ULTRASTRUCTURAL AND FLOW CYTOMETRIC ANALYSIS OF PLATELETS AND FIBRIN NETWORKS DURING THE MENSTRUAL CYCLE AND PREGNANCY

by

ALBE CARINA SWANEPOEL

Thesis submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

(PhD)

in the

FACULTY OF HEALTH SCIENCES

DEPARTMENT OF PHYSIOLOGY

UNIVERSITY OF PRETORIA

2013

ULTRASTRUCTURAL AND FLOW CYTOMETRIC ANALYSIS OF PLATELETS AND

FIBRIN NETWORKS DURING THE MENSTRUAL CYCLE AND PREGNANCY

by

ALBE CARINA SWANEPOEL

Supervisor: Prof. E. Pretorius

Department: Physiology

Co-supervisor: Prof. B.G. Lindeque

Department of Obstetrics and Gynaecology

Degree: PhD (Physiology)

ABSTRACT

INTRODUCTION: The menstrual cycle and pregnancy are processes unique to women. Both these processes involve various hormones as well as the coagulation system. Throughout normal pregnancy, platelet activation and increase in blood coagulation factors contributes to the hypercoagulable state observed on a physiological level. **METHODS:** Fibrin networks and platelets were analysed by electron microscopy and flow cytometry to determine any differences found in different phases of pregnancy compared to healthy control individuals. The fibrin networks from different phases of the menstrual cycle as well as different phases of pregnancy were investigated. **RESULTS:** It was found that ultrastructural changes in fibrin fiber morphology result from estrogen changes during the menstrual cycle. During pregnancy the minor thin fibers were prominent and thick matted layers of coagulant formation were evident. A large quantity of protein globular clusters similar to those seen in the menstrual cycle was present. Changes observed in platelet ultrastructure during pregnancy showed pregnancy-specific modifications. Platelets were activated and internal

organelles showed variation from control participants. Flow cytometric analysis of platelets verified pregnancy-specific modifications. Close interactions between platelets and erythrocytes were evident. CONCLUSION: The female body is equipped to handle alterations in the coagulation system as can be extrapolated from the pregnancy-specific modifications. This study is the first to show alterations in fibrin network and platelet ultrastructure during and after pregnancy when compared to non-pregnant controls. The physiological changes during normal pregnancy can be used as a standard for comparison to abnormal or ailing pregnancy.

Declaration

© University of Pretoria

I, Albe Carina Swanepoel, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University

Signed: ……………………..

Date: ………………………

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

South Africa

Acknowledgements

I am grateful for the support and input of several individuals, without whom this dissertation would not have been possible.

First of all my God, my Saviour, my Heavenly Father, my Provider, Protector and Counsellor. In You I live, and move and have my being. I want to quote Psalm 127:1 (paraphrase my own): If the Lord does not *lead and guide the researcher*, their work is useless. I stand amazed at your wonderful creation, and the fact that you entrusted me with insight into the wonderful workings of the female body and how You maintain and preserve pregnancy. I now know for sure that You are always in control, You are close to us and you answer our prayers with awesome deeds. Without your grace and wonderful love, I could not and do not want to exist.

My dear husband, Rikus Swanepoel. I thank the Lord daily for you! Without your love, support, prayers and wonderful counsel I would not have been where I am today. You are a man after God's heart. It is such a blessing to be married to my best friend, I love you very much.

My parents, Izak and Laetitia de Lange. I could not have asked for better parents and role models. You show the character of God to me on a daily basis, may I follow in your steps of righteousness and Godly wisdom. Your encouragement never wavered and I know you spent a lot of time on your knees for me. Thank you that you always listened, gave Godly counsel and that I know that I am loved. My sister, Estelle de Lange. Your love, support and encouragement really carried me through this time. The good laughs we had together were a real "pick-me-up". My grandparents, Daan and Ina Nolte, for your support and all your love. My parents-in-law, Johan and Ina Swanepoel. Thank you for your love and support throughout all my studies. I truly appreciate it. I love you all very much.

All my friends, especially Amoré Dippenaar, Irene Schoeman, Paula Wessels and Christel Venter. I couldn't have asked for a more loyal support group. Your thoughtfulness, love and prayers carried my through.

My supervisor, Professor Resia Pretorius. There is certainly no greater mentor. Your enthusiasm, motivation and wonderful insight that you share so abundantly are a real inspiration to me.

My co-supervisor, Professor B.G. Lindeque. Thank you for your support and wonderful insight.

The Netcare Femina Hospital and staff, especially the following Doctors: Dr PJ Swart, Dr Z Abdool, Dr S Suliman, Dr JF Postma. Thank you for your patience and assistance in this study. Also Renate, Claire and Boithumelo. Your wonderful assistance made this study possible – I couldn't have done it without you. The staff at Ampath (Drs Du Buisson, Kramer, Swart, Bouwer Inc) especially Sonja, Rika, Karen, Chanell, Lizzy and Ashley. Your friendliness and assistance (always with a smile) really brightened my day and gave me new hope.

The talented people at the Microscopy and Microanalysis Unit at the University of Pretoria. Chris van der Merwe, Allen Hall and André Botha have all provided wonderful advice and guidance in this project. Antoinette Buys, for your patience and assistance in the lab. I am grateful for all your help.

All participants of this study. Without your willingness to assist in further investigation into the wonderful workings of the human body, these findings would have remained a mystery.

Table of Contents

List of Tables and Figures

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Chapter 10

Abbreviations, Symbols and Chemical Formulae

%	Percentage
°C	degrees Celsius
α-granules	Alpha granules
δ-granules	Delta-granules/ Dense bodies/Dense core granules
λ-granules	Lysosomal granules
β	Beta
μl	Microliter
μm	Micrometer
ml	Milliliter
nm	Nanometer
ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
D-Dimer	Coagulation fragments indicating thrombin and plasmin activity

DTS	Dense tubular system
et al.	Et alia (and other)
Factor V, VIII, XI, XIII, VII, XII, XIII	Coagulation factors involved in the coagulation cascade
FC	Flow cytometry
FEG SEM	Field emission gun scanning electron microscope
FITC	Fluorescein isothiocyanate
FL	Flow cytometric channel
FS	Forward scatter
FSH	Follicle stimulating hormone
GDP	Guanosine diphosphate
GnRH	Gonadotropin
GP	Glycoprotein
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
hCG	Human chorionic gonadotropin
hPL	Human placental lactogen

IBM SPSS Statistics 21	Statistical analysis and graphic software
kV	Kilovolt
LH	Luteinizing hormone
M	Molar
MCFI	Mean channel fluorescence intensity
Mg^{2+}	Magnesium
MoAbs	Monoclonal antibodies
OCS	Open canalicular system
OsO_4	Osmium tetraoxide
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PBS	Phosphate buffered saline
PC	Protein C
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PF-4	Platelet factor 4

PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Protein S
PSM	Platelet specific marker
rpm	Revolutions per minute
rtPA	Recombinant tissue plasminogen activator
SCCS	Surface-connected canalicular system
SEM	Scanning electron microscope
SS	Side scatter
TAFI	Thrombin activatable fibrinolysis inhibitor
TEM	Transmission electron microscope
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
tPA	Tissue plasminogen activator
t-test	Statistical hypothesis test
U/ml	Units per milliliter
uPA	Urokinase

vs.	versus
vWF	von Willebrand factor

CHAPTER 1: INTRODUCTION

The menstrual cycle and pregnancy are processes unique to women. Both these processes involve various hormones as well as the coagulation system. The fluctuation in estrogen and progesterone levels through the menstrual cycle may possibly have an effect on platelets and the fibrin network. Since elevated levels of these hormones are also found in pregnancy, this may also affect the fibrin network and platelets during different phases of pregnancy.

Estrogen treatment has been shown to shift the haemostatic balance toward a hypercoagulable state.[1] The hypercoagulable state associated with pregnancy is well established. The elevated estrogen concentrations associated with pregnancy may influence the coagulations system resulting in hypercoagulability.

Thrombophilia is the propensity to thrombosis. Thromboembolism in pregnancy is associated with thrombophilia.[2] During pregnancy the haemostatic balance shifts toward thrombophilia. This is to prepare the female body for delivery, a great haemostatic challenge for the body.[3]

Throughout normal pregnancy, platelet activation contributes to the hypercoagulable state observed on physiological level.[4] Platelet activation is said to increase as the pregnancy progresses.[5]

During normal pregnancy various blood coagulation factors, such as factors XIII, XII, X, VIII and von Willebrand factor along with fibrinogen are increased.[6-10]

Pregnancy-induced haemostatic alterations therefore promote the hypercoagulable state associated with pregnancy.[11] This explains the increased risk of venous thromboembolism throughout pregnancy.[12]

It was hypothesised that ultrastructural and flow cytometric analysis of fibrin networks and platelets will exhibit alterations in the menstrual cycle and different phases of pregnancy.

Three phases of pregnancy, namely early pregnancy (8 – 14 weeks), late pregnancy (36 – 40 weeks) and post-partum (6 – 8 weeks after birth) were compared to non-pregnant healthy

1

controls. The control participants also participated in the menstrual cycle study where fibrin networks during different phases of the menstrual cycle were investigated.

Scanning electron microscopy was employed to investigate the external fibrin network morphology. Scanning and transmission electron microscopy was used to investigate the external and internal platelet structure respectively. Flow cytometric analysis was used to establish platelet architecture while scanning electron microscopy was employed to investigate erythrocyte/platelet interactions.

Ultrastructural and flow cytometric investigation concerning the menstrual cycle and pregnancy contribute significantly to the understanding of these processes and in addition advance further research to the benefit of all women.

"Women are always beautiful." ~Ville Valo~

2

CHAPTER 2: LITERATURE REVIEW

2.1 CHAPTER OBJECTIVES

In this thesis, the possible morphological alterations and flow cytometric changes of platelets and fibrin networks associated with different stages of the menstrual cycle as well as various phases of pregnancy are discussed.

The menstrual cycle and pregnancy are both physiological phenomena unique to women. Both these physiological events involve various hormones as well as the coagulation system.

The adaptations which a healthy female body undergoes throughout a normal pregnancy are dramatic. All systems in the body are affected by pregnancy. It is only the extent and timing of these alterations that will vary from system to system. [13]

It is also known that the coagulation profile changes during pregnancy and that risk of thrombo-embolic and haemorrhagic complications increase. Therefore, the current chapter provides an overview on the coagulation process, the menstrual cycle, pregnancy as well as all factors involved. This chapter concludes with a discussion on the basic principles of flow cytometry and how it was used in this study.

2.2 COAGULATION

Injury to the endothelium gives rise to the liberation of a cell-membrane-bound protein, tissue factor (TF), by perivascular cells throughout the body except for cells that are in contact with the circulation, for example endothelial cells. This triggers the coagulation and fibrinolytic systems to be activated. [14]

Coagulation entails an intricate interaction of substrates and soluble enzymes to ultimately produce thrombin, the catalyst involved in the conversion of fibrinogen to fibrin. Fibrin stabilizes the platelet clot formed at the site of the vascular disruption. When a vessel is damaged, the immediate adherence of platelets to the macromolecules in the subendothelial tissue and its subsequent aggregation leads to the formation of the primary haemostatic plug. The accumulated platelets are responsible for the local activation of the plasma coagulation factors. [14]

In the coagulation process the product of each reaction acts as an enzyme in a subsequent reaction, resulting in a cascade system. Two pathways of the coagulation cascade can be distinguished, namely the intrinsic pathway and the extrinsic pathway. In these two pathways a number of cofactors and regulation pathways are shared. Once the wound starts to heal, the platelets accumulate and the fibrin clot undergoes fibrinolysis. To maintain blood fluidity, the formation of platelet aggregates and fibrin clots are restricted by certain mechanisms. A multifaceted system of feedback loops is strictly controlled by the coagulation cascade. This constitutes the body's natural anticoagulation system. [14]

Fibrin

There are various overlapping functions of fibrinogen and fibrin. These include blood clotting, fibrinolysis, cellular and matrix communication, the inflammatory response, wound healing as well as neoplasia. These roles have evolved as a result of complementary interactions concerning particular binding sites on fibrin(ogen) and extrinsic molecules in addition to fibrin formation. Interactions sites on fibrin(ogen) regulate the above-mentioned roles. [15]

In the clotting process, fibrinogen is converted to fibrin by the cleavage of fibrinopeptides from the central domain. This cleavage exposes protrusions that subsequently interact with cavities that are constantly exposed at the extremities of the molecule. A half-staggered configuration known as a protofibril is produced by this process. [16]

The protofibrils form twisted arrangements once they have elongated adequately, aggregated laterally in a specific manner and twisted around each other. These structures are then referred to as fibrin fibers. [16,17]

Since the 22.5 nm periodicity needs to be preserved, with the addition of each new protofibril to the existing fiber and increase in length of the fiber diameter the newly added protofibril must be stretched. This results in the exterior protofibrils to be under the great tension. The fiber stops the progress of elongation when the energy necessary to stretch an additional protofibril surpasses the energy of bonding. [18]

A branch point is the point where the laterally aggregated protofibrils diverge and thus leads to branching. [19]

The branching of these fibrin fibers generates a three-dimensional arrangement. In actual fact, fiber ends are not often observed in a typical, intact fibrin clot. [19]

It is the branched taut fibers that construct a three-dimensional fibrin network. These branches and specifically the branch points influence the fibrinolytic properties of the fibrin network and therefore the stability of the clot. Ryan et al. established that practically every branch point in clots is composed of three fibers at an intersection. [20]

Weisel further explained that these fibrin branches are not like the branches of a tree. The three fibers at a branch are usually of analogous diameter. There is no distinct systematic pattern of one fiber having a greater width than another. [21]

However, the expanse between branch points as well as the number of branch points in total can differ considerably. This is due to the variability of the fibrinogen structure and various conditions of polymerization. [19]

Therefore, branching and lateral aggregation are in essence conflicting processes. The diameter of fibers is thus influenced by the specific process that produces them. Thick fibers with only a small number of branch points are produced by circumstances that inhibit branching, whereas conditions that favor branching and inhibits lateral aggregation have a tendency to yield coagulants assembled of thin fibers with numerous branch points. [19]

Clot stability refers to the resistance of the coagulum to dissolution by fibrinolysis and mechanical tension. [22]

Another aspect of fibrin vital for its actions is its mechanical properties. To prevent blood loss, a coagulum is necessary to form a haemostatic plug. This clot must be resilient to arterial pressure. If a thrombus is inflexible or fragile the risk is greater for the thrombus to embolize. A thrombus may have the ability to change form if it has more flexible or viscous properties. However, little is understood about the correlation concerning fibrin's mechanical properties and these pathological traits. [22]

If a thrombus is inflexible or fragile the risk is greater for the thrombus to embolize. A thrombus may have the ability to change form if it has more flexible or viscous properties. However, little is understood about the correlation concerning fibrin's mechanical properties and these pathological traits. Another essential factor that influences thrombus formation is the fibrinolytic attributes of fibrin. For the establishment of haemostasis and wound healing the coagulum has to temporarily form a plug. For efficient haemostasis, it is vital that there exists a

5

balance between clotting and lysis. After wound healing, the plug has to be disintegrated in order to prevent thrombosis. [22]

Platelets

In vitro, the aggregation of platelets occurs in two possible ways namely primary and secondary aggregation [23]. Primary aggregation occurs once an aggregating agent directly interacts with its corresponding receptor. [24,25]

Secondary aggregation is precipitated by the liberation of aggregating agents, serotonin and ADP among other constituents from the platelet dense granules [26], along with production of prostaglandins from arachidonic acid. [27]

2.3 MENSTRUAL CYCLE

The Stedman's Medical Dictionary defines the menstrual cycle as the period which an ovum matures, is ovulated and enters the uterine lumen through the fallopian tubes. Ovarian hormonal secretions effect endometrial changes such that, if fertilization occurs, nidation will be possible. In the absence of fertilization the endometrium sloughs and menstruation begins. This cycle lasts an average of 28 days, with day 1 of the cycle designated as the day when menstrual flow begins. [28]

Various systems are involved in the hormonal regulation of the ovarian and uterine cycles. The hypothalamus secretes gonadotropin (GnRH) while the anterior pituitary is responsible for follicle stimulating hormone (FSH) and luteinizing hormone (LH) release. Estrogen is the principal steroid hormone of the follicular phase, while progesterone is the dominant hormone of the luteal phase. Both estrogen and progesterone are secreted by the ovaries. [28]

In the female body, two elliptically-shaped ovaries are present. These reproductive glands, of about 2 – 4 cm in length, contain within its inner connective tissue structure called the stroma numerous oocytes or germ cells enclosed in ovarian follicles. These ovarian follicles exhibit different stages of maturity or regression. The ovaries are also responsible for specific hormone production. [28,29]

Within the menstrual cycle, two additional cycles can be distinguished namely the ovarian cycle and the uterine cycle. [29]

6

Ovarian cycle

The ovarian cycle concerns the changes observed in the ovary's follicles. The ovarian cycle is divided into three phases namely the follicular phase, ovulation and lastly the luteal phase. [29]

The follicular phase makes up the first section of the ovarian cycle. This is the period characterized by the growth of the follicle in the ovary. This phase can persist for a period of approximately 10 days up to 3 weeks and is therefore the most variable in duration. [29]

Ovulation is the second phase of the ovarian cycle. This is the process of expulsion of one or more oocytes form the ripened follicles. [29]

Sex steroids like estrogen and progesterone prepare the adult female for reproduction while the ovums, the female gametes, are liberated at specific intervals all through a woman's reproductive years. [30]

Ovulation is followed by the last phase of the ovarian cycle known as the luteal or postovulatory phase. The ruptured follicle transforms into the corpus luteum, characterized by its yellow coloring and accumulation of lipids (corpus = body, luteum = yellow). Hormones secreted by the corpus luteum assist the body's preparation for pregnancy. If the released oocyte is not fertilized and implanted in the uterine wall, the corpus luteum will terminate its function of hormone secretion after approximately 14 days and the ovarian cycle will commence once more. [29]

Uterine cycle

The lining of the endometrium goes through its own cycle known as the uterine cycle. The uterine cycle is regulated by the ovarian hormones and consists of menses, the proliferative phase followed by the secretory phase. [29]

Menses marks the beginning of the uterine cycle. This period of menstrual bleeding from the uterus corresponds to the initial part of the ovary's follicular phase. [29]

The second phase of the uterine cycle, the proliferative phase, is responsible for the expansion of the endometrial lining. Additional cells are added in layers to the endometrium to prepare for possible pregnancy. This phase coincide with the late phase of the follicular phase in the ovarian cycle. [29]

The secretory phase is the final stage of the uterine cycle. Following ovulation, the corpus luteum secretes hormones responsible for converting the condensed endometrial lining into a secretory

7

structure. The secretory phase of the uterine cycle and the luteal phase of the ovarian cycle therefore overlap in anticipation of pregnancy. If no fertilization and subsequent implantation occur, the surface coating of the secretory endometrium will slough off and be expelled as menses at the beginning of the subsequent uterine cycle. [29]

Hormonal control of the menstrual cycle

Various systems are involved in the hormonal regulation of the ovarian and uterine cycles. The hypothalamus secretes GnRH while the anterior pituitary is responsible for FSH and LH release. The principal steroid hormone of the follicular phase, estrogen, and the dominant hormone of the luteal phase, progesterone, is secreted by the ovaries. [29]

Early follicular phase

Day 1 of a cycle is indicated by the commencement of menstruation. Since the beginning of menstrual bleeding is a definite observation point, it was chosen to be the starting point of the menstrual cycle. Prior to the onset of each new cycle, the anterior pituitary starts to secrete increasing amounts of gonadotropin (FSH and LH). Maturation of several follicles is initiated by the increased FSH secretion. Steroid hormones synthesis is initiated in the granulosa and thecal cells of the maturing follicles, under the influence of FSH and LH respectively. Androgen produced by the thecal cells is transferred to the granulosa cells by means of diffusion, where they are converted to estrogens by aromatase contained within the granulosa cells. [29]

The increasing concentration of circulating estrogen is involved in various systems. Estrogen inhibits additional follicle development in the current cycle, as it exerts negative feedback on secretion of FSH and LH by the anterior pituitary. Concomitantly to its inhibiting actions, estrogen also stimulates additional estrogen production by the granulosa cells. Continued estrogen production is thus established by this positive feedback although concentrations of FSH and LH are reduced. [29]

Fluid secreted by the granulosa cells of the proliferating follicles accumulate in the antrum, a cavity in the follicle. This antral fluid holds enzymes and hormones essential for ovulation. Only a small number of follicles will reach complete maturation, since several follicles are obliterated at each stage of follicular development by hormonal regulated cell death, referred to as atresia. As a rule only a single dominant follicle will ripen until ovulation. [29]

8

Menstrual bleeding comes to an end in the course of the early follicular phase. Proliferation of the endometrium is initiated by the increasing estrogen levels. The thickening endometrium becomes more vascularized and cell numbers increase as new layers are added unto the endometrial lining. The cervix also exude clear, watery mucous under the influence of estrogen. [29]

Late follicular phase

Ovarian estrogen secretion peaks near the end of the follicular phase. A single follicle is still present. At the end of the follicular phase inhibin and progesterone are secreted by the granulosa cells of this single dominant follicle, in addition to estrogen. At that time, the negative feedback estrogen earlier exerted on GnRH is converted into positive feedback. [29]

Just prior to ovulation, the responsiveness of the anterior pituitary to GnRH is enhanced by the elevated estrogen concentration in conjunction with the increasing progesterone levels. Consequently, a significant amount of LH is secreted by the anterior pituitary, referred to as the LH surge. There is an increase in FSH too; however, since inhibin and estrogen generally inhibit the secretion of FSH, it is to a lesser degree than the LH surge. [29]

The surge in LH concentration is essential for ovulation. The final oocyte maturation stages will not come to pass exclusive of the LH surge. In the developing follicle, meiosis resumes with the first meiotic division. The primary oocyte is converted into a secondary oocyte or egg along with a polar body, which is expelled. [29]

At this stage the follicle is prepared for release of the ovum. The antral fluid accumulates and the follicle expands to its maximum dimensions as division is proceeding. The uterus is primed by the elevated estrogen concentrations in the follicular phase for a potential pregnancy. The final thickness of the endometrium is approximately 4 mm prior to ovulation. The cervical glands also start to secrete mucus with a thin, stringy consistency right before ovulation to assist entry of sperm. [29]

Ovulation

Ovulation transpires approximately 16 – 24 hours after LH reaches a peak. Collagenase, produced by the mature follicle, disperses collagen within the connective tissue securing the follicular cells en masse. An inflammatory response is initiated by collagen's breakdown products and leukocytes are attracted to the follicle. The activated leukocytes secrete

9

prostaglandins into the follicle, possibly causing contraction of smooth muscle cells in the outer theca. [29]

Once pituitary gonadotropins flood the ovary, healthy cells on the surface of the mature ovarian follicle is stimulated to disintegrate. [30]

Various studies have indicated a rapid response to gonadotropin stimulus. It has been shown that the associated biochemical alterations transpire uniformly all the way through the follicle wall, all over its whole circumference. [31-35]

The most apical point on the follicle is considered to be the rupture site, since this region is the most delicate morphological segment of the wall of the follicle. [30,36]

The follicle wall ruptures and the antral fluid, along with the ovum, is spurted out. The ovum is accompanied by two or three layers of granulosa cells upon expulsion, and is subsequently gathered into the Fallopian tube. [29]

The LH surge is also responsible for the migration of the follicular thecal cells into the antral space to amalgamate with the preceding granulosa cells to fill up the cavity. This mixture of thecal and granulosa cells subsequently change into luteal cells of the corpus luteum by the process of luteinization. Various morphological as well as biochemical changes take place in this process. Lipid and glycogen particles are deposited into the cytoplasm of the newly formed luteal cells. The luteal cells start to release progesterone and the production of estrogen is reduced. [29]

Early to Mid-luteal phase

Following ovulation, gradually increasing quantities of estrogen and progesterone is secreted by the corpus luteum. In the luteal phase, the prevailing hormone is progesterone, while the increasing estrogen concentration does not attain the observed culmination prior to ovulation. [29]

Negative feedback is exerted on the hypothalamus and anterior pituitary by the progesterone and estrogen combination. This negative feedback along with the production of luteal inhibin ensures the secretion of gonadotropin is inhibited for the duration of the luteal phase. [29]

The endometrium is stimulated by progesterone to continue preparing for pregnancy. Endometrial glands develop into spiral structures while added blood vessels expand into the

10

connective tissue layer. Lipid droplets and glycogen granules are accumulated in the endometrial cells' cytoplasm. These particles within the endometrial cells will supply sustenance for a developing embryo while the fetal-maternal tie, namely the placenta, is being established. [29]

Cervical mucus acquires a thickened texture under the influence of progesterone. In this way the cervical opening is blocked by a thick mucus plug which inhibits the entry of sperm and bacteria into the uterus. [29]

Progesterone also has thermogenic properties. During the luteal phase, after ovulation, the basal temperature of a woman increases with up to 0.5°C and will remain elevated until menstruation. Although this variation in temperature setpoint cannot be utilized in the effective prediction of ovulation, it is an easy method of assessing ovulatory or anovulatory cycles of a woman. [29]

Late luteal phase and menstruation

The inherent lifespan of the corpus luteum is about 12 days. The corpus luteum will automatically go through apoptosis, if pregnancy is not established, to develop into a dormant structure known as the corpus albicans. With degeneration of the luteal cells the production of progesterone and estrogen will also decrease. As a result, the negative feedback signal to the hypothalamus and pituitary is removed and increased secretion of FSH and LH can be initiated. [29]

The preservation of a secretory endometrium is dependent on the presence of progesterone. As progesterone production decreases subsequent to the degeneration of the corpus luteum, the blood vessels in the surface layer of the endometrium will constrict. The lack of oxygen and nutrients leads to cell death and the endometrium then sloughs its external layers. Menstruation thus begins approximately 14 days after ovulation, or two days after the termination of the corpus luteum. [29]

A total volume of 40 ml of blood and 35 ml of serous fluid and cellular debris is discharged from the uterus. A few clots can occur in the menstrual flow since plasmin, responsible for the disintegration of clots, is present. Menstrual bleeding continues for a period of 3 – 7 days, distinctly into the follicular phase of the successive ovulatory cycle. [29]

11

Period

The study of Cole, Ladner and Byrn indicated that a typical menstrual cycle transpires every 23–32 days. The follicular phase last 10–20 days after the commencement of the last menses, and the luteal phase lasts 9–17 days following the assumed ovulatory window. [37]

2.3.1 Thrombin and the menstrual cycle

The ovarian follicles are continuously being remodeled.   Cellular growth, migration, functional modifications as well as apoptosis constitute this continual cycle of proceedings concerned with the ovarian cycle. [38]

The quantity of fibrinogen contained within human follicular fluid is up to half of the amount of fibrinogen that is held in plasma [39,40]. Within plasma thrombin rapidly converts fibrinogen, its fundamental protein substrate, into an insoluble fibrin coagulate [41,42]. The fluid contained within healthy pre-ovulatory follicles however, is not prone to create fibrin fibers when exposed to synthetic surface as seen in plasma [39]. The presence of antiproteinases, including antithrombin, may explain the absence of fibrin strands in the follicular fluid [39,43,44].

The follicular fluid contains coagulation factors V, VII and X, while it lacks other haemostatic proteins such as factors VIII and IX along with von Willebrand factor. Gentry et al. stated that proteins concerned with the generation of thrombin and its modulatory pathways could be elicited from ovarian cells. This implicates thrombin as an essential part of folliculogenesis. [38]

2.3.2 Platelets and the menstrual cycle

Very little is known about the platelets and their specific role throughout the menstrual cycle. Only a few studies have focused on platelet function throughout the normal menstrual cycle. These studies investigated the correlation between the menstrual cycle and factors like short-term exercise [45], arachidonic acid-induced platelet aggregation and intrinsic platelet thromboxane production [46], migraine [47], plasma levels of nitric oxide [48] and plasma B-thromboglobulin and platelet factor 4 [49].

Platelet activation, triggered predominantly by the process of secondary aggregation, is further facilitated by ovarian hormones, essentially estrogen.[50]   The late follicular phase of the menstrual cycle is characterized by a surge in estrogen, while an increase in progesterone dominates the midluteal phase.[51]

12

2.4   THE MENSTRUAL CYCLE AND PREGNANCY

Cole, Ladner and Byrn specified the time of implantation as either of the following:

- 20–30 days following the commencement of the last menses
- or 5–14 days following the assumed ovulatory window
- or 9 days prior to the day of missing the succeeding menstrual period. [37]

2.5   PREGNANCY

Pregnancy is defined by Stedman's Medical Dictionary as the state of a female after conception until the termination of the gestation. [52]

Less than 35% of all naturally fertilized ova will yield a successful pregnancy. Most of the unsuccessful fertilizations terminate near the point of implantation owing to chromosomal defects. A successful pregnancy is characterized by delivery of a single offspring after 9 months.[53]

Fertilization

Upon release of an ovum from the ruptured follicle, the swaying action of the cilia gathers the ovum into the Fallopian tube. Sperm deposited in the vagina at that point undergo capacitation, the final maturation phase to promote sperm movement and fertilization of the ovum. [29]

Only by means of a chance encounter will the sperm fertilize the ovum. This process of sperm and ovum fusion is possibly facilitated by chemical attractants secreted by the ovum. Only a small window of opportunity exists for a sperm to fertilize the ovum. Although sperm remain viable in the female reproductive tract for up to six hours, it is only the period between 12 – 24 hours subsequent to ovulation that fertilization optimally will occur. [29]

Implantation

As soon as the ovum is fertilized it develops into a zygote, and mitotic division is initiated. The dividing zygote proceeds down the Fallopian tube to the uterus. It takes about five days for the dividing zygote to reach the uterine cavity. The smooth muscles of the Fallopian tube relax under the influence of progesterone. The movement of the zygote along the Fallopian tube is therefore a leisurely process. When the dividing zygote arrives at the uterus it has developed into a blastocyst, a hollow sphere consisting of roughly 100 cells. For the remainder of the gestation period the zygote will settle in the uterus. [29]

13

Placenta

Approximately 7 days after fertilization the blastocyst will implant into the uterine wall. The blastocyst will divide and develop into an embryo. Some of the blastocyst cells will transform into finger-like chorionic villi that will form part of the placenta. Nutrients, gases and waste can be exchanged over this fetal-maternal connection, although the blood of the mother and the embryo will never mix. [29]

Throughout pregnancy the placenta persist to expand until it attains a diameter of approximately 20 cm. With the implantation of the blastocyst along with the formation of the placenta the pre-programmed lifespan of the corpus luteum comes to an end. With disintegration of the corpus luteum, the placenta starts to secrete various hormones to prevent menstruation and subsequent loss of the embryo. [29]

Placental hormones

Human chorionic gonadotropin

Human chorionic gonadotropin (hCG) is a peptide hormone produced by the developing placenta. It is responsible for preserving the corpus luteum during early pregnancy. The corpus luteum can continue secreting progesterone thus hCG indirectly ensures that the endometrium remain intact. [29]

However, around week 7 of development the corpus luteum is not considered necessary to any further extent since the placenta assumes the production of progesterone. The corpus luteum finally disintegrates. At three months of development placental hCG reaches it maximal secretion concentration. [29]

Human chorionic gonadotropin is also responsible for stimulating the developing testes of male fetuses to produce testosterone. This will ensure that the testes descent into the scrotum before birth and establish male characteristics. [29]

Human placental lactogen

Human placental lactogen (hPL), also known as human chorionic somatomammotropin (hCS), is secreted by the placenta. The maternal metabolism of glucose and fatty acids is altered by this peptide hormone, in order to support fetal growth. The maternal utilization of glucose is reduced while the conversion of stored fat to fatty acids is promoted. This ensures that greater amounts of glucose along with free fatty acids can be supplied to the developing fetus. [29,54]

14

Estrogen and Progesterone

Both estrogen and progesterone are continuously secreted during pregnancy. The first source of these two steroid hormones is the corpus luteum, under the influence of hCH, followed by the placenta. These hormones prevent development of any additional follicles during pregnancy by suppressing the anterior pituitary function. [29,54]

Estrogen promotes expansion of the myometrium, the smooth muscle layer of the endometrium, to improve uterine strength for parturition. Progesterone preserves the endometrium, inhibits uterine contractions and prevents uterine contamination by promoting cervical mucus plug formation. Together progesterone and estrogen also assist in the preparation of mammary glands for lactation. [29,54]

Parturition

Typically parturition takes place between week 38 and week 40 of gestation. The uterus contracts at increasing intervals to force the fetus through the cervical canal. The initiating stimulus for these contractions could originate from fetal signals, maternal signals or a combination of the two. Estrogen and progesterone concentrations will not diminish until parturition is well-advanced. [29]

As soon as labor contractions commence, several mechanisms are initiated. Relaxin, a peptide hormone secreted by the placenta and ovaries, softens the cervix to promote cervical dilation and relaxes the ligament attachments between the pelvic bones. The fetus repositions itself head first lower in the abdomen and starts pushing on the softened cervix. As the cervix is stretched more uterine contractions are generated. As these contractions intensify, the fetus is pushed through the vagina into the world. At this stage the placenta is still attached to the newborn. Shortly after birth, the placenta separates from the wall of the uterus and is finally expelled. Even though approximately 240ml of blood is lost by the mother, excessive bleeding is reduced by uterine contractions which compress the maternal blood vessels. [29,54]

2.5.1 Coagulation and pregnancy

The possibility of haemorrhage is augmented throughout and even after parturition. For this reason, the quantity of blood coagulation precursors and fibrinogen are increased in pregnancy to maintain haemostasis and prevent hemorrhagic disaster.[55]

The risk of both thromboembolic and haemorrhagic complications is augmented during pregnancy. In the course of delivery, platelets and blood coagulation factors, such as fibrinogen, are utilized. The associated, enhanced fibrinolysis promotes subsequent childbirth as well as placental expulsion giving rise to elevated levels of D-dimer. With normal delivery these haemostatic alterations are self-regulating, and return to normal within 4 to 6 weeks following delivery. [55]

The risk of venous thrombosis is increased in pregnancy. The incidence of venous thromboembolism is six times higher during normal pregnancy than in the general female population of child-bearing age, although this occurrence is low given the increased release of haemostatic activation markers detected during normal healthy pregnancy. A balance between coagulation and fibrinolysis is required for normal haemostasis and for maintaining vascular integrity. The constant balance of coagulation and fibrinolysis is ensured by the complex physiological changes evident during pregnancy.[56]

Approximately 1 out of every 1000 deliveries is associated with venous thromboembolism during pregnancy.[57]

Haemostasis incorporates a multifaceted system of interactions, integrating coagulation factors, coagulation inhibitors, fibrinolysis, platelets and blood vessels. This system, with its positive and negative feedback mechanisms, has progressed to sustain the vascular integrity.[58]

Blood coagulation factors

In normal pregnancy, alterations occur in all aspects of haemostasis. The amplified release of several clotting factors, the diminished concentrations of certain natural anticoagulants as well as the reduced fibrinolytic activity all contribute to sustaining placental function throughout pregnancy and meet the haemostatic challenge of delivery. However, these alterations also increase the risk of thrombosis and placental vascular complications. [59]

Normal pregnancy is characterized by discernible increases in various blood coagulation factors, including Factors XIII, XII, X, VIII and von Willebrand factor along with fibrinogen. The most distinct variations are discerned in the third trimester. [6-10]

Increased estrogen levels are implicated in the haemostatic changes observed in the progression of pregnancy. In normal pregnancy, Factor II, V and IX remain unchanged or may be moderately

16

increased [6], while the only blood coagulation factor to decrease during pregnancy is Factor XI.[7]

Blood coagulation typically returns to normal subsequent to the fourth to sixth week following delivery.[60]

Fibrin

Liu and associates investigated the changes in coagulation of pregnant Chinese women. They found a significant increase in fibrinogen concentration in the first trimester of pregnancy compared to non-pregnant Chinese women, and reached the peak concentration at the end of pregnancy in the third trimester.[61]

Excessive bleeding for the period of parturition and after is possibly inhibited by the elevated levels of fibrinogen. [16,62]

Fibrinolysis

The activated fibrinolytic system restricts fibrin formation in occluded vessels, thus enhancing or even reinstating blood circulation. During normal pregnancy, there is an increase in the inactive proenzyme plasminogen. Also, tissue-type and urokinase-type plasminogen activator is elevated. [63,64]

The overall diminished fibrinolytic capability is attributable to the amplified release of numerous inhibitors. Combined oral contraceptives produce the exact opposite effect, with fibrinolysis increased due to decreased PAI-1 concentrations along with the absence of PAI-2. The increased incidence of complications from thromboembolism in pregnancy compared to the use of combined oral contraceptives may be explained by this occurrence. [55]

Decreased t-PA activity is responsible for reducing fibrinolysis in pregnancy. Fibrinolysis activity only returns to normal one hour postpartum. [14]

Impairment of the fibrinolytic system is rapidly amplified subsequent to separation of the placenta. Postpartum, fibrinolysis resembles a nonpregnant state within 24 to 28 hours after delivery.[8]

17

Delivery

In the late third trimester, the most pronounced hemostatic changes occur. This presumably prepares the body for haemostasis during the bleeding from placental separation. This initiates a hypercoagulable state. In the course of uterine contraction and delivery, blood coagulation is activated along with the utilization of platelets, blood coagulation factors and inhibitors. [65]

Bellart et al. reported a simultaneous increase in fibrinolysis and D-dimer levels. [64]

Maternal morbidity and mortality, stemming from bleeding complications, are prevented by local haemostasis and fibrin formation along with contractions of the uterine wall. [66]

Haemostasis can be excessively activated by complications during pregnancy and delivery resulting in a variety of consequences ranging from intravascular coagulation (DIC) to multi-organ failure. It is therefore vital to improve or preferably attempt to bring haemostasis to normal function ahead of delivery to avoid anomalous haemostasis. [55]

Postpartum

Within the first week after delivery, C-reactive protein, fibrinogen, platelets and antithrombin levels become elevated, an indication of acute phase reactions [7]. Even though free protein S levels can be diminished up to 8 weeks postpartum [8], blood coagulation typically only returns to normal after the fourth to sixth week after delivery [60].

Approximately 12 weeks after delivery, platelet function returns to normal levels comparable to platelet functions in a nonpregnant state. [67]

Impairment of the fibrinolytic system is rapidly amplified subsequent to separation of the placenta. Postpartum, fibrinolysis resembles a nonpregnant state within 24 to 28 hours after delivery. [8]

2.5.2   Platelets and pregnancy

Pregnancy is associated with a platelet count that is lower than the value normally observed for a general populace. In 2000 Boehlen and co-workers studied the platelet counts observed at term pregnancy. According to Boehlen et al. a two-mode distribution of platelet counts in pregnant women explains the low platelet count in pregnancy. They found that several pregnant women suffered from severe thrombocytopenia, while others showed a normal distribution. They added that, in spite of the above mentioned observations, a histogram of the platelet count in pregnancy

18

indicated a significant shift to the left of the curve for the whole distribution when compared to women who were not pregnant. They concluded that their study population illustrated a high prevalence (11.6%) of maternal thrombocytopenia. [68]

The platelet activation and adhesion associated with pregnancy are exaggerated with elevated blood pressure and increase in gestational age. [69]

Sejeny, Eastham and Baker reported a significant decrease in mean platelet counts from the second to the third trimesters, while no significance was observed during the period of the first to second trimester. They added that the plasma volume increases more than the red cell volume and, along with the reported progressively decreasing platelet count; this possibly explains the relative increase in plasma volume providing platelet production remains reasonably stable throughout pregnancy. [70] Increased platelet volume distribution width is a sensitive measure of macrothrombocytosis. [71]

Platelet reactivity is increased in the second and third trimester. Flood et al. has indicated an overall platelet under-reactivity in early pregnancy. This could possibly be an adaptive mechanism allowing successful implantation. [72] Platelets appear activated throughout normal pregnancy, contributing to the state of extensive hypercoagulability on physiological level.[4] Normal pregnancy is associated with progressive platelet destruction during the third trimester. This is compensated for by increased platelet production.[71,73]

Fay, Hughes and Farron investigated platelets in pregnancy, specifically platelet hyperdestruction. They found a noteworthy decrease in platelet counts in the last 8 weeks of gestation, while mean platelet volume significantly increased in the last 4 weeks. The platelet volume increased continuously through pregnancy. These observations suggest hyperdestruction of platelets, the increased consumption of platelets, during normal pregnancy. [71]

Approximately 12 weeks after delivery, platelet function returns to normal levels comparable to platelet functions in a nonpregnant state.[67]

2.5.3 Erythrocytes and Pregnancy

Erythrocytes are responsible for delivering oxygen to body cells. The unique cells lose their nuclei and other intracellular components during their maturation process in the bone marrow. The mature red blood cell, which is bi-discoidal in shape, is thus comprised of only a cell

19

membrane, hemoglobin and various enzymes. During the limited life span of these cells in the circulating blood, they undergo several changes including structural, compositional, biochemical, biophysical and immunological. The cells become smaller and more opaque. [74,75]

To facilitate growth and nutrition of the developing fetus, significant changes need to occur in the maternal hematopoietic system during pregnancy. [76]

Hypervolemia is possibly the most essential hematological modification during pregnancy, since it decreases the risk of hypotension in late pregnancy when blood flow to the lower extremities is obstructed during standing, sitting or lying in the supine position and during delivery. [76-78] Hypervolemia is also required to preserve the fetal-placental unit. [79]

An increased aggregation of erythrocytes will also occur during the course of pregnancy. [80] Alterations in erythrocyte aggregation are associated with elevated plasma fibrinogen levels. [81] Erythrocyte sedimentation rate increases throughout pregnancy. [82]

Hormones

With the commencement of pregnancy important changes occur in the female body. The levels and spectrum of circulating hormones are the most significant changes in the first trimester. Human chorionic gonadotropin (HCG), a hormone typically associated with pregnancy, as well as estrogen and progesterone increase in the first trimester. Human placental lactogen (HPL) is also present in the blood during pregnancy. These hormonal changes may influence the blood's hemorheological properties.[83]

Hemorheology

Since 1985, intensive studies have been conducted concerning blood hemorheological changes during pregnancy.[84-87]

Blood hemorheology, the relationship between blood flow and factors like blood volume and blood pressure, is influenced by two factors namely plasma viscosity [88,89] and the deformability of erythrocytes [90-92].

