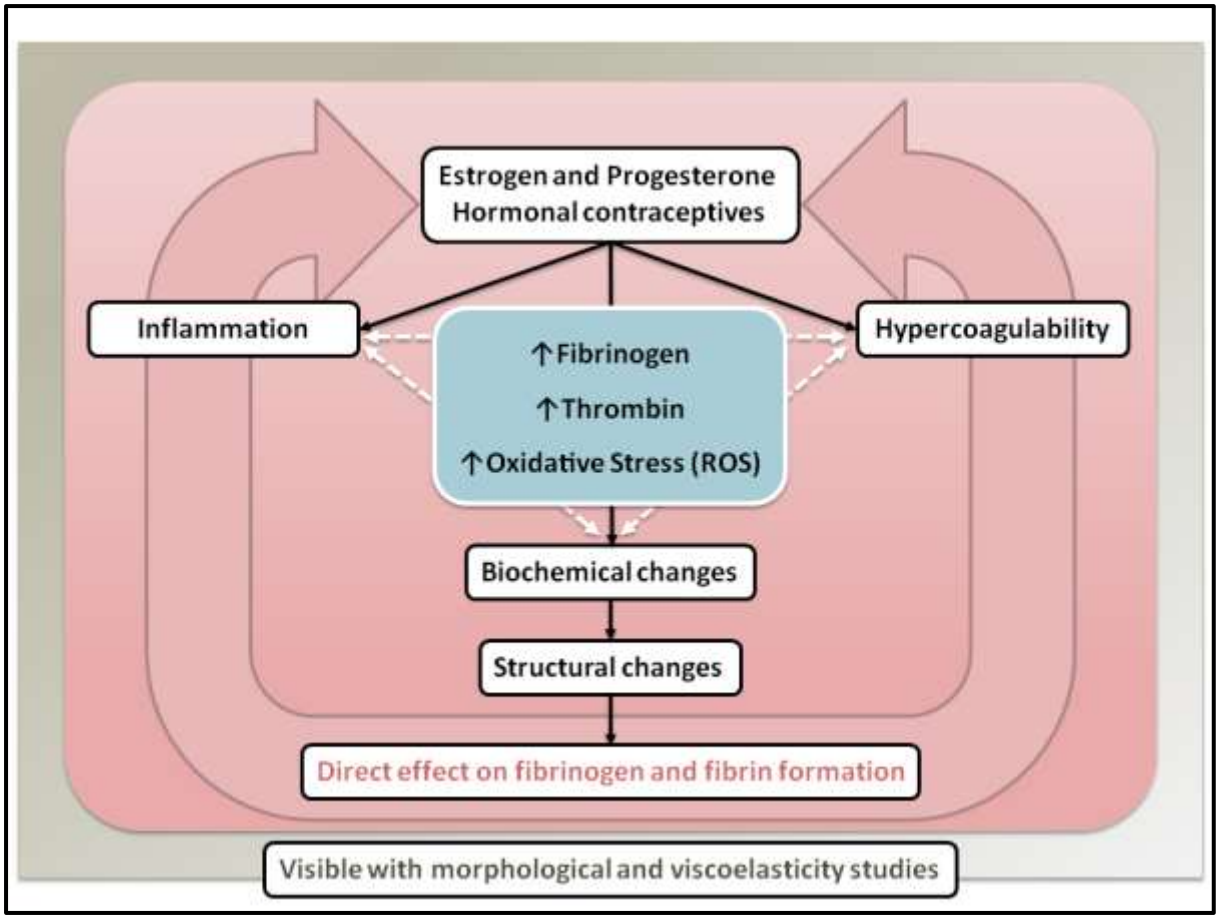

Figure 1. Effect of estrogen and progesterone in fibrinogen and fibrin network formation.

The binding of these hormones to the fibrin precursor, fibrinogen, before clot formation could lead to biochemical changes that result in ultrastructural changes, which can be visualized with morphological and viscoelastic studies. Therefore the possible pro-inflammatory properties of these hormones can trigger a prothrombotic state (see Figure 1).

A Pubmed search showed that the effect of endogenous hormone concentrations, specifically 17β-estradiol and progesterone, on fibrin network formation has not received attention thus far in the scientific community. However the individual effects of 17β-estradiol and progesterone need to be determined**.** In the current study, males were used to study the effect of 17β-estradiol and progesterone on the ultrastructure of the fibrin network since these hormones occur at lower concentration and with less fluctuation in the plasma of males compared to that of females [16].

It is essential to understand natural hormone mechanisms since these hormones are still present in circulation while hormonal contraceptives, which have been shown to indeed negatively affect fibrin clot features and lysability [17-20], are used. Although natural female hormones like 17β-estradiol and

progesterone decrease with the use of hormone therapies, specifically combined oral contraceptives, it is still essential to understand the natural hormone mechanisms at physiological concentrations since this could give more insight into possible interaction of any remaining natural hormones with synthetic hormones introduced with combined oral contraceptives. Due to the fact that 17β-estradiol and progesterone are known to increase hypercoagulability and the prothrombotic state we used scanning electron microscopy (SEM), atomic force microscopy (AFM) and thromboelastography (TEG) to investigate the morphology, surface roughness and viscoelastic properties of plasma fibrin clots and turbidity to measure clot formation and lysis.

Materials and methods

Blood collection

Ethical clearance was obtained to collect blood from 10 healthy male individuals between the age of 18 and 30 years (Ethics number 154/2014, University of Pretoria Ethics Committee). Exclusion criteria for this study were: abnormal BMI (obesity), smoking, chronic medication use, thrombotic disease. All participants were non-smokers, had a normal BMI, 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 aspirin or aspirin analogues within 48 hours before sampling.

Blood was collected in serum gel tubes (containing clot activator and gel for serum separation) for iron and hormonal profiles and citrate tubes (containing 0.5mL of 3.8% sodium citrate) for viscoelastic, morphological and turbidimetric analysis. Iron profiles were performed to assess the inflammatory status of each participant, while the hormonal profiles were determined to ensure endogenous hormones would not influence the results.

Sample preparation for viscoelastic and morphological analysis

Citrated whole blood was firstly centrifuged for 10 min at 1000 rpm (maximum RCF = 1250*g*) after which the supernatant plasma was transferred to Eppendorf tubes and centrifuged for a second time at 1250*g* for another 5 minutes to obtain platelet poor plasma (PPP). The PPP samples were transferred to new Eppendorf tubes 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 each hormone (17β-estradiol and progesterone separately) to attain the final concentrations as indicated in Table 1. The hormones powders obtained from Sigma Aldrich were initially dissolved in 100% ethanol at a concentration of 1mg/ml and subsequently diluted with distilled water to eliminate the effect of ethanol on the samples.

Table 1. Final concentrations of hormones in PPP (incubated at 37°C for 15 minutes) as determined from literature [21, 22].

	17β-estradiol final concentration in PPP ($pg.mL^{-1}$)	Progesterone final concentration in PPP ($ng.mL^{-1}$)
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 and morphological analysis.

Viscoelastic studies

Thromboelastography (TEG) was employed to investigate the effect of 17β-estradiol and progesterone on the viscoelasticity of fibrin clot formation. The different PPP-hormone mixtures (concentrations indicated in Table 1) were analysed in the following manner: 340µL of each PPP-hormone mixture was placed in a TEG cup and 20µl of 0.2M $CaCl_2$ was added to reverse the effect of the sodium citrate and subsequently initiate coagulation. The samples were placed in a Thromboelastograph 5000 Hemostasis Analyzer System to be analysed.

Morphological analysis

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to investigate the effect of 17β-estradiol and progesterone on fibrin network surface morphology. The different PPP-

hormone mixtures (concentrations indicated in Table 1) were used to prepare fibrin clots in a similar manner for both SEM and AFM analysis.

Thrombin (provided by the South African National Blood Services or SANBS) was prepared in biological buffer containing 0.2% serum albumin with a final concentration of 20U/mL. Thrombin converts fibrinogen to fibrin; when added to PPP it ultimately results in the formation of an expansive fibrin fiber network.

The different PPP-hormone mixtures (10µL) were individually mixed with thrombin (5µL) on round glass coverslips and immediately placed on a filter paper dampened with 0.075 M sodium potassium phosphate buffer solution (pH = 7.4) inside an airtight container to create a humid environment. The enclosed samples were incubated at 37°C for 10 minutes and then washed in 0.075 M sodium potassium phosphate buffer solution (pH = 7.4) for 20 minutes using a plate shaker to remove any uncoagulated proteins to prevent them from forming a layer on top of the fibrin network.

Samples subsequently underwent a primary fixation step with 4% formaldehyde for 30 minutes, followed by three rinsing changes with washed in 0.075 M sodium potassium phosphate buffer solution (pH = 7.4) of 5 minutes each. A secondary fixation step with osmium tetraoxide (1% OsO_4) was done for 15 minutes followed again by three more rinsing changes as mentioned before. Procedures were concluded with dehydration of the samples with a series of ethanol concentrations (30%, 50%, 70%, 90% and three times 100%) for 5 minutes each, 100% hexamethyldisilazane (HDMS) submersion for 30 minutes and lastly air-drying of samples in a flow hood.

For samples analysed with SEM, the glass coverslips were mounted with carbon tape on an aluminium platform and coated with carbon before it was examined with a Zeiss Ultra plus FEG SEM at 1kV. SEM micrographs were then taken at 40 000x machine magnification.

The samples analysed with AFM were not mounted or coated, but placed directly in the AFM Dimension Icon with ScanAsyst for surface roughness investigation. At each concentration of both 17β-estradiol and progesterone 5 randomly selected fibers were identified per participant for evaluation by AFM software for the specific surface roughness properties.

Sample preparation for Turbidimetric analysis

The frozen PPP samples were thawed before analysis 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 (described below) to give a more detailed account of these hormones' effect on clot formation and also clot lysis.

Plasma fibrinolytic potential of tissue factor induced clots, lysed by exogenous tissue plasminogen activator (tPA) [23-25] were determined for control plasma and plasma with different concentrations of 17β-estradiol (30, 60, 140, 220, 300 pg/ml and 1ng/ml) and progesterone (30, 60, 140, 220 and 300 ng/ml). 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 (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and 10 µmol/l phospholipid vesicles (Rossix, Mölndal, Sweden). Final plasma concentration for controls was 50% and 47% where 17β-estradiol or progesterone was added.

Statistical analysis

The 7 parameters measured with TEG are explained in Table 2. Figure 2 indicates how the mentioned parameters either decrease or increase in hypercoagulability. For the different concentrations of each hormone all parameter values were obtained for statistical analysis.

Table 2. Summary of viscoelastic parameters.

Parameter	Abbreviation	Unit of measurement	Description
Reaction time	R	Minutes	Time of latency from start of test to initial fibrin formation i.e. initiation time
Kinetics	K	Minutes	Time taken to achieve an amplitude of 20 mm
Angle (also known as alpha; slope R and the beginning of the decrease in amplitude as speed of clot growth decreases)	α	Degrees	The angle measures the speed at which fibrin build up and cross linking takes place, hence assesses the rate of clot formation
Maximal amplitude	MA	mm	Maximum strength/stiffness of clot. Represents the ultimate strength of the fibrin clot, i.e. overall stability of the clot
Time to maximum rate of thrombus formation	TMRTG	Dynes/cm^2/sec	The time interval observed before the maximum speed of the clot growth
Maximum rate of thrombus formation	MRTG	Minutes	The maximum velocity of clot growth
Total thrombus generation	TTG	Dynes/cm^2	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.

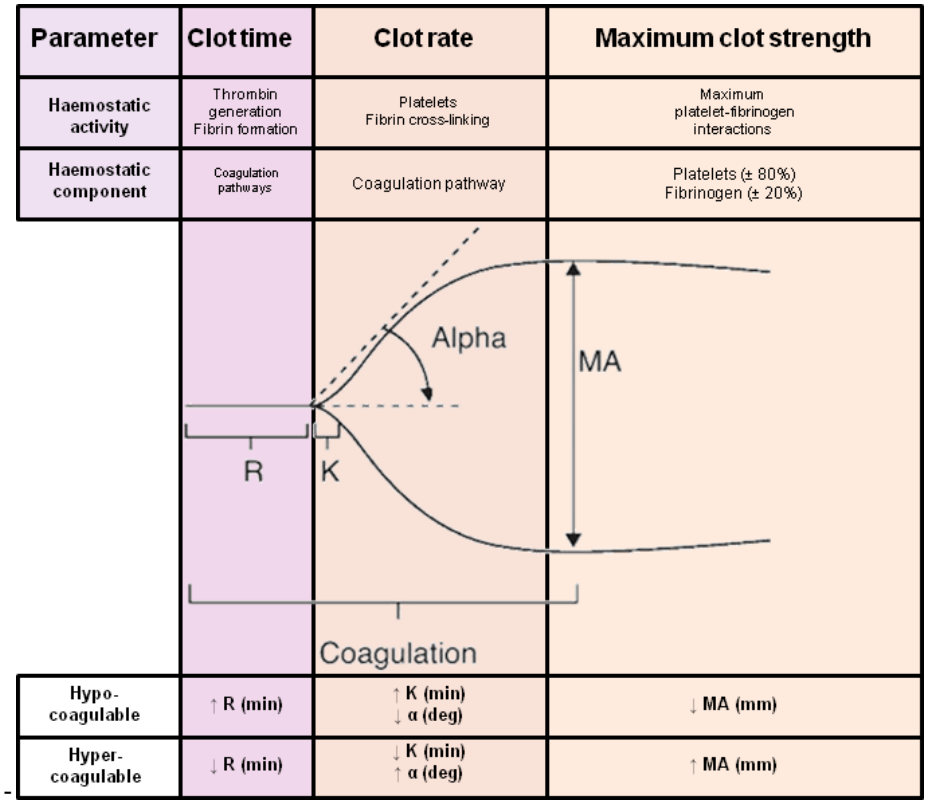

Figure 2. Viscoelastic parameters indicate hypo- and hypercoagulability.