It is through microcirculation that both these factors have an effect on blood perfusion. Perfusion is decreased when the blood viscosity is increased while the erythrocyte deformability is decreased. The biophysical properties of the erythrocyte membrane directly influence the ability of the erythrocyte to deform. Two key components namely the spectrin cytoskeleton

20

along with the phospholipid bilayer of the red blood cell have an effect on the deformability of the cell. [93-95]

Erythrocyte population

In late pregnancy the erythrocyte population consists of younger cells than their non-pregnant counterparts. [96] This shift towards a younger erythrocyte population is seen from early pregnancy, and becomes significant at week 20 – 28 of the pregnancy. The erythrocytes become less dense as the pregnancy progresses. [97,98] Similar observations have been made in rats and mice.[99]

A constant rise in mean corpuscle volume is accompanied by the decrease in erythrocyte diameter and increase in thickness leading to a more spherical shape.[96,97,100]

Erythrocyte deformability has also been shown to be lower in pregnancy compared to non-pregnant females.[101] This may be due to the younger population of cells, which are not as deformable as more mature cells, seen in pregnancy. [99] More mature, denser cells have the highest deformability compared to the younger, less dense cells which have the lowest deformability.[102] This association between density and deformability is possibly the most essential influence on erythrocyte senescence. [103]

The sequential younger erythrocyte population associated with late pregnancy implies a shorter life span of these cells.[104] This may be due to elevated erythropoietin concentrations resulting in 'emergency hemopoiesis'. [105]

Erythropoietin is a glycoprotein that regulates the process of red blood cell production, known as erythropoiesis.[106] Erythropoietin is produced upon physiological stimulus of tissue hypoxia. The synthesis of erythropoietin is down-regulated once erythrocyte cell mass is elevated. [107]

Elevated erythropoietin leads to the formation of erythrocytes with a shorter lifetime. [108] When erythropoietin concentrations are elevated the erythroid line expels the nucleus prematurely. This results in a population of younger reticulocytes released into circulation. [109] This population of cells will be much larger than normal erythrocytes once they have matured and are therefore removed much earlier from circulation than normal cells. [105,108,109]

Elevated erythropoietin concentrations are associated with pregnancy. [110,111] Elevated erythropoietin levels in pregnancy are most likely resulting from increased secretion of

21

pregnancy hormones including estrogen, progesterone and HPL. [79,112] Placental lactogen promotes erythrocyte production through erythropoietin action. Estrogen annuls the stimulus of placental lactogen and inhibits erythropoietin. Progesterone in turn cancels the inhibitory effect of estrogen and therefore promotes erythrocyte production.[79,113] This theory has been supported by the prolonged life span of erythrocytes seen in hypophysectomised rats.[114]

Hematocrit and Hemoglobin

Throughout pregnancy and increased production of erythrocytes persist although cell mass per unit of body weight remains constant, and hematocrit and hemoglobin levels continue to decrease.[76,78,97,115]

Concurrently, a change occurs in the quality of the composition of the plasma, particularly the blood plasma and whole blood viscosity. The viscosity of whole blood and the plasma are both essential for the non-Newtonian behaviour of the mother's circulating blood. These factors are mainly influenced by hematocrit and erythrocyte changes, as well as protein increases (fibrinogen concentration in the blood plasma in particular). [80]

This phenomenon of hemodilution, incorrectly referred to as the 'physiological anemia of pregnancy', can be due to a increase of plasma volume resulting in decreased hemoglobin in dilution [76,79,116,117] or partially due to the shorter life span of erythrocytes in the circulating blood [104].

2.6 FLOW CYTOMETRY

Flow cytometry (FC) is utilized to perform a rapid measurement of specific characteristics of a great quantity of single cells. Cells in suspension are firstly labeled with a fluorescent probe, and then channeled through a flow chamber to pass in single file through a focused laser beam at a frequency of up to 2000 cells each second. The laser beam activates the fluorophore at the excitation wavelength and the emitted fluorescence along with the light scatter properties of every cell can then be detected and processed. The intensity of the light emitted from the fluorophore is directly proportional to the specific characteristic being measured in the cell, since the amount of fluorescence is an indication of the amount of antigen present in the cell. [118]

One of the numerous advantages of evaluating platelet function by flow cytometry is the direct analysis of platelets in their physiological environment of whole blood. [119,120]

22

The decrease in manipulation of the sample is another advantage of FC. Minimal handling assists the prevention of platelet activation due to artifacts that are not found in whole blood along with possible loss of platelet subpopulations. [121-124]

Platelet activation

In vivo, the primary function of platelets is to obstruct bleeding from injured blood vessel walls. The collagen exposed at the point of injury activates platelets, triggering the aggregation of platelets at the site consecutively activating clotting factors to produce fibrin. Upon activation, platelets develop spiny spheres to facilitate the aggregation of platelets to each other. Throughout this process, granule membrane proteins fuse with the external membrane of the platelet. The membrane protein expression of CD62p and CD63 are subsequently increased. [125,126]

CD42b expression will however decrease upon activation, since it is dispersed within the open canalicular system. [125]

With platelet activation various changes take place at the platelet surface. The most sensitive detection technique of measuring amplified surface exposure of activation antigens on the surface of platelets is Flow cytometry (FC). Flow cytometric analysis of whole blood can decrease platelet activation resulting from artifacts like is often seen with other techniques, for example platelet aggregation and evaluation of β-thromboglobulin in plasma. [121]

Labelling specific membrane epitopes with monoclonal antibodies (MoAbs) enables flow cytometry to be a sensitive technique for examining alterations in antigenic determinants. [127-129]

Various fluorophores can be conjugated to the Flow cytometry MoAbs including phycoerythrin (PE) and fluorescein isothiocyanate (FITC). [118,130]

Platelets also have a characteristic profile of forward scatter and side scatter, corresponding to their cell size and complexity respectively. This enables the differentiation of platelets from erythrocytes and leucocytes. [130]

Platelet specific probes

Platelet activation in extracorporeal circulation has been principally investigated by the employment of CD41, CD42, CD62p and CD63. [127-129]

23

Resting and activated platelets can be identified by the use of CD41 and CD42b while the activation-dependent antigens can be detected by CD62p and CD63. [130]

CD41

CD41, also known as the membrane glycoprotein (GP) IIb, is found on megakaryocytes and platelets. It forms a complex with CD61 (platelet GPIIIa) to form the integrin GPIIb/IIIa (αIIbβ3). [131]

GPIIb/IIIa is an important factor in platelet function since it acts as a receptor for several adhesion molecules such as fibrinogen, fibronectin, von Willebrand factor as well as thrombospondin. [132]

Platelet aggregation, which is a principle function of the CD41/CD61 integrin, is triggered by the binding of fibrinogen to the complex, while the attachment of fibronectin and vWF to GPIIb/IIIa can result in platelet adhesion and spreading on the subendothelium. [131,133,134]

An upregulation of the integrin GPIIb/IIIa is therefore exhibited upon platelet activation. [135].

This integrin complex is predominant expressed on the platelet cell surface, while a small amount is dispersed between the membranes of the α-granules found in the cytoplasm and that of the canalicular system connected to the platelet surface. It is upon activation of the platelet that the complex will induce the release of the constituents of α-granules. [136-138]

Therefore, the key role of this complex is facilitating the enlargement of the forming coagulum and the adhesion of platelets to the microvascular system. [139-141]

CD42b

CD42b (GPIb) is a membrane glycoprotein found mainly on the platelet exterior. It plays a significant role in haemostasis and thrombosis since it is a receptor for various proteins, mainly von Willebrand factor (vWF). [142,143]

It is known as von Willebrand factor-dependent adhesion receptor [144-148]. The initial aggregation of circulating platelets at the site of vessel injury is mediated by the interaction between CD42 and vWF [149]. CD42 expression is down-regulated in activated platelets. [135]

24

CD62

CD62P is also referred to as P-selectin, GMP-140 and PADGEM. This member of the selectin family can be found in megakaryocytes and in platelet α-granules. [150,151]

Upon platelet activation, GMP-140 is translocated to the membrane surface [152]. CD62P can therefore be detected on the surface of activated platelet on account of degranulation of the alpha granules referred to as release reaction. [134,153,154]

CD62P plays a role in platelet-monocyte and platelet-neutrophil interactions [152]. CD62P appear to be the molecular connection between the thrombotic system, the inflammation and wound-healing. [150]

CD63

CD63 is part of the tetraspanin family and referred to as lysosomal membrane associated glycoprotein 3 (LAMP3) or GP53 [155]. In resting platelets, it is found within the cytoplasmic granules while activated platelets express these CD63 on the membrane surface [156].

Since CD63 is thus associated with the release reaction of platelet lysosomal content the surface expression of CD63 is indicative of platelet activation. [134,157]

2.7   CONCLUDING REMARKS

Very little is known about the possible morphological alterations and flow cytometric changes of platelets and fibrin networks associated with different stages of the menstrual cycle as well as various phases of pregnancy including early pregnancy, late pregnancy and post-partum.

The fluctuation in estrogen and progesterone levels through the menstrual cycle may have an effect on platelets and the fibrin network. These hormones in modified concentrations are also involved in preserving pregnancy and may thus have an influence in pregnancy as well.

2.8   AIMS AND OBJECTIVES

This study aims to demonstrate the morphological changes in fibrin network structure, platelets and erythrocyte interaction with platelets during pregnancy. Possible alterations in platelet architecture throughout the menstrual cycle and pregnancy will also be investigated.

The following research objectives direct this research:

25

1. Using Scanning electron microscopy (SEM) to investigate the ultrastructure of the fibrin network at three phases of pregnancy namely early pregnancy (8 – 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).

2. Using SEM and TEM to establish the external and internal morphology of platelets at three phases of pregnancy namely early pregnancy (8 – 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).

3. Using FC to establish the platelet architecture at three phases of pregnancy namely early pregnancy (8– 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).

4. Using SEM to investigate the ultrastructure of erythrocytes at three phases of pregnancy namely early pregnancy (8 – 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).

2.9 HYPOTHESIS

H_0: Ultrastructural and flow cytometric analysis of fibrin networks and platelets will exhibit no alterations in the menstrual cycle or different phases of pregnancy.

H_A: Ultrastructural and flow cytometric analysis of fibrin networks and platelets will exhibit alterations in the menstrual cycle and different phases of pregnancy.

CHAPTER 3: MATERIALS AND METHODS

3.1 CHAPTER OBJECTIVE

All methods and procedures employed in this thesis are provided in this chapter.

3.2 PARTICIPANTS

3.2.1 Menstrual Cycle

A total number of 30 healthy females between the age of 18 and 35 years were employed in the study. Volunteers were recruited from the University of Pretoria. Students from the Faculty of Health Sciences were asked to voluntarily participate. All information concerning the participants was handled anonymously. Informed consent was obtained from each participant before commencement of the part of the study where they were involved.

The participants were non-smokers, did not have a history of thrombotic disease or used any chronic medication known to interfere with coagulation factors and/platelet function. The participants have never used contraceptive medication; they neither used aspirin or aspirin analogues within 48 hours before sampling.

Between day 1 and day of 5 of the menstrual cycle, during the menstrual phase, 5ml of blood were drawn into a citrate tube by a qualified phlebotomist. The lowest levels of the gonadotropic hormones FSH and LH as well as the ovarian hormones estrogen and progesterone are exhibited during the period of menses [29]. Since the effect of these hormones on the fibrin network and platelets ultrastructure during the menstrual cycle was investigated, this stage of the menstrual cycle was chosen to draw blood.

This citrated blood was used for ultrastructural and flow cytometric analysis. The blood was prepared for SEM investigation of the fibrin network, SEM and TEM analysis of the external and internal morphology of platelets, SEM investigation of erythrocytes along with flow cytometric analysis of platelets.

All participants that exhibited a normal fibrin network structure formed part of the study population. Fibrin network ultrastructure has been intensely investigated by Pretorius and colleagues.[158-161] The fibrin network structure is made up of thick, major fibers and thin, minor fibers contributing to a mesh-like arrangement when examined with a scanning electron

27

microscope. Pretorius and co-workers have additionally quantified alterations in the fibrin fiber network morphology [160], compared human fibrin networks to various animal models [161] and established the role of fibrin morphology in identifying disease patterns [158].

Since none of the first 30 volunteers exhibited any abnormal fibrin network morphology, they were all employed for this study.

One subsequent blood collection was done from all qualified participants during the pre-ovulatory phase (day 12 – 14, during the first and highest estrogen peak).

The blood was collected by finger-prick and was utilized for flow cytometric analysis of platelets.

Figure 1 shows the method of evaluating volunteers to participate as controls for the menstrual cycle and pregnancy studies.

In order to evaluate if a volunteer would form part of the menstrual cycle study and also serve as a control for the pregnancy study, the morphology of the fibrin network was studied by use of (SEM) as described under Laboratory procedures. If a volunteer was deemed suitable for both studies (i.e. if a normal fibrin network was distinguished), the morphology of the platelet internal and external structure as well as platelet flow cytometric analysis was performed as explained under Laboratory procedures.
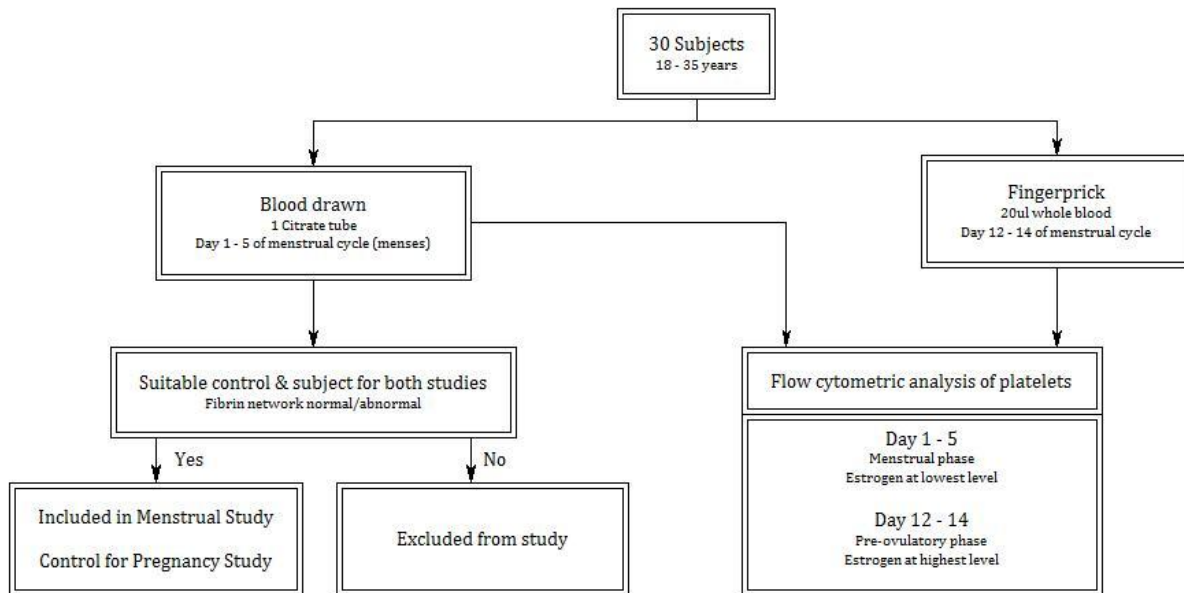
Figure 1. Diagrammatic layout of Evaluation of Controls for the Pregnancy study and Participants in the Menstrual Cycle Study.

3.2.2 Pregnancy

Sixty pregnant volunteers were used and two groups were distinguished: the first group included 30 women in the early phase of pregnancy (8 – 14 weeks) and the second group included 30 women in the late phase of pregnancy (36 – 40 weeks). The second group also participated in the follow-up phase post-partum (6 – 8 weeks after birth). Women between the age of 18 and 35 years were employed for the study, and all participant information was handled anonymously. Volunteers for this part of the study were recruited from the Femina Clinic, Pretoria. Informed consent was obtained from each participant.

The participants were non-smokers, did not have a history of thrombotic disease or used any chronic medication known to interfere with coagulation factors and/platelet function. They neither used aspirin or aspirin analogues within 48 hours before sampling.

29

5ml of blood were drawn by a qualified nurse at Ampath (Drs Du Buisson, Kramer, Swart, Bouwer Inc.) from each women participating in the study. Blood was drawn only once from the 30 women in the first pregnancy group. Women forming part of the second group (late pregnancy) had blood drawn on their last visit to the gynaecologist before birth, as well as their first visit again to the gynaecologist post-partum.

The citrated blood collected was used for ultrastructural as well as flow cytometric analysis. The blood was prepared for SEM investigation of the fibrin network, SEM and TEM analysis of platelets' external and internal morphology respectively, SEM investigation of erythrocytes as well as flow cytometric analysis of platelets.

Females from the menstrual cycle study served as controls for the pregnancy study. Data pertaining to SEM and TEM analysis of fibrin networks, platelets and erythrocytes, along with flow cytometric analysis of platelets obtained from the blood drawn during menses, the same data analyzed to determine whether a participant is a control or not, were used as control data for comparison to the two pregnancy groups. During menses, the levels of all gonadotropic hormones (FSH and LH) as well as the ovarian hormones (estrogen, progesterone and inhibin) are at their lowest [29]. Since pregnancy is characterized by elevated levels of estrogen and progesterone [29], it was best thought to use blood obtained during menses, so the effect of these hormones on platelets, fibrin networks and erythrocytes can be studied.

In Figure 2 the layout of methods followed for the pregnancy study is shown.

30
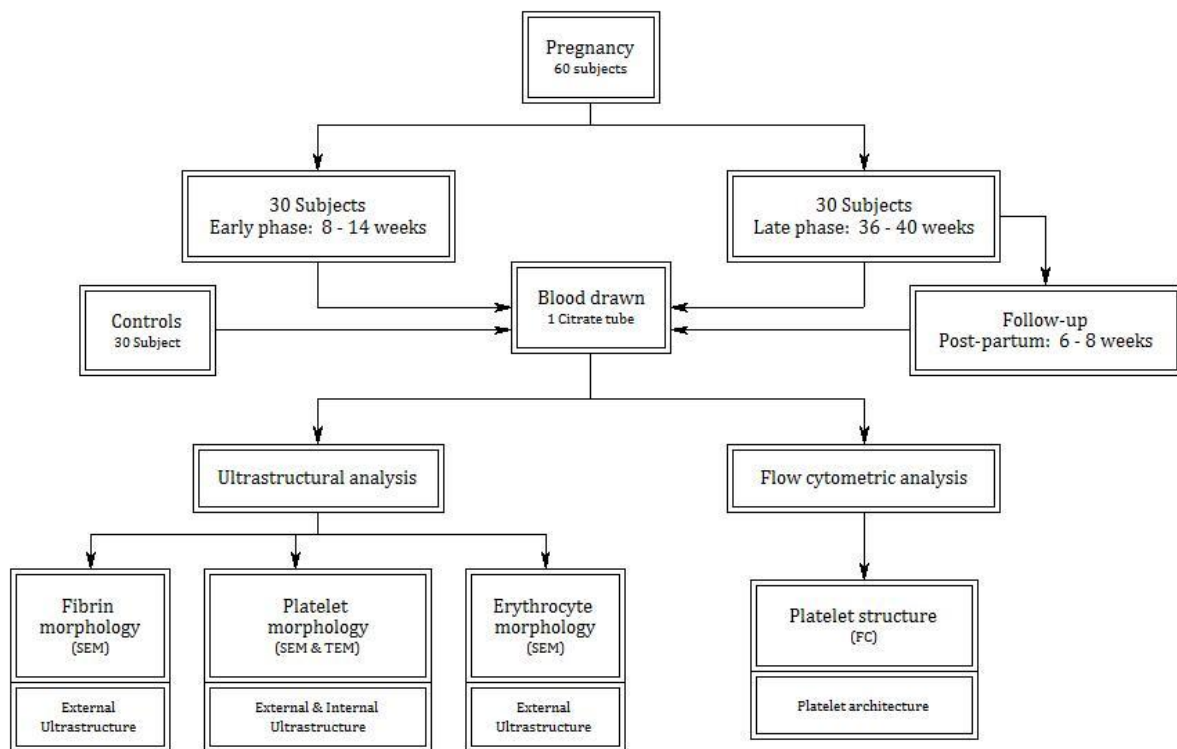
Figure 2. Diagrammatic layout of Study involving pregnant individuals.

The modus operandi for the ultrastructural analysis of the fibrin network, platelets and erythrocytes along with the flow cytometric analysis of platelet organelles are discussed in the following section namely: Laboratory procedures.

31

3.3 LABORATORY PROCEDURES

Since only one tube of blood was used per participant for all the laboratory procedures, the following modus operandi was followed for all samples:

3.3.1 Flow cytometry

3.3.1.1 Platelet architecture

In order to address the third research objective namely: **Using FC to establish the platelet architecture at three phases of pregnancy namely early pregnancy (8– 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).**

Preparation of citrated whole blood for flow cytometric (FC) analysis of platelets
For FC analysis, 20μl of the citrated whole blood was transferred to an eppindorf tube containing 1ml sheath fluid.

A volume of 200μl of the blood and sheath fluid mixture was place in 1ml sheath fluid in a flow tubes. This was done for four flow tubes separately. To each of the flow tubes a different flow cytometric probe was then added (namely CD41-PE, CD42-PE, CD62P-PE and CD63-PE). Each tube was subsequently stained with CD41-FITC. The samples were incubated in the dark and at room temperature for 20minutes, before they were analysed by the FC500.

Preparation of citrated whole blood for flow cytometric (FC) analysis of platelets
In the later sampling of blood for the analysis of platelets during the menstrual cycle, finger-pricks were used to collect blood instead of drawing blood in citrate tubes.

For this procedure, the same method was used as described above. The only difference was that 10μl citrate, an anticoagulant, was added to the 1ml sheath fluid in the eppindorf tube before the finger-prick blood was added. The remaining methods remained the same.

32

3.3.2 Electron microscopy

3.3.2.1 Whole blood

In order to address the fourth research objective namely: **Using SEM to investigate the ultrastructure of erythrocytes at three phases of pregnancy namely early pregnancy (8 – 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).**

Preparation of whole blood for Scanning ultrastructural investigation

After flow cytometric analysis, 20μl of the whole blood collected in the citrate tube was used to make a whole blood smear on a glass coverslip. The sample was placed on filter paper dampened with PBS. This created a humid environment. The sample was placed at 37°C for 5 minutes. Following incubation the samples was placed in PBS and placed on a plate shaker for 20 minutes. This washing process assisted in the removal of excess blood cells and plasma.

The whole blood sample was then fixed in a solution of 2.5% gluteraldehyde for 30 minutes and rinsed three times in 0.075M sodium potassium phosphate buffer solution (PBS) with a pH of 7.4 for 5 minutes. Thereafter, the sample was placed in secondary fixative, 1% osmium tetraoxide (OsO$_4$) solution, for 15 minutes, rinsed again as previously described and then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol for 5 minutes in each concentration.

The SEM procedures were completed by critical point drying of the material, mounting and coating the sample with carbon and examining the fibrin clot with a Zeiss Ultra plus FEG scanning electron microscope. Photomicrographs were taken at 1kV.

3.3.2.2 Blood plasma

Following the whole blood smear, the whole blood collected in the citrate tube was centrifuged at 1250rpm for 10 minutes. The plasma supernatant was transferred to an eppindorf tube and centrifuged for a further 4 minutes at 1250rpm to obtain the supernatant platelet poor plasma (PPP) as well as the platelet rich plasma (PRP) pellet.   Firstly, the supernatant PPP was used to make the fibrin smear for SEM analysis. Secondly, the PRP pellet was used to prepare the platelet smear for SEM and platelet pellet for TEM analysis.

33

Fibrin network

In order to address the first research objective namely: **Using Scanning electron microscopy (SEM) to investigate the ultrastructure of the fibrin network at three phases of pregnancy namely early pregnancy (8 – 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).**

Preparation of fibrin clot for Scanning Electron Microscopy analysis

Blood was centrifuged at 1250rpm for 10 minutes. The plasma supernatant was transferred to an eppindorf tube and centrifuged for a further 4 minutes to obtain platelet poor plasma (PPP). Fibrin clots were prepared by using human thrombin (provided by The South African National Blood Services). The thrombin is 20 U/ml and was prepared in biological buffer containing 0.2% human serum albumin. With the addition of thrombin to the PPP, the conversion of fibrinogen to fibrin is triggered and the liberation of intracellular platelet components including transforming growth factor, platelet derived growth factor and fibroblastic growth factor into the coagulum follows.

A mixture of 10μl of PPP (obtained from centrifugation) with 10μl of human thrombin was made on a glass coverslip to form a fibrin clot (coagulum) on the surface. This glass coverslip containing the coagulum was then placed on filter paper dampened with PBS. This created a humid environment. The sample was placed at 37°C for 5 minutes. Following incubation the samples were placed in PBS and placed on a plate shaker for 20 minutes. This washing process assisted in the removal of any blood proteins possibly ensnared within the fibrin network.

The coverslip with the plasma and thrombin mix was then fixed in a solution of 2.5% gluteraldehyde for 30 minutes and rinsed three times in 0.075M sodium potassium phosphate buffer (PBS) with a pH of 7.4 for 5 minutes. After rinsing, the sample was left in secondary fixative, 1% osmium tetraoxide (OsO_4) solution, for 15 minutes. Following fixation, the sample was rinsed again as previously described. The sample was then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol for 5 minutes in each concentration.

34

The SEM procedures were completed by critical point drying of the material, mounting and coating the sample with carbon and examining the fibrin clot with a Zeiss Ultra plus FEG scanning electron microscope. Photomicrographs were taken at 1kV.

Once the individual was considered a healthy control subject, the following methods were followed to provide sufficient information for comparison with participants in the pregnancy study:

Platelets

In order to address the second research objective namely: **Using SEM and TEM to establish the external and internal morphology of platelets at three phases of pregnancy namely early pregnancy (8 – 14 weeks), late pregnancy (36 – 40 weeks) and post partum (6 – 8 weeks after birth).**

Preparation of platelet-rich plasma (PRP) for ultrastructural analysis

Blood was centrifuged at 1250rpm for 10 minutes. The plasma supernatant was transferred to an eppindorf tube and centrifuged for a further 4 minutes at 1250rpm to obtain the supernatant platelet poor plasma (PPP) as well as the platelet rich plasma (PRP) pellet. For SEM analysis, the PRP pellet obtained from centrifugation was used to make a plasma smear on a glass coverslip. No other chemicals were added to this smear. The remaining PRP in the eppindorf tube was once again centrifuged for 4 minutes at 1250rpm to obtain a pellet of platelets to be used for TEM preparation.

Preparation of PRP on a millipore membrane to exhibit platelets sample with Scanning Electron Microscope (SEM)

The plasma smear (consisting of 20μl of PRP) on the glass coverslip was placed on filter paper dampened with PBS to establish a humid environment and placed at 37°C for 5 minutes. After the 5 minute incubation period, the sample was placed in PBS and washed for 20 minutes. The sample was not placed on a plate shaker, to ensure that all the platelets remained on the coverslip. This washing process ensured the removal of any excess plasma and plasma proteins.

The sample consequently underwent fixation in solution of 2.5% gluteraldehyde for 30 minutes. Hereafter triplicate rinsing with phosphate buffer for 5 minutes, secondary fixation with 1%

35

Osmium tetraoxide (OsO_4) for 15 minutes and a second rinsing process as described above were followed. Dehydration in ethanol with concentrations of 30%, 50%, 70%, 90% and finally threefold with 100% ethanol for 5 minutes in each concentration completed the preparation process.

The sample finally underwent critical point drying, was mounted and coated with carbon and examined with a Zeiss Ultra plus FEG scanning electron microscope. Photomicrographs revealing the external morphology of the platelets were taken.

Preparation of PRP in epoxy resin to exhibit platelets with Transmission Electron Microscope (TEM)

The platelet-pellet was prepared as described in the SEM preparation until after the dehydration step. Following dehydration the sample was infiltrated in a mixture of one part ethanol and one part epoxy resin for 30 minutes and then stored in pure epoxy resin for four hours. The sample was then placed in a rubber mould filled with pure epoxy resin and left to set for three days overnight in an oven at 60 °C. Ultra-thin sections of 80-100nm were cut with a diamond knife using an ultramicrotome. The sections were contrasted with uranyl acetate for 7 minutes followed by contrasting with lead citrate for 5 minutes, after which samples were allowed to dry for a few minutes before examination with the JEOL transmission electron microscope (JEM 2100F). Photomicrographs revealing the internal structures of the platelets were taken.

36

CHAPTER 4: FLOW CYTOMETRIC COMPARISON OF PLATELETS FROM A WHOLE BLOOD AND FINGER-PRICK SAMPLE

4.1 CHAPTER OBJECTIVES

In this chapter platelets from whole blood and finger-prick samples were analysed by flow cytometry to determine if there were any differences between these two sample collection methods. The impact of 24 hour storage was also investigated.

4.2 INTRODUCTION

Flow cytometry is a powerful technique, and its importance in research and clinical investigation has long been established.[162] Markers used in flow cytometry may give us valuable information regarding cellular activity and since 1989 it has been deemed an essential tool for the investigation of platelets.[163] Flow cytometry thus provides a numerical technique, which is both objective and quantitative, to assess platelet function.[164]

Several surface glycoproteins (GP) are found on the membrane of platelets and flow cytometry has been used to a great extent in the immunophenotyping of these entities. The study of platelet function, physiology and their interaction with other cells have been advanced by investigating the recognition of these surface glycoproteins by specific monoclonal antibodies (MoAbs). Not only can the glycoproteins on the surface of the platelet membrane be detected by MoAbs, but molecules transferred to the surface from the internal platelet granules can be identified.[164] This is of particular interest in the study of platelet activation.

The application of a panel of MoAbs is preferred for flow cytometric analysis of platelets, since different flow cytometric probes reveal different characteristics of platelet function.[165] CD41 as well as CD42b are frequently used as platelet identifiers, since they are only present on platelets and not any other circulating blood cell.[163,166,167] Alterations in antigenic determinants can also be investigated and MoAbs are used to label epitopes specifically expressed on the platelet membrane. Studies of extracorporeal circulation have mainly used CD62P and CD63 to investigate platelet activation.[130] Therefore, flow cytometry can be employed to establish the amount of activated and non-activated platelets.[164]

Whole blood can be obtained from either blood drawn in blood tubes with added anti-coagulants (e.g. citrate tubes) or by sampling blood from a finger-prick. These methods have been used

37

previously by Wall and co-workers to investigate platelet dense granule release and uptake. Both whole blood collected in sodium citrate tubes and 20 μl of blood from a "fingerstick", as they referred to it, without added anticoagulant were used. After collection, the samples were immediately diluted with Hanks balanced salt solution (HBSS) and a fluorescent marker, mepacrine, was added.[168]

In research where a sample population needs to be followed over a prolonged period of time, or over consecutive days, it is not practical to frequently draw blood. It is much more acceptable for the participants to donate a finger-prick sample. The question also arose whether storage times would influence results. Therefore, in the current chapter we investigate the repeatability of flow cytometry results when using blood drawn in citrate tubes and samples from finger-pricks - either used immediately, or after samples were stored for 24 hours.

4.3 MATERIALS AND METHODS

4.3.1 Blood collection

Blood was collected from 3 healthy, control male individuals. Ethical approval was obtained from the University of Pretoria, Human Ethics Committee and all participants completed informed consent forms. The participants were non-smokers, did not use any chronic medication and did not have a history of thrombotic disease. None of the participants were using aspirin or aspirin analogues. Firstly, 5ml blood was drawn into a citrate tube (0,5 ml of Sodium Citrate (3,8%) for 4,5ml of blood) for the determination of the platelet gate. Another 5ml of blood was drawn into a citrate tube for the subsequent experiments. Sodium citrate is an anticoagulant usually used for flow cytometric analysis of whole blood.[165,169] A volume of 20μl of blood was collected from finger-pricks. For each concentration of citrate analyzed, a separate finger-prick was taken to ensure that any possible platelet activation after the first sampling was eliminated. Procedures were done in duplicate.

4.3.2 Sample preparation

4.3.2.1 Platelet rich plasma preparation

Whole blood was left to stand for 45 minutes to obtain platelet rich plasma (PRP) by self-sedimentation. 20μl PRP was transferred into a 1ml aliquot of Isoflow™ Sheath Fluid. Isoflow™ Sheath Fluid, an isotonic fluid used exclusively for flow cytometry, is specifically formulated to

38

ensure low particle and fluorescence backgrounds to guarantee superior signal to noise ratio measurements. This mixture of PRP and Isoflow™ was stained with the 20μl (the quantity of product sufficient to stain, as prescribed by Beckman Coulter) of one of the following probes separately: CD41-PE (clone P2), CD42b-PE (clone SZ2), CD62P-PE (clone CLB-Thromb/6) and CD63-PE (clone CLBGran/12).

4.3.2.2 Whole blood preparation

Experiment 1

From the 5ml of citrated blood, 20μl was transferred into 1ml aliquots of Isoflow™ Sheath Fluid. The 20μl of blood was chosen as to be able to compare the whole blood sample to that of the 20μl of blood collected from the finger-prick.   100μl of the mixture was placed into four flow tubes. The remainder of the blood mixture was placed in the fridge at 6°C. Each tube was stained with the 20μl of one of the following probes separately: CD41-PE, CD42b-PE, CD62P-PE and CD63-PE (Beckman Coulter). The flow tubes containing CD62P-PE and CD63-PE were also stained with 20μl of CD41-FITC.

Experiment 2

20μl of the 5ml whole blood drawn in the citrate tube was placed into 1ml aliquots of sheath fluid. 100μl of the mixture was placed into four separate flow tubes. Each tube was stained with 20μl of CD41-FITC and 20μl of one of the following probes separately: CD41-PE, CD42b-PE, CD62P-PE and CD63-PE (from Beckman Coulter).

24 hours after sampling, the remaining blood mixture, placed in the fridge at 6°C overnight, was prepared in the same manner as described above.

Experiment 3

From the 5ml of citrated blood, 20μl was transferred into 1ml aliquots of sheath fluid containing various concentrations of thrombin namely i) 5μl thrombin, ii) 10μl thrombin and iii) 20μl thrombin. This volume of blood (20μl) was chosen as to be able to compare the whole blood sample to that of the 20μl of blood collected from the finger-prick. 100μl of the mixture was placed into four separate flow tubes. Each tube was stained with 20μl of CD41-FITC and 20μl of each of the following probes separately: CD41-PE, CD42b-PE, CD62P-PE and CD63-PE (from Beckman Coulter).

39

4.3.2.3 Finger-prick preparation

Experiment 1

For the finger-prick sample collection, three separate finger-pricks were done with a lancet. 20µl of blood was collected from each finger-prick and immediately placed in 1ml aliquots of sheath fluid containing various volumes of citrate (3,8% Sodium Citrate) namely: i) No citrate, ii) 5µl of citrate and iii) 10 µl of citrate. 100µl of the blood, sheath fluid and citrate combination was transferred to four individual flow tubes from each sample. The remaining blood, sheath fluid and citrate combination was placed in the fridge at 6°C. 20µl of the following probes were added to each tube separately: CD41-PE, CD42b-PE, CD62P-PE and CD63-PE (from Beckman Coulter). The flow tubes containing CD62P-PE and CD63-PE were also stained with CD41-FITC.

Experiment 2

The 20µl samples of blood collected from three separate finger-pricks were immediately placed in 1ml aliquots of sheath fluid containing various volumes of citrate (3,8% Sodium Citrate) namely: i) No citrate, ii) 5µl of citrate and iii) 10µl of citrate . 100µl of the blood, sheath fluid and citrate combination was transferred to four individual flow tubes. Each tube was stained with 20µl of CD41-FITC and 20µl of one of the following probes: CD41-PE, CD42b-PE, CD62P-PE and CD63-PE (from Beckman Coulter).

24 hours after sampling, the remainder of the blood, sheath fluid and citrate mixture, placed in the fridge at 6°C overnight, was prepared in an identical manner as described above.

4.3.3 Flow cytometric analysis

The flow cytometer was calibrated and standardized before use with fluorochrome-labeled bead (FlowCheck™ Fluorospheres, Beckman Coulter). Samples stained with the different probes, were incubated at room temperature in the dark for 20 minutes before analyzed by a flow cytometer (FC 500, Beckman Coulter). Forward scatter and 90° side scatter were displayed on logarithmic scales. Two platelet gates were set. The first gate was set according to the morphological characteristics of platelets in the PRP sample (forward scatter and side scatter) while the second gate was set according to CD41-FITC fluorescence, a platelet specific marker. For the duplicate procedures 60 000 platelets were counted and analyzed per phycoerythrin-labelled monoclonal antibody (including CD41-PE, CD42b-PE, CD62P-PE and CD63-PE).

40

Instrument configuration and settings are shown in Table I. The fluorescence of the different antibodies was plotted on 256-channel log histograms. The acquired Listmode data was analyzed with CyflogicTM software, CyFlo Ltd, Finland. The results were expressed in arbitrary units as mean channel fluorescence intensity (MCFI).

4.3.4   Statistical analysis

Results from the flow cytometric analysis were compared by using the paired two-sided Student's t-test. MCFI results are represented as mean ± standard deviation (SD). A p-value of ≤0.05 was considered significant.

Since 3 similar participants were chosen, the MCFI observations for the 3 participants can be regarded as being independent and identically distributed random variables for each of the different blood-preparation techniques. Furthermore, since the MCFI for each participant was calculated as the mean fluorescence of a large sample of platelets (10 000 platelets per individual per sample), the well-known Central Limit Theorem assures us that the Normal distribution is a close approximation for the distribution of the MCFIs. This allows us to make use of the paired two-sided Student's t-test to compare the results from the flow cytometric analysis at a 5% level of significance.

4.4   RESULTS
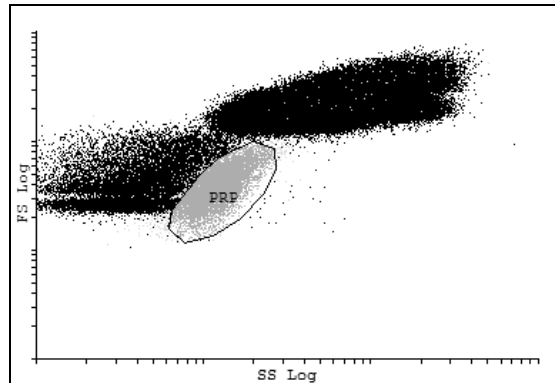
Figure 1. PRP-gate. Platelet gate set according to forward scatter (FS) and side scatter (SS) of platelet rich plasma (PRP). This dot plot shows the PRP gate when a whole blood sample was analysed. The other populations outside the PRP gate represent the other blood cells found in the whole blood sample.
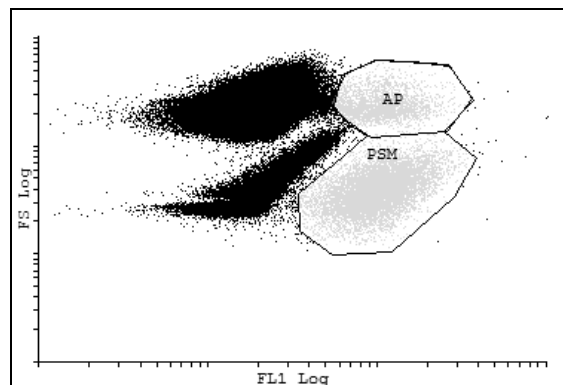
Figure 2. PSM gate. Platelet gate set according to FL1 of CD41-FITC platelet specific marker (PSM). Activated platelets (AP) are also incorporated in the PSM gate. The other populations outside the PRP gate represent the other blood cells found in the whole blood sample.

42
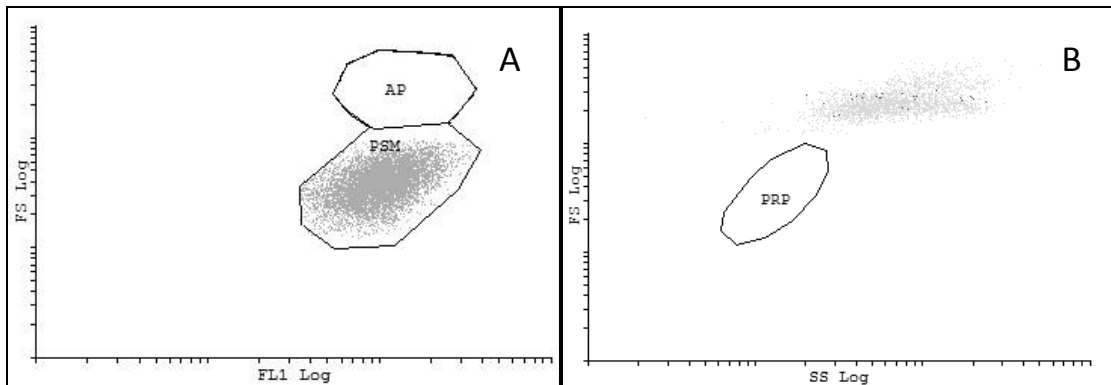
Figure 3. **Overlay of Platelet gates***.* The PRP sample not containing CD41-FITC was analysed by use of the PSM gate while the whole blood sample that did contain CD41-FITC was analysed by the use of the PRP gate. A = PRP only corresponds to the non-activated platelets of the PSM-gate. B = When the activated platelets are represented on the dot plot of the PRP-gate, these cells are not incorporated in the PRP-gate but are clustered on the upper right part of the dot plot.

43

Table 1. Instrument configuration and settings

Acquisition setup - Discriminator:			
	FS = 1	FL3 = OFF	Compensation:
	SS = OFF	FL4 = OFF	None
	FL1 = OFF	FL5 = OFF	
	FL2 = OFF	AUX = OFF	

Acquisition setup - Detectors:		Volt	Gain
	FS	816	1
	SS	251	5
	FL1	553	1
	FL2	639	1
	FL3	677	1
	FL4	450	1
	FL5	577	1
	AUX	300	1

44

Table 2. Analysis immediately after sampling: whole blood (WB) sample and finger-pricks (FP) containing various concentrations of citrate. Results represented as mean ± SD of MCFI. (n = 60 000 platelets analysed for each of the sampling procedures)

MoAb	WB No thrombin	FP No citrate	FP 5µl citrate	FP 10µl citrate
PRP gate				
CD41-PE	24.04 ± 2.71	17.74 ± 0.38	23.02 ± 3.12	24.36 ± 4.27
CD42b-PE	29.68 ± 3.37	28.17 ± 1.32	30.81 ± 2.85	32.54 ± 3.17
CD62P-PE	79.94 ± 2.15	78.16 ± 4.61	77.20 ± 3.32	76.99 ± 2.16
CD63-PE	49.54 ± 2.09	47.01 ± 0.72	48.61 ± 1.09	45.88 ± 4.88
PSM gate				
CD41-PE	47.72 ± 6.04	70.51 ± 1.24	64.13 ± 2.52	52.82 ± 9.45
CD42b-PE	35.07 ± 3.97	47.58 ± 2.35	40.39 ± 1.74	36.44 ± 3.55
CD62P-PE	115.49 ± 5.95	100.97 ± 3.24	99.15 ± 0.47	107.55 ± 4.86
CD63-PE	63.52 ± 2.53	54.86 ± 7.26	49.12 ± 6.35	57.31 ± 5.94

45

Table 3. Analysis immediately after sampling: whole blood (WB) samples containing various concentrations of thrombin to evaluate activation. Results represented as mean ± SD of MCFI. (n = 60 000 platelets analysed for each of the sampling procedures)

MoAb	WB No thrombin	WB 5µl thrombin	WB 10µl thrombin	WB 20µl thrombin
PSM gate				
CD41	47.72 ± 6.04	54.46 ± 18.28	53.90 ± 18.70	71.24 ± 5.72
CD42b	35.07 ± 3.97	37.75 ± 6.66	39.26 ± 6.82	40.11 ± 6.48
CD62P	115.49 ± 5.95	148.57 ± 19.97	155.92 ± 25.96	174.92 ± 9.40
CD63	63.52 ± 2.53	76.07 ±13.55	72.73 ± 9.00	84.13 ± 12.49

46

Table 4. Analysis 24 hours after sampling. Results of the whole blood (WB), first finger-prick containing no citrate, the second finger-prick containing 5μl of citrate and the third finger-prick containing 10μl of citrate analysed 24 hours after the sample was taken. Results are represented as mean ± SD of MCFI. (n = 60 000 platelets analysed for each of the sampling procedures)

MoAb	WB No thrombin	FP No citrate	FP 5μl citrate	FP 10μl citrate
PRP gate				
CD41-PE	25.99 ± 1.84	46.32 ± 0.44	48.93 ± 8.39	28.07 ± 1.64
CD42b-PE	37.88 ± 6.60	38.79 ± 0.38	36.40 ± 7.59	39.76 ± 0.81
CD62P-PE	81.32 ± 0.84	75.98 ± 4.76	72.87 ± 0.82	73.53 ± 2.28
CD63-PE	45.63 ± 1.95	42.84 ± 6.05	46.63 ± 0.50	47.25 ± 1.51
PSM gate				
CD41-PE	51.41 ± 1.58	70.51 ± 1.24	64.13 ± 2.52	60.82 ± 3.41
CD42b-PE	32.01 ± 0.31	47.58 ± 2.35	40.39 ± 1.74	39.13 ± 2.62
CD62P-PE	118.69 ± 4.61	100.97 ± 3.24	99.15 ± 0.47	104.59 ± 4.12
CD63-PE	62.86 ± 1.17	54.86 ± 7.26	49.12 ± 6.35	53.98 ± 7.42

47

4.4.1 Gating strategies

Two platelet gates were set, according to the scatter characteristics and specific platelet marker utilized. The first platelet gate was set according to the forward scatter (FS) and side scatter (SS) properties of platelets found in the PRP obtained from self-sedimentation. The PRP contained only platelets and no other blood cells. Therefore, a pure platelet population could be distinguished without additional MoAb staining. Figure 1 represents a subsequent analysis where whole blood (containing all blood cells) were analysed by the PRP gate.