SEM micrographs taken at 40 000x machine magnification were used to measure fibrin strand thickness. ImageJ (http://rsb.info.nih.gov/ij/download.html) was used to measure a total of 1000 fibrin fibers (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 [26]. 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) is then used as statistical measure [26]. AFM software was used to evaluate the surface roughness of 150 areas on individual fibrin fibers (3 points on 5 randomly selected 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, lysis time which is the midpoint of maximum turbidity to clear, minus clot formation time – midpoint of clear to maximum turbidity) [27].

GraphPad Prism 5 was employed to perform one-way analysis of variance (ANOVA) for all statistical analysis, with a p-value of ≤ 0.05 considered significant.

Results

Rational for using male plasma

Since we chose to use males for this study (males have lower concentration and less fluctuating levels of 17β-estradiol and progesterone in the plasma compared to that of females [16]) we wanted to ensure that the results would be comparable to that of females. The ideal time for sampling blood from females is during the menstrual phase when the above-mentioned hormones are at their lowest levels [10]. When samples from females were prepared in a similar manner as described in the materials and method section, the results were similar to that seen for males (discussed later in the results section, see Figure 5 for representative micrographs of male plasma with added hormones and supplementary Figure 1 for representative micrographs of female plasma with added hormones). Since the effect of these hormones was similar for males and females we decided to rather focus on the male plasma to prevent any possible effect of endogenous hormones in female samples.

Analysis of male plasma

Table 3 shows all participant information, including the iron profile as well the hormone profile summary (mean ± standard error of the mean). The iron panel and all hormones were in the normal ranges.

Table 3. Summary of the participant information, iron and hormone profiles with reference ranges indicated as mean ± standard error of the mean.

Variables	Healthy individuals (n = 10)	Reference ranges Males	Reference ranges Females
Age (years)	24.00 (±0.72)		
Gender	Males		
Iron profiles			
Iron (µmol. L^{-1})	15.65 (± 1.40)	11.60 - 31.30	
Transferrin (g.L^{-1})	2.47 (± 0.06)	2.20 - 3.70	
Saturation (%)	25.40 (± 2.33)	20.00 – 50.00	
Serum Ferritin (ng.mL^{-1})	98.50 (± 15.72)	20.00 – 250.00	
Hormone profiles			
Estradiol (pmol. L^{-1})	62.52 (± 7.84)	28.00 – 156.00	46.00 – 1828.00
Progesterone (nmol. L^{-1})	1.75 (± 0.25)	0.70 – 4.30	0.60 – 86.00
Estriol (ng.mL^{-1})	<0.07	<0.07	<0.08
Testosterone (nmol. L^{-1})	17.11 (± 1.77)	8.64 – 29.00	0.29 – 1.67
SHBG (nmol. L^{-1})	39.23 (± 3.77)	14.50 – 48.40	26.10 – 110.00

Table 4. Summary of the effects of different concentrations of 17β-Estradiol and Progesterone on fibrin diameter, surface roughness of the fibrin fibers and viscoelastic parameters indicated as mean ± standard error of the mean.

	Control	Lowest physiological concentration	Highest physiological concentration	F-value [F=3,36]	ANOVA p-value
17β-Estradiol					
Morphological analysis					
Fibrin diameter (nm)	136.20 (± 1.56)	***116.63 (± 1.46)***	***114.25 (± 1.47)***	**42.11**	**<0.0001**
Surface roughness (nm)	21.76 (±1.03)	19.96 (± 0.84)	***17.53 (± 0.76)***	**2.678**	**0.0463**
Viscoelastic parameters					
R (minutes)	9.04 (± 0.809)	9.40 (± 0.84)	***5.12 (± 0.57)***	**7.373**	**0.0006**
K (minutes)	5.50 (± 0.88)	3.77 (± 0.98)	***2.02 (± 0.34)***	**3.651**	**0.0214**
Angle (degrees)	64.20 (± 2.83)	62.96 (± 2.16)	71.85 (± 2.61)	**2.942**	**0.0460**
MA (mm)	23.82 (± 1.09)	26.95 (± 1.22)	***33.14 (± 1.72)***	**6.402**	**0.0014**
MRTG (Dyn.cm^{-2}.s^{-1})	4.18 (± 0.61)	4.24 (± 0.41)	***9.06 (± 1.43)***	**7.388**	**0.0006**
TMRTG (Minutes)	10.16 (± 0.86)	11.14 (± 0.98)	***6.55 (± 0.69)***	**6.109**	**0.0018**
TTG (Dyn.s^{-1})	157.94 (± 9.61)	186.29 (± 11.39)	***252.65 (± 21.05)***	**5.917**	**0.0022**
Progesterone					
Morphological analysis					
Fibrin diameter (nm)	136.20 (± 1.56)	***120.38 (± 1.84)***	***122.41 (± 1.68)***	**37.90**	**<0.0001**
Surface roughness (nm)	21.76 (± 1.03)	19.28 (± 0.67)	***15.70 (± 0.55)***	**13.94**	**<0.0001**
Viscoelastic parameters					
R (minutes)	9.04 (± 0.809)	7.99 (± 0.68)	7.34 (± 0.41)	**1.211**	**0.0006**
K (minutes)	5.50 (± 0.88)	***2.69 (± 0.30)***	***2.69 (± 0.31)***	**6.065**	**0.0214**
Angle (degrees)	64.20 (± 2.83)	65.42 (± 2.13)	68.01 (± 1.60)	**0.9516**	**0.0460**
MA (mm)	23.82 (± 1.09)	28.46 (± 1.41)	***30.22 (± 2.28)***	**2.595**	**0.0014**
MRTG (Dyn.cm^{-2}.s^{-1})	4.18 (± 0.61)	6.34 (± 0.89)	***7.00 (± 0.81)***	**2.838**	**0.0006**
TMRTG (Minutes)	10.16 (± 0.86)	10.16 (± 0.71)	9.03 (± 0.47)	**0.7013**	**0.0018**
TTG (Dyn.s^{-1})	157.94 (± 9.61)	198.68 (± 14.27)	***225.89 (± 23.90)***	**2.546**	**0.0022**

Table 5. Summary of the effects of different concentrations of 17β-Estradiol and Progesterone on turbidimetric parameters indicated as mean ± standard error of the mean (Std Error).

Turbidimetric parameters									
17β-estradiol	**Control**	**30pg.mL^{-1}**	**60pg.mL^{-1}**	**140pg.mL^{-1}**	**220pg.mL^{-1}**	**300pg.mL^{-1}**	**1ng.mL^{-1}**	**F-value [F = 6;63]**	**ANOVA p-value**
Lag time (minutes)	4.85 (± 0.17)	5.34 (± 0.21)	5.10 (± 0.18)	5.08 (± 0.18)	5.14 (± 0.20)	5.21 (± 0.14)	5.10 (± 0.19)	**0.662**	**0.681**
Slope (x10^{-3} au/s)	6.35 (± 0.64)	5.81 (± 0.39)	5.11 (±0 .20)	5.28 (± 0.22)	5.58 (± 0.44)	5.34 (± 0.18)	5.27 (± 0.22)	**1.381**	**0.236**
Maximum absorbance	0.50 (± 0.04)	0.44 (± 0.03)	0.44 (± 0.03)	0.44 (± 0.04)	0.45 (± 0.03)	0.45 (± 0.04)	0.44 (± 0.04)	**0.297**	**0.936**
CLT (minutes)	71.05 (± 0.18)	67.38 (± 1.22)	67.83 (± 1.23)	***66.37 (± 0.92)***	***66.52 (± 1.16)***	***66.06 (± 1.27)***	66.64 (± 1.44)	**2.007**	**0.078**
Progesterone	**Control**	**30ng.mL^{-1}**	**60ng.mL^{-1}**	**140ng.mL^{-1}**	**220ng.mL^{-1}**	**300ng.mL^{-1}**		**F-value [F = 5;54]**	**p-value**
Lag time (minutes)	4.85 (± 0.17)	5.01 (± 0.16)	5.13 (± 0.18)	5.06 (± 0.19)	5.06 (± 0.17)	5.26 (± 0.20)		**0.573**	**0.721**
Slope (x10^{-3} au/s)	6.35 (± 0.64)	5.48 (± 0.52)	5.30 (± 0.29)	5.20 (± 0.22)	5.70 (± 0.56)	5.72 (± 0.60)		**0.692**	**0.632**
Maximum absorbance	0.50 (± 0.04)	0.44 (± 0.04)	0.46 (± 0.04)	0.46 (± 0.04)	0.46 (± 0.04)	0.46 (± 0.04)		**0.176**	**0.971**
CLT (minutes)	71.05 (± 0.18)	68.36 (± 2.06)	67.27 (± 1.16)	68.82 (± 1.24)	70.14 (± 1.81)	68.79 (± 1.58)		**0.753**	**0.588**

An analysis of variance showed that fibrin diameter, surface roughness and all viscoelastic parameters were significantly influenced by both the highest physiological concentration of 17β-estradiol and progesterone (refer to Table 4 for mean ± standard error of the mean of each concentration evaluated for the mentioned hormones, F-values and p-values). Dunnett post hoc criterion for significance was performed to compare the effect of each hormone's concentration to the control group (significance indicated with blue, bold and italic in Table 4). The post hoc analysis indicated that 17β-estradiol altered all viscoelastic parameters, except the Angle; 17β-estradiol significantly decreased the R, K and TMRTG and significantly increased the MA, MRTG and TTG. The lowest and highest physiological concentrations of progesterone both significantly increased the K value while only the highest physiological concentration of progesterone increased the MA, MRTG and TTG. Progesterone had no effect on the R, Angle or TMRTG. Furthermore it indicated a decrease in fibrin diameter for both hormones at every concentration compared to that of control fibrin fibers with no hormones added. It also showed a significant decrease in surface roughness for 17β-estradiol and progesterone at the highest physiological.

With regards to turbidimetric parameters: an ANOVA showed no statistically significant overall change to CLT, however Dunnett post hoc criterion for significance indicated that only the 140-300pg.mL^{-1} concentrations of 17β-estradiol significantly decreased the CLT (significance indicated with blue, bold and italic in Table 5).

Viscoelasticity

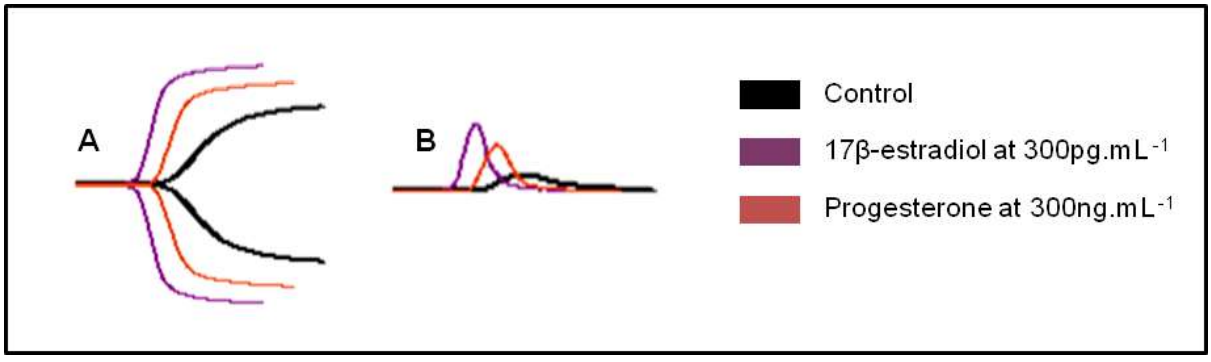

Figure 3. Representative viscoelastic traces. A = Traditional TEG waveforms, B = Clot growth velocity curves.