Figure 2 shows the second platelet gate set according to CD41-FITC fluorescence, a platelet specific marker on a dot-plot displaying FS on the y-axis and FL1 fluorescence on the x-axis. CD41 is used as a reliable platelet-specific marker. It enables the differentiation of platelets in a whole blood sample.[2] The CD41-FITC marker was used in conjunction with the mentioned phycoerythrin-labelled monoclonal antibodies (CD41-PE, CD42b-PE, CD62P-PE and CD63-PE). For the platelet specific marker (PSM) gate, a clear distinction can be made between the unactived platelets (to the lower right of the diagram) and the activated platelets (to the upper right part of the diagram).

An overlay of the two platelet gates is shown in Figure 3. In Figure 3 A it is shown that the PRP gate only corresponds to the non-activated platelets in the population while Figure 3 B shows the activated platelets are incorporated in the PSM gate but not the PRP gate. Therefore, from Figure 3 we can deduce that the gating strategy is very important if all parameters are to be fully evaluated.

Both gating strategies were used for analysis of the different phycoerythrin-labelled monoclonal antibodies used. The platelet rich plasma (PRP) gate showed a significantly lower MCFI value compared to the platelet specific marker (PSM) gate for all instances.

4.4.2 Comparisons

To determine the integrity of the different sampling methods, whole blood samples were compared to finger-prick samples containing various volumes of citrate immediately after sampling. The volumes of citrated added to the sheath fluid before sampling was: i) no citrate, ii)

48

5μl of citrate and iii) 10μl citrate. The comparison of whole blood samples with various volumes of thrombin added directly after sampling was done to determine the activation of platelets. Thrombin volumes added to whole blood samples before analysis were: i) 5 μl, ii) 10 μl and iii) 20 μl. The influence of storage time was compared for whole blood and finger-prick samples analyzed 24 hours after sampling.

The results from Experiment 1 and Experiment 2 were pooled and each of the mentioned phycoerythrin-labelled markers were used to analyse specific characteristic of platelets gated by CD41-FITC. For each marker, 10 000 platelets were counted and analyzed per person adding up to a total of 60 000 platelets for the duplicate procedures.

The following results, obtained immediately after sampling, were compared to determine any significant changes: Table 2 displays results from whole blood sample containing no thrombin (non-activated whole blood sample) compared to finger-pricks containing various concentrations of citrate. Table 3 displays results from whole blood samples containing various concentrations of thrombin.

Table 4 shows the results of immediate analysis compared to results of analysis after 24 hours: Whole blood analyzed immediately was compared to the whole blood sample stored for 24 hours. The finger-prick containing no citrate analyzed immediately was compared to the same sample stored for 24 hours. The same procedures were performed for the other finger-prick samples, containing 5μl and 10μl citrate respectively, immediately analyzed after collection and the aliquot stored for 24 hours.

4.4.3   Whole blood vs. Finger-prick

There was no significant difference between the whole blood samples and the finger-prick samples containing 10μl of citrate (p-values >0.05). However, significant changes were shown for the other finger-prick samples containing no citrate and 5μl of citrate separately (p-values <0.05).

Both gating strategies were used for analysis of the different PE-labelled monoclonal antibodies used to evaluate the different sampling methods. The platelet rich plasma (PRP) gate showed a significantly lower MCFI value compared to the platelet specific marker (PSM) gate for all instances.

49

4.4.4   Non-activated vs. Activated

Whole blood samples were activated by adding different volumes of thrombin to separate samples. The whole blood sample containing no thrombin (thus, the non-activated sample) was compared to whole blood samples containing 5μl, 10μl and 20μl of thrombin. A significant difference was found between the non-activated whole blood sample and the activated whole blood samples (p-value <0.05). This was also true when the activated whole samples were compared to the finger-prick sample containing 10μl citrate. The MCFI for all PE-labelled monoclonal antibodies (CD41-PE, CD42b-PE, CD62P-PE and CD63-PE) increased with the increase in thrombin added to the whole blood samples.

4.4.5   Immediate analysis vs. 24 hours

No significant difference was found for the MCFI of whole blood analyzed immediately compared to whole blood analyzed 24hours after sampling (p-value >0.05). This was also true for the finger-prick samples with 10μl of citrated added before sampling. The immediate analysis of the finger-prick samples with no citrate and 5μl of citrate added separately, showed significant differences from the same samples analyzed 24 hours after sampling (p-value <0.05).

4.5   DISCUSSION

The current investigation confirms that both a whole blood sample and a finger-prick can be used interchangeably for flow cytometric analysis of platelets. Storage time of up to 24 hours in a fridge at 6°C also does not influence the platelet activation in the blood sample. Aspects such as the strategy employed for gating the sample, the specific flow cytometer instrument used, the preparation of the samples, the specific probes used as well as the storage time will be discussed in greater detail.

Gating strategies

Flow cytometry enables the researcher to detect several parameters from a single sample. Different probes or markers can simultaneously be analysed by using multicolor flow cytometry.[170]

50

The function of "gating" or isolating particular cell clusters in flow cytometry enables classification and investigation of platelets in the mixed populations of cells like found in whole blood.[164] Two parameters are employed to facilitate platelet gating namely 1) forward light scatter (FS) which arranges cells according to size, and 2) the use of a platelet-specific antibody like CD41 or CD42b.[164]

Multicolour flow cytometry has the advantage of gathering more information from a single sample therefore minimizing the sample size and decreasing preparation effort. This technique also decreases variation since fewer sample preparation is required and is an effortless method to study platelet population.[170]

The reliability of the population of platelets in a whole blood sample is determined by the gating strategy employed.[121,171,172] Van Velzen and colleagues have recently investigated the effect of different platelet gating strategies. They stated that the changes in scatter characteristics after platelet activation will differ from the morphology of non-activated platelets and therefore a pure platelet population will not be selected. They asserted that utilizing a platelet antigen like CD41 or CD42b, which is commonly present on the platelet membrane, is preferred above the use of the forward scatter/side scatter strategy. By using a platelet specific marker not only eliminate coincidence but also excludes complexes platelets form with monocytes and decreases possible contamination.[170]

This research of van Velzen et al was done for a fixated whole blood sample. In this investigation similar results were found for an unfixed whole blood sample. Since the platelet rich plasma (PRP) gate only shows the unactived platelets in the absence of CD41-FITC, it is important to firstly stain the sample with CD41-FITC and secondly to set the platelet gate according to the FL1 information obtained from CD41-FITC i.e. the platelet specific marker (PSM) gate. This indicates the importance of multicolor gating for optimal analysis of platelets.

Instrumentation

Earlier flow cytometric analysis has been performed on FACScan cytometer (Becton Dickinson). Platelet microparticles [173] and magnetic labeling of platelets [174] have been studied on the FC500 (Beckman Coulter). Erythrocytes [175], dysfunctional T regulatory cells [176] and stem cells [177] amongst others has also been analyzed on the FC500 (Beckman Coulter). This is the first study conducted on the FC500 (Beckman Coulter) to investigate platelets in an unfixated whole blood and finger-prick samples as well as the effect of storage time on the platelets.

51

Blood preparation for flow cytometry

Whole blood was drawn in citrate tubes (0,5 ml of Sodium Citrate (3,8%) for 4,5ml of blood). For the finger-pricks, citrate had to be added to the sheath fluid to prevent coagulation of the blood sample. Weil stated that, if blood is mixed with a sodium citrate solution in appropriate portions, the blood will not clot since the calcium salts are not accessible for coagulation.[178] Weil added that blood mixed with sodium citrate can be kept in the fridge at 6°C for several days, and only slight changes in cell structure can be observed after one week of storage.[178] The results indicate that a volume of 10μl of citrate added to the sheath fluid aliquot is adequate to prevent coagulation of a finger-prick sample.

Typically, platelets have been studied after preparing platelet rich plasma or washing the platelets.[179,180] These separation procedures lead to *in vitro* activation of platelets due to the formation of artifacts. The pioneer use of whole blood for flow cytometric studies by Shattil and colleagues [121] was just the beginning for important improvements for the relevance of flow cytometry for clinical application.[165] The flow cytometric analysis of whole blood holds many advantages for the study of platelet activation. This method has been used for over 20 years to investigate platelet function. Ault et al. used the MoAbs specific for GPIb (CD42b) and GMP140 (CD62P) to measure platelet aggregation and release reaction in a whole blood sample.[181] Activation-dependent variations in multiple surface receptors can be determined in a whole blood sample. In vitro platelet activation is also prevented, since there is minimal manipulation of the samples and it will decrease the possible loss of platelet subpopulations.[165]

Probes

A Pubmed search revealed few references in recent literature that discuss the use of CD41-PE, CD42b-PE, CD62P-PE, CD63-PE and CD41-FITC in whole blood and finger-prick samples as well as the particular storage methods employed in this study. Current literature showed that flow cytometry has been employed to study platelet function testing in apheresis products, where all the above-mentioned probes were used in conjunction with secondary antibodies.[130] Other studies include flow cytometric analysis of platelet count [182], platelet reactivity [183], platelet function in children [184] and the importance of the sampling site in patient about to undergo surgery [185]; these studies used only one or two of the above-mentioned probes. All the mentioned studies were performed on a FACScan cytometer (Becton Dickinson).

52

The most recent investigation of platelet activation was done by van Velzen et al in 2012. They incorporated all 4 the mentioned antibodies; however, the conjugated fluorochromes differed from the probes used in this study and the samples were fixated before analysis.[170]

Sampling method

No significant difference was found between the whole blood sample and the finger-pricks containing 10μl of citrate. There was a significant difference between the whole blood sample and the finger-pricks containing no citrate and with 5μl of citrate added. This indicated that the method of sampling, both the whole blood drawn in the citrate tube as well as the finger-prick samples, was adequate. It also shows that 10μl of citrate is the preferred concentration to be added to the sheath fluid before adding blood from a finger-prick. The whole blood sample and each of the finger-prick samples were done on separate days. This was done to eliminate the possible effect of platelet activation after the first sample.

Activation of platelets

The activated whole blood samples showed significantly higher MCFI values when compared to the non-activated whole blood sample and the finger-prick sample containing 10μl of citrate. We can therefore deduce that the whole blood sampling containing no thrombin, as well as the finger-prick with 10μl of citrate added, did not contain an activated platelet population.

Once receptor activation occurs, the internal platelet granules are secreted and the cytoskeleton will be rearranged leading to signal transduction.[186,187]

Upon activation, platelets exhibit elevated levels of specific activation markers on the platelet surface for example CD62P [188] and CD63 [189]. Both these markers are dominant immunologic indicator of platelet activation.[137,189]

CD62P, also referred to as P-selectin, is an activation-dependent MoAb most extensively used in the study of platelet granule membrane proteins.[190] It is a constituent of a resting platelet's α-granule membrane.[137] It will only be expressed on the surface membrane once the contents of the α-granule are secreted.[151,191,192] For this reason, the MoAb specific for CD62P will not bind to resting platelets, only to degranulated, activated platelets.[165] Once activated, platelets also rapidly transfer CD63 from the lysosome-like granules to the platelet surface through the surface canalicular system.[163]

53

Van Velzen et al. also found the expression of CD62P and CD63 to be increased when platelets were exposed to thrombin activation in a fixated sample.[170]

Period between sampling and analysis

CD41 is a calcium-dependent compound of GPIIb/IIIa. The interactions between cells and the cells with the matrix are mediated by GPIIb/IIIa.[193,194] CD42b is a receptor for von Willebrand factor, which plays a critical role in the adhesion of platelets to the wall of injured blood vessels.[195]

CD41 and CD42b are reliable platelet-specific markers. These MoAbs are used to differentiate between platelets and "debris" or fragments.[163]

Since the expression of CD41 and CD42b in the whole blood samples was similar for immediate analysis of the sample and analysis 24 hours after sampling we can infer that these platelets identified by CD41 and CD42b expression were not activated after sampling and remained inactive for at least 24 hours. Michelson and associates indicated on several occasions that the binding of CD42b to resting platelets is noticeably increased compared to its binding with activated platelets.[196-198] The decreased expression on the surface of activated platelets possibly results from the translocation of this complex to the surface-connected canalicular system membranes.[198,199] This shows that CD42b could be a sensitive marker of in vivo activation of platelets.

Although platelet storage has been associated with increased expression of CD62 in the past [164,164] our results indicate that both an unfixated whole blood and a finger-prick sample can be stored in the fridge at 6°C if prepared appropriately. The time of analysis, whether it is immediately after the sample was take or 24hours after sampling appears to not have an influence on the expression of CD41 and CD42b. However, since the participants were all young and healthy individuals, the effect of age and disease will have to be investigated for this method.

Before conclusions can be drawn from these results, potential limitations need to be addressed. The small sample size, the use of only 4 MoAbs and the analysis at two time points can possibly constrain the findings of this study. A greater sample size, using different MoAbs and analyzing the samples over a greater time period or at more regular intervals can bring greater insight.

However, although the sample size is limited, the results indicate that the platelets are not additionally activated by this particular methodology. Therefore, these methods may be successfully implemented in clinical studies with antiplatelet agents.

4.6   CONCLUSION

Firstly, the importance of gating strategy for a unfixated sample was established. If all platelets, activated and non-activated, are to be taken into consideration, then the platelet specific marker (PSM) gate should be employed and all samples should be stained with CD41-FITC and not only with the specific phycoerythrin-labelled monoclonal antibodies (including CD41-PE, CD42b-PE, CD62P-PE and CD63-PE).

Secondly, we can conclude that an unfixated whole blood and a finger-prick sample are identical with regards to platelet function. Both sampling methods showed little activation when compared to activated whole blood samples. Therefore, a whole blood sample and a finger-prick sample can be used interchangeably for flow cytometric analysis of platelets. This is advantageous for research where a sample population needs to be examined over consecutive days or an extending time interval. In these cases, when it is not practical to repeatedly draw blood, a finger-prick will be sufficient.

And lastly, samples can be stored for 24 hours after sampling. Sheath fluid is a sufficient medium for storing unfixated whole blood and finger-prick samples in the fridge at 6°C for 24 hours, provided that a sufficient amount of citrate is added to the sheath fluid for a finger-prick sample. A volume of 10μl citrate provides sufficient anticoagulant action for a 20μl aliquot of finger-prick blood. Both whole blood samples and finger-prick samples can be kept in the fridge at 6°C for 24 hours before analysis since no platelet activation occurs. This will aid studies where analysis can't immediately be performed due to travelling distance from flow cytometer or time constraints.

CHAPTER 5: ULTRASTRUCTURAL CHANGES OF FIBRIN NETWORKS DURING THE MENSTRUAL CYCLE: A QUALITATIVE INVESTIGATION

5.1 CHAPTER OBJECTIVES

In this chapter the fibrin network ultrastructure of different phases of the menstrual cycle, namely the menstrual phase (day 1 – 5), the pre-ovulatory phase (day 12 – 14) and the luteal phase (day 20 – 25) will be compared to establish whether differences in fibrin network morphology exist during the menstrual cycle.

5.2 INTRODUCTION

Platelets and fibrin play an important role in the regular coagulation process where they are involved in the maintenance of haemostasis.[200] Fibrinogen is the major plasma protein coagulation factor and these fibrinogen molecules assemble to form a clot or thrombus, consisting of a fibrin fiber network. The ultrastructure of this network may change, mainly due to kinetic and modulating factors present in plasma. Also, hormonal fluctuations may influence fibrin structure. An example of this is during the menstrual cycle, when plasma fibrinogen levels change, mainly due to the variations of estrogen [201-203]; causing an altered fibrin assembly. Estrogen not only alters pro-coagulant protein expression [201,203-205]; but differences in platelet function and thrombosis activity have also been noted.[202,206,207]

Functionally, female platelet isolates, though capable of binding more fibrinogen and displaying a greater maximal aggregation extent than male platelet isolates [202,205], do not aggregate as quickly as the larger number of male platelets does. Thus, to some degree females are rendered less susceptible to thrombosis. However, in the absence of estradiol in acyclicity, female platelets are again more susceptible to thrombosis.[202,208,209] Estrogen is known to markedly decrease the risk of cardiovascular up to 50%, showing the benefit of estrogen.[210] Gebara et al showed estrogen to play a cardio-protective role by increasing the fibrinolytic potential.[211] Estrogen treatment has been shown to shift the haemostatic balance toward a hypercoagulable state.[1]

56

Therefore, a hyperestrogenic state in females results in higher coagulation factors and higher maximal platelet aggregation capabilities than males, rendering them more prone to thrombosis in an acyclic state. It is therefore widely known that estrogen has a protective effect on the coagulation system, and in its decrease leading to absence at menopause, females are again at higher risk to strokes than their male counterparts.

Throughout the female reproductive lifetime, estrogen plays a fundamental role, as gametes are liberated at specific intervals while estrogen and progesterone along with other sex steroids prepare her body for reproduction.[30] The menstrual cycle is the period characterized by ovum maturation, the release of the ovum where after it passes through the fallopian tubes to enter the uterine lumen. The endometrium undergoes changes to accommodate a fertilized ovum to sustain pregnancy. These changes are brought about by ovarian hormonal secretions. If fertilization does not occur ovarian secretions cause the endometrium to slough and menstruation commences. Menstrual flow also referred to as menses, marks the first day of the menstrual cycle. [28] If fertilization does take place, estrogen and progesterone both play critical roles in maintaining the pregnancy.

Two additional cycles can be distinguished within the menstrual cycle. The ovarian cycle relates to the alterations observed in the follicles of the ovary while the uterine cycle concerns the changes in the lining of the endometrium.[29] The ovarian cycle is divided into a further three phases starting with the follicular phase characterized by the growth of the ovarian follicle, followed by ovulation or the release of the matured ovum and ending with the luteal or postovulatory phase.[29] The follicular phase is characterized by elevated levels of the hormone estrogen while the luteal phase is dominated by elevated concentrations of progesterone. Both these essential steroid hormones are secreted by the ovaries. [28,29,52]

Throughout the menstrual cycle estrogen levels peak twice. The first estrogen peak is reached during the mid-follicular phase (between day 12 – 14 of the cycle) and then rapidly decreases. This estrogen peak triggers LH secretion which in turn leads to ovulation. During the luteal phase, the corpus luteum secretes progesterone and estrogen. Approximately eight to nine days after ovulation (day 22 – 23 of the menstrual cycle) progesterone levels reach a peak, along with a lower second peak of estrogen.[212-214]

57

The question that now arises is whether changes in particularly the fibrin network are visible at an ultrastructural level, due to the subtle estrogen changes during the menstrual cycle. In order to investigate this question, 6 healthy female participants were identified and the ultrastructure of their fibrin network studied at different intervals in the menstrual cycle where differences in estrogen levels are prevalent.

5.3 MATERIALS AND METHODS

This study is divided into three parts namely Experiment 1, analysing the fibrin external ultrastructural differences throughout the menstrual cycle; Experiment 2, which focused on the influence of estradiol on a purified fibrinogen model; and Experiment 3, analysing the internal structure of the fibrin network throughout the menstrual cycle. The second and third experiments were conducted to confirm results from Experiment 1.

5.3.1 Experiment 1

5.3.1.1 Blood collection

Blood was collected from 6 healthy, female participants. These individuals were non-smokers, did not have a history of thrombotic disease or used any chronic medication known to interfere with coagulation factors and/platelet function. The participants have never used contraceptive medication neither were they using aspirin or aspirin analogues within 48 hours before sampling. Blood was collected in a citrate tube containing 0,5 ml of sodium citrate (3,8% sodium citrate) on three occasions namely the menstrual phase (day 1 – 5, day 1 with the commencement of menses), the pre-ovulatory phase (day 12 – 14, first estrogen peak) and the luteal phase (day 20 – 25, progesterone peak and second estrogen peak). A total volume of 5ml of blood was collected per blood draw. The cycles of all the individuals were 28 days each.

5.3.1.2 Sample preparation

The citrated whole blood was centrifuged for 10 minutes at 1,000 rpm (maximum RCF = 17.523x g; 1 250 g). The plasma was transferred to a 1,5ml eppindorf tube and centrifuged once more for 10 minutes at 1 250g. The platelet poor plasma (PPP) obtained was used to make a fibrin smear on a round glass cover slip. 10μl of PPP was mixed with 10μl thrombin (provided by The South African

58

National Blood Services). The thrombin was 20 U/ml and was prepared in biological buffer containing 0.2% human serum albumin. When thrombin was added to PPP, fibrinogen was converted to fibrin to form an expansive fibrin fiber network.

5.3.2   Experiment 2

5.3.2.1 Sample preparation

Purified fibrinogen, with a concentration of 0.166mg/10ml, was used to make two smears. Fibrinogen is converted to fibrin by thrombin.[15] For the first smear 10μl of fibrinogen was placed on glass cover slip and mixed with 5μl thrombin to make a fibrin smear. For the second smear, to imitate the pre-ovulatory phase of the menstrual cycle, 10μl of fibrinogen and 5μl estrogen concentration was placed on a glass cover slip and mixed with 5μl thrombin. The estrogen concentration increases to almost 1200pmol/L in the days leading up to ovulation.[215]   This estrogen increase is the first and highest estrogen peak in the menstrual cycle. A concentration of 1000pmol/L was therefore used to imitate the pre-ovulatory estrogen peak. Since the estrogen volume was one quarter of the total volume of the fibrinogen, estrogen and thrombin mixture, the estrogen concentration added to the mixture was 4000pmol/L to make the final concentration of 1000pmol/L in the coagula.

5.3.2.2 Preparation of samples for scanning electron microscopy (SEM)

The samples on the cover slips were immediately placed in a 6-well plate on filter paper dampened with PBS to create a humid environment and placed at 37°C for 10 minutes. Following incubation the glass cover slips with the coagula were covered with PBS and placed on a plate shaker for 20 minutes. This washing process was done to remove any blood proteins trapped within the fibrin network.

After the washing process, the samples were fixated with a 2.5% gluteraldehyde solution for 30 minutes. After primary fixation, the samples were rinsed three times in 0.075M sodium potassium phosphate buffer (pH=7.4) for 5 minutes each. After rinsing, the sample was placed in 1% osmium tetraoxide (OsO_4) solution for 30 minutes. Following secondary fixation, the samples were rinsed once more as described above. The samples were then dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol. Procedures were concluded by critical point drying of the samples,

59

mounting the samples and coating it with carbon. The samples were examined with a Zeiss Ultra plus FEG scanning electron microscope.

5.3.3 Experiment 3

5.3.3.1 Blood collection

Blood was collected from the same 6 healthy, female participants as Experiment 1.

Blood was collected in a citrate tube containing 0,5 ml of sodium citrate (3,8% sodium citrate) at the same three occasions of the menstrual cycle as Experiment 1 namely the menstrual phase (day 1 – 5, day 1 with the commencement of menses), the pre-ovulatory phase (day 12 – 14, first estrogen peak) and the luteal phase (day 20 – 25, progesterone peak and second estrogen peak). A total volume of 5ml of blood was collected per blood draw.

5.3.3.2 Sample preparation

The citrated whole blood was centrifuged for 10 minutes at 1,000 rpm (maximum RCF = 17.523x g; 1 250 g). The plasma was transferred to a 1,5ml eppindorf tube and centrifuged once more for 5 minutes at 1 250g. The platelet poor plasma (PPP) obtained was used to make a fibrin clot in an eppindorf tube. 20μl of PPP was mixed with 20μl thrombin (provided by The South African National Blood Services) in an eppindorf tube.

5.3.3.3 Preparation of samples for transmission electron microscopy (TEM)

The fibrin clot samples in the eppindorf tubes were incubated at 37°C for 5 minutes. Following incubation the samples were washed for 20 minutes. This washing process was done to remove any blood proteins trapped within the fibrin network.

The samples were prepared as described in the SEM preparation until after the dehydration step. Following dehydration the samples were infiltrated in a mixture of one part ethanol and one part epoxy resin for 30 minutes and then stored in pure epoxy resin for four hours. The samples were then placed in a rubber mould filled with pure epoxy resin and left to set for three days overnight in an oven at 60 °C. Ultra-thin sections of 80-100nm were cut with a diamond knife using an ultramicrotome. The sections were contrasted with uranyl acetate for 7 minutes followed by contrasting with lead citrate for 5 minutes, after which samples were allowed to dry for a few

60

minutes before examination with the JEOL transmission electron microscope (JEM 2100F). Photomicrographs revealing the internal structures of the fibrin fibers were taken.

For all experiments a blinding procedure between observer and investigator was followed to minimize bias

5.4 RESULTS
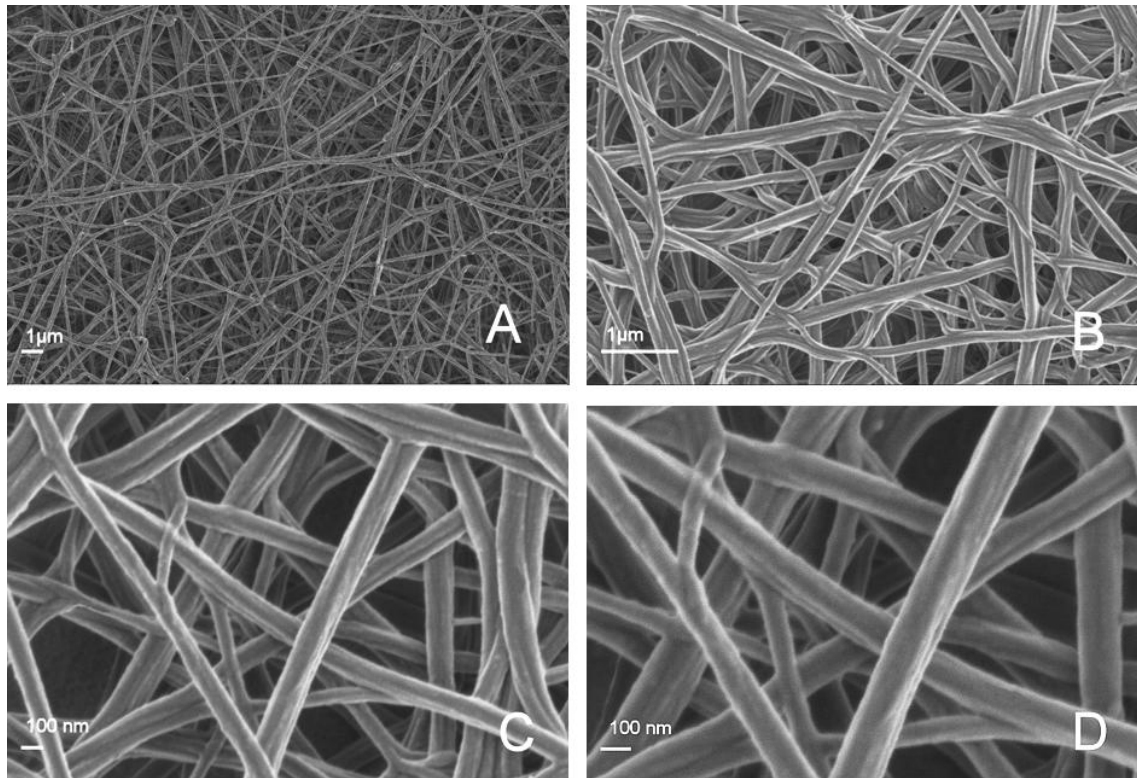
Figure 1. Scanning electron micrographs of fibrin networks from male participants.
Machine magnification: A=10 000x, B=40 000x, C=100 000x, D=150 000x.
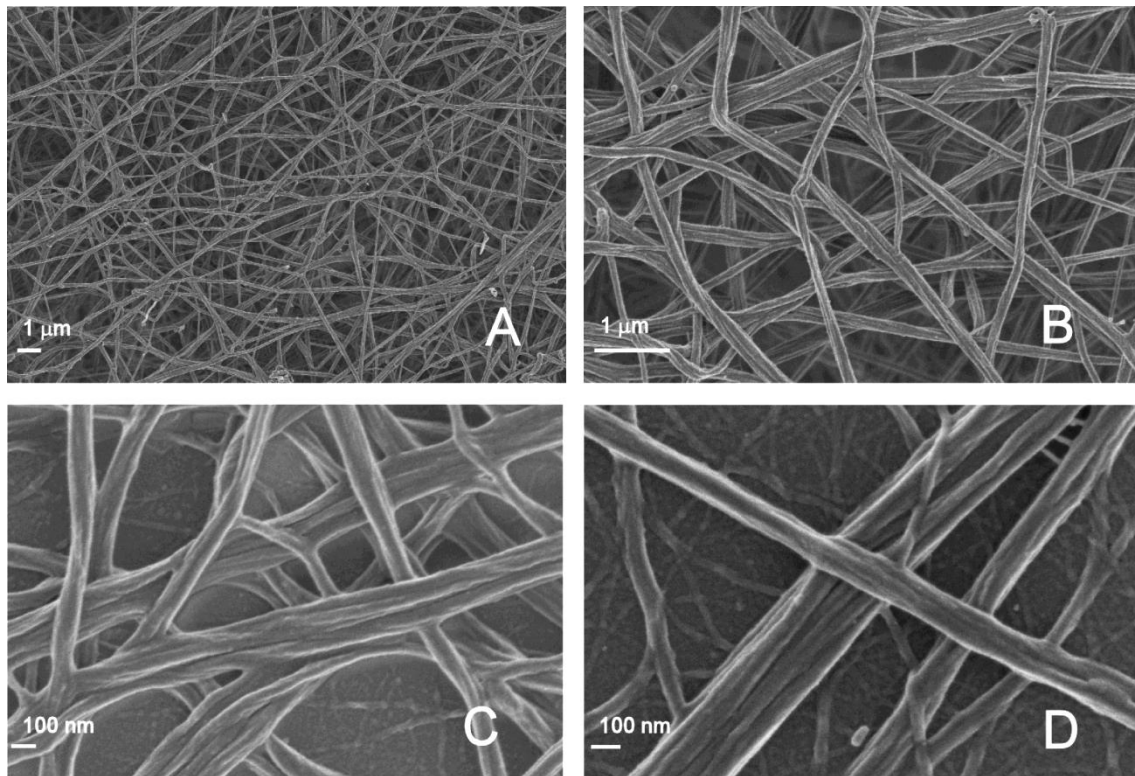
Figure 2. Scanning electron micrographs of fibrin networks observed during the menstrual phase (day 1 – 5) of the menstrual cycle.
Machine magnification: A=10 000x, B=40 000x, C=100 000x, D=150 000x.
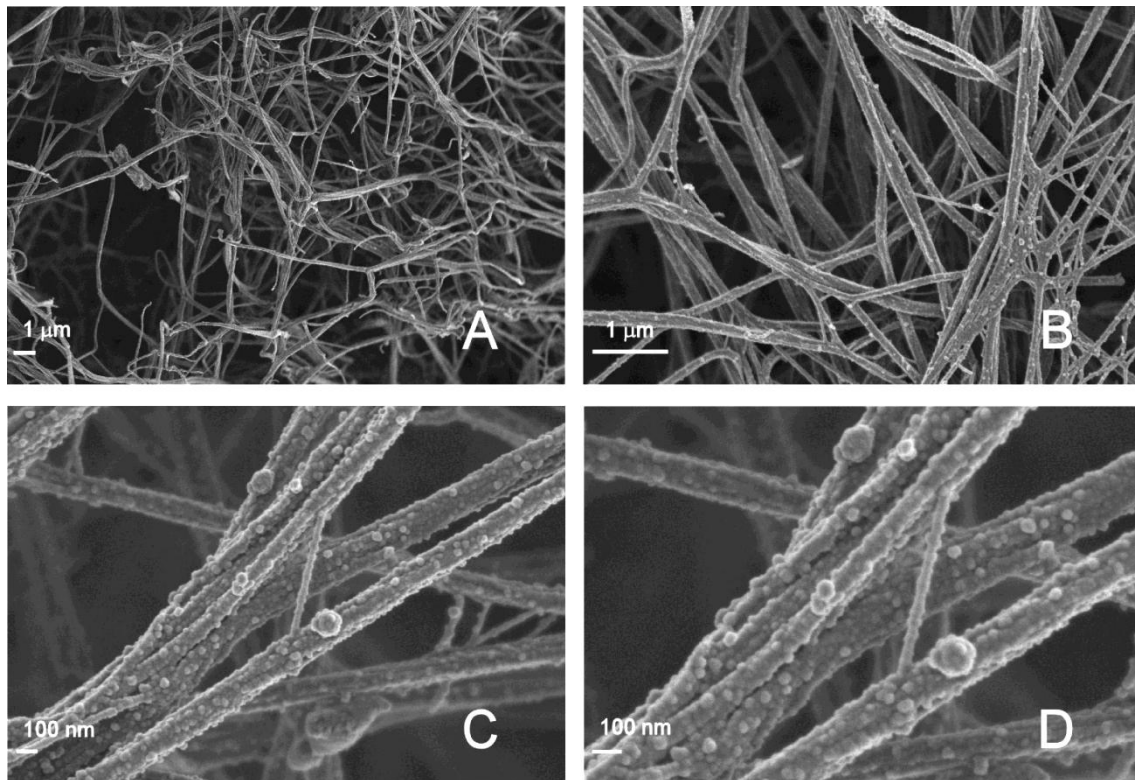
Figure 3. Scanning electron micrographs of fibrin networks observed during the period leading up to ovulation (day 12 – 14) of the menstrual cycle.
Machine magnification: A=10 000x, B=40 000x, C=100 000x, D=150 000x.

64
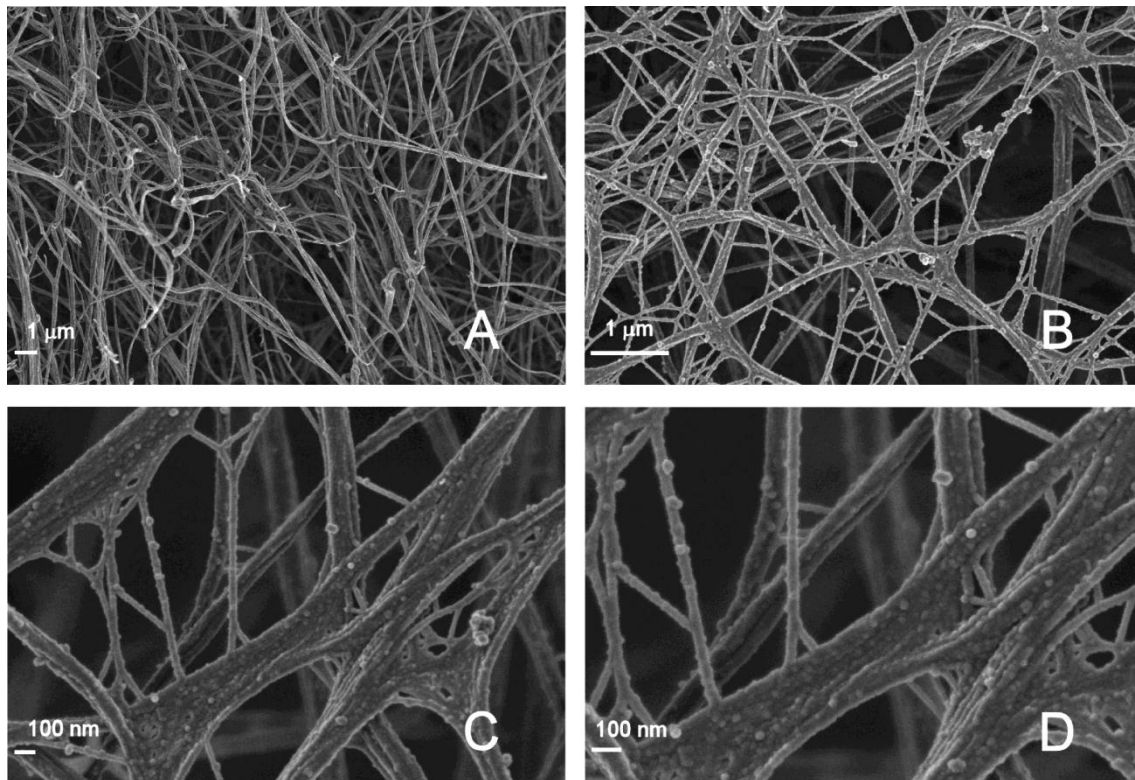
Figure 4. Scanning electron micrographs of fibrin networks observed during the luteal phase (day 20 – 25) of the menstrual cycle.
Machine magnification: A=10 000x, B=40 000x, C=100 000x, D=150 000x.

1.1
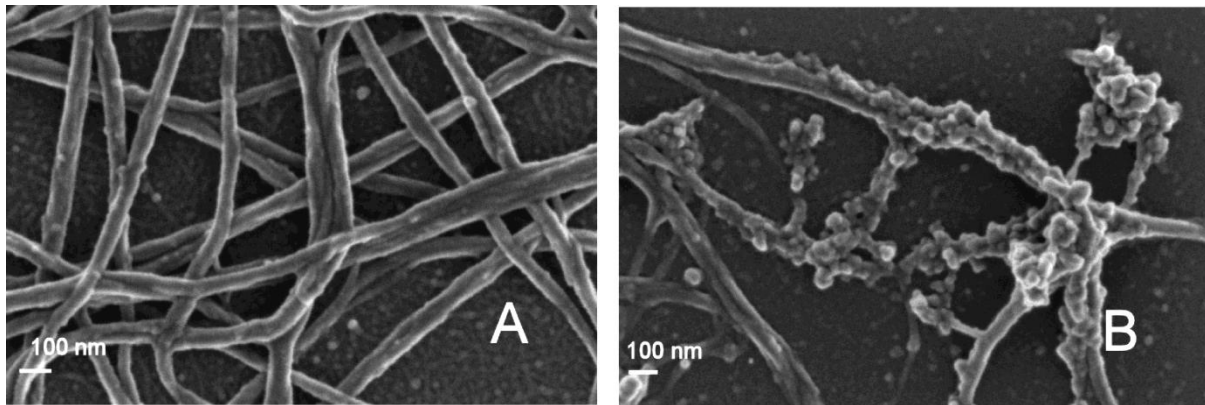
Figure 5: Scanning electron micrographs observed of fibrinogen model.
A=Fibrinogen without the addition of estrogen,
B=Fibrinogen and estrogen (pre-ovulatory concentration).
Machine magnification: 150 000x.
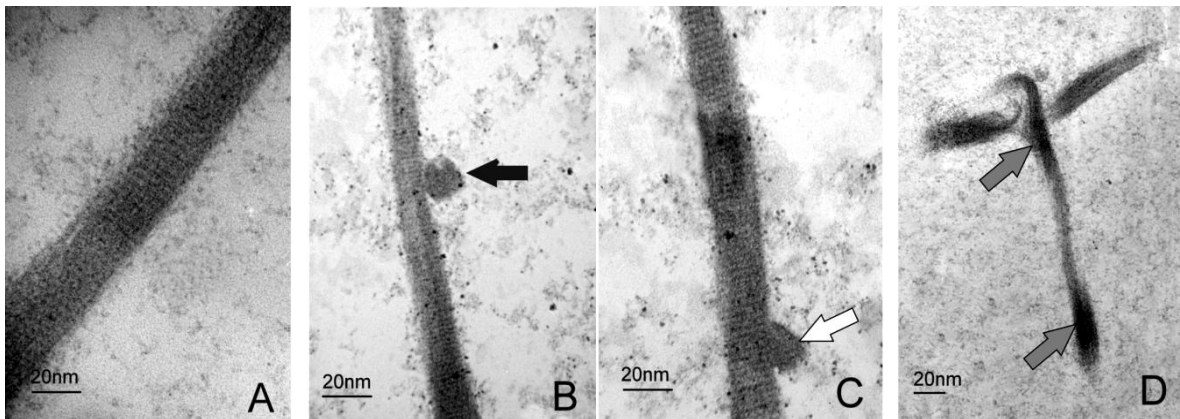
**Figure 6: Transmission electron micrographs of fibrin networks observed during
the pre-ovulatory phase (day 12 – 14) of the menstrual cycle.**
Black arrow = Globules on the surface of the fibrin fiber,
White arrow = Globules forming part of the fibrin fiber,
Gray arrows = dark globular clusters within the fibrin fibers.

66

5.4.1 Experiment 1

With the addition of thrombin to PPP, an extensive fibrin fiber network forms. This network typically consists of major, thick fibers with a few scattered minor fibers in between the thick fibers. In Figure 1A – D different magnifications of the typical fibrin network from male participants are exhibited. During the first phase of observation, fibrin from individuals was studied from the menstrual phase (day 1 – 5). Figure 2A – D shows different magnification from this phase. Both Figure 1 and Figure 2 show similar fibrin network ultrastructure. It is important to notice that the highest magnification (machine magnification 150 000x) showed smooth fibrin fibers for both the fibrin network from the male participants and the menstrual phase.

Figure 3A – D shows fibrin networks from the pre-ovulatory phase (day 12 – 14 of the menstrual cycle). During this phase, the fibers do not appear smooth, and it seems as if the fibrinogen, on activation with thrombin, causes a globular placement of the molecules that form the resulting fibrin fibers. Alternatively, the fibrin fibers might be stickier, attracting free plasma proteins to it, which stick to the individual fibers to form this globular ultrastructure. Also, a more flimsy fiber morphology is noted in figure 2A (if compared to Figure 2A) – which give a typical lower magnification view of the fibrin network.