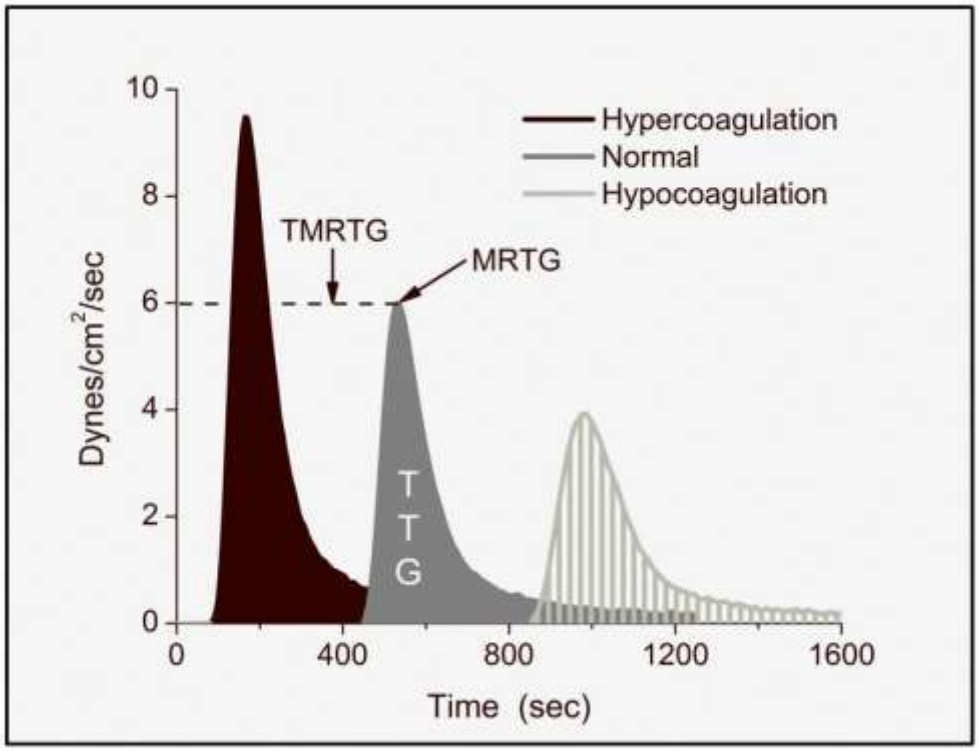

Figure 4. Representative clot velocity curve indicating the difference between normal coagulation, hypercoagulation and hypocoagulation.

17β-estradiol

The viscoelasticity study showed that only the highest concentration of 17β-estradiol (300pg.mL^{-1}) significantly influenced almost all the thrombo-elastic parameters analysed, while the lowest physiological concentration had no effect on these parameters. 17β-estradiol at 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 decrease and increase in the specific parameters is indicative of hypercoagulability (see figure 3 and 4, which are both representative figures), therefore 17β-estradiol at specifically 300pg.mL^{-1} produce hypercoagulable fibrin clots while 60pg.mL^{-1} produced fibrin clot formations similar to that of controls.

Progesterone

Progesterone did not influence fibrin clot formation in the same manner as 17β-Estradiol. It was mainly the K, MA, MRTG and TTG values that were influenced by different concentrations of the hormone. The amplification (K) was significantly decreased by both concentrations of progesterone, while the overall stability (MA), maximum velocity of clot growth (MRTG) and clot strength (TTG) was significantly increased by the highest physiological concentration.

TEG measures 'live' clotting parameters. Whilst this is a useful technique, other clotting assays like PT and aPTT should be considered in conjunction for future studies.

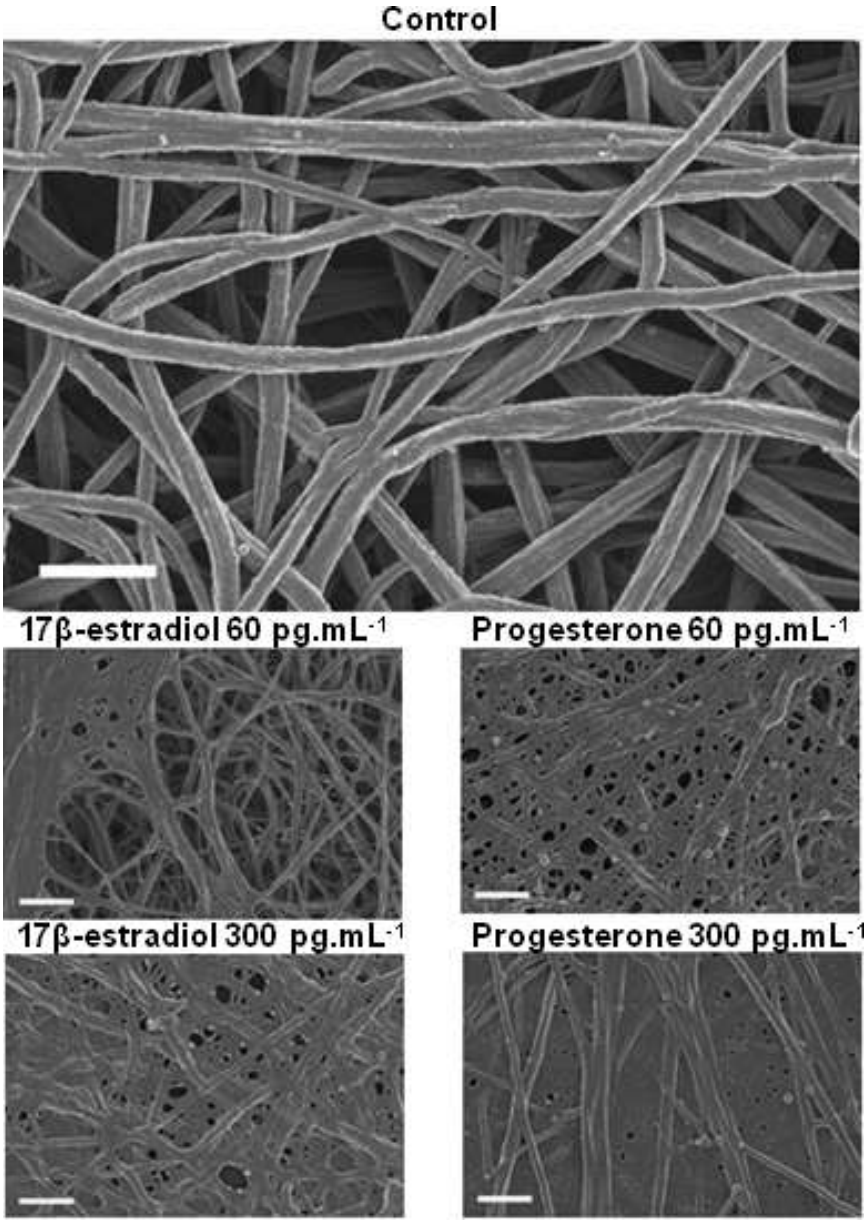

Figure 5. The effect of 17β-Estradiol on fibrin network morphology. Scale bar indicates 1µm.

Morphology of fibrin network and fibrin fiber diameter

17β-Estradiol

Figure 5 Control indicates fibrin networks without addition of hormones. The fibrin strands are evenly dispersed and mainly composed of thick fibers. Figure 5 also show the effect of the different concentrations of 17β-estradiol on fibrin network morphology. An accumulation of thin fibers forming dense matted deposits are present for all concentrations, but are most prevalent with the highest physiological concentration (300pg. mL^{-1} final concentration) covering most of the surface. These dense matted deposits give the fibrin network a sticky appearance.

Progesterone

Figures 5 show the effect of different concentrations of progesterone on fibrin network morphology. Like 17β-estradiol dense matted deposits are present for both concentrations but appear more closely packed for progesterone, with the highest physiological concentration (300ng.mL^{-1} final concentration) again having the greatest effect.

It appears as if 17β-estradiol at the lowest physiological concentration produce patches of dense matted deposits dispersed over thin fibers while 17β-estradiol at the highest physiological concentration has a similar effect on the morphology of the fibrin network as progesterone at the lowest concentration where dense matted deposits are prominent with holes still visible between very thin fibers; progesterone at the highest physiological concentration forms thick mass of fibrin with very few small holes in between the thin fibers.

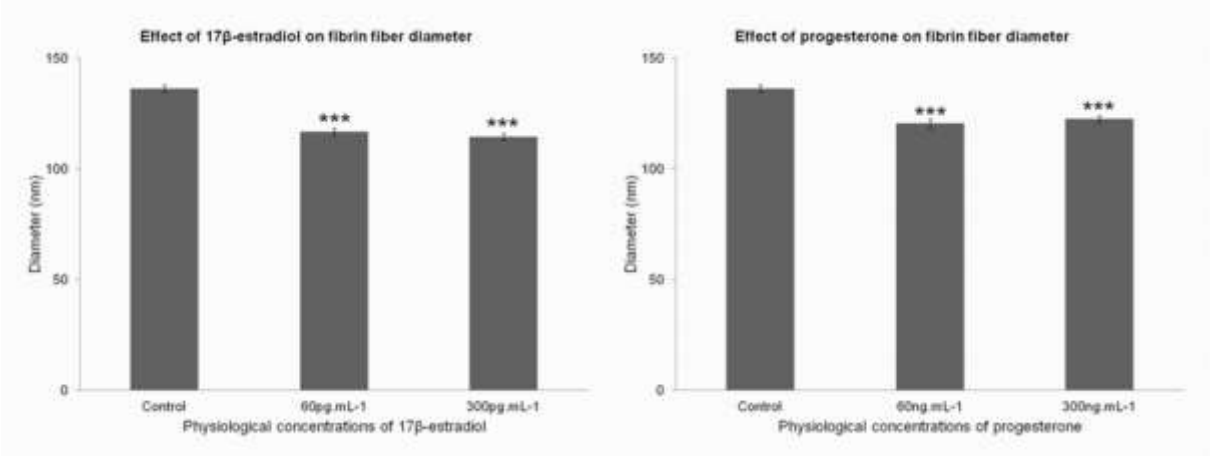

Figure 6. The effect of 17β-Estradiol and Progesterone on fibrin fiber diameter. Indicated the mean and standard error of the mean.

All concentrations of both 17β-estradiol and progesterone significantly decreased the diameters of individual fibers compared to the fibers formed without addition of hormones (see Table 4 and Figure 6).

Surface roughness

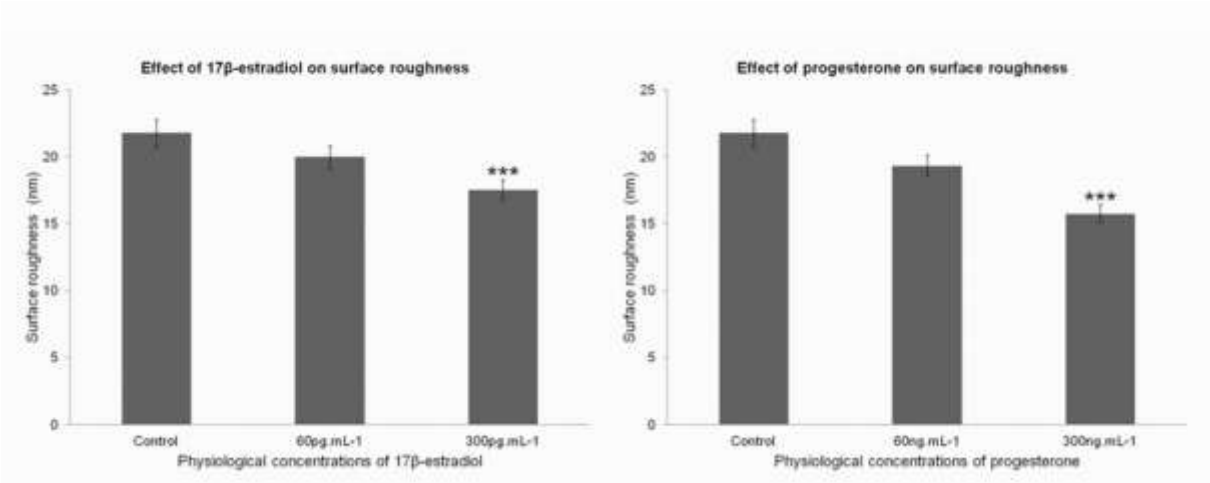

Figure 7. The effect of 17β-Estradiol and Progesterone on surface roughness of fibrin fibers. Indicated the mean and standard error of the mean.

17β-estradiol

AFM analysis revealed that the lowest physiological concentration of 17β-estradiol did not influence the roughness of the fibrin strands, while the highest physiological concentration of the hormone significantly decreased the roughness of the fibrin fibers (see Table 4 and Figure 7). Thus 17β-estradiol at 300pg.mL^{-1} had an effect on fibrin fiber roughness. At this specific concentration the fibrin fibers become smoother compared to fibers with no hormones added.

Progesterone

AFM analysis showed that the lowest physiological concentration of progesterone did not influence the fibrin strand roughness (see Table 4 and Figure 7). It was the highest physiological concentration of this hormone that followed the trend of the highest physiological concentration of 17β-estradiol by significantly decreasing the roughness of the fibrin fibers. Thus progesterone concentrations of 300ng.mL^{-1} affect fibrin fiber roughness.

Therefore 17β-estradiol at its highest physiological concentration of 300pg.mL^{-1} and progesterone at it's highest physiological concentration of 300ng.mL^{-1} produce fibrin fibers that are smoother compared to that of controls.

Turbidimetric parameters

17β-estradiol

Overall 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. CLT was also decreased for all concentrations of 17β-estradiol, although only statistically significant for the 140-300 pg.mL^{-1} concentrations.