The final phase of analysis was during the luteal phase (day 20 – 25) of the menstrual cycle. Figure 4A - D shows micrographs form this phase. The fibers appear more flimsy (compare 1A, 2A, 3A and 4A) and also have globular deposits (Figure 4D). During this phase, the minor, thin fibers are more prominent between the thick fibers if compared to the first phase (Figure 2B). Here it also seems as if the individual fibers are fused possibly because of a more sticky nature.

5.4.2 Experiment 2

Since the alterations in fibrin morphology seen in Experiment 1 coincided with the estrogen peaks in the menstrual cycle, estrogen was added to a fibrinogen model to determine the exclusive effect of estrogen on the fibrin network.

A normal fibrin network is shown in Figure 5A, where a network of smooth fibers is shown.

67

In Figure 5B the effect of exclusively estrogen on the fibrin network can be seen.   The fibers also have globular plasma protein deposits on the formed fibrin fibers, similar to the fibers in Figure 3 and Figure 4.

5.4.3   Experiment 3

Figure 6 displays different angles of sections through the fibrin fibers. In Figure 6A the fibrin internal structure during the menstrual phase (day 1 – 5) is shown, while Figure 6B – D shows the internal morphology of the fibrin fibers during the pre-ovulatory (day 12-14) and luteal (day 20 – 25) phases. The external and internal structure of the fibrin network appears smooth during the menstrual phase (Figure 6A). Globules similar in shape and size to those seen on the external fibrin network structure (Figure 3C and D) can be distinguished. Some globules appear to be on the surface of the fibrin strand (Figure 6B, black arrow), while others form part of the fiber (Figure 6C, white arrow). Some dark, globular clusters can also be distinguished within the lighter fibrin fibers (Figure 6D, gray arrows).

5.5   DISCUSSION

Haemostasis is the dynamic balance maintained between the coagulation system and the fibrinolytic system [14] and it is well-known that, in females, there are cyclical variability in estradiol and progesterone during the follicular and luteal phases.[216] Although the hormonal changes during the cycle are a well-known phenomenon, the question that arises is whether coagulation plasma protein concentrations vary significantly, and how hormonal changes influence this. Knoll and co-workers in 2012 attempted to answer this by systematically reviewing literature discussing variations and those reports suggesting no variation in coagulation protein concentrations during the different phases of the female cycle. From their review, they concluded that the optimal timing of haemostatic testing seems to be the menstrual and early follicular phase of the menstrual cycle.[217]

Some interesting results of variations in haemostatic variability include D-dimer differences during the follicular and luteal phases and as estradiol concentration increased, PAI-I decreased. D-dimer is a fibrin degradation product or a small protein fragment present in the blood after a blood clot is degraded by fibrinolysis. PAI is a serine protease inhibitor that is the principal inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), the activators of plasminogen and ultimately, fibrinolysis. During the menstrual cycle, changes have also been observed in levels of von

Willebrand factor (VWF), fibrinogen, and activated factor VII.[218] Kadir and co-workers in 1999 mentioned that in healthy individuals, fibrinogen, von Willebrand factor antigen and von Willebrand factor activity concentrations showed strong cyclic variations with peak values in the luteal phase.[203] Knoll and co-workers in 2012 reported that VWF-levels were mostly about 10% (range 2–24%) lower in menstrual/early follicular phase compared to the luteal phase. However, the authors found conflicting literature regarding the levels of fibrinogen during the menstrual cycle. The main constant factor of change during the menstrual cycle is therefore estrogen levels and it can be concluded that estrogen has an influence on the plasma proteins and their packaging during fibrin fiber network formation.[217]

Therefore, during the menstrual cycle of healthy individuals, the ultrastructural profiles of fibrin networks were analysed by following a qualitative analysis of fibrin fibers. In the current research, the fibrin fibers from male participants are smooth. This is also true for the fibrin networks from females during day 1 – 5 of the menstrual cycle. However, during day 12 – 14, the fibrin fiber morphology starts to change, becoming less smooth, and during the luteal phase of the cycle (day 20 – 25) the network appears sticky, where the minor, thin fibers are more prominent between the thick fibers if compared to the male participants and the menstrual phase in females.

The smooth fibrin networks from male participants showed typical fibrin network ultrastructure. Since the fibrin networks from the menstrual phase compared well to that of the male participants, it can be assumed that normal morphology for the fibers are exhibited in the absence of estrogen.

The changes noted in the fibrin networks during the pre-ovulatory and luteal phases were confirmed to be due to changes in levels of estrogen. As mentioned previously, there are two estrogen peaks, the first estrogen peak is between day 12 – 14 and the second peak occurs during the luteal phase.[212] These peaks coincide with the changes we see in the current qualitative research, where the fibrin morphology changes during the same time as the estrogen peaks occur. The effect of estrogen on the fibrin network in the menstrual cycle was also confirmed with a fibrinogen model, where the addition of estrogen altered the fibrin network morphology in the same manner as seen in whole blood.

Investigation of the internal structure of the fibrin fibers confirmed the presence of globules on the fibrin network. These globules were not only present on the surface of the fibrin fiber strand, but some of these globules formed part of the strand itself. This shows that the altered morphology is

69

not only confined to the outer surface of the fibers. Since dark globular clusters also appeared on the inside of the fibrin fibers, it can be assumed that the formation of the fibers is influenced by estrogen, not only the final structure.

The globules present on and within the fibrin network are thought to be due to a change in the packaging of the fibrinogen, as Knoll et al stated. [217]

These changes in fibrin ultrastructure may have a dual function. Firstly, if fertilization takes place this altered fibrin fibers may support the implantation of a fertilized ovum. Secondly, if fertilization does not take place, this changed morphology will ensure sufficient coagulation during menses to prevent excessive blood loss.

Some limitations exist for this study. Since these findings are only ultrastructural analysis of the fibrin network, biochemical analysis of the altered morphology may indicate the mechanisms involved in these unique results. It may shed some more light on the origin of the globules seen on and within the fibrin fibers.

Our research group has studied and compiled a database of healthy individuals with 1000's of fibrin network micrographs. Since the micrographs from the 6 participants compared well with our database and the same trends were seen for all the participants we expect the same results for additional participants. For further investigation, a larger sample size could be employed; however we believe that the results will remain the same for a larger participant group.

5.6 CONCLUSION

During the menstrual cycle, the fibrin fiber morphology differs. Estrogen causes the changes in the external and internal structure of the fibrin networks identified during the menstrual cycle. This research therefore is the first to show ultrastructural changes in fibrin fiber morphology resulting from the estrogen changes during the menstrual cycle.

70

CHAPTER 6: ULTRASTRUCTURAL ANALYSIS OF FIBRIN NETWORKS DURING THREE PHASES OF PREGNANCY: A QUALITATIVE INVESTIGATION

6.1 CHAPTER OBJECTIVES

In this chapter the fibrin network ultrastructure of different phases of pregnancy, namely early pregnancy (week 8 – 14), late pregnancy (week 36 – 40) as well as post-partum (week 6 – 8 after birth) will be compared to non-pregnant fibrin networks as well as each other to establish whether differences in fibrin network morphology exist during pregnancy.

6.2 INTRODUCTION

6.2.1 Haemostasis

Haemostasis is the physiological process that prevents exsanguination from a severed blood vessel. This is achieved by the rapid conversion of blood from its fluid phase into a clot localized at the site of vessel injury. Haemostasis is therefore essential to preserve life by minimizing blood loss from damaged tissue and assisting in the restoration of vascular integrity. [219]

An array of complex processes maintaining normal blood flow under physiological conditions, including the immediate response to vessel injury, is called the haemostatic system. Haemostasis requires swift reaction to tissue damage. [220] Procoagulant as well as anticoagulant mechanisms work in unison to maintain physiological balance in the haemostatic system. [221]

The interdependent systems that collectively ensure a balanced haemostatic system are divided into three parts namely primary, secondary and tertiary haemostasis. [222]

Upon vessel injury, the damaged vessel will constrict temporarily to decrease blood flow. Platelets adhere to the site of injury, aggregate and form the primary platelet plug. This is referred to as primary haemostasis. In secondary haemostasis a cascade of reactions referred to as coagulation is initiated by the exposed tissue factor of the severed vessel. This chain of proteolytic events results in the formation of fibrin. Fibrin networks formed during coagulation stabilizes the platelet plug. Tertiary haemostasis is the process where the fibrin clot will be dissolved by fibrinolysis once the endothelium has been restored and vascular integrity is regained to ensure normal blood flow. [222,223]

71
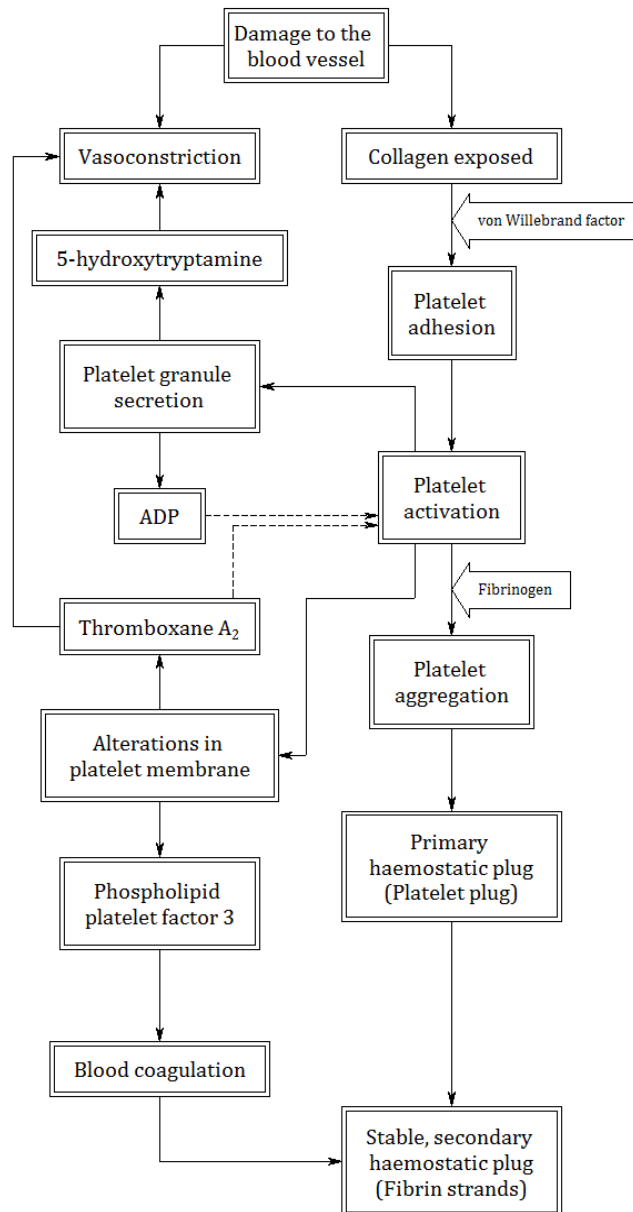
Diagram 1. Haemostatic system leading to plug formation. [224]

Plasma proteins, blood cells and the vascular system work in concert to maintain haemostasis. [222]

Four main components interact to control the thrombus development from the fluid phase of blood: The endothelial cell layer of the vascular system, platelets, coagulation factors and

72

fibrinolysis. Pathological thrombosis or haemorrhage may result from the disturbances in the strictly controlled haemostatic process. [220]

6.2.1.1 Vascular system

A single layer of endothelial cells are found inside all blood vessels. These cells are constantly in contact with the blood passing through the lumen of the vessel. The endothelial cells are heterogeneous with diverse physiological functions depending on the position of the cells in the vascular system. The endothelial cells however predominantly regulate haemostasis and vascular integrity. [225]

6.2.1.2 Platelets

When the vascular integrity is compromised platelets will adhere to the exposed collagen of the damaged endothelium. Platelets will then become activated and change their shape. The activated platelets will start to release their granules. This will trigger additional platelet activation and result in aggregation of the activated platelets. These actions also initiate the coagulation cascade responsible for fibrin formation. [226]

6.2.1.3 Coagulation factors

Coagulation is process during which the circulating blood protein fibrinogen is polymerized to the protein monomer fibrin. This process is catalysed by thrombin with a network of fibrin fibers as result.[227]

The formation of fibrin strengthens the primary haemostatic plug comprised of platelets. [228]

Several procoagulant plasma proteins are involved in the coagulation cascade. These are termed Factors with designated Roman numeral. The proteases concerned with thrombus formation are found in their inactive form in circulation (referred to as zymogen) and are only biologically activated once damage occurs to the vasculature. [229]

6.2.1.4 Fibrinolysis

Blood clots are resolved by the fibrinolytic system. The process of fibrinolysis involves the actions of activated plasmin. The pro-enzyme plasminogen is converted by tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator to its active form, namely plasmin. Polymerized fibrin is subsequently cleaved by plasmin as it binds to the fibrin fibers. [221]

73

6.2.2 Coagulation

A complex chain reaction is initiated when a blood vessel is damaged to curtail the loss of blood and aid the repair of the vessel. [223]

Primary haemostasis refers to the formation of the primary haemostatic plug comprised of platelets. Secondary haemostasis is the subsequent formation of fibrin strands as a result of complex interactions between plasma coagulation factors. These fibrin fibers strengthen the platelet plug to form the stable, secondary haemostatic plug. The process of coagulation and its association with platelets can be divided into a four steps namely initiation, amplification, propagation and stabilization. [219]

Initiation of the coagulation system is triggered by exposed tissue factor (TF) of the severed endothelium of the blood vessel. TF binds to the circulating factor VIIa and, along with factor V, converts factor IX and factor X to their activated states. Activated factor X (Xa) binds to prothrombin, which is converted to thrombin. Only a small amount of thrombin is generated in this manner, which unaided is not sufficient to generate fibrin. [230,231]

Thrombin generated in the initiation phase triggers a feedback mechanism. This permits the activation of factor VII bound to TF by the activated factors VII, IX and X. Activated factor VII is subsequently increased. Cofactors are also activated by the thrombin-triggered feedback mechanism. Factor V and VIII accelerate factor II by factor Xa which in turn accelerates factor IX (IXa). More factor IXa is generated from the activation of factor XI, also initiated by the thrombin-triggered feedback mechanism. This process is referred to as amplification. [219]

Propagation is the maintenance of factor Xa production by the intrinsic tenase complex to ensure thrombin generation is maintained. The intrinsic tenase complex consists of the cofactor for factor IXa, factor VIIIa. Factor IX activation initially results from the factor VIIa/TF complex. [219]

During the stabilization phase elevation levels of thrombin activate factor XIII. Factor XIIIa forms cross links between soluble fibrin monomers to produce a stable clot and increase the tensile strength of the fibrin polymers. The clot is protected from lysis by thrombin-activatable-fibrinolysis inhibitor and the α2-antiplasmin bound to fibrin strands. [219,228]

74
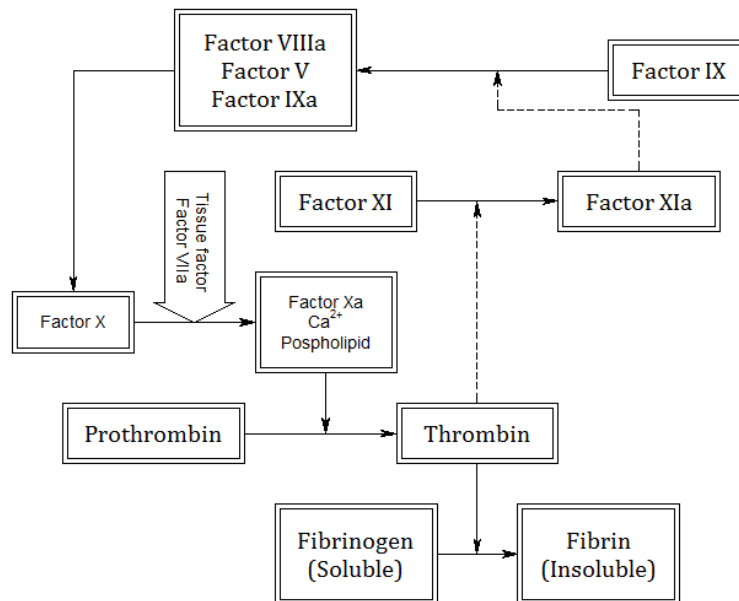
Diagram 2. Coagulation factors involved in clot formation. [226]

To prevent overwhelming thrombosis, protein C is activated. Activated protein C cleaves factor Va and factor VIIIa. Factor VIIa/TF and factor Xa is inhibited by tissue factor pathway inhibitor. Thrombin, factor IXa and factor Xa are inhibited by antithrombin. [230]

However, fibrinolysis has to be employed for haemostasis to be balanced. Endothelial cells release tPA which converts plasminogen to the serine protease plasmin. When tPA is bound to fibrin it is subject to positive feedback and the process of fibrinolysis is promoted. Since plasmin cleaves tPA into a two-chain molecule, exposure of the binding site is increased and complex formation is subsequently promoted. Arginine and lysine bonds are hydrolysed by plasmin. This results in proteolysis of several substrate like fibrinogen and fibrin along with factors V, VIII and XIII. The cleaved fibrin and fibrinogen form fragments. [228]

75
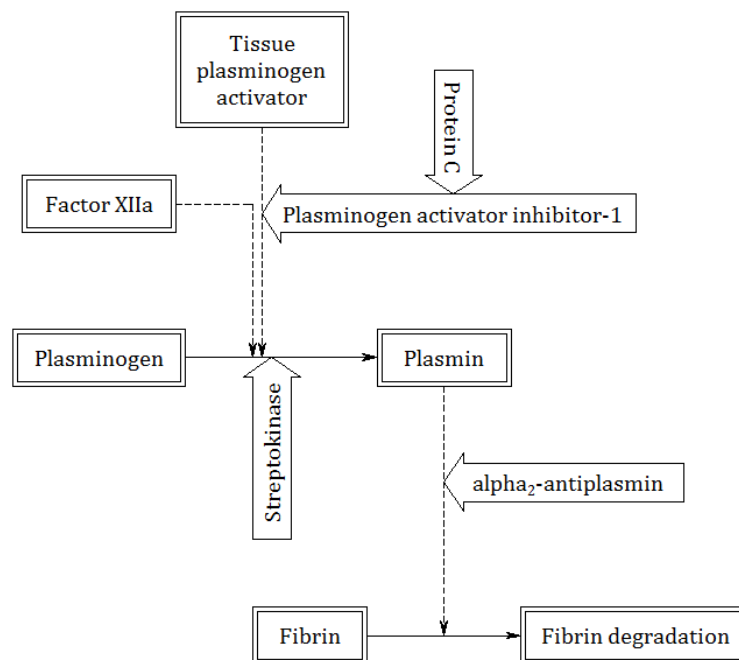
Diagram 3. Fibrinolysis [224]

A vast array of interactions between cellular and molecular components is employed in the process of haemostasis. Although clot formation is the major role of coagulation, it is also involved in defence mechanisms and cellular repair. [219]

6.2.3 Coagulation and pregnancy

Normal pregnancy is characterized by significant alterations in the haemostatic system accompanied by an augmented risk of thrombosis. An elevated coagulability of the blood in conjunction with a decrease in fibrinolytic activity contributes to the prothrombotic state attributed to pregnancy. It is specifically during the third trimester that these changes manifest. [232,233]

Fibrinogen concentrations may increase up to 200% above normal levels during pregnancy. This is also true for some coagulation factors. [11,234] Factor VII concentrations gradually increase as the pregnancy progresses, and by term reaching its highest levels of up to 1000%. [235,236] Thrombin levels are also increased during uncomplicated pregnancy. [237]

76

The concentrations of endogenous anticoagulants however decrease or remain unaffected. These pregnancy-induced haemostatic alterations therefore promote the hypercoagulable state associated with pregnancy. [11,234]

Fibrinolytic activity is decreased during pregnancy, however returns to normal swiftly after delivery. The decreased fibrinolytic activity is caused by the large quantities of placental derived plasminogen activator inhibitor type 2 (PAI-2) present during pregnancy.[59]

Tabel 1. Summary of haemostatic changes during normal pregnancy.

Systemic changes	Increased	Decreased	No Change
Procoagulant factors	I, V, VII, VIII, IX, X	XI	
Anticoagulant factors	Soluble TM	PS	PC
Adhesive proteins	vWF		
Fibrinolytic proteins	PAI-1, PAI2	t-PA	TAFI

[14,59,232,233,238-240]

Haemostasis is under a lot of strain during placental separation. It is a rapid process during which the maternal blood flow to the placental site (nearly 700ml of blood passes through every minute) needs to be curbed. This is achieved by thrombotic plug formation at the sites where the maternal vessels were severed and compression of the myometrium. [14]

Pregnancy is an independent risk factor for thrombosis. Venous thromboembolism, one of the foremost obstetric causes of maternal death, is estimated to affect almost 1 in 1000 deliveries, with an increased incidence in particularly the third trimester. [239,241,242]

77

Thrombotic risk is increased by 4 to 10 fold throughout the gestation period as well as post-partum. [59] This is attributed to the alterations in haemostatic factors as well as physiological vasodilation associated with pregnancy. [238]

In 2005 Uchikova and Ledjev studied the haemostatic changes during the late phase (week 35 – 40 of gestation) of normal pregnancy. They found that significant changes were apparent during pregnancy, particularly the increase in fibrinogen, activity of factor VII, fact X and alpha2-antiplasmin and the decreased activity of protein C and S. They confirmed that both the coagulation and fibrinolytic systems were activated during pregnancy. However, regardless of the profound changes seen in the coagulation and fibrinolytic systems none of the pregnant participants developed thrombo-embolic disease during their pregnancies. They concluded that a newly established equilibrium is established to maintain the haemostatic balance during pregnancy. [239]

All the changes in the coagulation system during pregnancy form part of an intricate physiological adaptation essential for two related purposes. Firstly the maternal and fetal circulations at the uteroplacental interface can expand during pregnancy without complications. Secondly, during placental separation bleeding from the placental site is sufficiently controlled. [14]

To summarize, two complementary processes are involved in haemostasis: Blood clot formation and dissolution of the clot. Thrombus formation stops blood loss from a severed vessel while clot dissolution, also referred to as fibrinolysis, cleaves the clot once the vessel is repaired. [229]

A finely controlled network of interaction between pro- and anticoagulant factors contributes to the coagulation system. [221]

During pregnancy several procoagulant factors, including fibrinogen and thrombin, are increased while the fibrinolytic activity is decreased. [14,59,233,238,239]

Pregnancy is an independent risk factor for thrombosis and venous thrombo-embolism is the foremost obstetric cause of maternal mortality. [239,241]

The impact of pregnancy on the coagulation system, especially the morphology of the fibrin network, needs to be investigated to establish the cause of the increased risk for thrombosis.

78

6.3 MATERIALS AND METHODS

The external, structural alterations in the fibrin networks of plasma from different phases of pregnancy were analyzed by preparing the samples for scanning electron microscopy (SEM) as described in Laboratory procedures under 3.3.2.2 Blood plasma: *Fibrin network* in Chapter 3.

6.4 RESULTS
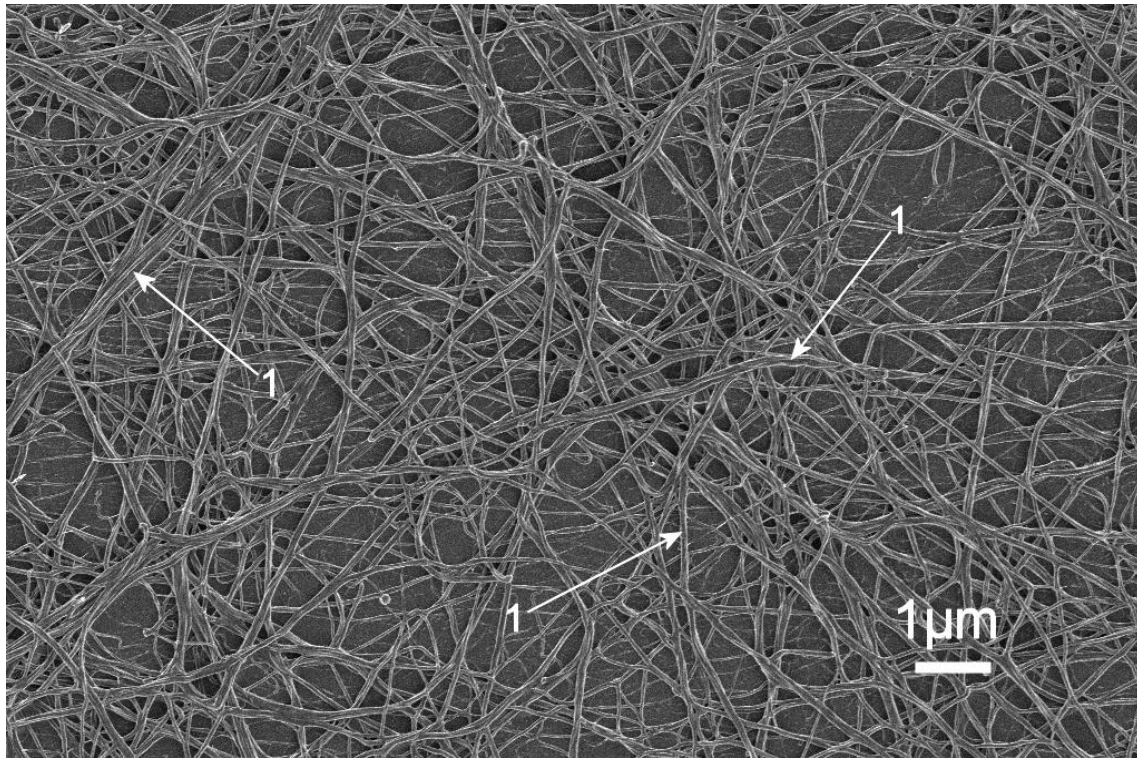
Figure 1.A Control fibrin network. Machine magnification=10 000x
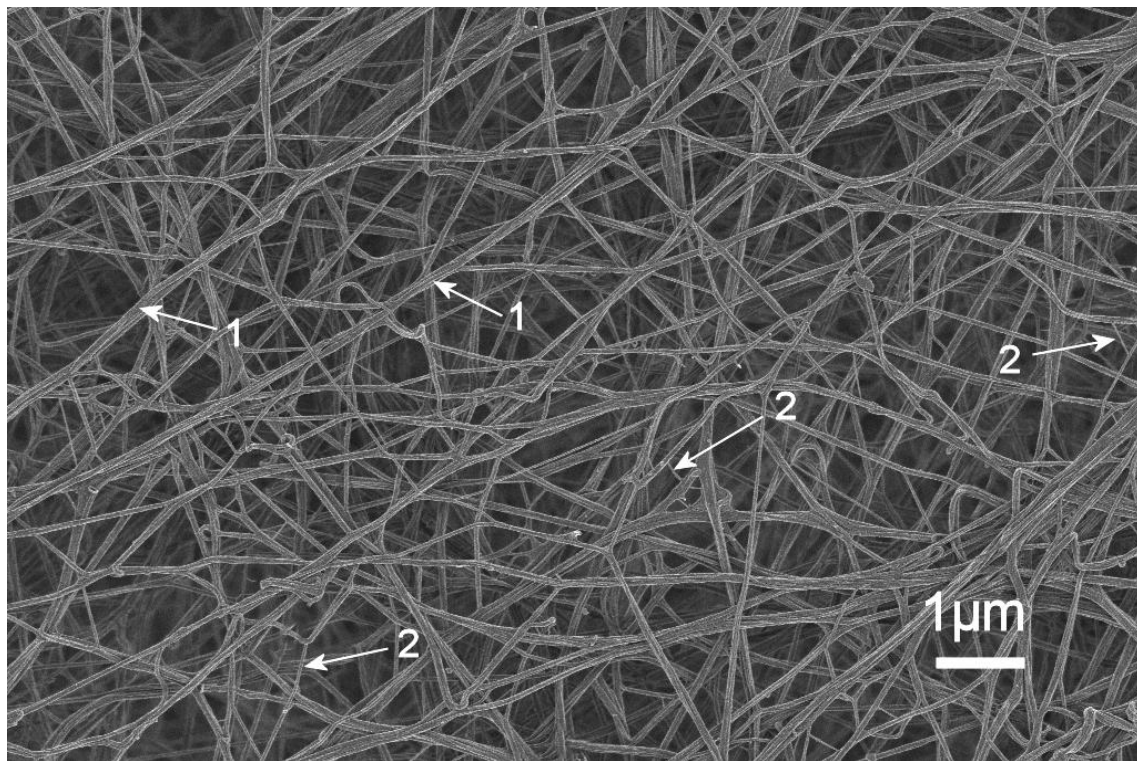
Figure 1.B Control fibrin network. Machine magnification=10 000x

80

1 – Major, thick fibers	3 – Coagulant formation
2 – Minor, thin fibers	4 – Protein globular clusters
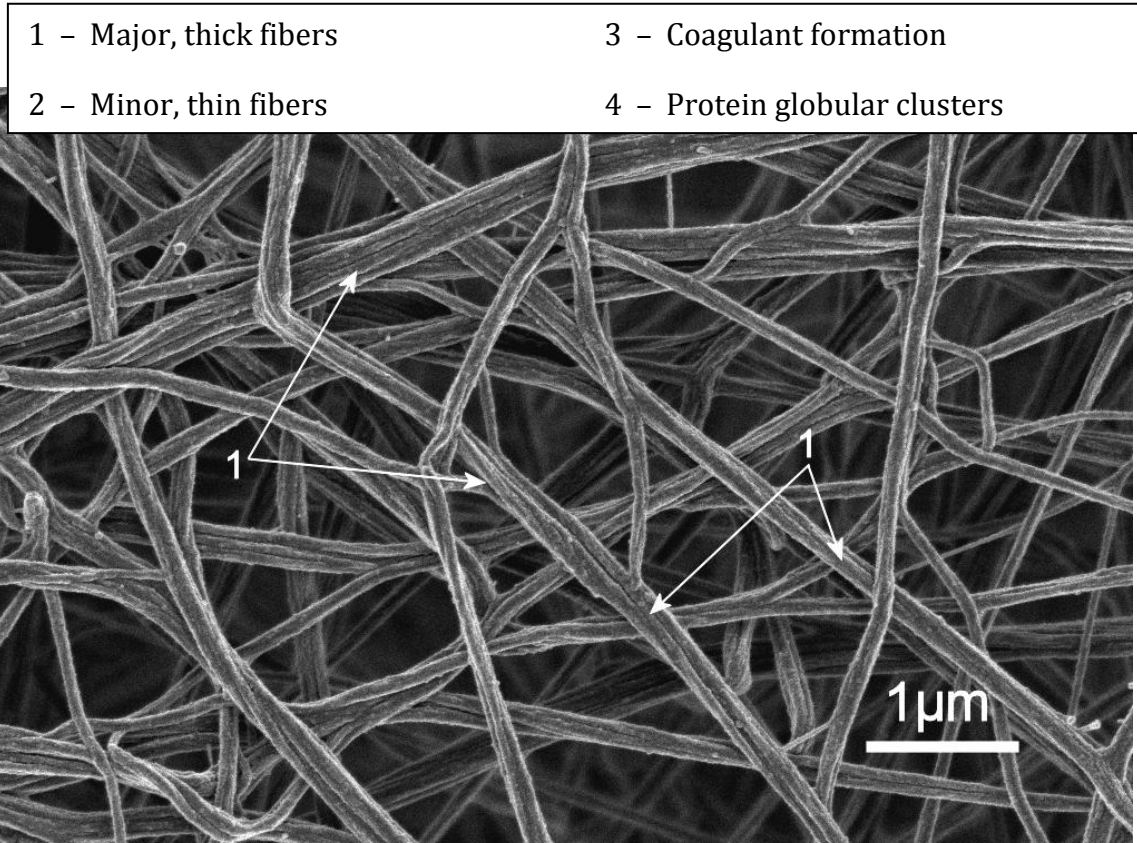
Figure 1.C Control fibrin network. Machine magnification=40 000x
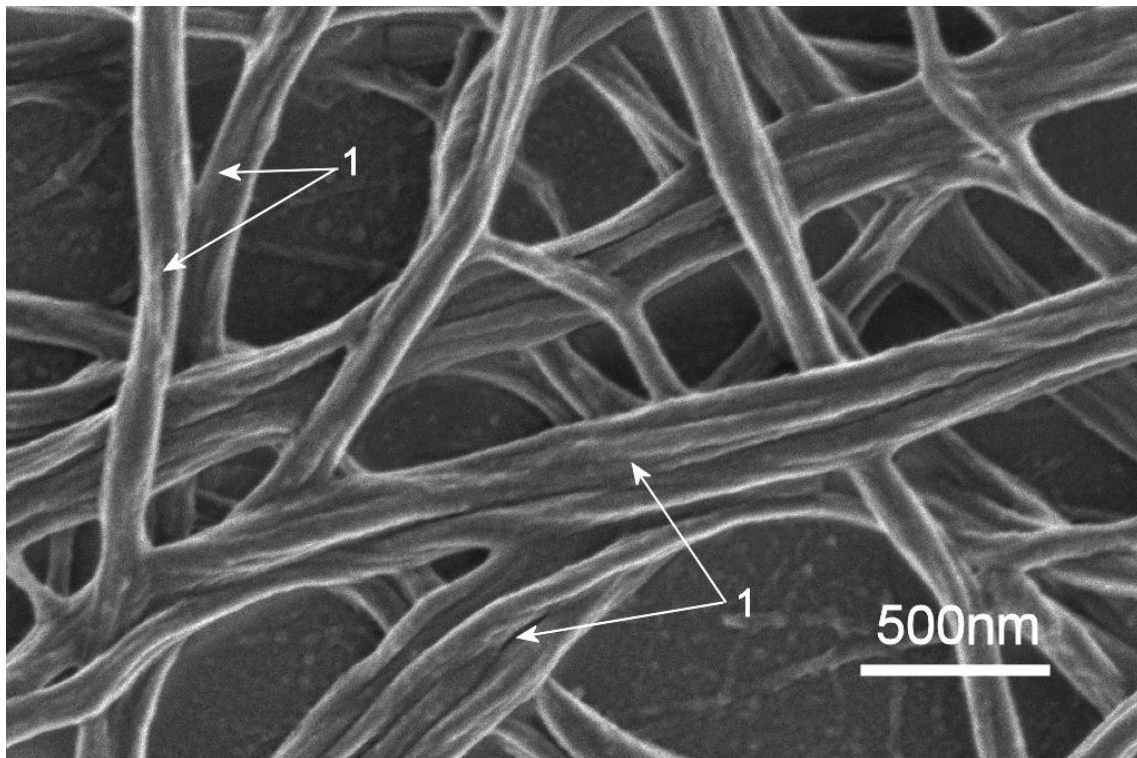
Figure 1.D Control fibrin network. Machine magnification=100 000x
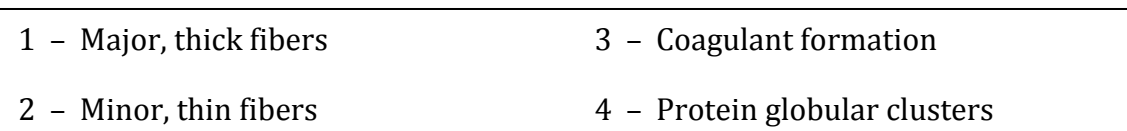
1 – Major, thick fibers	3 – Coagulant formation
2 – Minor, thin fibers	4 – Protein globular clusters

81
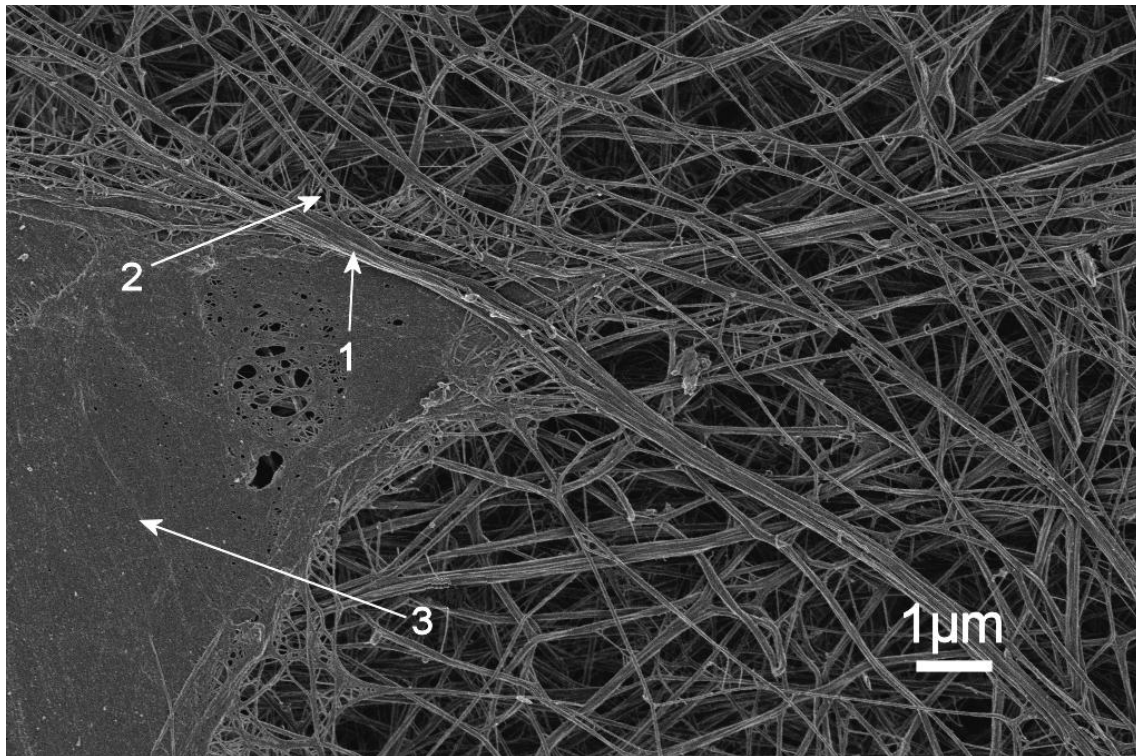
Figure 2.A Fibrin network of early pregnancy. Machine magnification=10 000x
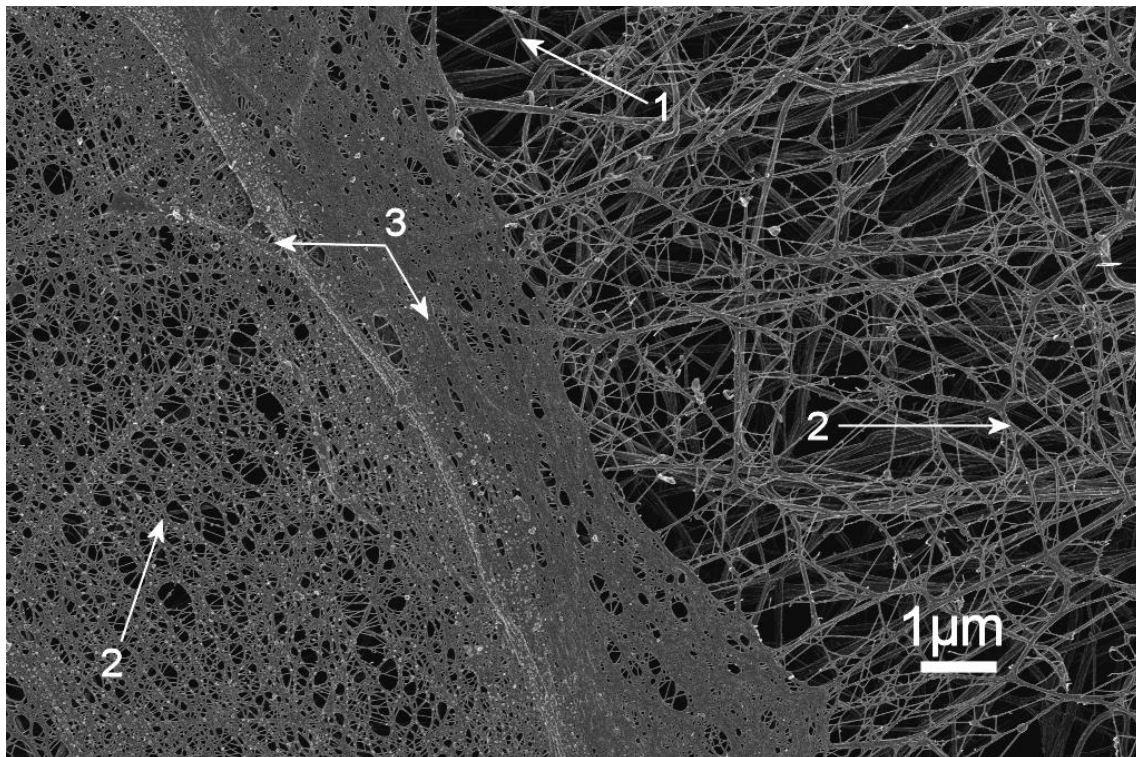
Figure 2.B Fibrin network of early pregnancy. Machine magnification=10 000x

1 – Major, thick fibers	3 – Coagulant formation
2 – Minor, thin fibers	4 – Protein globular clusters

82
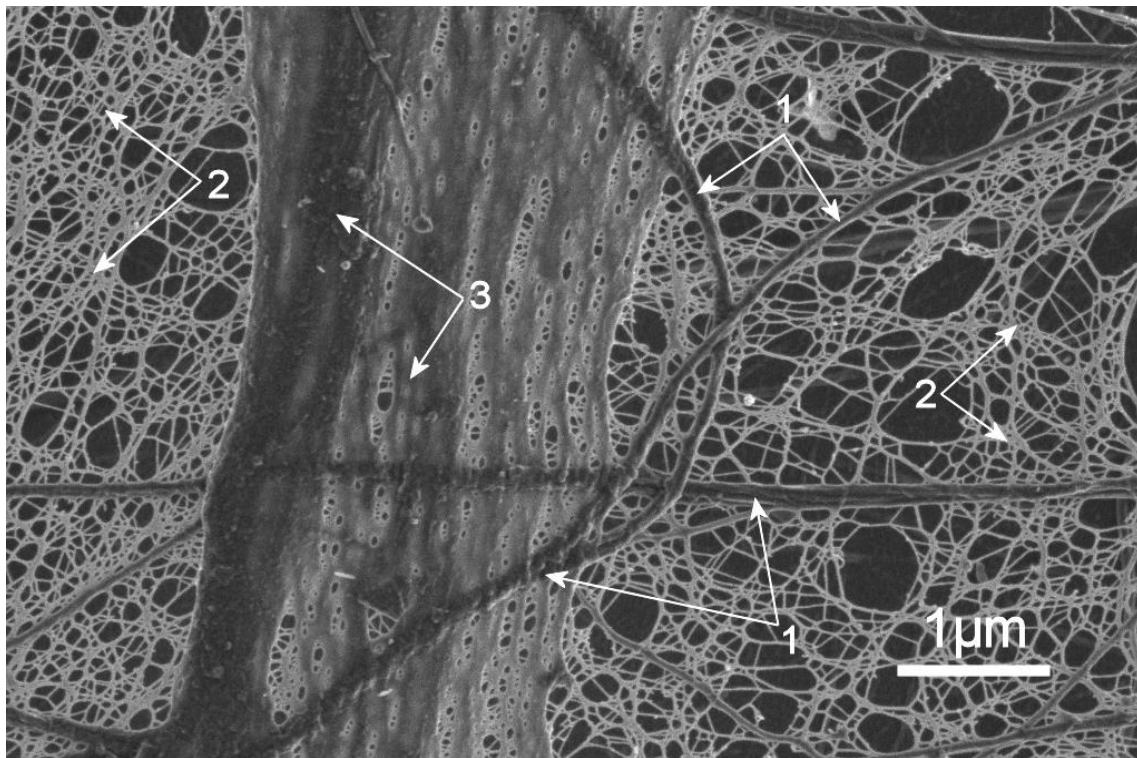
Figure 2.C Fibrin network of early pregnancy. Machine magnification=40 000x

Figure 2.D Fibrin network of early pregnancy. Machine magnification=100 000x

1 – Major, thick fibers		3 – Coagulant formation	
2 – Minor, thin fibers		4 – Protein globular clusters	

83
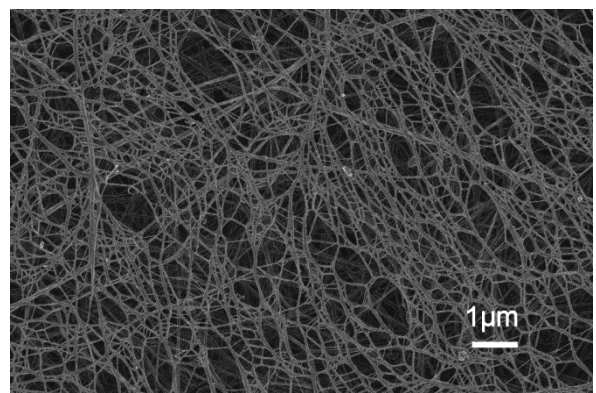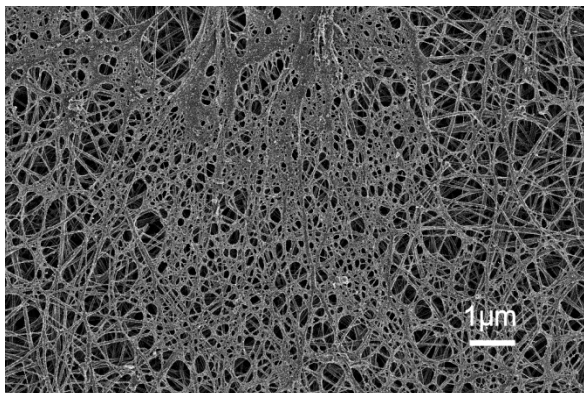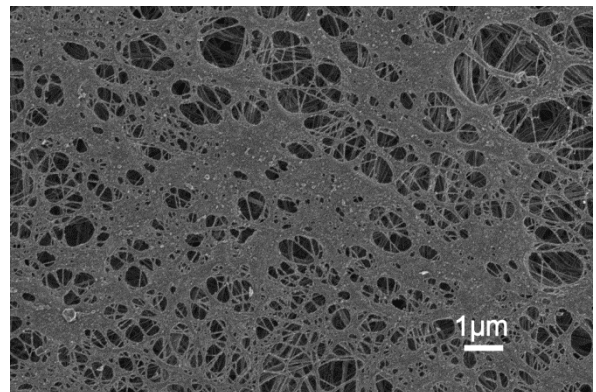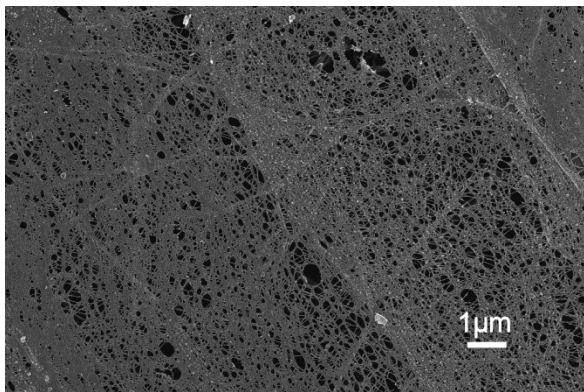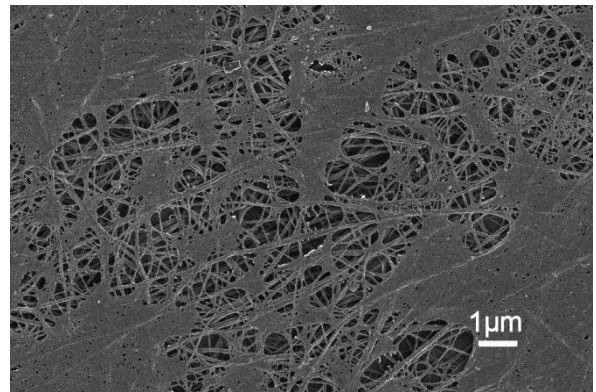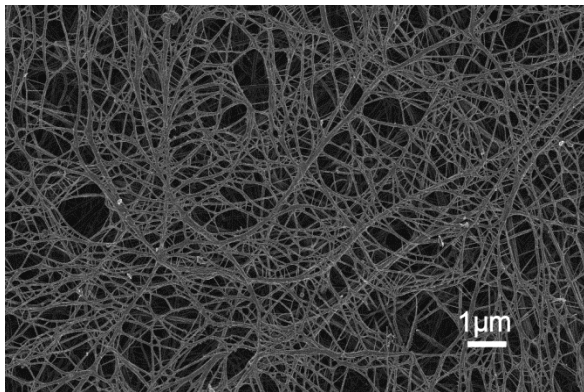
Plate 1.A Fibrin micrographs of early pregnancy.
Machine magnification=10 000x
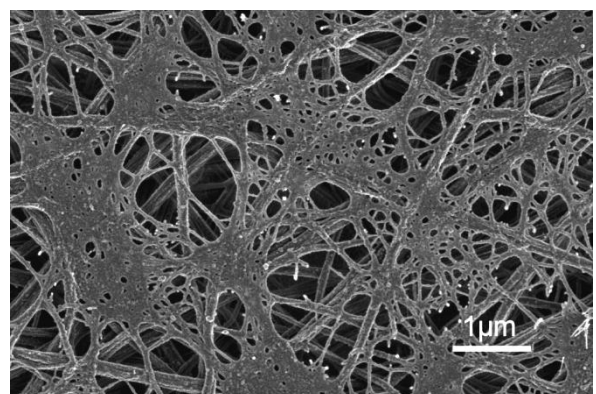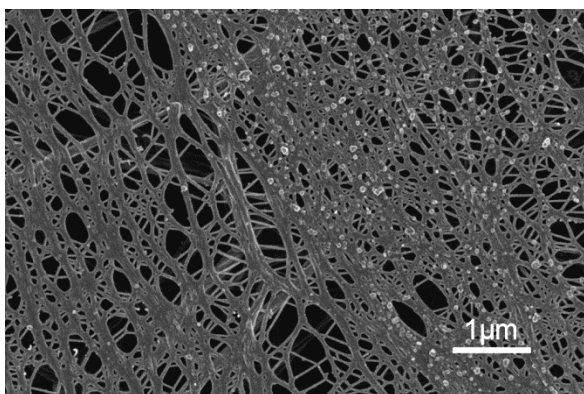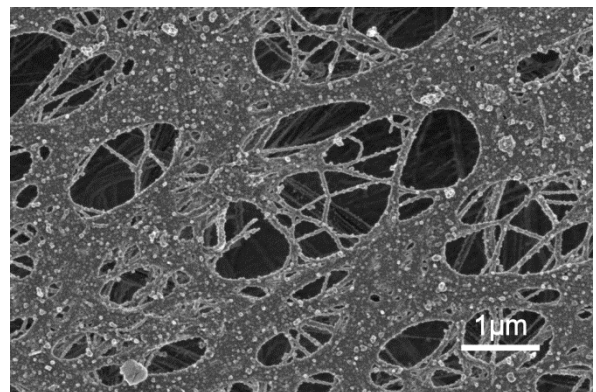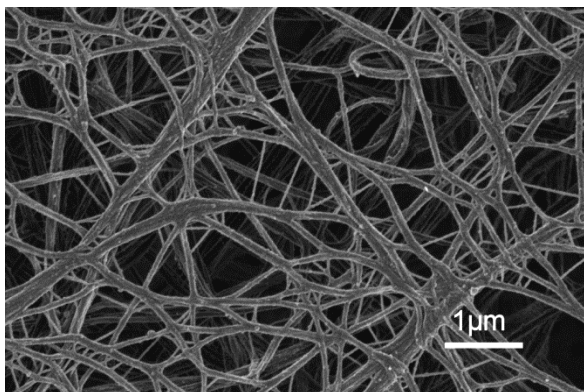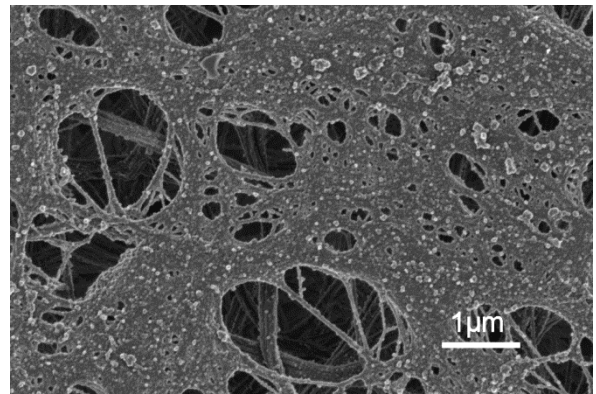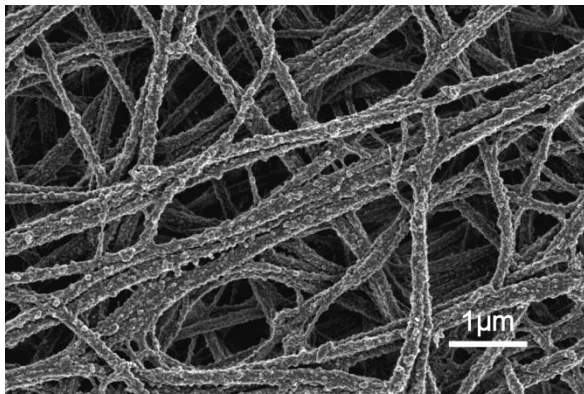
Plate 1.B Fibrin micrographs of early pregnancy.
Machine magnification=40 000x

85

Plate 1.C Fibrin micrographs of early pregnancy.
Machine magnification=100 000x

Figure 3.A Fibrin network of late pregnancy. Machine magnification=10 000x

Figure 3.B Fibrin network of late pregnancy. Machine magnification=10 000x

1 –	Major, thick fibers	3 –	Coagulant formation
2 –	Minor, thin fibers	4 –	Protein globular clusters

87
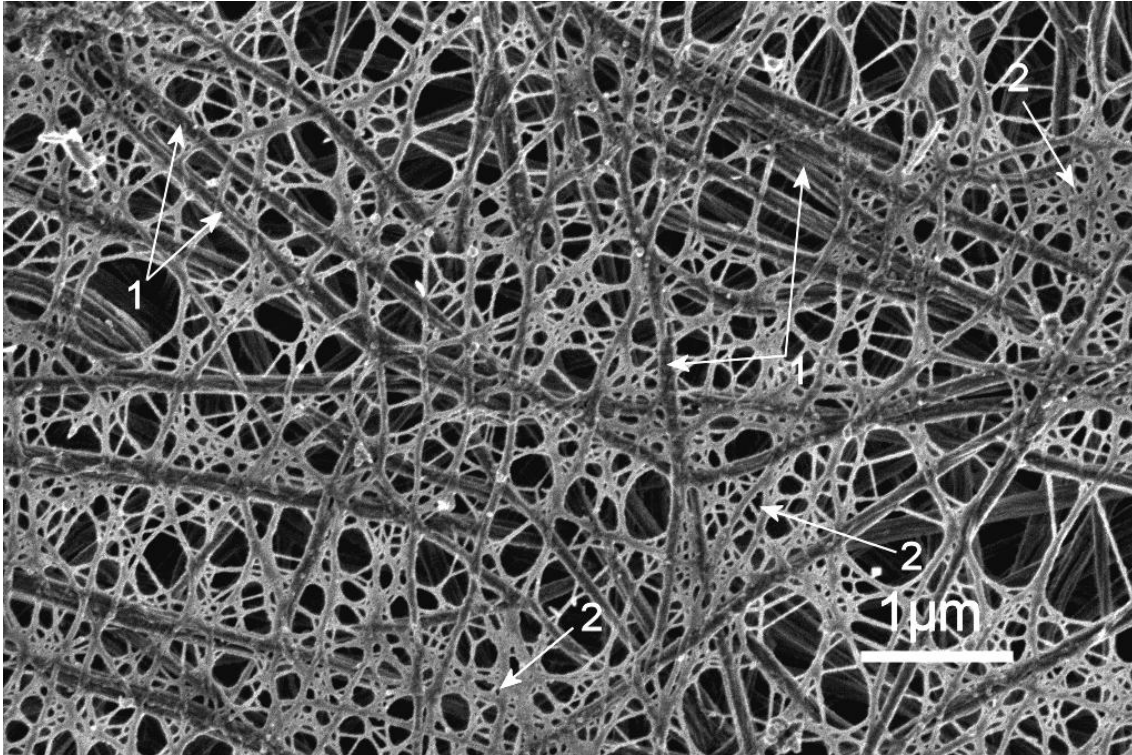
Figure 3.C Fibrin network of late pregnancy. Machine magnification=40 000x

Figure 3.D Fibrin network of late pregnancy. Machine magnification=100 000x

1 – Major, thick fibers	3 – Coagulant formation
2 – Minor, thin fibers	4 – Protein globular clusters
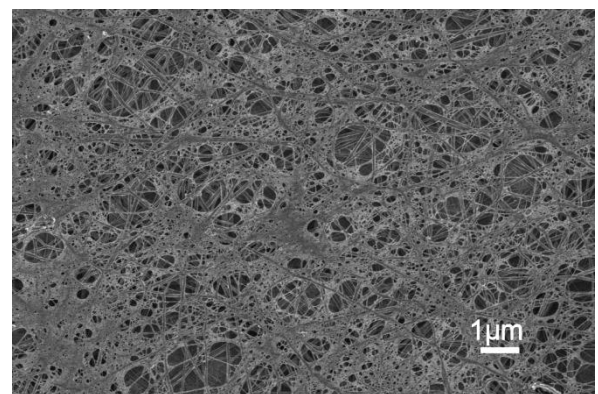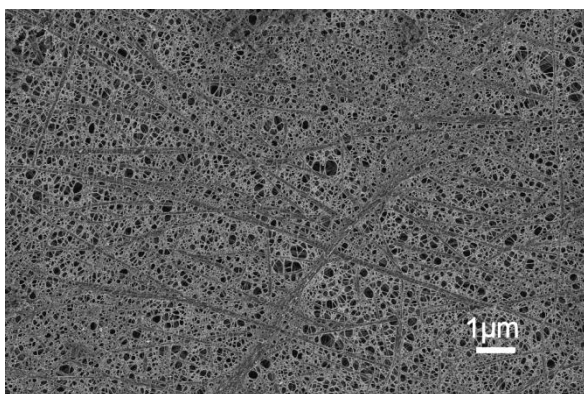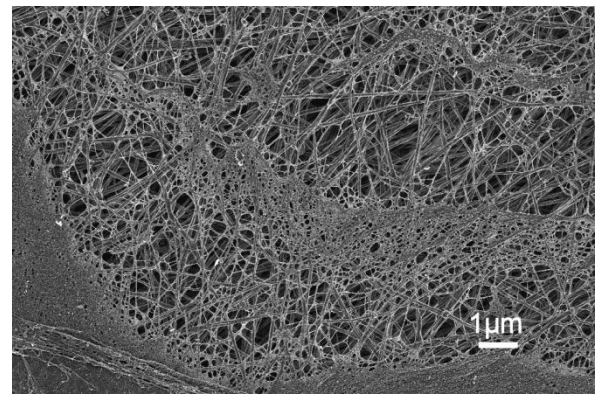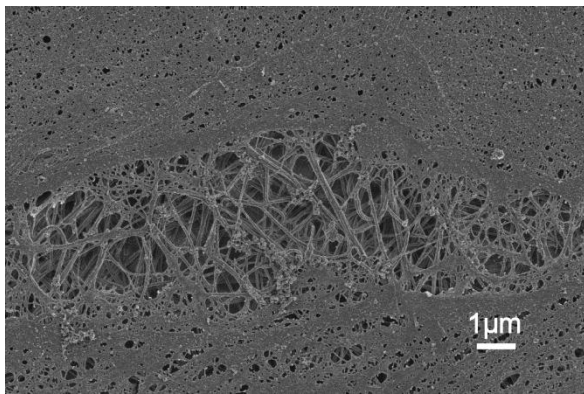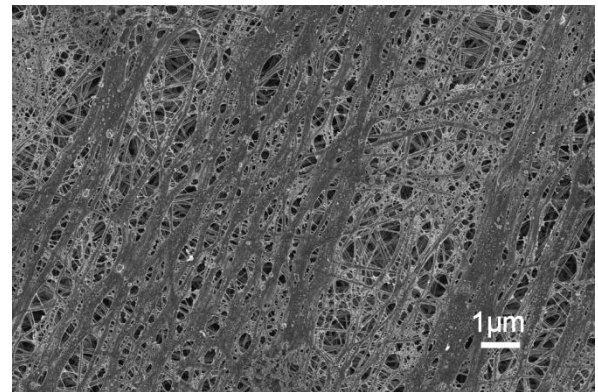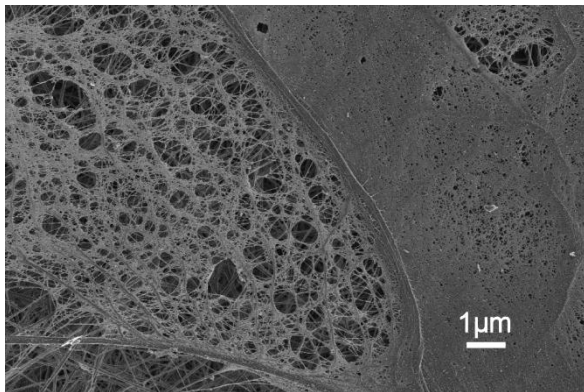
Plate 2.A Fibrin micrographs of late pregnancy.
Machine magnification=10 000x
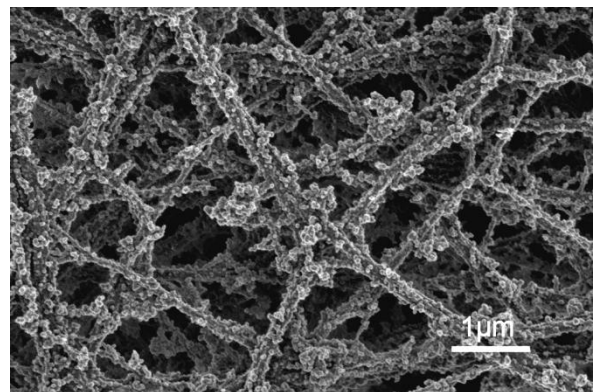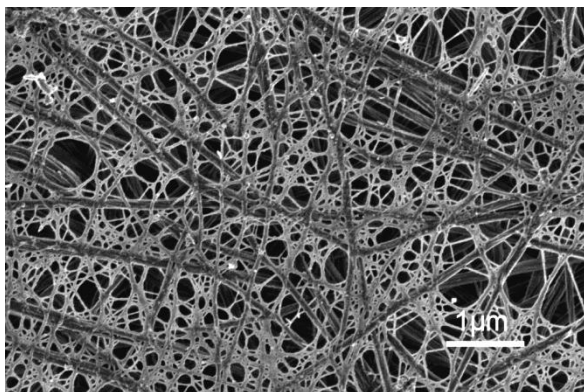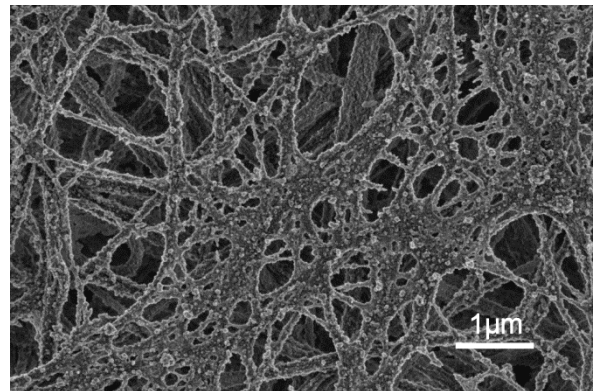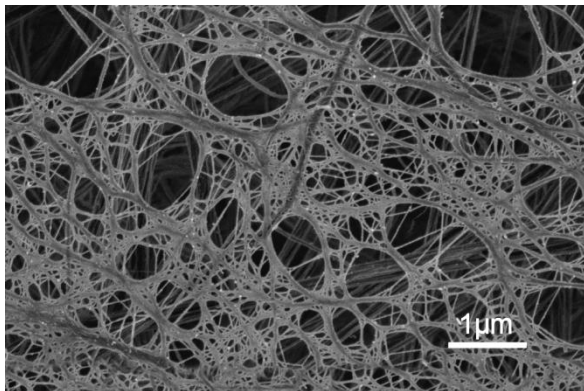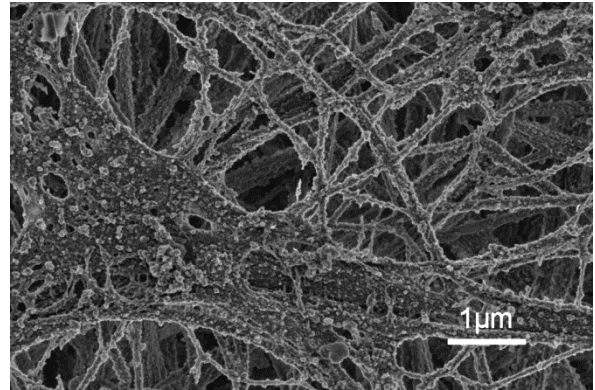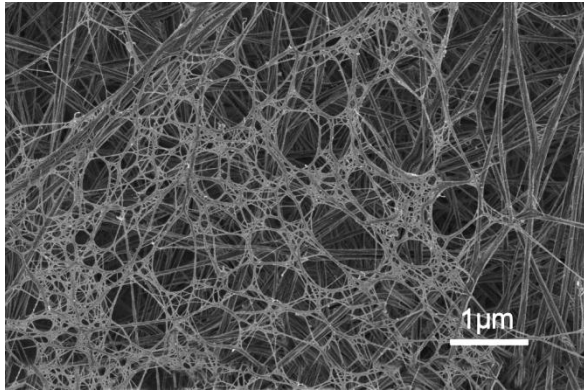
Plate 2.B Fibrin micrographs of late pregnancy.
Machine magnification=40 000x
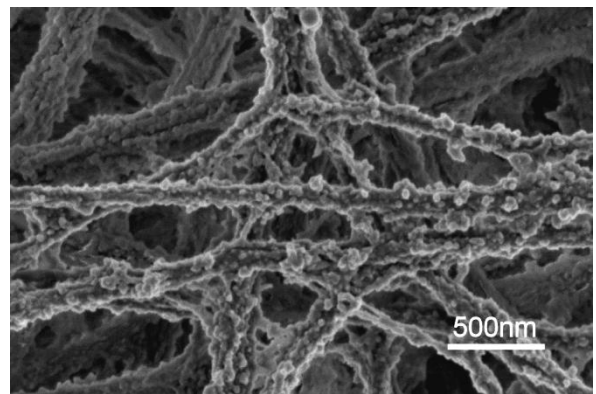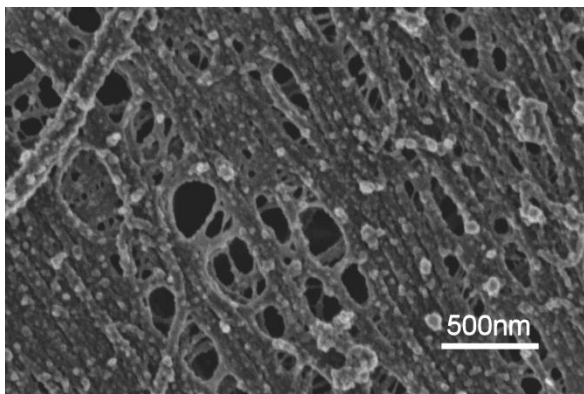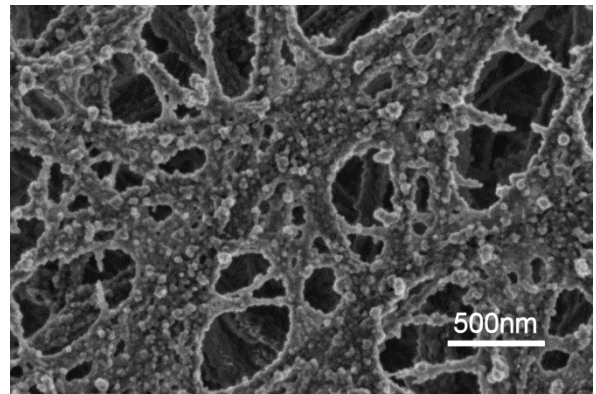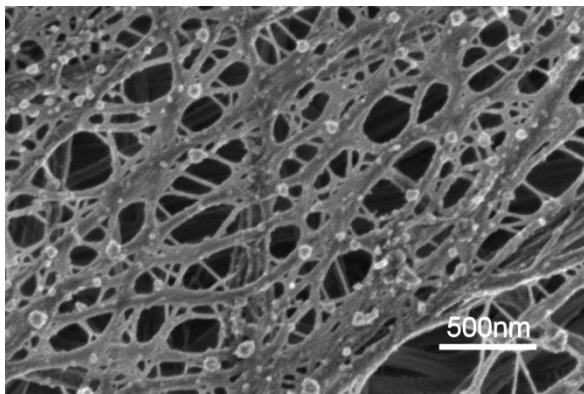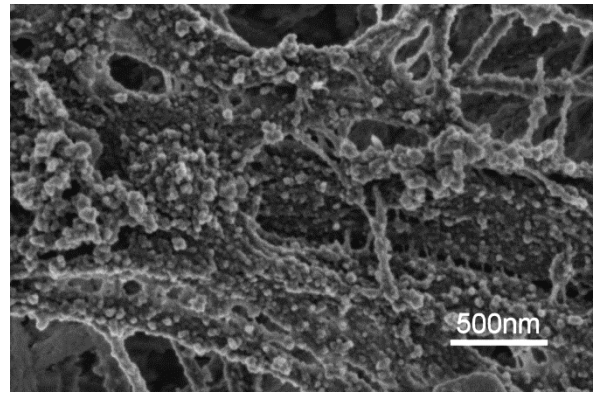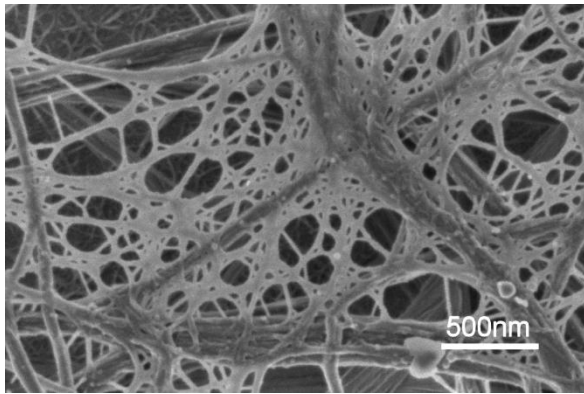
Plate 2.C Fibrin micrographs of late pregnancy.
Machine magnification=100 000x

Figure 4.A Fibrin network post-partum. Machine magnification=10 000x

Figure 4.B Fibrin network post-partum. Machine magnification=10 000x

1 – Major, thick fibers	3 – Coagulant formation
2 – Minor, thin fibers	4 – Protein globular clusters

92
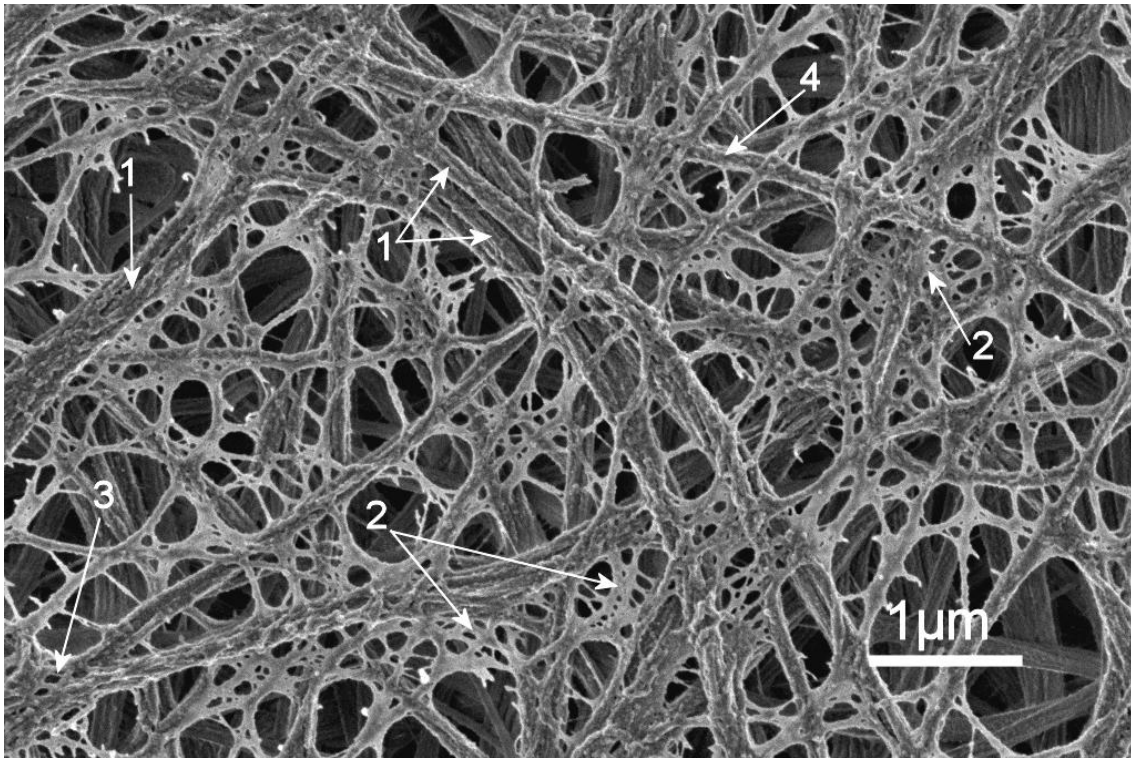
Figure 4.C Fibrin network post-partum. Machine magnification=40 000x

Figure 4.D Fibrin network post-partum. Machine magnification=100 000x

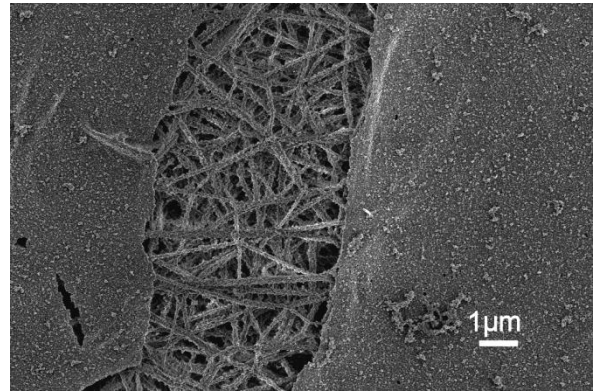| 1 – Major, thick fibers	3 – Coagulant formation
2 – Minor, thin fibers	4 – Protein globular clusters

93
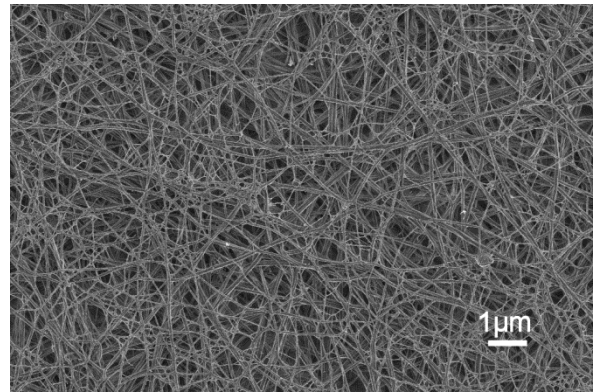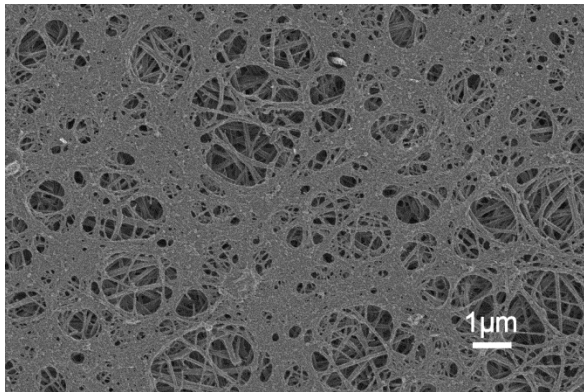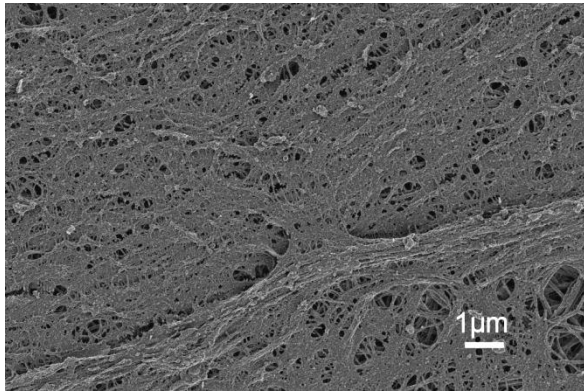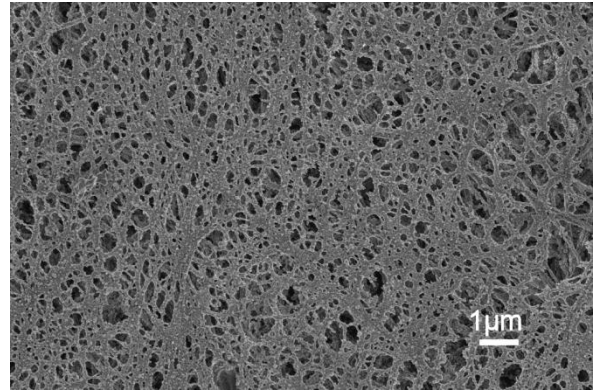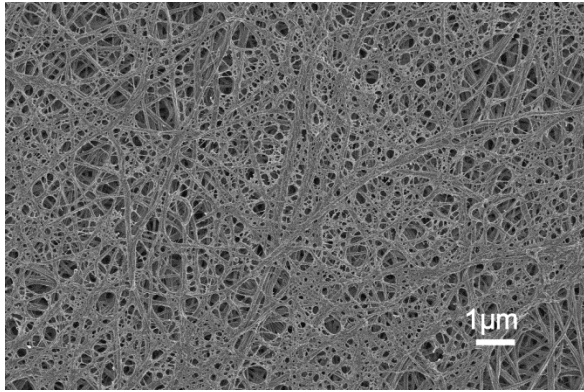
Plate 3.A Fibrin micrographs of post-partum.
Machine magnification=10 000x

94
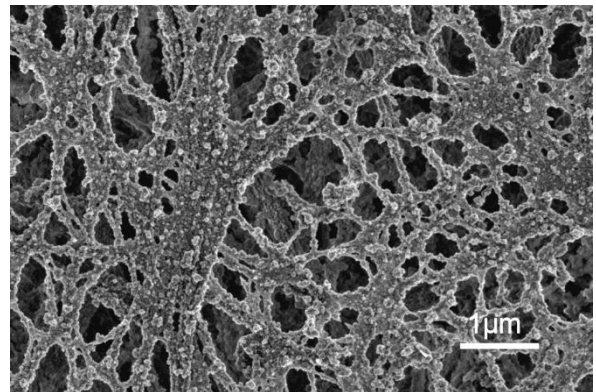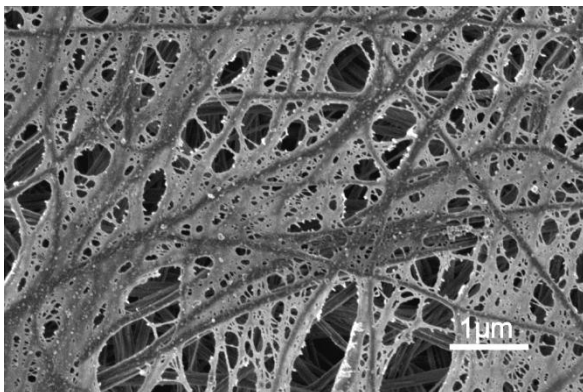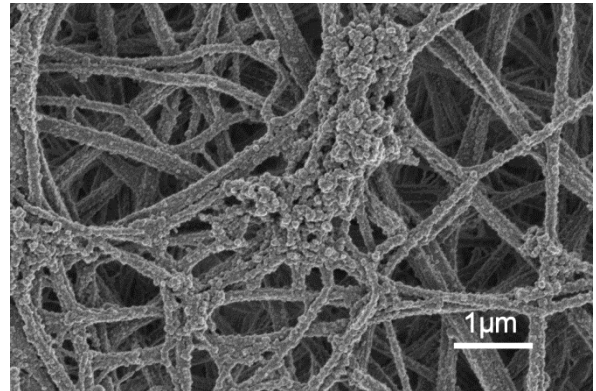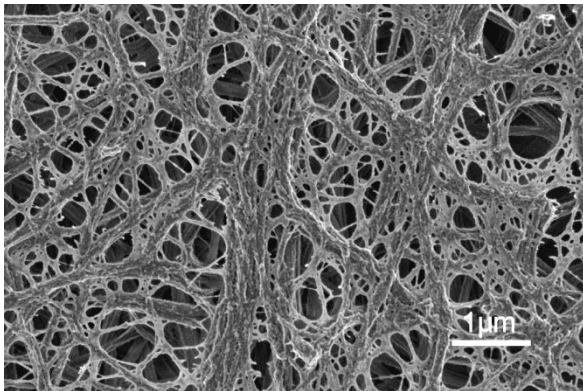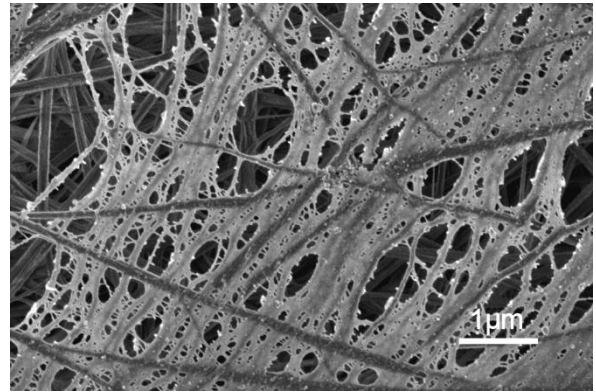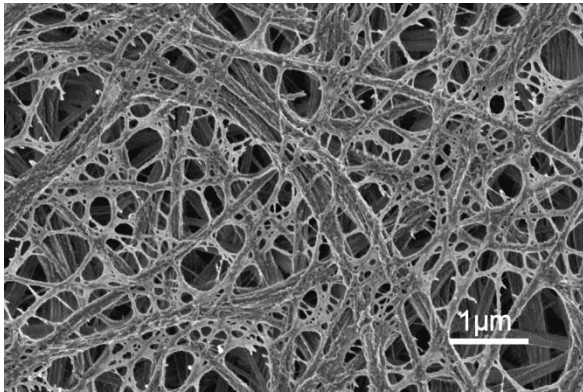
Plate 3.B Fibrin micrographs of post-partum.
Machine magnification=40 000x

Plate 3.C Fibrin micrographs of post-partum.
Machine magnification=100 000x

Figures 1.A-D show micrographs of fibrin networks from non-pregnant participants. These networks are typical of healthy control individuals exhibiting several major, thick fibers and some minor, thin fibers between the thick fibers. The fiber strands are all smooth.

Figures 2.A-D exhibit the fibrin networks from early pregnancy (week 8 – 14) participants. The minor, thin fibers are more prominent and thick, matted layers of coagulant formation can be distinguished. The fibrin strands have a granular morphology and individual granular globules are clearly visible. Plates 1.A-C confirm the altered morphology.

Figures 3.A-D show the late pregnancy (week 36 – 40) fibrin networks. Thick, matted coagulant areas are also seen in this group. The minor, thin fibers are also prominent and thickly packed between the major, thick fibers. The same granular globules can be distinguished on the higher magnifications. Plates 2.A-C verify the changes in fibrin network morphology.

Figures 4.A-D show fibrin networks from the post-partum group of participants (week 6 – 8 after birth). Coagulant formation was very prominent. Minor, thin fiber formation between the major, thick fibers is also distinctive. On higher magnification the granular globules seen in the early and late pregnancy groups is once again exhibited. Plates 3.A-C support the variations in ultrastructure.
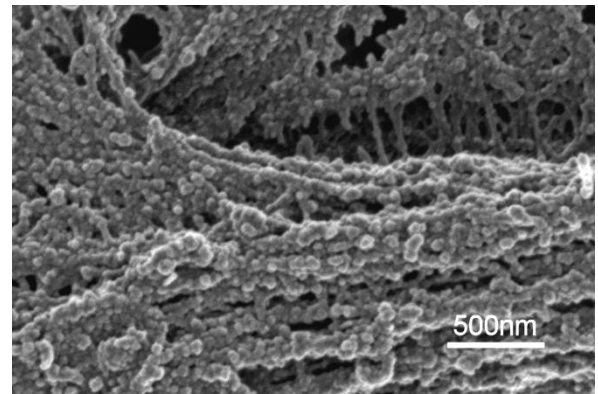
Overall, the fibrin networks from pregnancy appear similar to each other, exhibiting prominent coagulant formation, an increase in the formation of minor, thin fibers, and the presence of granular globules. Al three phases however differ from the typical fibrin network ultrastructure exhibited by the fibrin networks from non-pregnant individuals.