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.

Progesterone

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 (no statistically significant changes).

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.

Discussion

The viscoelastic studies revealed that 17β-estradiol at 300pg.mL^{-1} influenced the rate of clot formation. The reaction time, kinetics and time interval observed before the maximum speed of the fibrin clots growth was decreased relating to more rapid clot formation compared to that of controls. Not only was clot formation faster, the overall velocity of clot growth and the stability and strength of the clot was increased. Therefore 17β-estradiol at its highest physiological concentrations resulted in more rapid formation of strong, thick fibrin clots. Progesterone on the other hand did not affect the initiation time of clot formation. All concentrations tested increased clot amplification, while only progesterone at its highest physiological concentration increased the maximum velocity of the clot growth and overall stability and strength of the clot. Therefore progesterone does increase clot amplification but at 300ng.mL^{-1} increase the formation rate of fibrin clots that are stronger and denser. These viscoelastic alterations were confirmed with SEM. Piechocka et al [28] recently showed that the hierarchical architecture of the fibrin fibers are reflected by the strain-stiffening response of fibrin networks. Duval et al [29] has suggested a connection between clot structure and viscoelastic properties via fibre tautness.

Both 17β-Estradiol and Progesterone decreased the fibrin fiber diameter while forming a more closely-packed arrangement known as dense matted deposits (DMDs). Fibrin network architecture is closely associated with fibrinolysis. Although individual thinner fibers lyse more easily [30] 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 [31]. Since both hormones decrease the

fibrin fiber diameter it could be assumed that in turn this would decrease the local enhancement and acceleration of fibrinolysis [31]. However, turbidimetric analysis indicated that both 17β-Estradiol and Progesterone decreased the CLT, although only significantly for 17β-Estradiol at concentrations between 140 – 300 pg.mL^{-1}. Therefore, both these hormones decrease the fibrin diameter and result in more densely packed clots (visualised with SEM and confirmed with TEG and turbidimetric analysis) accompanied by an increased ability to lyse the formed clot. Since the concentrations tested were physiological concentrations this may explain why not all females, who are continually exposed to these hormonal concentrations, suffer thrombotic events (as could be assumed by the morphological effects of these hormones at high physiological concentrations).

Elevated fibrinogen [32, 33] and thrombin levels [34] are associated with denser fibrin clot structure and reduced susceptibility to lysis. Plasma fibrinogen is susceptible to oxidative stress [35], and fibrinogen exposed to reagents present during oxidative stress resulted in decreased fibrin fiber diameter [36].

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 17β-estradiol is more commonly known to attenuate oxidative stress [37] it has recently been shown that chronic 17β-estradiol exposure induces oxidative stress in the hypothalamus [38] and even in organisms that do not possess estrogen receptors like *Eisenia fetida* [39].

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 prooxidant [40].

It is interesting that, although both hormones at all concentrations decreased the fibrin diameter and resulted in the formation of DMDs that the fibrin fiber roughness was not influenced by all concentrations of both hormones.

Fibrinogen is a large centrosymmetric glycoprotein [41, 42]. It contain three pairs of polypeptide chains (Aα, Bβ and γ polypeptides) which are curved into a central E-region with two distal D-regions [43, 44] (see Figure 8). Fibrinogen orientation is influenced by fibrinogen concentration [45, 46] as well as the surface charge [47]. 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 [45-47] (see Figure 9). Hydrophobicity has a direct correlation to the degree of conformational alterations [48, 49]. Clark et al attributed these variances to altered binding mechanisms and post-adsorption conformational changes [50]. Crosslinking of the Aα and γ constituent chains in fibrinogen can also occur in different formations as indicated in Figure 10 [51].

17β-estradiol 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 [52]. 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. 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 [53]. Since the ultrastructural and viscoelastic studies showed hypercoagulable clot formation, which is associated with increased polymerization, at the highest physiological concentration of 17β-Estradiol we can hypothesise that this specific concentration of 17β-Estradiol alters Trp residues in the D domain.

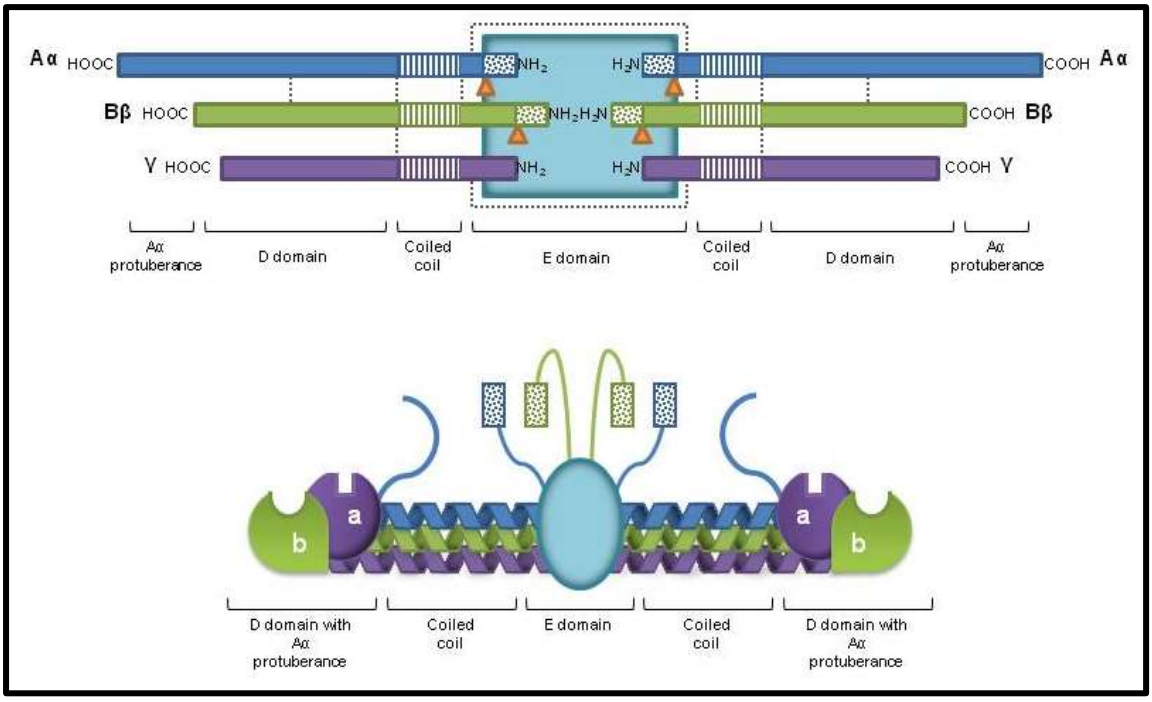

Figure 8. Fibrinogen structure. The top diagram indicates the polypeptide organisation of fibrinogen with the three chains (α,β and γ) while the bottom diagram depicts domain organisation (D domain and E domain) and binding sites of fibrinogen. Adapted from [54, 55]