6.5   DISCUSSION

R. Virchow was the first to formulate the basic concept of the pathophysiology of thrombosis in 1856. [243] Vasculature, blood cells and coagulation factors are the three physiological mechanisms referred to as the Virchow's triad, and are involved in the pathophysiology of thrombosis. Increased thrombosis incidence can be caused by changes in the endothelial lining of the vessel, blood flow or blood coagulability. [222,243]

97

The fibrin network ultrastructure has been shown to influence the progression of disease like thrombosis and vascular disease.[244] Wolberg emphasised the increasing importance of fibrin network in the investigation of health and disease. [245]

Fibrin network formation occurs in a specific manner. Thrombin cleaves fibrinopeptides, forming double stranded half-staggered oligomers. These oligomers lengthen into protofibrils that accumulate and diverge to produce a three dimensional clot network of fibrin networks. The newly formed fibrin strands are stabilized by factor XIII cross-links. This protects the clot from proteolytic assault as well as mechanical stress. However, the mechanical properties of the fibrin clot should still allow the penetration of cells while preventing exsanguination. [244]

Fiber length and diameter are generally directly proportional to each other i.e. increased fiber length is associated with increased fiber diameter. Fiber length and diameter on the other hand are inversely proportional to the degree of branching. Only when branching is minimal will the fibers grow to maximal length and diameter and vice versa. [22]

The complex relationship between the stiffness of the clot and the fibrin structure is dependent on the length and thickness of the fibers, the density of the network along with the amount of branching and cross-linking between the fibers. It is only when the length, diameter, density and degree of branching of the fibers are in equilibrium that the clot will have maximal rigidity. [22,246]

Clot stability includes both the viscoelastic and fibrinolytic properties of the clot. The viscoelasticity maintains the mechanical functions of the clot while it is effectively disintegrate in a timely fashion by the process of fibrinolysis. Both properties are directly affected by the ultrastructure of the fibrin network. [22]

Plasma proteins can directly or indirectly influence fibrin network properties. Alterations in the formation, structure and stability of the fibrin network can occur if the concentration of fibrinogen, fibrin(ogen) binding proteins and thrombin generation is aberrant. [247]

Fibrinogen serves as precursor for the fibrin network [248]. Fibrinogen expression and its interactions with other proteins are essential for homeostasis as well as haemostasis. Wolberg recently reviewed fibrin formation, structure and function. [245,245] She found that alterations in

98

normal fibrinogen structure accompanied by increased formation of fibrin strands and greater stability of the network promote thrombosis. [245]

When fibrin(ogen) synthesis, formation and function is altered both thrombotic and bleeding disorders can occur. [249]

Elevated levels of fibrinogen increase the strength and stability of the clot by increasing the density of the fibers as well as the branch points. [22,249]

Pregnancy is associated with a significant increase in fibrinogen concentration. [11,234,239] This increased fibrinogen levels during pregnancy may be responsible for the altered fibrin network morphology observed. A greater degree of branching point between the multitudes of minor, thin fibers is evident. The fibrinogen concentration however is not the only possible factor to influence the fibrin network morphology. It most likely the collaboration of various factors that affect the formation of the fibrin network.

The fibrin network formation is also indirectly influence by pro- and anticoagulant plasma protein through their involvement in the generation of thrombin in situ. [247]

Thrombin mediates the conversion of fibrinogen to fibrin to form a haemostatic clot. The fibrin network structure is greatly influenced by the concentration of thrombin available during gelation, since both the thickness of the fiber strands as well as the density of the clot are influenced by thrombin. [246,250]

A very small amount of thrombin is necessary for the cleavage of fibrinopeptides and the subsequent catalyse of fibrin polymerization. [246,250] When thrombin concentrations are low thick, loosely-packed fibrin strands are formed with a high susceptibility to fibrinolysis. High concentrations of thrombin on the other hand will produce thin, tightly-packed fibrin fibers that are not as resistant to fibrinolysis. [246,247,250]

Abnormal thrombin generation can lead to variation in fibrin network ultrastructure associated with thrombosis. [250]

Thrombin is integrated into the fibrin clot and can remain clot-bound. Since this activated fibrin-bound thrombin not inhibited by heparin or antithrombin it can potentially have a prothrombotic role in haemostasis. [250] The quality of the fibrin network determines the binding of thrombin to

99

it. More thrombin is trapped in thin fibrin strands typical of the fibrin network associated with the site of vessel injury. [250] Thinner fibrin strands additionally trigger increased synthesis of vWF by endothelial cells. [251]

The risk of re-thrombosis is therefore increased if formation of the primary clot is compromised. [250]

The elevated levels of thrombin associated with pregnancy [237] could therefore be implicated in the ultrastructural changes in the fibrin network seen during pregnancy since a majority of minor, thin fibers are observed. The matted coagulant formation may therefore also be attributed to the increased thrombin concentration. The increased thrombin concentration along with the increase in fibrinogen may play an equal role in the increased formation of the minor, thin fiber network. The thinner fibers, which trap thrombin leading to a greater amount of fibers being formed, also release trigger the release of vWF. The increase in minor, thin fibers may therefore explain the increase in vWF associated with pregnancy.

Lui et al. have stated that both coagulation and fibrinolysis are activated during normal pregnancy. [61]

Fibrinolysis is the breakdown of a fibrin clot. It plays an important role in haemostasis since it prevents occlusion of blood vessels and thus maintaining blood flow. A series of proteins and their specific inhibitors tightly regulate the fibrinolytic system. [244]

Fibrin acts as both cofactor and substrate for plasmin, a fibrinolytic enzyme, during clot dissolution. Therefore the susceptibility of a clot to fibrinolysis is greatly influenced by the fibrin network morphology. [250]

Tissue plasminogen activator (tPA)-mediated plasmin generation will be slower when the fibrin strands are thinner compared to a faster rate when the fibrin network consists of thick fibers. Therefore the overall fibrinolytic activity is reduced for a fibrin network consisting of thinner fibrin fibers.[250]

Thinner fibrin strands are also more resilient to fibrinolysis than thicker fibrin fibers. Although an individual thick fiber is more slowly lysed than a thin fiber, the clots produced by low thrombin concentrations consist of thick, loosely woven fibrin fibers with fewer individual fibers present for

100

a given amount of fibrinogen. These clots therefore have a greater susceptibility to lysis than tightly-packed thin fibers, with subsequently more individual fibers per fibrinogen, produced by higher thrombin concentrations. While plasmin generation occurs on individual fibers, the rate of plasmin activity is limited by the plasmin movement through the three-dimensional fibrin network. [247,250,252,253]

The configuration and especially the number of fibers per volume in a fibrin clot therefore have a greater influence on the rate of fibrinolysis than the fibers as such. It is particularly a fibrin network that is composed of a large amount of thin fibers that readily binds recombinant tissue plasminogen activator (rtPA). The bound rtPA then diffuses into the clot at a slower rate compared to a network consisting of a smaller amount of thicker fibrin strands. [254]

The increased incidence of minor, thin fibers associated with pregnancy therefore influences fibrinolytic activity. The rate of fibrinolysis is decreased since the network is closely packed and plasmin movement through the three-dimensional network is limited.

The increased fibrinogen and thrombin concentrations result in the formation of a matted, tightly-packed network of mainly minor, thin fibers which are more resilient to fibrinolysis. These changes in fibrin network ultrastructure therefore increase the risk of thrombosis, since fibrinolysis is not able control the coagulant formation.

Venous thrombosis or thromboembolism is characteristic of plasma hypercoagulability. Triggered expression of procoagulant activity on intact endothelium is the proposed mechanism. This can be caused by inflammation or reduced blood flow after prolonged immobility. [249]

Venous and/or arterial coagulopathies have been associated to various haematological factors including irregular pro- and anticoagulant protein levels, thrombin concentration, the activity of clotting factors or the resistance of these clotting factors to activation and fibrinolysis inhibitors. [249]

Increased levels of fibrinogen are an invariable risk factor for venous and arterial thrombosis. [249] Increased levels of prothrombin also increase the risk of venous thrombosis. [255]

In pregnancy the risk for venous thrombosis is augmented. A six-fold increase in incidence of venous thromboembolism is associated with pregnancy. [56]

101

The variation in fibrin ultrastructure observed in pregnancy may explain how thrombin and fibrinogen increase the risk of venous thromboembolism. The network of minor, thin fibers trap additional thrombin, laying down more layers of fibrin originating from the increased amount of fibrinogen and may ultimately occlude a vessel. The proposed mechanism of venous thrombosis where procoagulant activity is a trigger on the intact endothelial wall of a blood vessel could be ascribed to the pro-thrombotic condition of the fibrin network observed in this study.

In 2009 Pretorius et al compared the fibrin networks from a healthy pregnant individual and a pregnant individual with dysfibrinogenaemia using scanning electron microscopy. Samples were taken at 30 weeks as well as 3 months post-partum. The fibrin networks of both individuals displayed a fine, dense net of minor, thin fibers distributed evenly over the major, thick fibers. And since this net was not present in healthy non-pregnant individuals or non-pregnant individuals with dysfibrinogenaemia, these changes were specific to pregnancy. Three months post-partum the morphology of the fibrin network returned to normal. Pretorius and her colleagues suggested that this altered morphology could contribute to increased risk of thrombosis since the denser network of minor, thin fibers may influence the rate fibrinolysis.   [256]

It is evident from the SEM micrographs that apparent morphological changes can be seen in early pregnancy, late pregnancy and are still evident 6 – 8 weeks post-partum. This indicates that the coagulation system is activated from the commencement of pregnancy up until at least 8 weeks after birth.

Hormonal changes, especially an increase in estrogen, increase the activity of certain coagulation factors such as XII, VIII, X and IX. [56]

The increase in coagulation factors brought on by the increase in estrogen during pregnancy could be responsible for the formation of granular globules on the fibrin strands.

When thrombin converts fibrinogen to fibrin it cleaves fibrinopeptides from the central domain. This exposes knobs that can then interact with holes that are always exposed at the ends of the molecule. [257]

It may be that the exposed knobs themselves extent or aggregate to give rise to the granular globules, or the altered fibrin structure could yield areas to which clusters of coagulation proteins more readily attach to the fibrin strand.

102

The same granular globules were seen during different phases of the menstrual cycle. It was concluded that estrogen causes the alterations in the internal and external structure of the fibrin strands since it coincide with estrogen peaks in the menstrual cycle.

6.6   CONCLUSION

The coagulation as well as fibrinolytic system is activated during pregnancy. Several procoagulant factors, including fibrinogen, are increased along with elevated concentrations of thrombin and vWF and a decrease in fibrinolytic activity. The increase in estrogen associated with pregnancy may cause the increase in coagulation factors and ultimately the pro-thrombotic state characteristic of pregnancy.

The fibrin networks from pregnancy exhibit a noticeable increase in the formation of minor, thin fibers and prominent coagulant formation. Large quantities of granular globules are also present. Al three phases differ from the typical fibrin network ultrastructure consisting of mainly major, thick fibers and a few minor, thin fibers. There are no apparent differences between early phase, late phase or post-partum fibrin networks. This suggests that activation of the coagulation system commences with pregnancy and this pro-thrombotic state continues till at least 8 weeks after birth.

CHAPTER 7: ULTRASTRUCTURAL ANALYSIS OF PLATELETS DURING THREE PHASES OF PREGNANCY: A QUALITATIVE AND QUANTITATIVE INVESTIGATION

7.1 CHAPTER OBJECTIVES

In this chapter the platelet internal and external ultrastructure of different phases of pregnancy, namely early pregnancy (week 8 – 14), late pregnancy (week 36 – 40) as well as post-partum (week 6 – 8 after birth) will be compared to platelets from non-pregnant individuals as well as each other, to establish whether differences in platelet morphology exist during pregnancy.

7.2 INTRODUCTION

7.2.1 Haemostasis

The coagulation cascade is initiated by thrombin production induced by the activation and aggregation of platelets. [243]

Platelets are small, anucleated blood cells that are discoid in shape. They are derived from megakaryocytes in the bone marrow and have a lifespan of 8 – 14 days. They move freely through circulation until the integrity of the vascular system is compromised. These cells play an integral role in the maintenance of haemostasis since they form the first line of haemostatic defence by formation of the primary haemostatic plug. [219,228]

Upon vessel injury a series of events trigger the formation of a platelet-rich clot. Platelet function during haemostasis can be divided into four parts namely adhesion, activation, secretion and aggregation. [219]

The adhesion of platelets to the exposed subendothelial connective tissue of the injured vessel is a critical step in primary haemostasis. von Willebrand factor (vWF) acts as the link between the subendothelium and platelet receptors. [221]

Following platelet adhesion, various biochemical agonists like collagen and thrombin along with platelet-produced agonists like adenosine diphosphate (ADP) bind to glycoprotein receptors. Platelets are subsequently activated. The activated platelets will undergo structural changes. The platelets become more spherical and the formation of pseudopodia is initiated to increase surface area for interaction between platelets. [219]

104

Two types of storage granules are contained within platelets. Alpha granules enclose platelet-specific proteins like thombospondin and coagulation proteins like factor V. Dens granules, or dense bodies, hold non-metabolic adenines like ADP along with divalent cations like 5-hydroxytryptamine (5-HT, serotonin). [219] Once the platelet shape is altered the platelets start to secrete the contents of their internal granules. Platelets also produce elevated amounts of thromboxane A2 (TxA2) through the metabolism of platelet arachidonic acid. The released products stimulate the activation of additional platelets. The activated platelets spread out on the surface of the subendothelium and subsequently activate and recruit more platelets through their released products. [220,221]

The activated platelets aggregate to form a platelet-rich plug at the site of injury. Fibrin fibers are employed to reinforce the plug. [221]

7.2.2 Platelet morphology

Platelets contain various types of organelles. Three particular granule populations can be distinguished and these contain several kinds of constituents. [258]

7.2.2.1 Granule populations

Alpha granules

With a diameter of 200-400nm, the alpha granules are the largest granules found in human platelets. The size and abundance of these single membrane rotund organelles afforded them the name of alpha granules (or α-granules). A collection of large adhesive proteins as well as proteins involved in tissue repair and cell-matrix interaction are contained within the alpha granules. These constituents are released at the location of vascular damage upon degranulation. [258-260]

Dense granules

These hefty, electron dense granules are also referred to as delta granules (δ-granules) or dense bodies. They are the smallest granules found in the platelet cytoplasm, with a diameter of only 150nm. They are dense core granules enveloped within a translucent region surrounded by a single membrane. Dense granules hold non-protein particles like ADP, calcium and serotonin. These molecules are responsible for the recruitment of additional platelets to the injury site. [258-260]

105

Lysosomal granules

The third type of granule population is referred to as the lysosomal or λ-granules. The size of these granules is intermediate to that of the alpha granules and dense granules, with an average diameter of around 200nm. Lysosomal granules contain exclusively acid hydrolases proficient for the hydrolytic degradation of platelet aggregates in circulation. These acid hydrolases are only released upon strong stimulus. [258-260]

Table .1 Platelet granules and cytoplasmic content.

Alpha granules	Dense granules	Lysosomes
Platelet specific proteins	**Nucleotides**	**Glycosidases**
vWF	*ADP and ATP*	
Thrombospondin-1 (TSP-1)	*GDP and GTP*	
Fibronectin		
Platelet factor 4 (PF-4)		
Mitogenic factors	**Amines**	**Proteases**
Platelet-derived growth factor (PDGF)	*Serotonin*	
	Histamine	
Coagulation factors	**Bivalent cations**	**Cationic proteins**
Fibrinogen	*Mg^{2+}*	
Factor V, VII, XI, XIII	*Ca^{2+}*	
Protease inhibitors		
Plamsinogen activator inhibitor-1 (PAI-1)		
Tissue factor pathway inhibitor (TFPI)		
Granule membrane-specific proteins		
P-selectin (CD62P)		
CD63		

[260,261]

106

7.2.2.2 Other components

Mitochondria

Only a few mitochondria are found within a platelet, and their average diameter is approximately 500nm. Mitochondria provide energy necessary for platelet reactivity. [258] Since anaerobic glycolysis has no effect on the level of ATP in the platelet, the entire energy requirement of the platelet can solely be supported by mitochondria. [262]

Glycosomes

Glycosomes are similar in size to alpha granules. They have a round to oval shape and contain glycogen. [262] Under basal settings accumulated glucose not employed for metabolic purposes is converted into glycogen. [263] Glucose is the main source of energy for platelets. [262]

Membranous system

The membranous system comprises of two subunits namely the open canalicular system (OCS) and the dense tubular system (DTS). The OCS channels are the connection between the cytosol of the platelet and the surrounding region while the channels of the DTS store essential metabolic enzymes. [259]

The OCS is also referred to as the surface-connected canalicular system (SCCS). The dilated channels of the OCS permeate the cytosol within the platelet and demarcated openings are found on the surface of the platelet membrane. Most granular constituents are released into the OCS during platelet activation. The OCS is also implicated in the accumulation and transport of fibrinogen to the alpha granules from the plasma. [264]

The dense tubular system forms the messenger pathways responsible for platelet reactivity. [258]

7.2.3 Platelets and pregnancy

During normal pregnancy platelet aggregation and secretion of ATP are increased compared to non-pregnant individuals, reaching a prominent peak in the third trimester but returning to normal by 6 – 12 weeks post-partum. [67,265] Platelet binding to fibrinogen remains unchanged. [265,266]

107

Fitzgerald et al. indicated an elevated biosynthesis of thromboxane during pregnancy. They concluded that this increase was caused by platelet release of thromboxane and that it was consistent with platelet activation associated with pregnancy. [267]

Platelet count remains normal during pregnancy, although benign gestational thrombocytopenia may present in the third trimester. Platelet activation along with β-thromboglobulin and PF-4 release are also found. Platelet and fibrinogen consumption is increased during delivery. Most haemostatic changes return to normal within 6 weeks after birth, however, the platelet count and protein S levels may be altered for a longer period. [232]

Most research concerning platelets and pregnancy do not focus on normal pregnancy. The majority focus is on preeclampsia [65,266,268-270], HELLP syndrome [271,272] and hypertension [69,273].

A Pubmed Central search yielded no research focusing on the platelet internal and external structure observed with scanning and transmission electron microscopy during normal pregnancy.

To sum up, platelets play an important role in primary haemostasis. Platelets adhere to the injury site and are activated. The activated platelets will then secrete there granular constituents to employ additional platelets to form a platelet aggregate known as the primary haemostatic plug. [219]

Various granule populations are found within platelets. Alpha granules contain proteins involved in coagulation, adhesion and wound healing. Dense granules mainly contain ADP, calcium and serotonin. Alpha and dense granules release their constituents upon platelet activation while the lysosomal granules secrete acid hydrolyses only upon strong stimuli. [259,260]

Since very little is known about platelet morphology during normal pregnancy, this study aimed to establish platelet internal and external structures during pregnancy.

108

7.3   MATERIALS AND METHODS

The internal and external changes in platelet ultrastructure from different phases of pregnancy were analyzed by preparing the samples for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described in Laboratory procedures under 3.3.2.2 Blood plasma: *Platelets* in Chapter 3.

7.4   RESULTS

7.4.1   Scanning electron microscopy

109
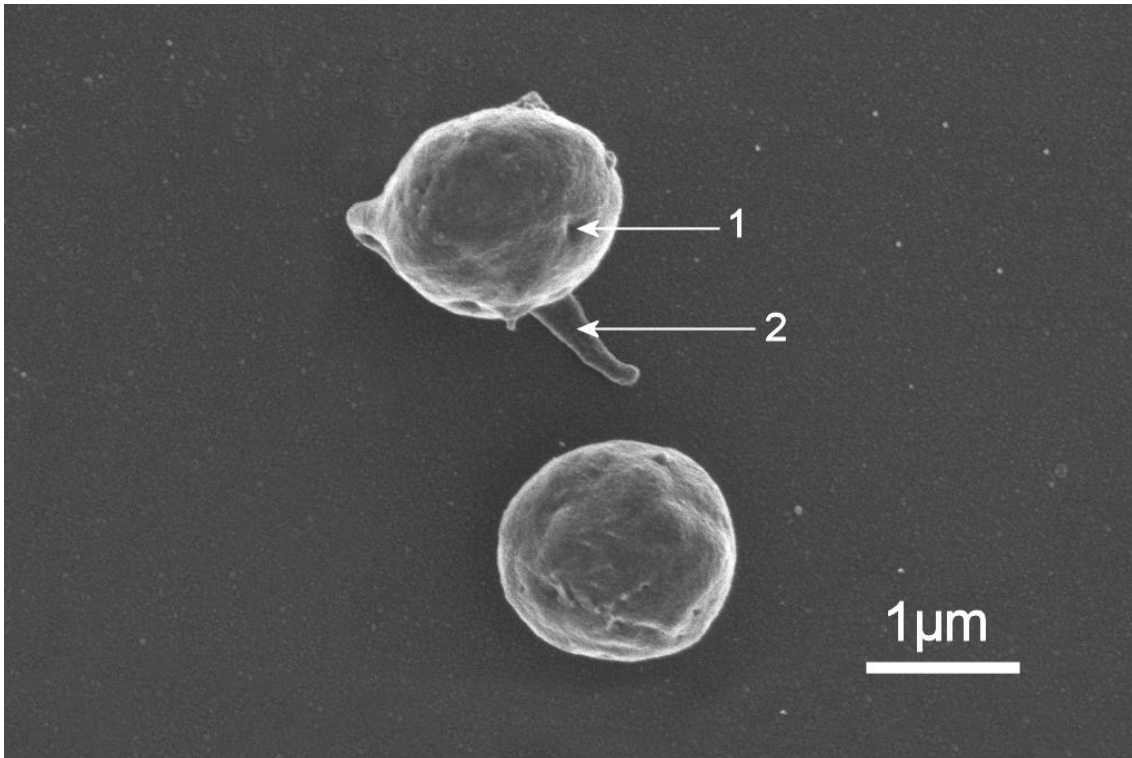
Figure 1.A Control platelets.
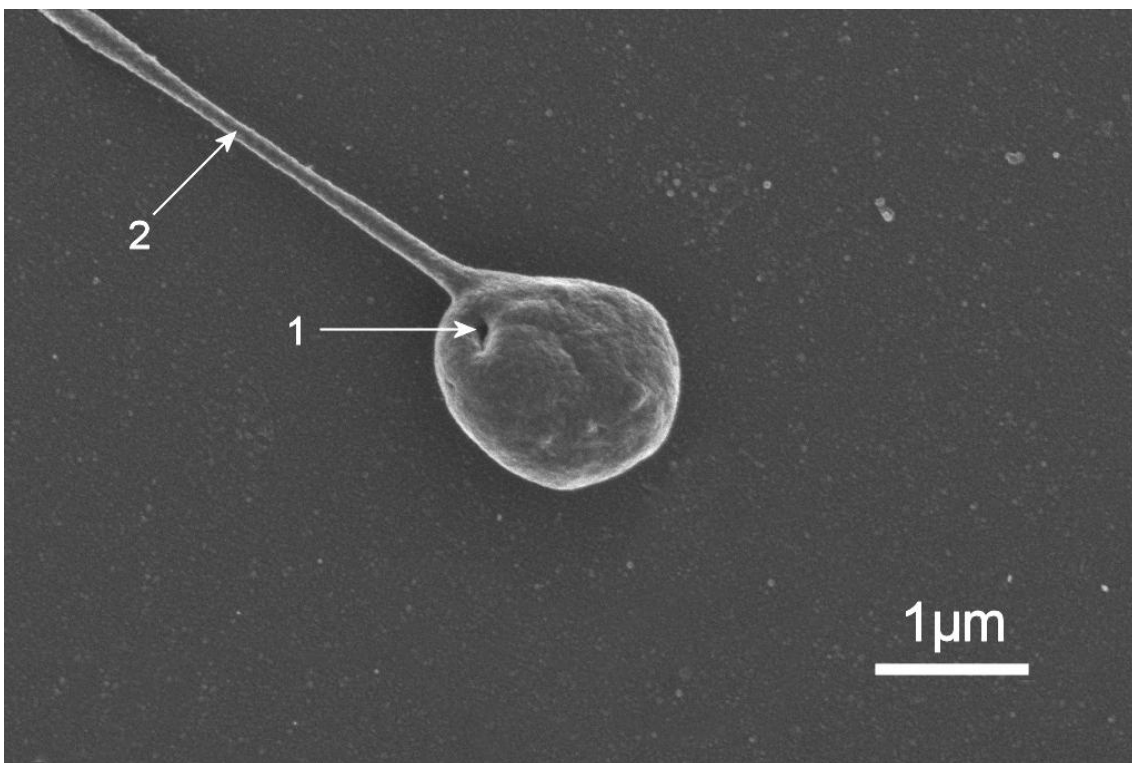
Figure 1.B Control platelet.

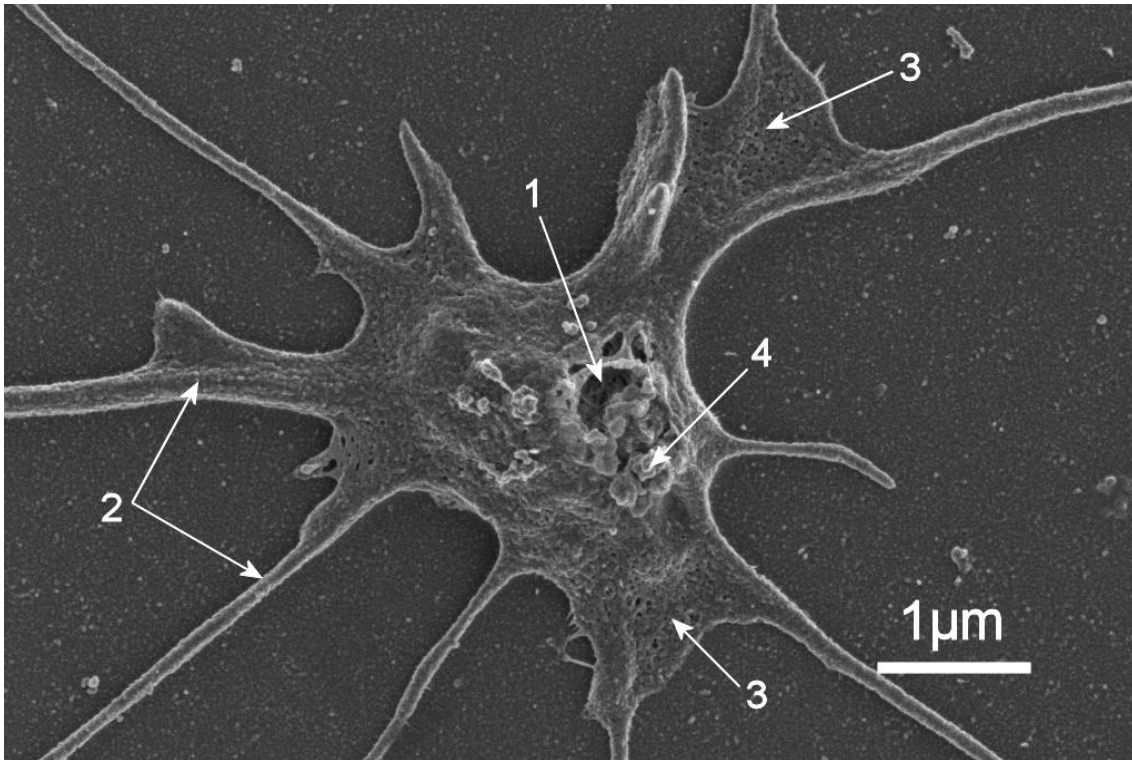| 1 – Open canalicular system	3 – Platelet spreading
2 – Pseudopodia	4 – Membrane blebbing

10

Figure 2.A Platelet of early pregnancy participant.
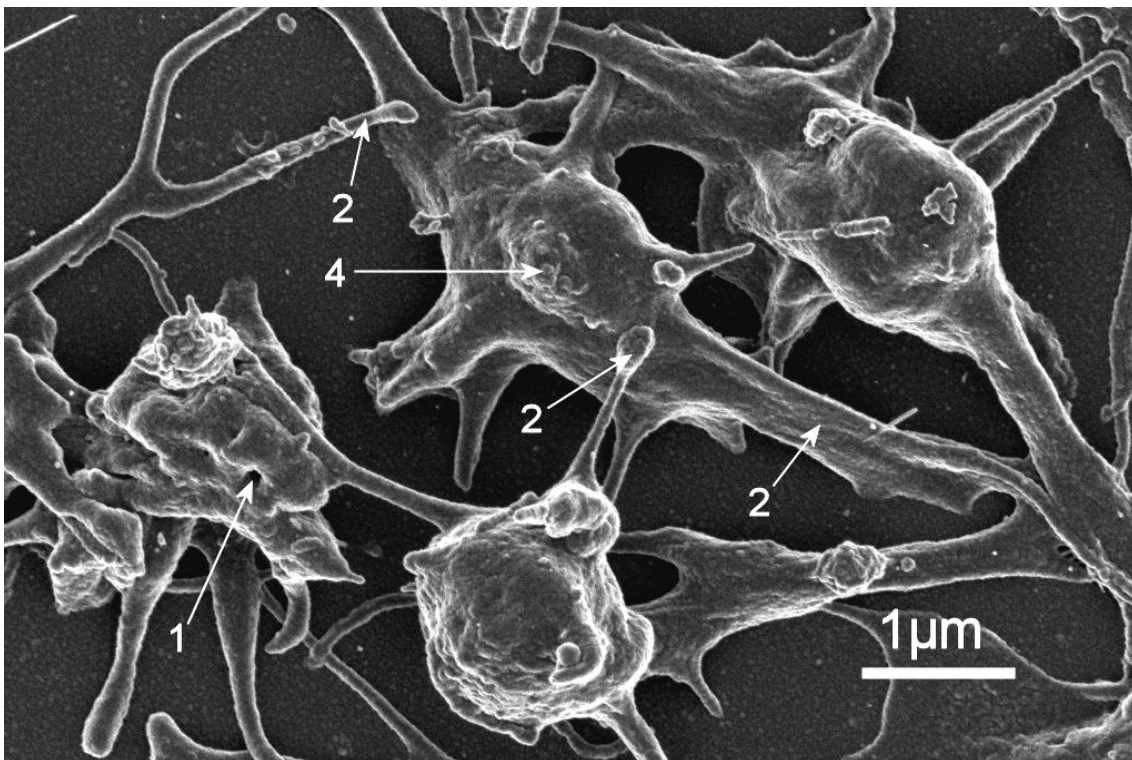
Figure 2.B Platelets of early pregnancy participant.

1 – Open canalicular system		3 – Platelet spreading	
2 – Pseudopodia		4 – Membrane blebbing	

11

112
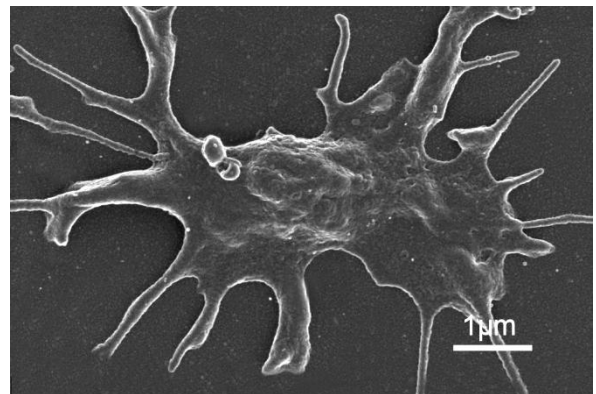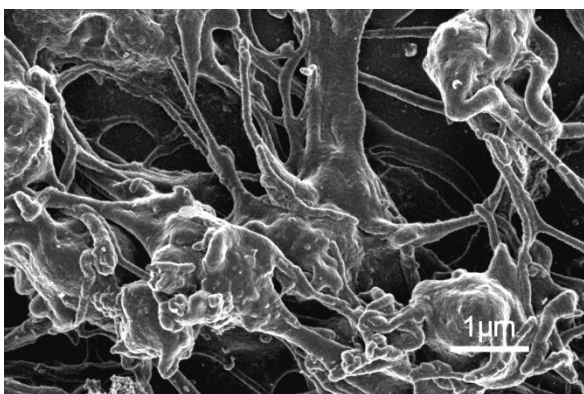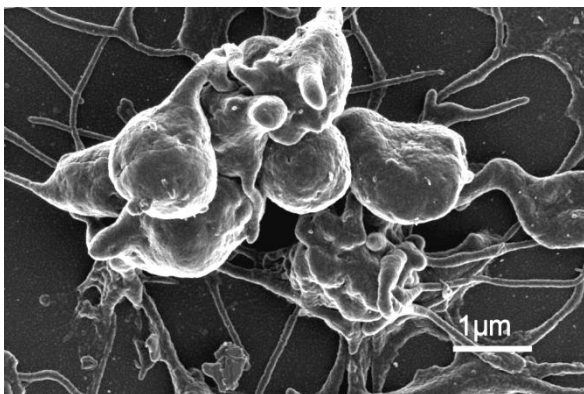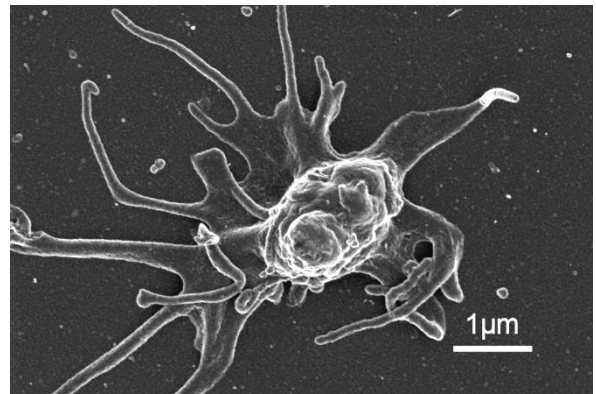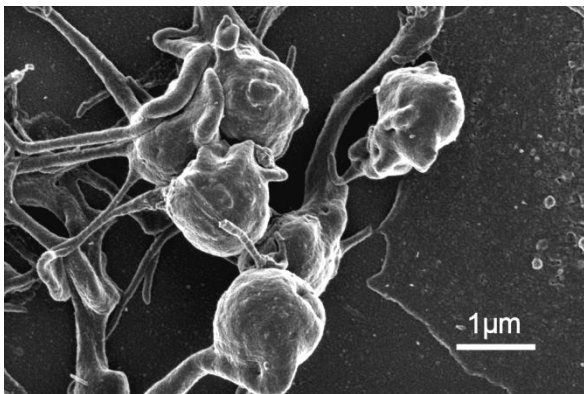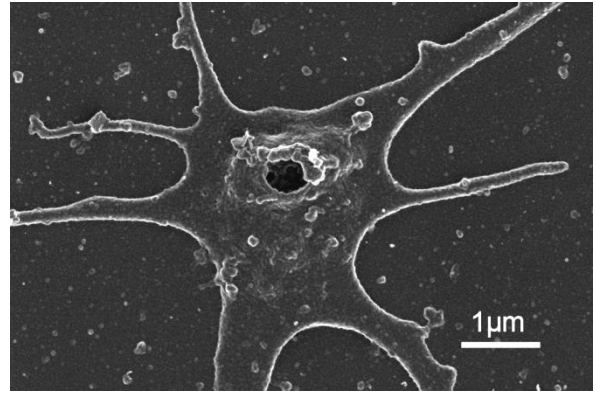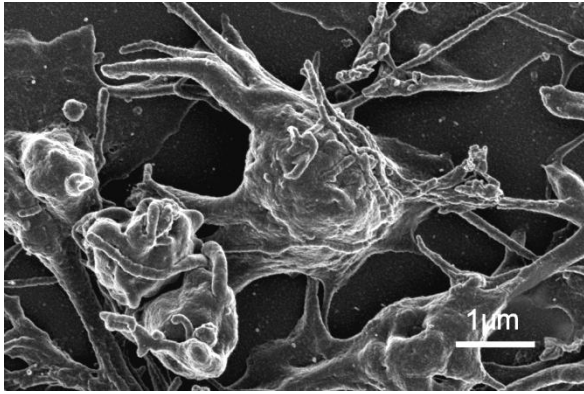
Plate 1. Platelets of early pregnancy participants.
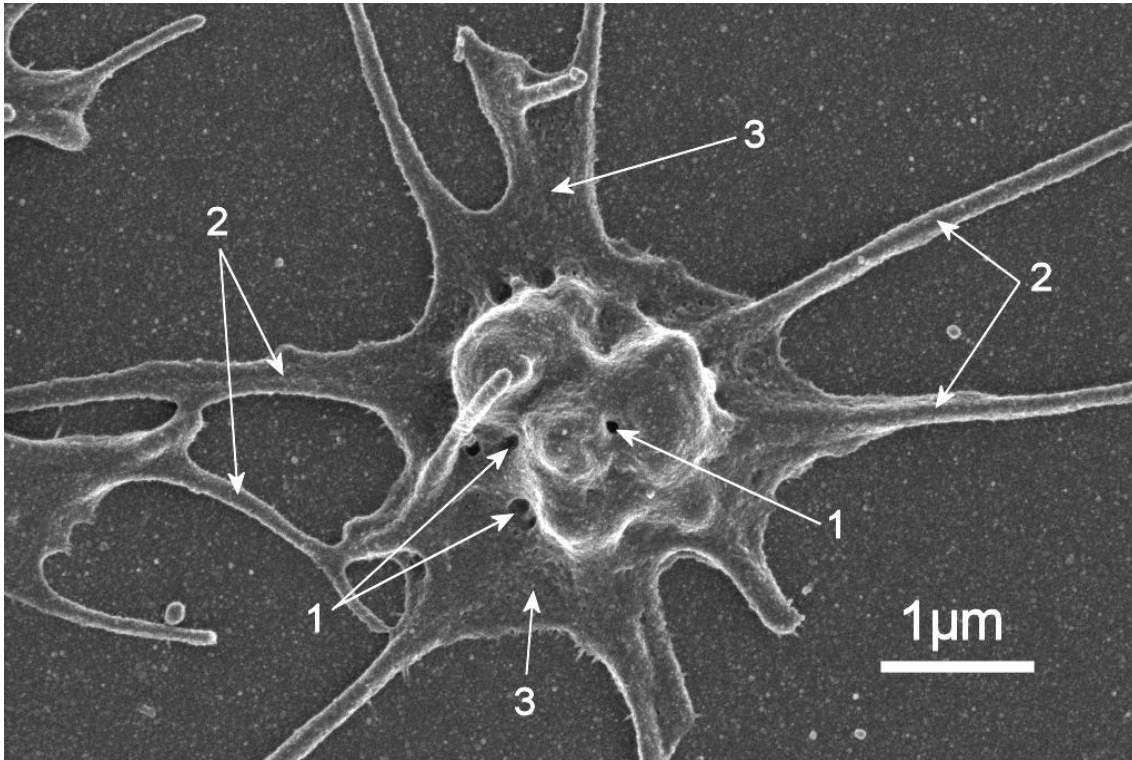
Figure 3.A Platelet of late pregnancy participant.
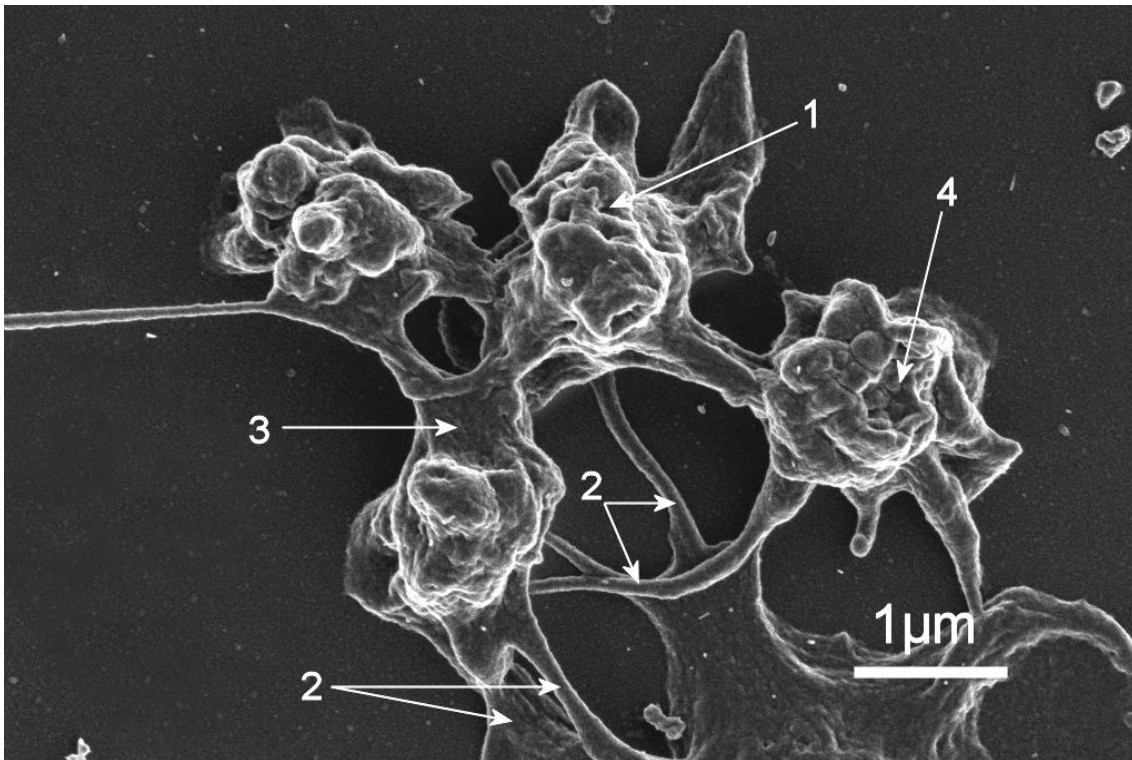
Figure 3.B Platelets of late pregnancy participant.
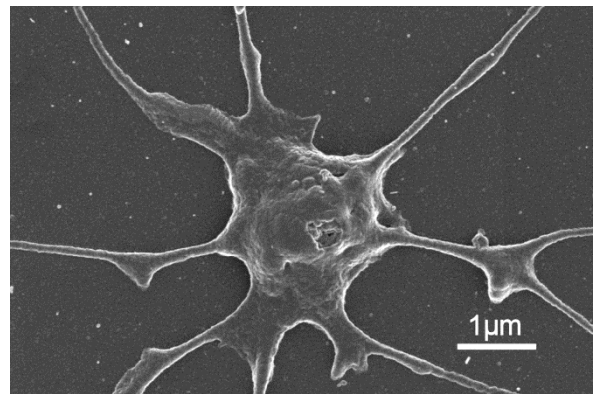
1 – Open canalicular system	3 – Platelet spreading
2 – Pseudopodia	4 – Membrane blebbing

13

114
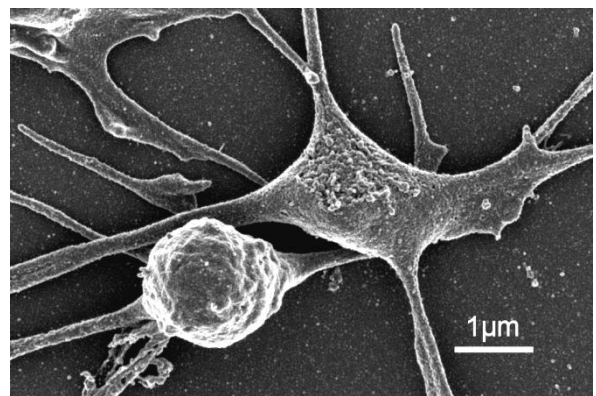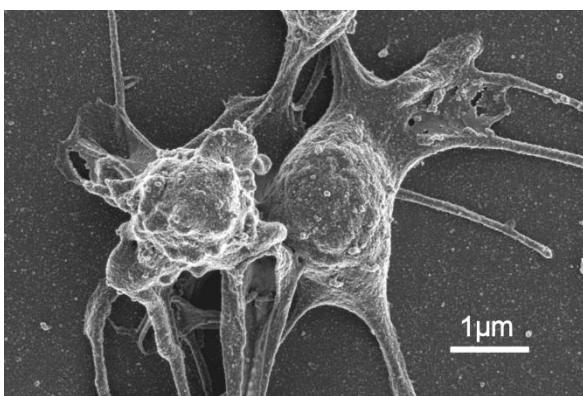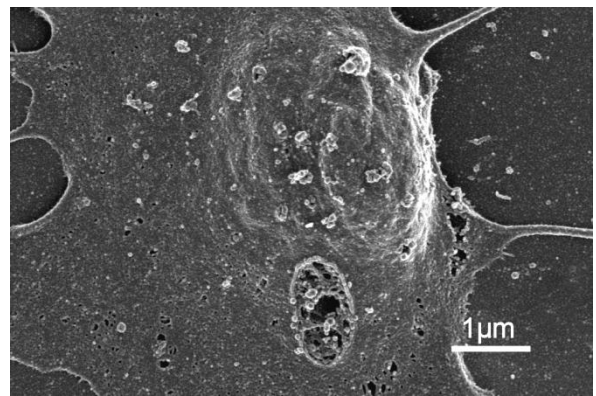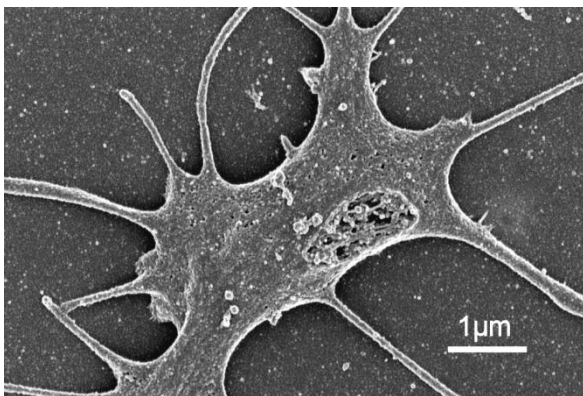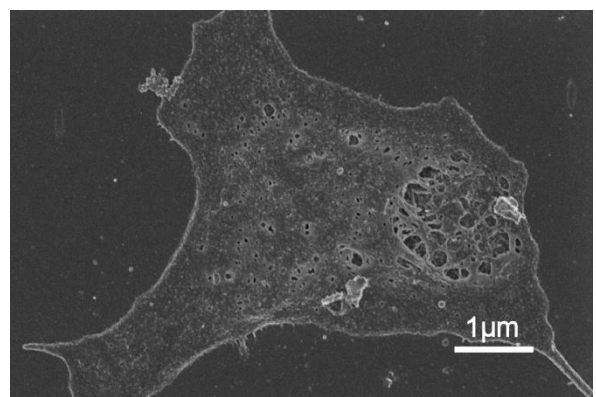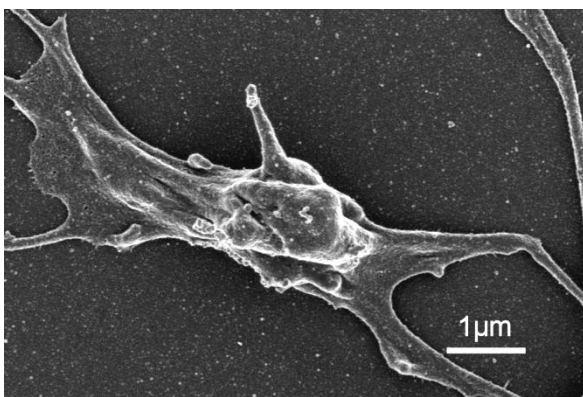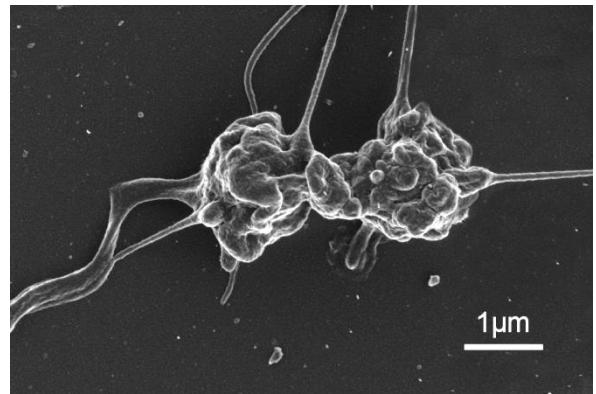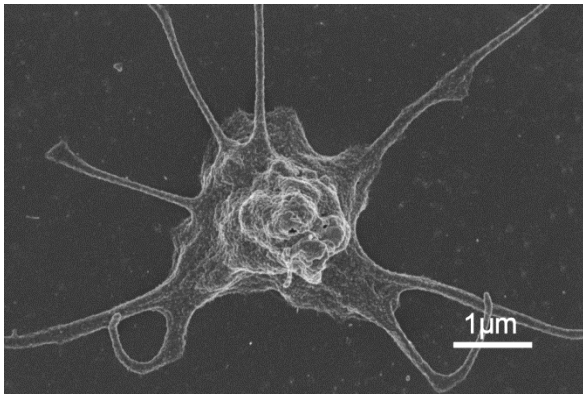
Plate 2. Platelets of late pregnancy participants.
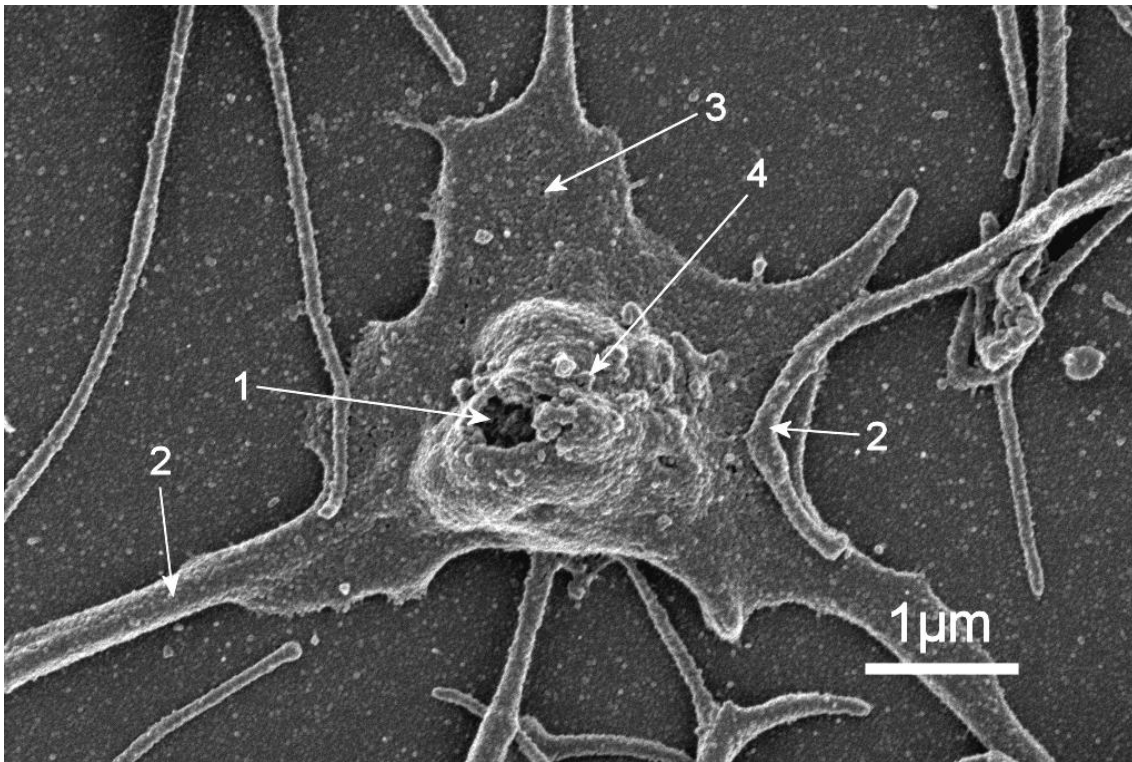
Figure 4.A Platelet of post-partum participant.
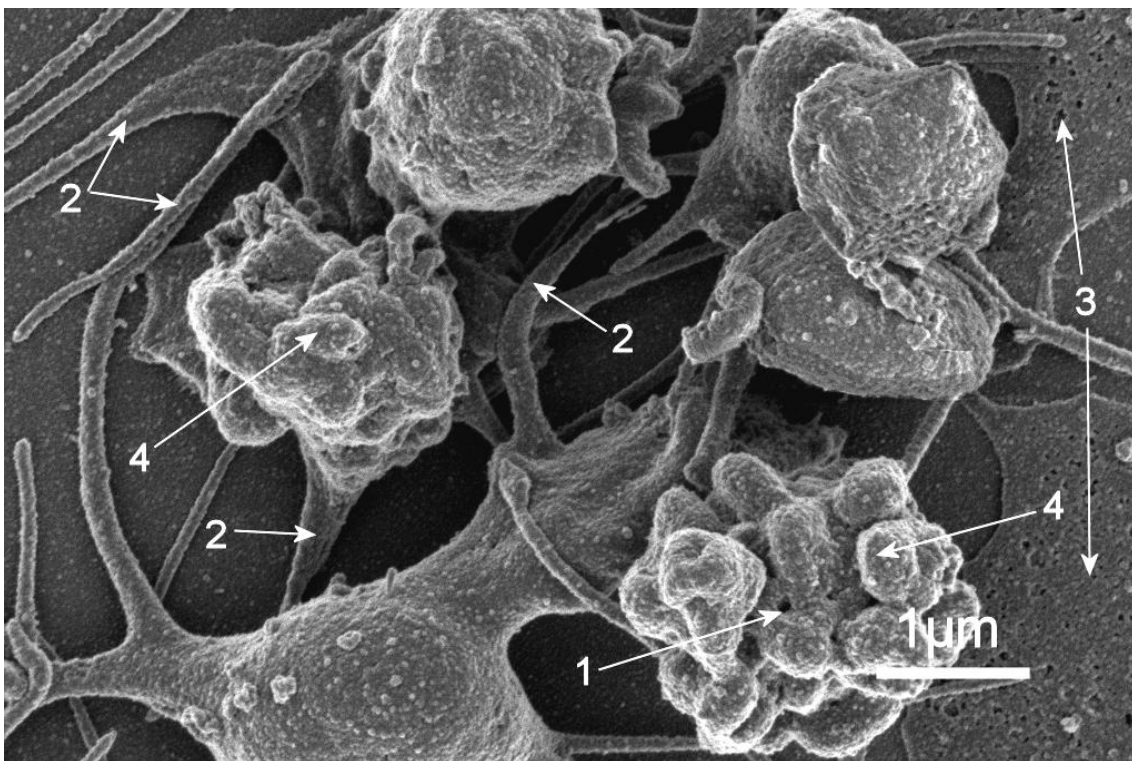
Figure 4.B Platelets of post-partum participant.

115

1 – Open canalicular system	3 – Platelet spreading
2 – Pseudopodia	4 – Membrane blebbing

116
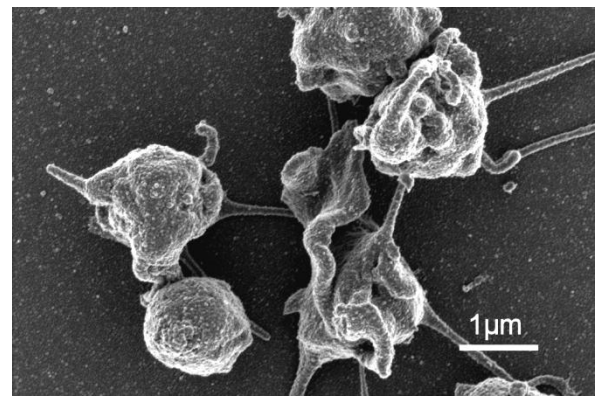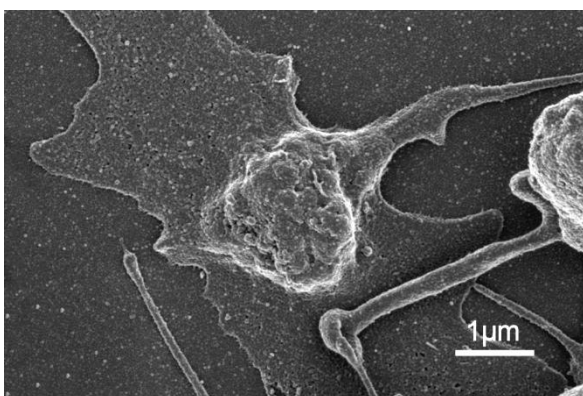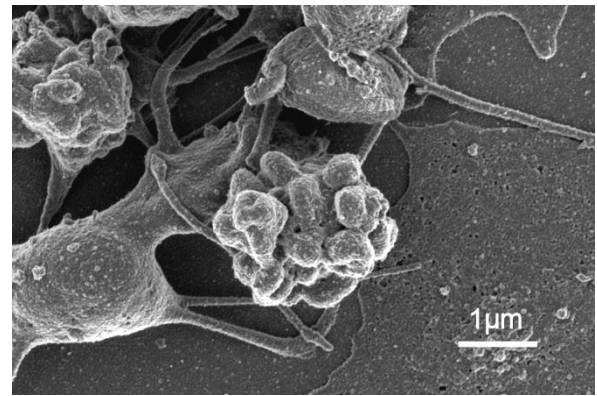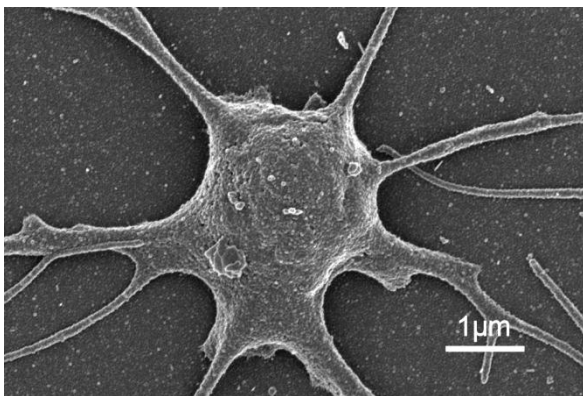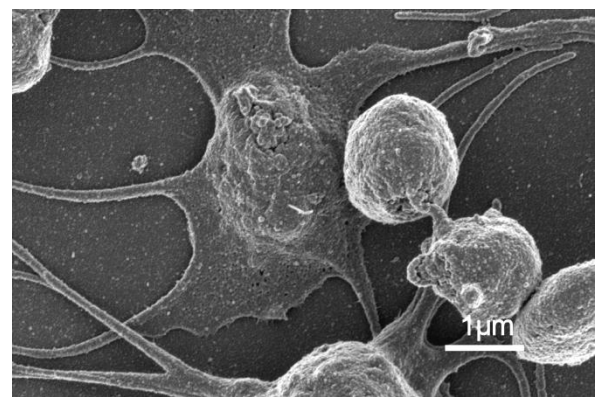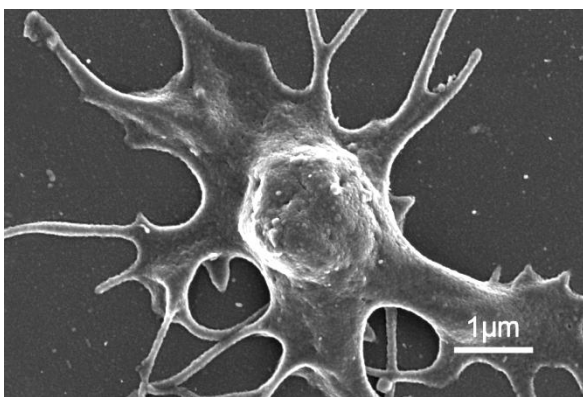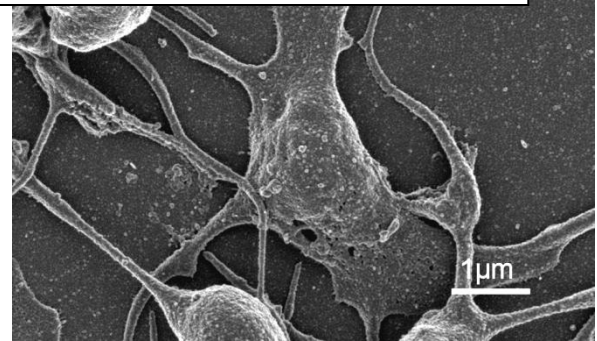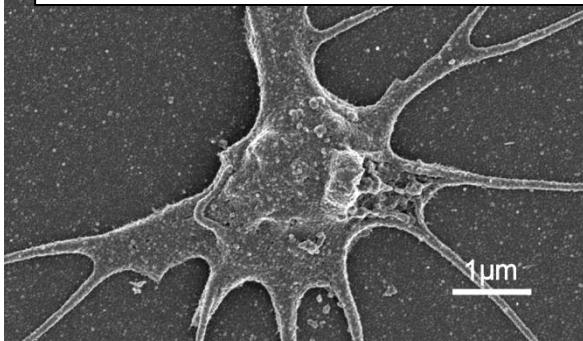
Plate 3. Platelets of post-partum participants.

Figure 1.A and Figure 1.B are SEM micrographs of platelets from the non-pregnant participants. They are typical control platelets. They are spherical and have no or only one pseudopodium extending from the body of the platelet.   A few small pores of the open canalicular system are also shown on the membrane.

Figure 2.A shows a platelet from an early pregnancy participant. Figure 2.B shows a collection of platelets also associated with early pregnancy. The pores of the open canalicular system are much larger in Figure 2.A when compared to the control platelets. Several pseudopodia extend from the body of all the platelets, and in Figure 2.B the interaction of these pseudopodia with the adjacent platelets is clearly visible.   These platelets are not as compact as the control platelets but are more spread out. Blebbing of the membrane is also seen in both figures. The additional micrographs in Plate 1 confirm the described morphology.

Figure 3.A is a platelet from a participant in the late pregnancy phase. Figure 3.B shows the interaction between several platelets from a late pregnancy participant. Additional pores of the canalicular system are exhibited in Figure 3.A. Again several pseudopodia extending from the platelet body and Figure 3.B once again shows interaction of the pseudopodia with adjoining platelets. Platelet spreading is also exhibited along with platelet membrane blebbing. Plate 2 verifies the described changes in morphology.

Figure 4.A and Figure 4.B show a single platelet and several platelets interacting respectively. Both micrographs represent the platelets from participants 6 – 8 weeks after birth. A large open canalicular system pore can been seen in Figure 4.A. Several extended pseudopodia and platelet spreading are again prominent. Membrane blebbing is also seen. Plate 3 confirms the ultrastructural changes described.

7.4.2 Transmission electron microscopy
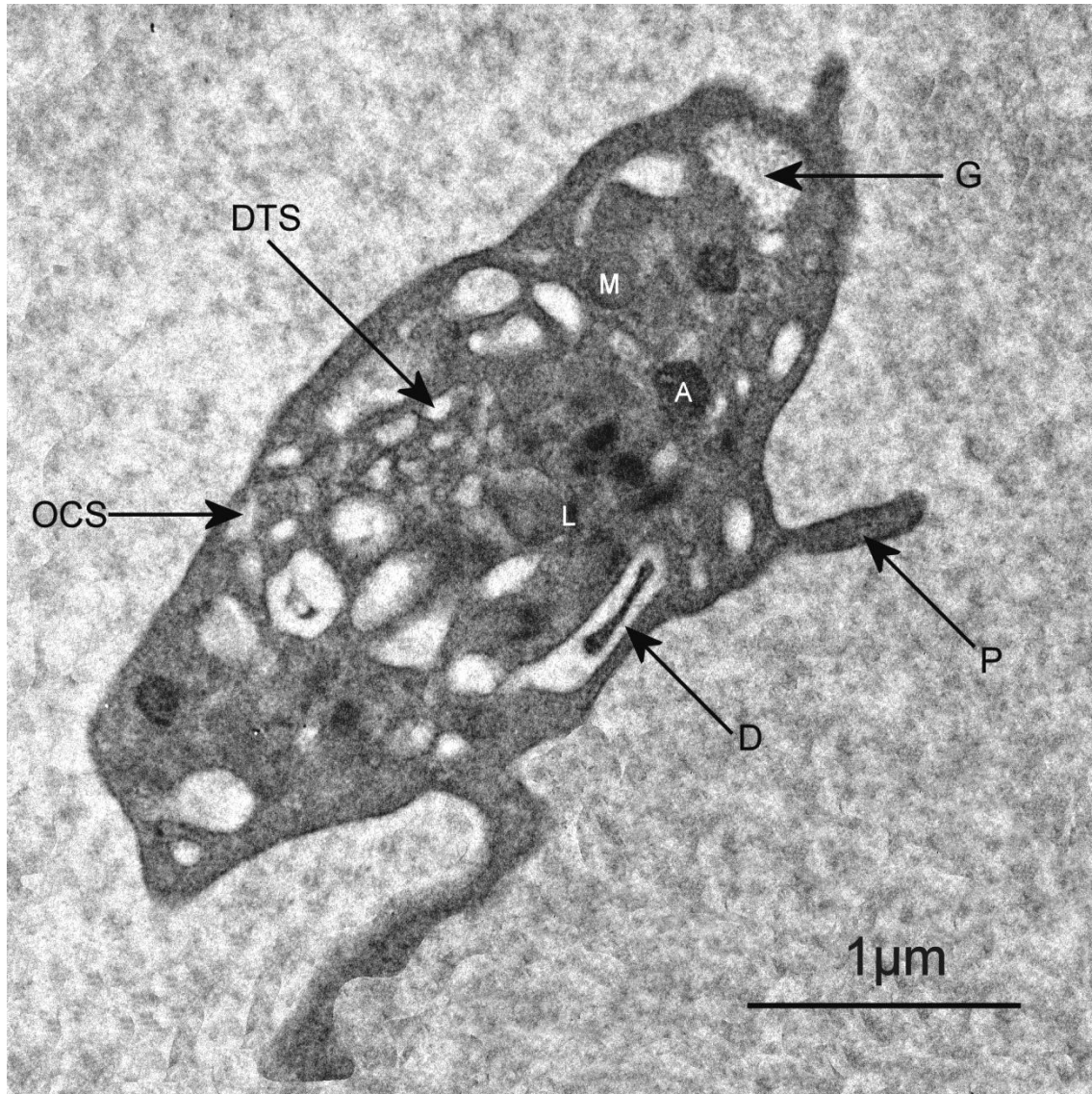
Figure 5. Control platelet.

|	
A – Alpha granules	G – Glycogen
D – Dense granules	DTS – Dense tubular system
L – Lysosomal granules	OCS – Open canalicular system
M – Mitochondria	

118
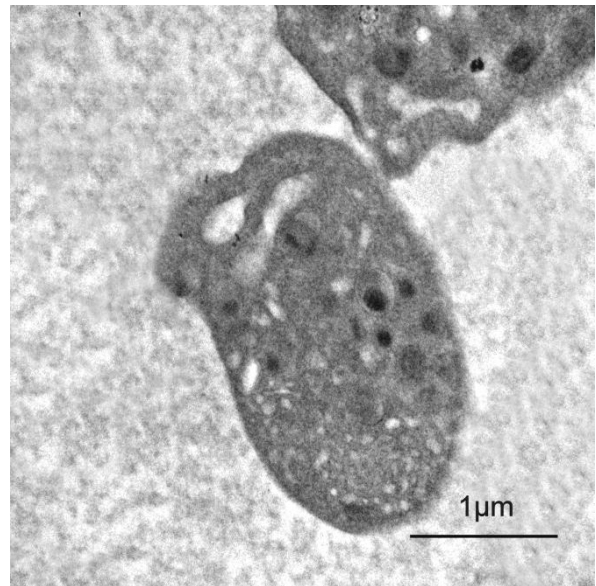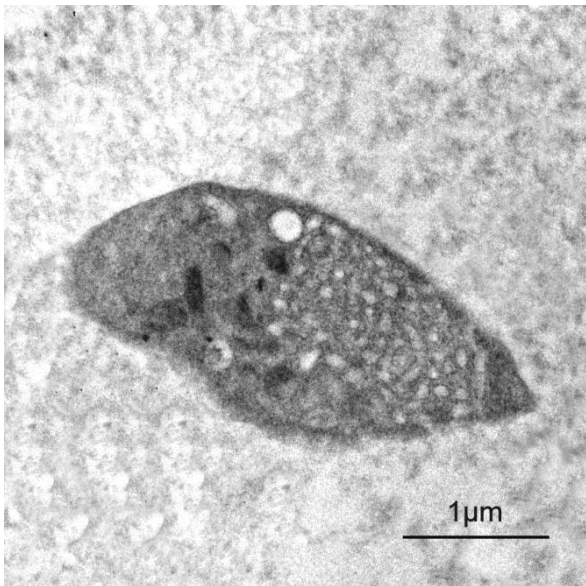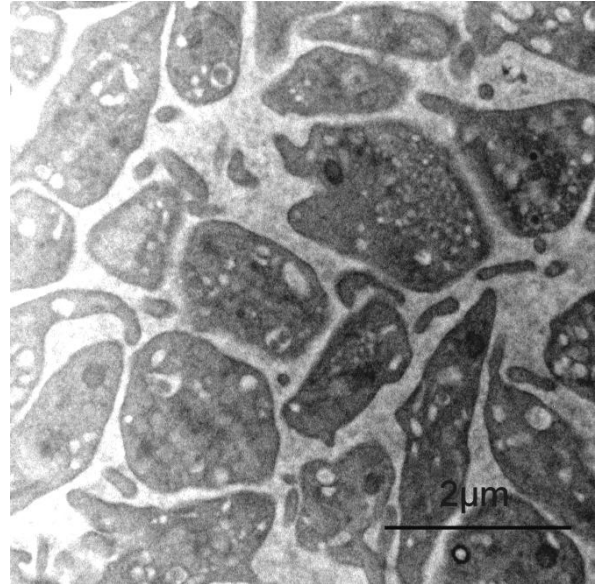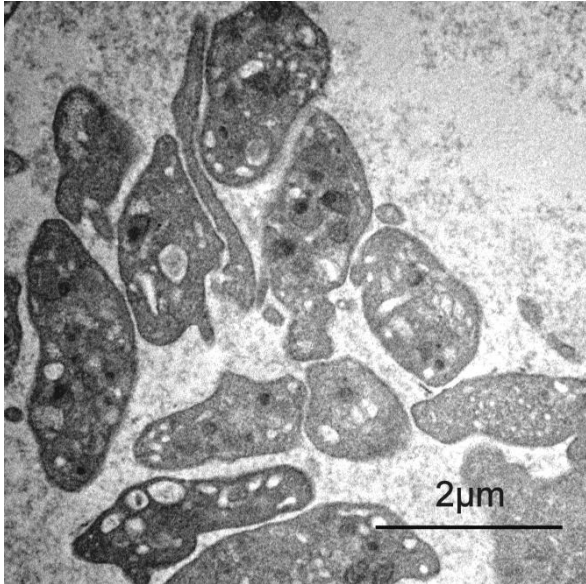
Plate 4. Platelets of control participants.
Machine magnificiation=30 000x

Figure 6. Platelet of early pregnancy participant.

A – Alpha granules	G – Glycogen
D – Dense granules	DTS – Dense tubular system
L – Lysosomal granules	OCS – Open canalicular system
M – Mitochondria	

120

Plate 5.A Platelets of early pregnancy participants.
Machine magnificiation=15 000x

Plate 5.B Platelets of early pregnancy participants.
Machine magnificiation=30 000x

Plate 5.C Platelets of early pregnancy participants.
Machine magnification=60 000x

123

Figure 7. Platelets of late pregnancy part*icipant.*

A – Alpha granules	G – Glycogen
D – Dense granules	DTS – Dense tubular system
L – Lysosomal granules	OCS – Open canalicular system
M – Mitochondria	

124

Plate 6.A Platelets of early pregnancy participants.
Machine magnification=15 000x

125

Plate 6.B Platelets of early pregnancy participants.
Machine magnification=30 000x

126

Plate 6.C Platelets of early pregnancy participants.
Machine magnificiation=60 000x

Figure 8. Platelet of post-partum participant.

A – Alpha granules	G – Glycogen
D – Dense granules	DTS – Dense tubular system
L – Lysosomal granules	OCS – Open canalicular system
M – Mitochondria	

128

Plate 7.A Platelets of post-partum participants.
Machine magnificiation=15 000x

Plate 7.B Platelets of post-partum participants.
Machine magnificiation=30 000x

© University of Pretoria

Plate 7.C Platelets of post-partum participants.
Machine magnificiation = 60 000x

132

Figure 5 shows the internal structure of a platelet from a non-pregnant participant. This is the typical composition of internal compounds from a healthy individual. Alpha granules, dense granules and lysosomal granules can be distinguished. Glycogen and mitochondria are present. The OCS and DTS are seen. A few pseudopodia are also visible. Plate 4 shows more platelets from non-pregnant participants. The platelets are loosely-packed together and each platelet can be distinguished separately.

Figure 6 shows the platelet internal structure of an early pregnancy participant. No mitochondria or glycogen can be distinguished. The OCS and DTS are also not visible. More alpha granules can be seen with only one dense body. Lysosomal granules are present. In Plates 5.A – C the same increase in alpha granules and lysosomal granules occur with a decrease in dense granules and mitochondria are visible. The platelets are also closely packed together.

Figure 7 shows the internal constituents of platelets from the late pregnancy phase. Again no mitochondria, glycogen or channels from the OCS or DTS are visible. An increased amount of alpha granules and decreased amount of dense granules are again exhibited. Lysosomal granules can be seen. Plates 6.A – C show an increased occurrence in alpha granules and lysosomal granules with decreased incidence of the other two components. The platelets are again packed closely together.

Figure 8 shows platelets from post-partum participants. No dense bodies can be seen but lysosomal granules are present. No mitochondria and glycogen is seen. The channels of OCS and DTS are also not visible. An increase in alpha granules is again evident. Plates 7.A – C show the closely packed platelets with increased incidence of alpha granules and lysosomal granules.

7.4.3 Statistical analysis

The internal components, specifically the alpha granules, dense granules, lysosomal granules as well as mitochondria, of a 100 platelets were counted for each of the following groups: controls, early pregnancy, late pregnancy and post-partum. All values in Table 2 represent mean ± standard deviation. Statistical modelling was performed by employing a computerised software system (IBM SPSS Statistics 21) for statistical analysis of the data. One-way ANOVA was used to compare results. A level of significance was set at $p < 0.05$.

Figure 9, 10, 11 and 12 show the differences in mean values of each platelet component.

133

Table 2. Mean values of different platelet components in non-pregnancy and different phases of pregnancy.

	Control	Early pregnancy	Late pregnancy	Post-partum
Alpha granules	3.24 ± 2.32	11.67 ± 5.21	12.94 ± 6.26	10.39 ± 5.21
Dense granules	1.46 ± 1.34	0.99 ± 1.15	0.37 ± 0.75	0.23 ± 0.53
Lysosomal granules	3.09 ± 2.52	5.67 ± 3.35	8.53 ± 3.37	9.12 ± 3.79
Mitochondria	0.75 ± 0.93	0.12 ± 0.46	0.18 ± 0.56	0.08 ± 0.27

134

Figure 9. Alpha granule content of platelets from non-pregnant individuals and different phases of pregnancy.

135
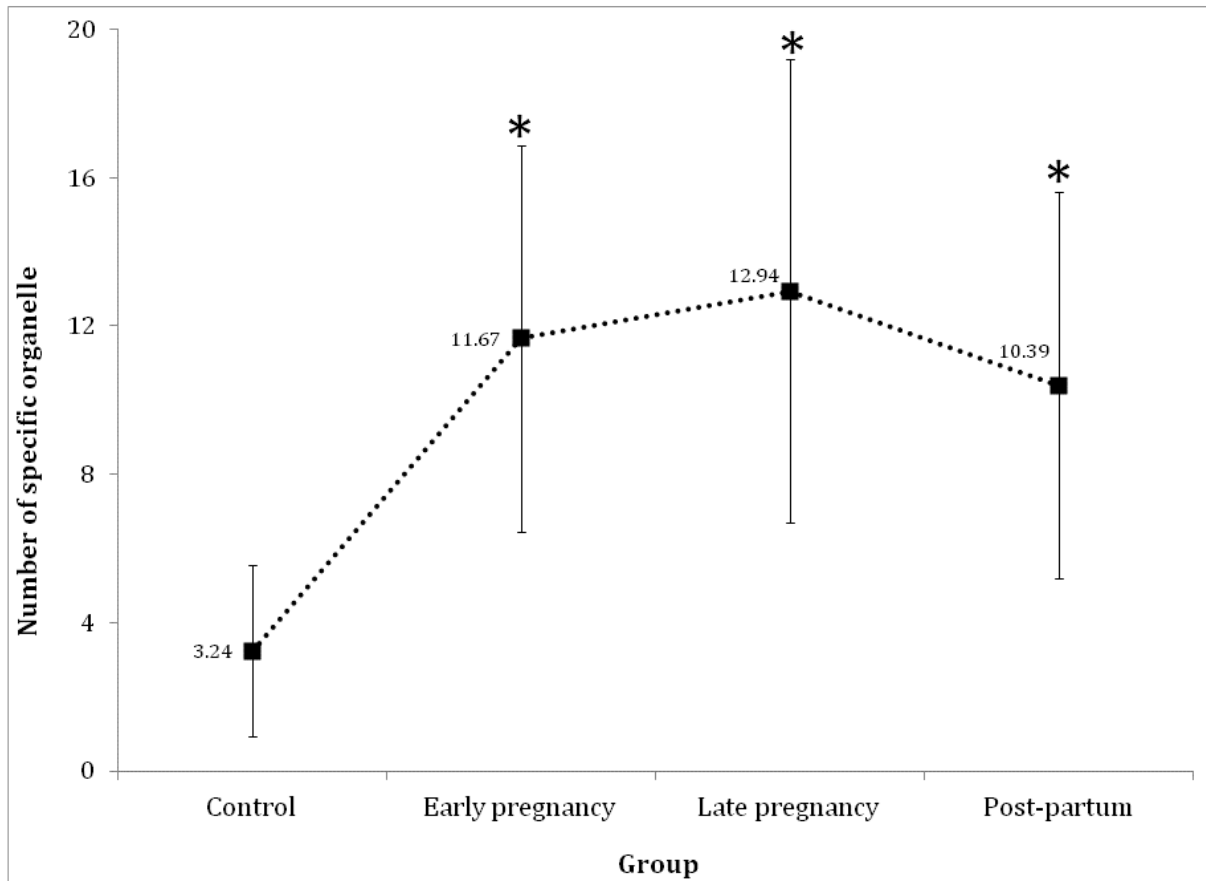
Figure 10. Dense granule content of platelets from non-pregnant individuals and different phases of pregnancy.

136
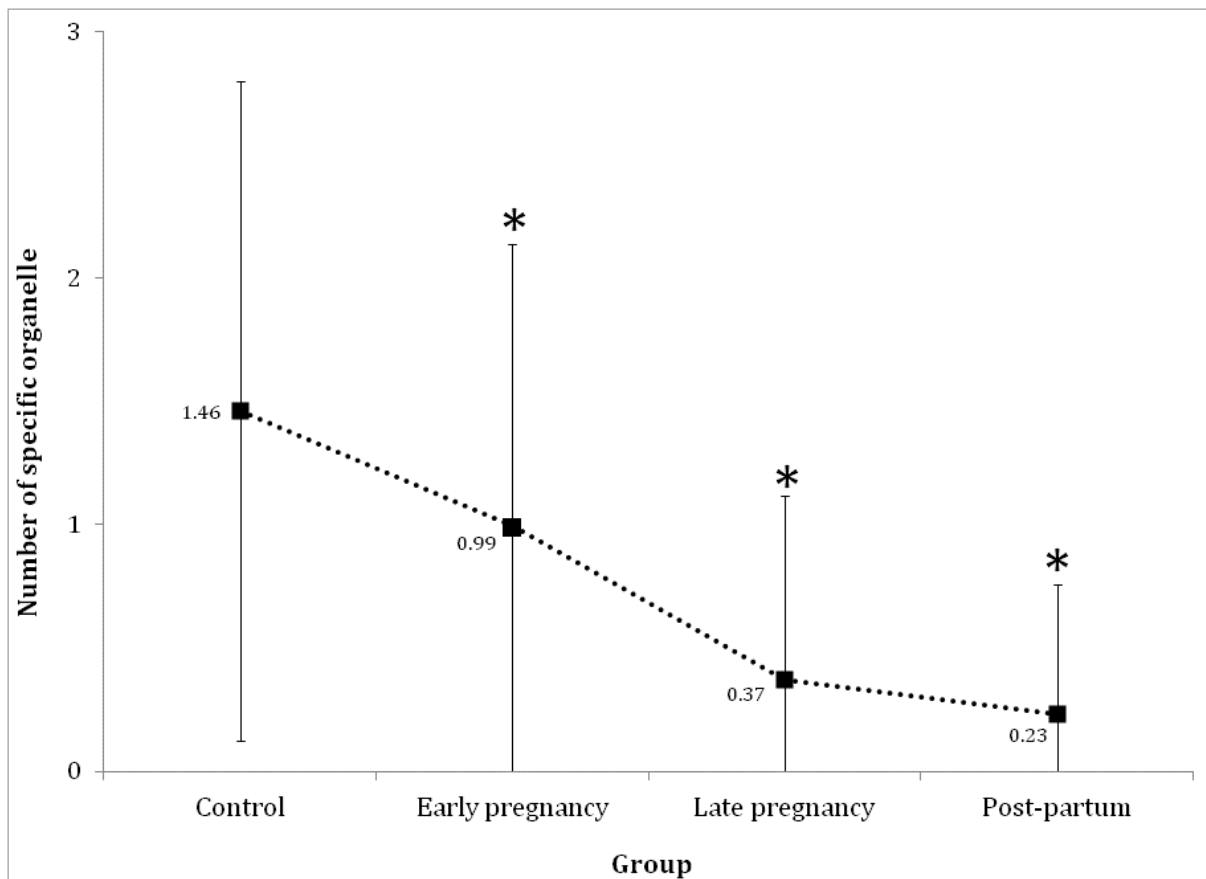
Figure 11. Lysosomal granule content of platelets from non-pregnant individuals and different phases of pregnancy.

137

Figure 12. Mitochondrial content of platelets from non-pregnant individuals and different phases of pregnancy.
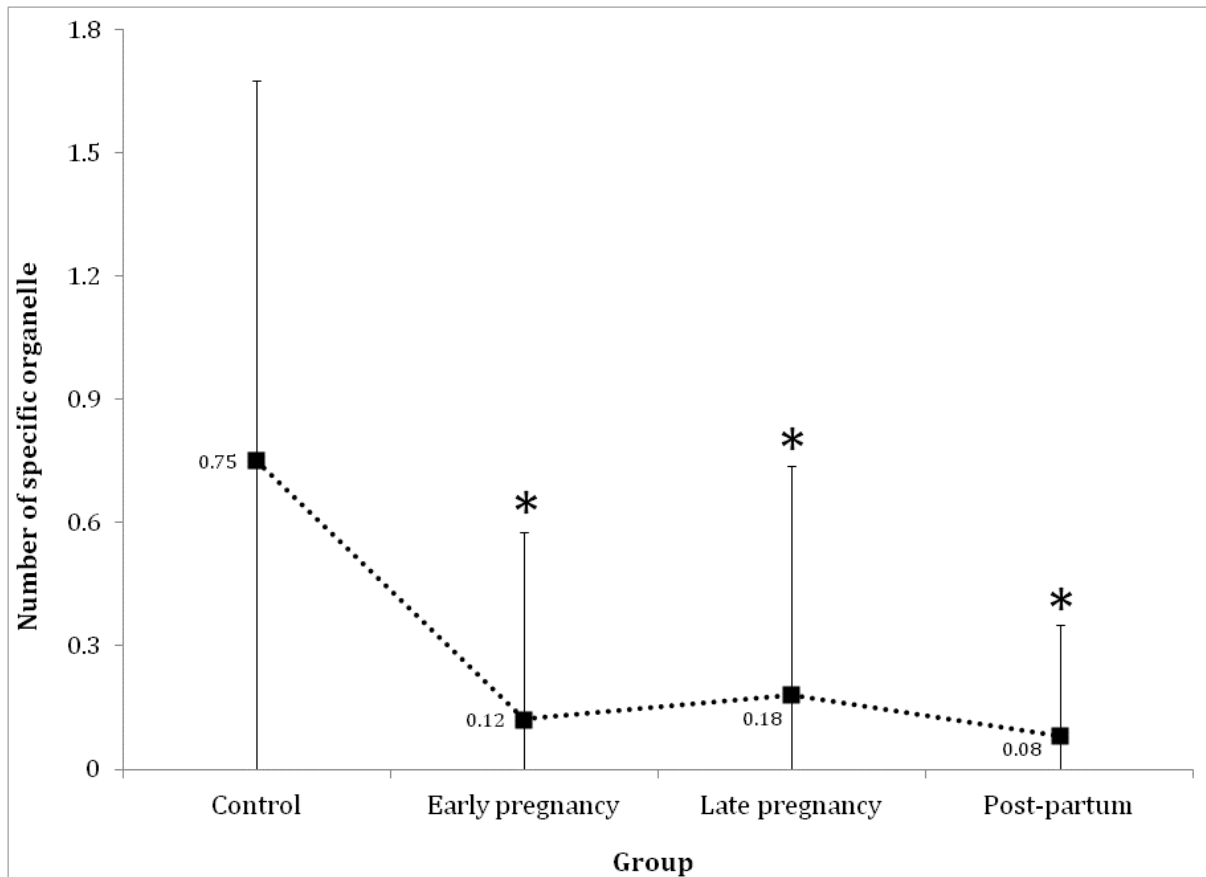
138

A one-way ANOVA revealed significant differences between the control values and all three phases of pregnancy for all organelles (alpha, dense, lysosomal and mitochondria) with all p-values less than 0.05.

Alpha granules

A one-way ANOVA showed a significant difference in the amount of alpha granules during pregnancy compared to the control group, $F(3, 396) = 76.239$, $p < 0.05$. Post hoc comparisons using Tukey HSD test showed an increase in alpha granule count. This increase seen in pregnancy was almost four-fold to that of the control group. There was also a significant difference between the alpha granule count of the late pregnancy group and the post-partum group ($p < 0.05$), but no significant difference between the early pregnancy values and either the late pregnancy or post-partum values.

Dense granules

One-way ANOVA indicated a significant difference in dense granule numbers during pregnancy when compared to the control group $F(3, 396) = 32.958$, $p < 0.05$. Post hoc comparison using Tukey HSD test indicated a significant decrease in dense granule count for all pregnancy groups. This decrease seen in pregnancy ranged from nearly twofold to six fold when compared to the non-pregnant group. There was also a significant difference between the dense granule count of early pregnancy compared to both the late pregnancy and post-partum groups (individually and combined) with a p-value less than 0.05 but no significant difference between late pregnancy and post-partum.

Lysosomal granules

A one-way ANOVA revealed significant differences in lysosomal counts from all pregnancy groups compared to the control group $F(3, 396) = 71.674$, $p < 0.05$. Post hoc comparisons using Tukey HSD test showed a significant increase in number of lysosomal granules for all pregnancy groups. This increase seen in pregnancy was nearly threefold to that of the non-pregnant, control participants. There was also a significant difference between the lysosomal granule numbers of early pregnancy compared to both the late pregnancy and post-partum groups (individually and combined) with $p < 0.05$. However, no significant difference between late pregnancy and post-partum was found.