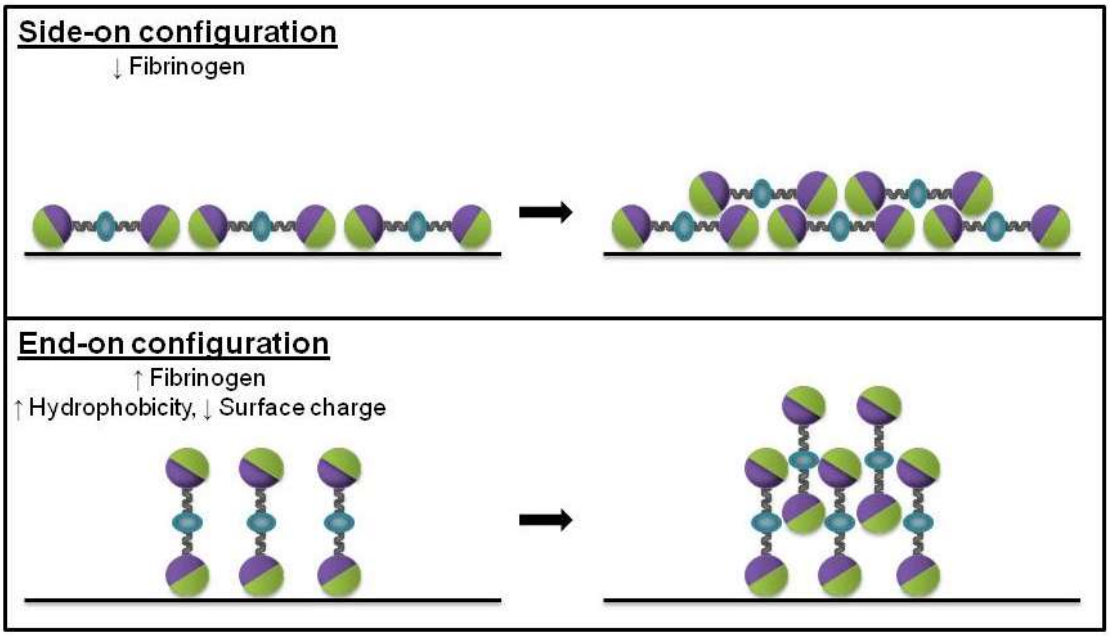

Figure 9. Two possible configurations of fibrinogen packaging. Adapted from [45-49]

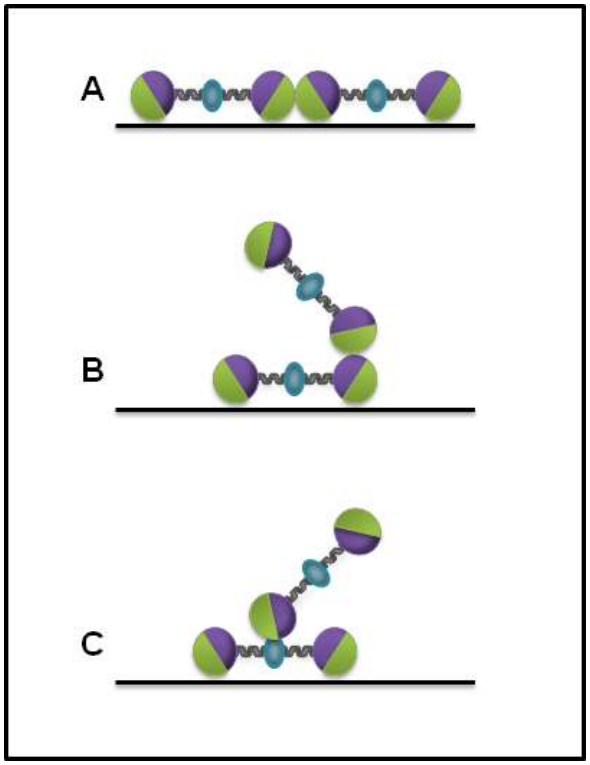

Figure 10. Different configurations of 2 fibrinogen molecules. (A) Linear end-to-end. (B) Angled end-to-end. (*C*) End-to-center. Figure adapted from [51]

Tabel 5. Past knowledge, current findings and future work on estradiol and progesterone.

Past What did we know before we started?	**Present** What knowledge did this paper add?	**Future** What needs to be done?
• Cardioprotective role [8, 56] • Prothrombotic [11] • Pro and anti-inflammatory properties [15]	• ↓Fibrin diameter with ↓CLT • ↑DMDs • ↓Fibrin fiber surface roughness • Resulting in increased systemic hypercoagulability, confirming the prothrombotic and pro-inflammatory properties	• Determine possible ROS generation during fibrin packaging after exposure to hormone • Determine specific orientation and conformation of altered fibrinogen packaging after exposure to hormone

		• Determine the specific Trp residues that are exposed by 17β-estradiol and how it influences fibrinogen packaging • Determine whether progesterone also influence Trp residues (similar to 17β-estradiol)

Table 5 provides and overview of this paper. Here we reviewed the existing knowledge and added new insights into the effect of estradiol and progesterone on fibrin packaging and coagulation properties. We also suggest future research endevours 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.

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 estrogen and progesterone, 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.

Conclusion

From a historical clinical perspective, the relative hypercoagulability associated with estrogen and progesterone has caused both adverse and adventagous outcomes in a variety of settings. Pathological thrombosis in the setting of oral contraception [57], assisted reproductive technology [58], or postmenopausal hormone replacement [59] have been well documented. However, female gender has been found to bestow better outcomes after trauma, with associated superior post traumatic fibrinogen concentrations [60]. 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 [61]. While some works have implicated roles for decreased tissue factor pathway inhibitor concentrations [62], activated protein C resistance [62], and elevated heparanase procoagulant activity [63] 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.

Conflict of interest

The authors have no conflict of interest to report.

Authorship

A.C. Swanepoel: Contributed to the concept and design, analysis and interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published.

A. Visagie: Contributed to the concept and design, analysis and interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published.

Z. de Lange: Contributed to the concept and design, analysis and interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published.

O. Emmerson: Contributed to the concept and design, analysis and interpretation of data; and final approval of the version to be published.

V.G. Nielsen: Contributed to the concept and design, analysis and interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published.

E. Pretorius: Contributed to the concept and design, analysis and interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published.

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