139

<u>Mitochondria</u>

A one-way ANOVA revealed significant differences in the number of mitochondria from all pregnancy groups compared to the control group $F_{(3, 396)}$ = 27.285, $p < 0.05$. Post hoc comparisons using Tukey HSD test showed a significant decrease in mitochondria count for all pregnancy groups. This decrease seen in pregnancy ranged from fourfold to nine fold when compared to the non-pregnancy group. No significant difference was found between the different phases of pregnancy.

Taken together, these results suggest that pregnancy influence platelet components including all populations of granules (alpha, dense and lysosomal) and also mitochondria. Early pregnancy differs significantly from late pregnancy and post-partum (combined and separately) for the dense granules and lysosomal granules but not for alpha granules and mitochondria. Late pregnancy differs from post-partum only for alpha granules but not for dense granules, lysosomal granules or mitochondria. Significant increases and decreases can be seen in Figures 8 – 11.

7.5 DISCUSSION

Platelet external structure

The OCS and alpha granules are closely related. They support platelet function in two ways. For alpha granules to accomplish their physiological tasks, their constituents need to be liberated from their intracellular storage areas. The α-granule membrane fusion to the surface-connected membranes of the OCS or the plasma membrane will trigger granule secretion. [274] Therefore the OCS firstly links the alpha granules to the external medium. Secondly the OCS channels and pores along with alpha granules provide additional membrane surface area to the platelet upon stimulation. The platelet surface area can increase up to four fold in size and result in platelet spreading. [275] Since alpha granules facilitate the aggregation of platelets, the increased incidence and enlargements of the pores may support increased secretion of alpha granule constituents resulting in the close association of platelets exhibited.

Platelet pseudopodia formation is the initial morphological change associated with adhesion and aggregation. [276] These processes are suggested to aid adherence to other platelets and the forming fibrin strands. [277] The increased pseudopodia formation seen during pregnancy and the

140

visible interaction of the processes with other platelets therefore support platelet adhesion and aggregation.

With platelet adhesion to the injury site the platelet will be activated by various signalling events. Once activation is triggered the round shape of the resting platelet need to be altered swiftly to its active form to accommodate primary haemostasis. The compact platelet will start to flatten, a process referred to as platelet spreading. Spreading facilitate the platelet in covering the damaged area while the constituents liberated from the platelet will recruit additional platelets and trigger the formation of the fibrin network to form the stable, secondary haemostatic plug. [278]

Membrane blebbing is one of the traits of apoptosis. [279] Elevated thrombin induces platelet activation resulting in blebbing of the membrane similar to apoptosis. [280] The membrane blebbing seen during pregnancy can therefore be the result of elevated thrombin concentrations. Since no thrombin was added to the platelet samples, it can be deduced that elevated thrombin concentrations were present in the plasma.

Platelet internal structure

Various blood coagulation mediators are secreted by platelets. Alpha granules contain large polypeptides that play a role in primary as well as secondary haemostasis. Fibrinogen and vWF along with adhesive proteins are secreted by alpha granules. These mediate primary haemostasis by supporting both interactions between platelets as well as platelet interaction with the endothelium. [275]

Platelet alpha granules also contribute to secondary haemostasis since they secrete numerous coagulation factors, including factor V, XI and XIII upon activation. Prothrombin, the inactive precursor of thrombin, is also contained within the alpha granules. Platelet alpha granules also play a role in the inhibition of fibrinolysis by secreting plasminogen activator inhibitor-1 (PAI-1) and a2-antiplasmin. [275]

Alpha granules furthermore support haemostatic balance. Antithrombin and C1-inhibitor are both involved in controlling clot formation and are stored in the alpha granules. Plasmin along with its inactive precursor (plasminogen) is stored in the platelet alpha granules. Alpha granules therefore regulate coagulation by playing a significant role as both procoagulant and anticoagulant. [281]

141

The increased number of alpha granules seen during all three phases of pregnancy may point to a pregnancy-specific modification. Since haemostatic factors are altered from the commencement of pregnancy to preserve the pregnancy and prepare the female body for delivery, changes within the platelets may also take place. More alpha granules are produced to increase the production of their constituents involved in coagulation and wound healing. It may be that the alpha granules are responsible for secreting a constant stream of haemostatic factors, thus promoting coagulation throughout pregnancy. The increase in alpha granules thus contributes to the pro-thrombotic state associated with pregnancy. The alpha granule count increases during pregnancy, but starts to decrease 6 – 8 weeks post-partum. Normal alpha granule counts are however not achieved within 8 weeks after birth. This shows that the pro-thrombotic state of pregnancy persists till at least two months after birth. It also shows that this pregnancy-specific modification may return to normal levels or levels close to normal counts and not persist throughout the female's life.

Serotonin, calcium, pyrophosphate and a non-metabolic adenine nucleotide pool consisting of ATP and ADP are contained within the dense granules. [282,283] ADP and collagen trigger the secretion of dense granules contents. [284] These constituents play a role in the recruitment of additional platelets during platelet activation. [258-260]

The decreased numbers of dense granules may also be a pregnancy-specific modification. Along with the steady stream of alpha granule constituents maintaining the pro-thrombotic state of pregnancy, the decreased dense granule numbers may act as a countermeasure for the increase in coagulation. Since dense granule constituents promote platelet recruitment and ultimately platelet aggregation, the decreased numbers may prevent an "over-aggregation" of platelets in circulation. Early pregnancy dense granule count is higher than late pregnancy and post-partum. It may be that this modification progresses with gestation. However, it does not return to normal within two months after delivery.

Lysosomes are present in all types of cells. They enclose acidic hydrolase, responsible for the degradation of proteins. [285] The increase in lysosomal granule count may also be a pregnancy-specific countermeasure like the dense granules. The acidic hydrolase within the lysosomal granules are only liberated during strong stimulus. Increased lysosomal granule numbers will increase the amount of acidic hydrolase which in turn will increase the degradation of fibrin clots. Once again the coagulation system is controlled to maintain a

142

distinctive haemostatic balance that will prepare the body for delivery but not destroy it in the process.

Mitochondria supply the necessary energy needed for platelet reactivity. [258] The decrease in mitochondrial numbers seen throughout pregnancy may result from the pro-thrombotic state of pregnancy. Joseph et al. attributed decreased mitochondrial count to the possible release or consumption of mitochondria in activated platelets compared to resting platelets. [286] Platelet activation is apparent during all three phases of pregnancy. Mitochondrial release and/or consumption may therefore explain the decreased counts.

7.6   CONCLUSION

Platelets play an important role in haemostasis. They form the primary haemostatic plug which is the first line of defence against blood loss from a severed blood vessel. They adhere to the injury site and are activated. Secretion of granular content and subsequent aggregation will result in the formation of the platelet plug.

In the past platelet morphology during normal pregnancy has not been given much attention.

In this study ultrastructural changes pertaining to the external as well as the internal arrangements of platelets are visible. External alterations observed by SEM include increase and enlargement of the OCS pores, pseudopodia formation, platelet spreading and membrane blebbing. TEM investigation showed that platelets from pregnancy were tightly packed and internal structures were different from the non-pregnant group. The internal granules, including alpha granules, dense granules and lysosomal granules, along with mitochondria showed modification in their occurrence within the cell. Alpha granule and lysosomal granule counts were significantly increased during pregnancy while dense granule and mitochondrial numbers were significantly decreased. This may point to a pregnancy-specific modification. The increased alpha granule numbers might promote the pro-thrombotic state associated with pregnancy while the decreased dense granule count along with increased numbers of lysosomal granules act as countermeasures to ensure coagulation is not pathologically activated. Mitochondrial decrease may be caused by the increase in platelet activation. All ultrastructural alterations associated with pregnancy persist for at least two months after birth.

143

CHAPTER 8: FLOW CYTOMETRIC ANALYSIS OF PLATELETS DURING THREE PHASES OF PREGNANCY:

A QUANTITATIVE INVESTIGATION

8.1 CHAPTER OBJECTIVES

In this chapter the platelet architecture of different phases of pregnancy, namely early pregnancy (week 8 – 14), late pregnancy (week 36 – 40) as well as post-partum (week 6 – 8 after birth) will be compared to platelets from non-pregnant individuals as well as each other to determine whether changes in platelet architecture exist during pregnancy.

8.2 INTRODUCTION

8.2.1 Flow cytometry

Flow cytometry is a sensitive and specialized method to detect surface antigens. Several antibody combinations can be used simultaneously. [121,125,169,171]

Flow cytometric analysis of platelets, particularly platelet activation, has been progressively studied. [125,165]

8.2.2 Whole blood

Whole blood can be used to establish the activation state of platelets in circulation. By employing an activation dependent monoclonal antibody (moAb), platelet function can be assessed *in vivo*. [169]

Flow cytometry can be used to evaluate specific characteristics of various individual cells in a whole blood sample. For whole blood samples, buffered sodium citrate is added to serve as anticoagulant. The samples are diluted to reduce possible platelet aggregate formation. Fluorophores like phycoerythrin (PE) and fluorescein isotheiocyanate (FITC) are conjugated to specific antibodies. The sample is stained with the fluorescently labelled monoclonal antibodies (MoAbs). The cells are then channelled into single file within the flow cytometer and passes through a focused laser beam.

The amount of florescent emission and light scatter properties of each cell is detected and analysed. [169,287]

Platelets are identified by the characteristic forward and light scatter associated with typical platelet morphology. A "platelet identifier" MoAb can also be used to identify the platelet population in whole blood. Additional MoAbs are added to investigate platelet function. [169]

Antibody binding can be conveyed in two ways. The mean channel fluorescence intensity (MCFI) refers to the arbitrary unit for measuring the intensity of fluorescence in the sample. Also the percentage of positive platelet staining for a specific antibody can be used. This is based on the positive analysis region positioned on the right of the negative control fluorescence histogram. The second method of data expression, although not affected by variation in signal amplification and more sensitive for subpopulation of platelets arising from in vivo insult, may give a false indication of the amount of antigen expressed on the platelet surface. Therefore using MCFI is preferred if the total amount of platelet surface antigen need to be determined. [169] Using both data presentation methods may provide a more complete representation of the results.

8.2.3 Platelet activation markers

Changes in platelet morphology and function can be triggered by various physical, chemical or metabolic factors. [288,289] The internal granules will be secreted and neoantigens will be expressed on the platelet surface. Receptor activation also triggers rearrangements in the cytoskeleton leading to platelet shape changes which mediates signal transduction by binding coagulation factors. [156,186,187,290-292]

Activation markers can be used to study platelet responsiveness to different agonists. [186,187]

Platelet activation markers comprise of activation-dependent alterations in the GPIIb-IIIa complex, granule proteins exposed on the membrane surface, secretion and binding of platelet proteins as well as procoagulant surface development. [169] Activation-dependent MoAbs directed against granule membrane proteins expression and conformational GPIIb-IIIa complex alterations are the most prominent markers studied. [131]

CD41, also known as platelet membrane glycoprotein (GP) IIbIIIa or integrin $\alpha_{IIb}\beta_3$, and CD42b, referred to as GPIb, are platelet identifying markers for both resting and activated platelets. CD62P

145

(P-selectin) and CD63 (lysosomal integral membrane protein or LAMP-3) are activation-dependent antigens. [130]

Various internal and external control mechanisms are employed to maintain the resting state of platelets within circulation. These mechanisms also prevent unsuitable activation of integrins through intracellular signalling. [293]

CD41 is found on the membrane of megakaryocytes and platelets. It forms a complex with CD61 to form the integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$). [131]

GPIIb-IIIa complex play a critical role in platelet aggregation since it serves as the receptor for several coagulation factors such as fibrinogen and vWF. The GPIIb-IIIa complex-specific MoAbs bind to mainly resting platelets. [131] After vascular damage platelets depend on integrin $\alpha_{IIb}\beta_3$ to assist in the securing and repairing the injured vessel. [294]

CD42b, also known as GPIb, is an important receptor for vWF [170] and thrombin [295]. It mediates cell-matrix and cell-cell interaction. [145,193,194]

With disruption of vessel subendothelium, von Willebrand factor (vWF) is rapidly deployed to the site of vessel injury where collagen is exposed. VWf molecules join to the GPIba integrin subunit (CD42 complex) and platelets will start to adhere to the wound. As platelets adhere to each other and the vessel wall, the platelets will be activated and platelet integrin GPIIb/IIIa is upregulated. With platelet activation the integrin changes its conformation to facilitate platelet adherence to each other by either fluid phase fibrinogen or vWF molecules that acts as a link between GPIIb/IIIa integrin molecules. This causes the thrombus to expand rapidly. [243]

Expression of GPIIbIIIa (CD41) and GPIb (CD42b) therefore represent the adhesion and aggregation of platelets respectively.

CD62P, forming part of the α-granule membrane, and CD63, a lysosomal integral membrane protein, are two of the leading platelet activation markers. [137]

The platelet surface plays an important role in pro-coagulation. On the surface of activated platelets negatively charged phospholipids are exposed. These phosphololipids, which include phosphatidylserine, provide the surface whereupon the clotting factors can gather. [221]

146

CD62P (P-selectin) is the most important activation-dependent marker directed against granule membrane proteins. CD62P is responsible for platelet adhesion to neutrophils and monocytes. [296]

In resting platelets, CD62P forms part of the α-granule membrane. Upon platelet activation and subsequent degranulation, CD62P is expressed on the surface of the platelet membrane. [151] As a result the CD62P specific MoAb only binds to platelets after degranulation and not to platelets in their resting state. [297]

In vivo degranulated platelets swiftly lose their surface P-selectin however remains in circulation and will continue to perform normal platelet function. [298,299] Platelet surface P-selectin is therefore only a useful marker for detecting degranulated platelets in circulation if the platelet activation is a continuous process. [169]

CD63 is the flow cytometric marker for lysosomal liberation resulting from platelet activation. [134,157] Platelet activation initiates the translocation of CD63 from the cytoplasmic granules to the plasma membrane. [300]
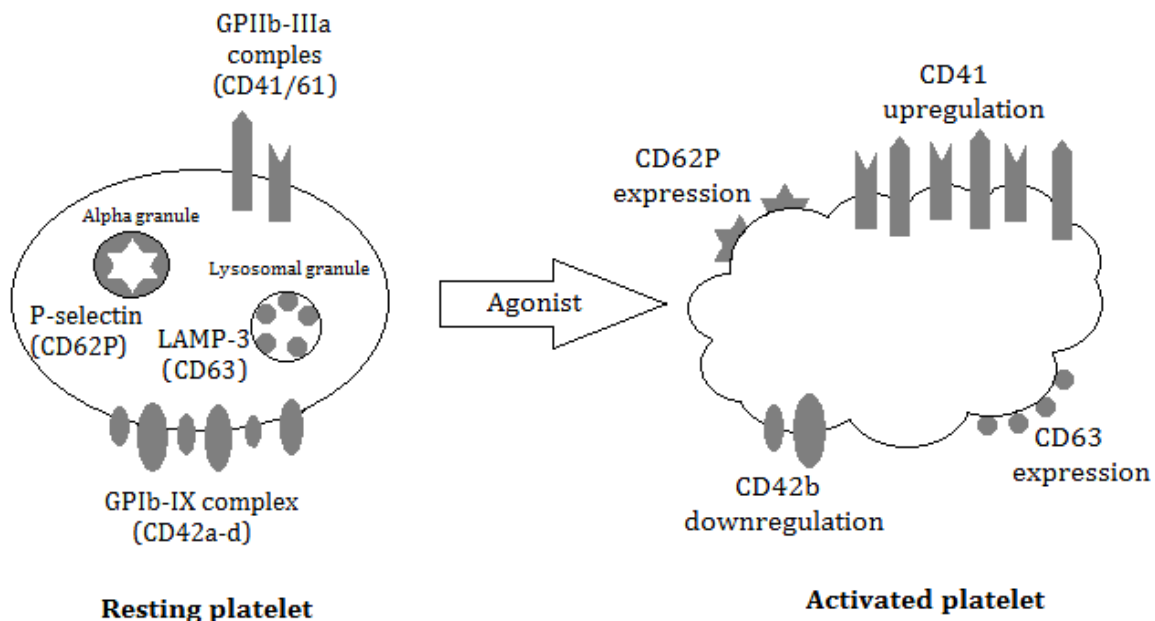
Figure 1. The effect of platelet activation on MoAb binding.[169,301]

147

8.2.4   *Platelet flow cytometry and pregnancy*

Activated platelets are associated with CD41 (GP IIbIIIa, fibrinogen binding site) upregulation and CD42 (GPIb, vWF and thrombin binding site) downregulation. [135]

Expression of CD62 and CD63 on the plasma surface increased during pre-eclampsia compared to normal pregnancy. [302,303]

Flow cytometric analysis of platelet activation pertaining to normal pregnancy has been inconclusive.

Star et al. found that the platelets in the circulation were not influenced by pregnancy. Activation was not increased and binding of MoAbs were not significantly different from that of non-pregnant individuals. Star did not find any significant increase in activated platelets circulation at any stage of healthy pregnancy. [301]

Jane et al. however found platelet activation in the second and third trimester of pregnancy. Surface levels of lysosomal granule membrane antigen were also detected. They concluded that platelet degranulation was increased during pregnancy, independent of the binding of fibrinogen. [266]

To summarize, platelet function has been studied by flow cytometry.[125,165] Various fluorescent labelled MoAbs can be used in conjunction to investigate different aspects of the studied cell population. [121,125,169,171]

CD41 and CD42b, are platelet specific markers used to identify both resting and activated platelets. CD62P and CD63 are activation-dependent antigens. [130] CD62P forms part of the α-granule membrane while CD63 is a lysosomal integral membrane protein. [137] Both these markers are expressed on the platelet surface membrane following degranulation of activated platelets.

Conflicting evidence exist for platelet specific markers during pregnancy. Normal pregnancy has been compared to pre-eclampsia and hypertension and studied during the three trimesters, but not post-partum.

This study aimed to investigate the effect of different phases of pregnancy on platelet specific markers by employing flow cytometry.

148

8.3   MATERIALS AND METHODS

The platelet architecture from different phases of pregnancy were analyzed by preparing the samples for flow cytometric (FC) analysis as described in Laboratory procedures under *3.3.1.1. Platelet architecture* in Chapter 3.

8.4   RESULTS

The platelet specific marker CD41-FITC was used to set the platelet gate as described in Chapter 4. The instrument configurations and settings are summarized in Table 1.

Table 1. Instrument configuration and settings.

Acquisition setup - Discriminator:			
	FS = 1	FL3 = OFF	Compensation:
	SS = OFF	FL4 = OFF	None
	FL1 = OFF	FL5 = OFF	
	FL2 = OFF	AUX = OFF	

Acquisition setup - Detectors:		Volt	Gain
	FS	816	1
	SS	251	5
	FL1	553	1
	FL2	639	1
	FL3	677	1
	FL4	450	1
	FL5	577	1
	AUX	300	1

150

8.4.1 Statistical analysis

Table 2 represents the mean ± standard deviation of flow cytometric analysis of platelets from non-pregnant controls and three different phases of pregnancy. Statistical modeling was performed by employing a computerised software system (IBM SPSS Statistics 21) for statistical analysis of the data.

The paired two-sided Student's t-test was employed to compare the control group at different phases in the menstrual cycle. A p-value of < 0.05 was considered significant.

After Levene's test gave no indication that the different experimental groups had different variances at a 5% level of significance, a One-way ANOVA was deemed suitable to use to compare results of the control group during the menstrual phase and the results from the different phases of pregnancy. A level of significance was set at $p < 0.05$.

One-way ANOVA was used to compare results of the control group during the menstrual phase and the results from the different phases of pregnancy. The lowest concentrations of the gonadotropic hormones (Follicle stimulating hormone (FSH) and Luteinizing hormone (LH)) as well as the ovarian hormones (Estrogen and Progesterone) are exhibited during the period of menses [29]. Since the possible effect of the pregnancy hormones on the platelet architecture is investigated, the menstrual phase was chosen as control group. A level of significance was set at $p < 0.05$.

151

Table 2. Flow cytometric analysis of platelets from controls during different phases of the menstrual cycle.

	CM	CP
CD41-PE	22.22 ± 5.05	24.31 ± 2.48
CD42-PE	14.62 ± 1.52	14.36 ± 1.56
CD62P-PE	37.59 ± 2.47	39.16 ± 3.81
CD62P-PE Activation	77.99 ± 3.69	79.30 ± 4.21
CD63-PE	18.37 ± 1.32	17.51 ± 2.28
CD63-PE Activation	46.31 ± 9.25	41.46 ± 9.92

CM = Control, Menstrual phase; CP = Control, Pre-ovulatory phase;

152

Table 3. Flow cytometric analysis of platelets from different phases of pregnancy.

	EP	LP	PP
CD41-PE	22.13 ± 3.42	21.76 ± 3.50	19.67 ± 2.97
CD42-PE	13.51 ± 1.82	14.80 ± 2.21	14.38 ± 1.57
CD62P-PE	36.19 ± 2.60	35.20 ± 2.34	35.66 ± 2.90
CD62P-PE Activation	76.46 ± 4.05	75.82 ± 3.18	75.49 ± 4.01
CD63-PE	19.21 ± 1.73	18.83 ± 1.75	18.90 ± 1.26
CD63-PE Activation	49.37 ± 8.82	50.72 ± 8.70	47.61 ± 6.33

EP = Early pregnancy; LP = Late pregnancy; PP = Post-partum

153

The student t-test showed no significant differences between the different phases of the menstrual cycle for the control group (p-value > 0.05). One-way ANOVA revealed significant differences between groups for CD41, CD42 and CD62P (p-value < 0.05). No significant difference was found between groups for CD62P activation, CD63 and CD63 activation (p-values > 0.05).

CD41

A one-way ANOVA showed a significant difference in the MCFI of CD41-PE between groups, $F(3,116) = 2.794$, $p < 0.05$. Contrast test showed significant decrease in MCFI of CD41-PE for the post-partum group compared to the controls and early pregnancy, but no significant difference between any of the other groups.

CD42b

A one-way ANOVA showed a significant difference in the MCFI of CD42b-PE between groups, $F(3,116) = 3.257$, $p < 0.05$. Tukey HSD test indicated a significant increase in MCFI for the early pregnancy group compared to the controls (p-value < 0.05). Early pregnancy MDFI was also significantly lower than the MCFI of the late pregnancy group (p-value < 0.05). The MCFI of the late pregnancy group was significantly higher than the post-partum group (p-value < 0.05). No significant differences were seen in the other group comparisons.

CD62P

A one-way ANOVA showed a significant difference in the MCFI of CD62P-PE between groups, $F(3,116) = 3.842$, $p < 0.05$. Contrast test revealed differences between the control group and pregnancy as a whole as well as controls and late pregnancy. Tukey HSD test showed a significant decrease in MCFI between the control group and late pregnancy with a p-value less 0.05, but no significant differences in any of the other group comparisons.

A one-way ANOVA showed no significant difference in the percentage activation of CD62P-PE between groups (p-value > 0.05).

CD63

A one-way ANOVA showed no significant difference in the MCFI of CD63-PE between groups, with a p-value greater than 0.05.

A one-way ANOVA showed no significant difference in the percentage activation of CD63-PE between groups, with a p-value greater than 0.05.

Taken together, these results suggest that some differences exist between the MCFI of CD41, CD42b and CD62P between the groups. For CD41 the post-partum group differs from both the control and early pregnancy groups. For CD42b an increase from control group to early pregnancy to late pregnancy is seen. Post-partum the CD42b decreases from late pregnancy. For CD62P the control group differs from pregnancy as a whole, and specifically late pregnancy. No significant differences exist between the MCFI of CD63 or the percentage activation of CD62P and CD63 for all groups.

8.5 DISCUSSION

GPIIb/IIIa is a receptor for several adhesion molecules like fibrinogen and von Willebrand factor. [132] The principle function of this complex is platelet aggregation. The binding of fibrinogen to the complex will initiate aggregate formation. VWF binding initiates platelet adhesion and spreading. [131,133,134] Upregulation of this complex is therefore associated with platelet activation. [135].

Although it is mainly expressed on the platelet cell surface, a small amount of this complex is circulated within the α-granule membrane and the open canalicular system. The CD41/61 complex induces secretion of alpha granule components following platelet activation. [136-138]

Since CD41 and platelet alpha granules are closely related, the results of the internal ultrastructural changes associated with pregnancy as discussed in the previous chapter should be taken into account. Increased CD41 in combination with CD61 triggers the secretion of alpha granule constituents. The decrease in CD41 MCFI may have two possible explanations. The increased alpha granule count seen during pregnancy may be caused by the decrease in CD41, and CD41 is therefore directly affected by pregnancy. The other possibility is that the same amount of CD41 is expressed on the platelet membrane but at a lower fluorescent intensity.

The decrease in CD41 MCFI of the post-partum group compared to the control group and early pregnancy group may be caused by the strain that delivery puts on the haemostatic system.

155

Thrombin-induced platelet activation has been shown to down-regulate the surface expression of CD41. [197,199] During delivery the thrombotic system is practically "overactived" to prevent exsanguination. It may be that this shock to the haemostatic system results in the depletion of CD41 after birth.

CD41 may therefore contribute to the pregnancy-specific modifications seen in the granular content of platelets discussed in the previous chapter.

CD42b is found on the platelet membrane [142,143]. It is known as von Willebrands's factor adhesion receptor [144-148]. Primary platelet aggregation is mediated by CD42 and vWF interaction; downregulation of CD42 expression is associated with activation [135,149].

CD42b MCFI follows a significant increasing curve as pregnancy progresses. Downregulation of CD42b is associated with platelet activation. The increase in CD42b may form part of the pregnancy-specific modifications explained in the previous chapter. Although pregnancy is associated with increase in von Willebrand factor [14,59,232,233,238-240], the optimal binding of vWF to Cd42b may be inhibited in some way. This will ensure that platelet aggregation is not pathologically augmented throughout pregnancy. Since post-partum CD42b expression is decreased compared to late pregnancy, it may indicate that this mechanism associated with pregnancy will return to normal after birth.

CD62P is found in platelet α-granules [150,151]. CD62P is translocated to the surface of activated platelets as a result of alpha granule release [134,152-154].

Given that the expression of CD62P is closely associated with alpha granules, the increased alpha granule count seen in platelet TEM micrographs need to be taken into consideration.

Increased alpha granule counts could indicate decreased or slower expression of the granule contents and therefore less CD62P expression on the platelet membrane. Therefore activated platelets with decreased expression of CD62P would present similar to the non-activated platelets of the controls participants.

In the third trimester the female body reaches its final preparations for delivery and the coagulation system reaches its hyperactivity peak. This may be the reason that the CD62 MCFI is

156

significantly increased in late pregnancy compared to the control group, since the pro-thrombotic state of pregnancy is augmented.

MCFI and percentage activation of CD62P convey different aspects of activation. The MCFI which conveys the intensity of fluorescence and the percentage of platelets positive for CD62P have to be viewed collectively. Although the percentage positive cells is more sensitive for platelet subpopulation and is not influenced by variation in signal amplification, it may provide false indication of the degree of antigen expression on the platelet membrane. Elevation in CD62P MCFI can thus be accompanied by unchanged CD62P activation.

CD62P activation is not significantly influenced by pregnancy. This may be accredited to the pregnancy-specific modifications referred to in the previous chapter. Since pregnancy is a normal biological process, for which the female body is well equipped, the increase in CD62P expression when associated with CD62P activation may show that less CD62P is actually expressed. The CD62P that is expressed, although fewer, has a greater fluorescence intensity providing a false elevated CD62P expression.

Since CD62P mediates platelet interactions with monocytes and neutrophils [152], CD62P serves as the link between thrombosis, inflammation and wound-healing [150]. The unchanged CD62P activation percentage may ensure that the inflammatory response is not augmented while the body is in the prothrombotic state.

Although the expression and activation percentages differ, CD62P can be employed as a useful marker for detecting activated platelets in circulation of pregnancy since platelet activation has been shown to be a continuous process throughout gestation.

This pregnancy-specific modification may be a mechanism to maintain the pro-thrombotic state of pregnancy to assist in delivery and maternal health without augmenting the process to a pathological condition.

CD63 is found within the cytoplasmic granules of resting platelets, but are translocated to the platelet membrane of activated platelets [156]. Since release reaction of platelet lysosomal content are associated with CD63, the expression of CD63 on the platelet surface is indicative of platelet activation [134,157]

157

CD63 is in close association with the lysosomal granules found within platelets. In the previous chapter, an increase in lysosomal granules during pregnancy was established. Therefore an increase in CD63 should accompany the increase in lysosomal granules. This is however not the case.

Platelets can lose their surface P-selectin while still performing their normal functions in circulation. [298,299] The same could be true for CD63. It is possible that the increased lysosomal granule counts result in increased expression of CD63 on the platelet membrane which is subsequently lost while the platelets remain in circulation.

It is evident that platelets can still perform their primary biological functions of activation, degranulation and aggregation even though CD63 expression is not increased or swiftly lost following expression.

8.6 CONCLUSION

Flow cytometry is a sensitive method to study platelets. Various fluorescent labeled MoAbs can be used in conjunction to investigate platelet function. [121,125,169,171]

Platelet specific probes, CD41 and CD42b for example, identify both resting and activated platelets. Activation-dependent markers like CD62P and CD63 form part of the granular components within the platelet and are only expressed following degranulation of activated platelets. [130,137]

Conflicting evidence exist for platelet specific markers during pregnancy. Normal pregnancy has been compared to pre-eclampsia and hypertension.

The MCFI of CD41, CD42b and CD62P show significant differences between groups. The MCFI of CD63 and the activation percentages of CD62P and CD63 are not significantly influenced by pregnancy. Downregulation of CD41 and CD62P along with the upregulation of CD42b may play a role in the pregnancy-specific modifications discussed in the previous chapter. The unchanged CD63 MCFI and activation percentages of CD62P and CD63 may also attribute to the preservation of female health during pregnancy.

158

CHAPTER 9: ULTRASTRUCTURAL ANALYSIS OF ERYTHROCYTES INTERACTION WITH PLATELETS DURING THREE PHASES OF PREGNANCY: A QUANTITATIVE INVESTIGATION

9.1 CHAPTER OBJECTIVES

In this chapter the whole blood of different phases of pregnancy, namely early pregnancy (week 8 – 14), late pregnancy (week 36 – 40) as well as post-partum (week 6 – 8 after birth) will be investigated to determine possible interaction between erythrocytes and platelets during pregnancy.

9.2 INTRODUCTION

9.2.1 Haemostasis

Erythrocytes are bidiscoidal blood cells responsible for supplying oxygen to the body cells. The nucleus, intracellular organelles and mitochondria are lost during the maturation process, therefore consisting of only a cell membrane, haemoglobin and some enzymes. During the finite lifespan that erythrocytes circulate, a range of structural, compositional, biochemical and immunological alterations can take place. [74] The interaction between platelets and the blood vessel wall is influenced by physical and chemical properties of erythrocytes. [304]

Erythrocytes contribute to haemostasis. Under experimental conditions erythrocytes have shown pro-aggregatory properties by liberating ADP, a platelet activating agent. [305]

While endothelial cells and neutrophils have a downregulating effect on the thrombotic system [306], erythrocytes have prothrombotic properties. [307]

The interaction of activated platelets with intact erythrocytes therefore amplifies the activation and pro-aggregatory function of platelets. [119,308]

9.2.2 Erythrocytes and pregnancy

The haematopoietic system undergoes changes to suffice the requirements of the growing fetus and the mother. [76]

159

Throughout normal pregnancy, platelet activation contributes to the hypercoagulable state observed on physiological level. [4] Platelet activation is said to increase as the pregnancy progresses. [5]

In pregnancy the risk for venous thrombosis is increased. A six-fold increase in incidence of venous thromboembolism is reported in normal pregnancy compared to the female population of child-bearing age. [56]

Erythrocyte abnormalities have been shown in severe preeclampsia. Cunningham et al. speculated that pregnancy-associated changes in the erythrocyte membrane composition, induced by plasma/erythrocyte lipid interchange, are augmented in severe pre-eclampsia. [309] Erythrocyte interaction with platelets during normal pregnancy, especially during different phases of normal pregnancy, has not been investigated.

Erythrocytes therefore contribute to haemostasis. They liberate ADP, a platelet activating agent. [305]

Platelet activation also contributes to the prothrombotic state observed in normal pregnancy. [4] Activated platelets interact with intact erythrocytes to amplify platelet activation and pro-aggregatory function. [119,308]

Abnormality in erythrocyte morphology has been established in pre-eclampsia and eclampsia. [309] Platelet interaction with erythrocytes in whole blood smears during pregnancy has not been investigated. This investigation aimed to investigate this platelet/erythrocyte interaction.

9.3   MATERIALS AND METHODS

The interaction between platelets and erythrocytes in whole blood smears from different phases of pregnancy was analyzed by preparing the samples for scanning electron microscopy (SEM) as described in Laboratory procedures under *3.3.2.1 Whole blood* in Chapter 3.

9.4   RESULTS
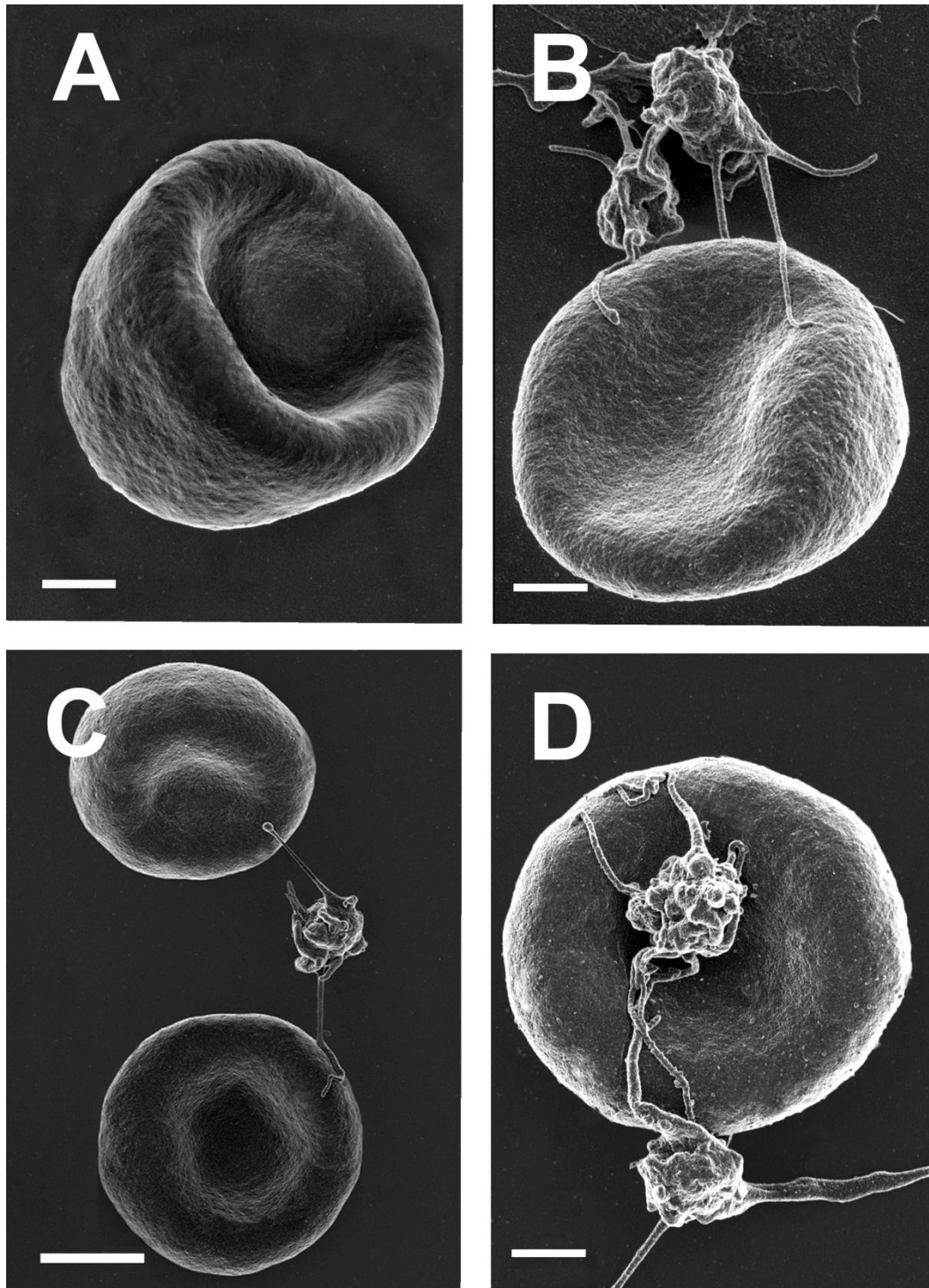
**Plate 1. Erythrocyte and platelets interactions in whole blood smears
(grey scale micrographs). Scale bar = 1µm**
A = Healthy, non-pregnant control; B = Early pregnancy;
C = Late pregnancy; D = Post-partum.
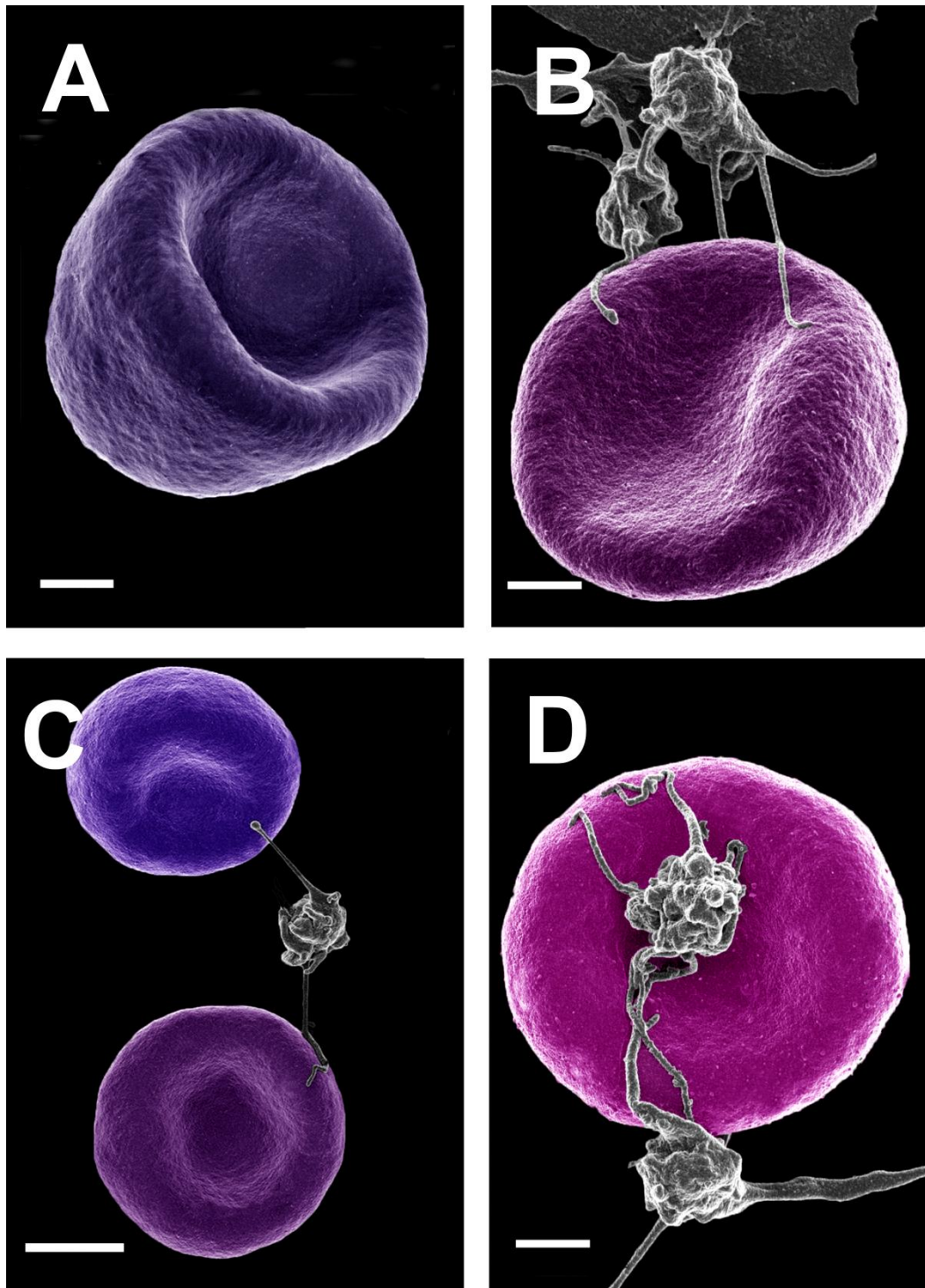
Plate 2. Erythrocyte and platelets interactions in whole blood smears (colour micrographs). Scale bar = 1µm
A = Healthy, non-pregnant control; B = Early pregnancy;
C = Late pregnancy; D = Post-partum.

Plate 1A shows a typical RBC from a female of child-bearing age – typically with no platelet association, while Plate 1B-D shows early and late pregnancy, as well as six to eight weeks post-partum. During these three stages almost all erythrocytes in the whole blood smears are closely associated with platelets, showing platelet spreading and pseudopodia closely associated with the erythrocyte membranes.

Although, in all samples, one erythrocyte is typically involved with only one platelet, a single platelet may have multiple interactions with erythrocytes as shown in Plate 1C. Plate 1 shows micrographs in typical grey scale. Plate 2 shows the same interactions in coloured micrographs to emphasize the platelet and erythrocyte interactions.

9.5   DISCUSSION

In response to vascular injury erythrocytes may enhance the adherence and aggregation of platelets in haemostatic primary plug formation.[310-312] Erythrocytes also influence the rate of platelet removal from the blood by increasing the transport of platelets to the endothelial surface. [313]

Platelet reactivity is significantly influenced by cell-cell interactions between platelets and erythrocytes.[119,308,314] Cell-cell interaction between activated platelets and erythrocytes indicates that biochemical communication between these cell types is initiated upon platelet activation. This is because erythrocytes cannot promote platelet activation or the recruiting activity of cell-free releasates without a platelet agonist.[119,308]

The platelet/erythrocyte interactions exhibited may be the mechanism through which these cell types communicate to initiate platelet activation and support aggregation to assist thrombus formation.

Several processes may influence thrombocytopenia in pregnancy. These processes may be physiological or pathological in nature. Preeclampsia, a syndrome unique to pregnancy, increases the risk for the development of thrombocytopenia. The pathophysiology of thrombocytopenia is mediated by complex interactions between platelets and haemostatic factors.[315]

Platelet/erythrocyte interactions may play a role in thrombocytopenia. The increased interaction will lead to increased platelet activation, degranulation and aggregation. This will decrease the amount of platelets in circulation, resulting in thrombocytopenia.

163

9.6 CONCLUSION

Erythrocytes not only provide oxygen to the body, they also contribute to haemostasis. Erythrocytes have shown pro-aggregatory properties by liberating ADP, a platelet activating agent, and resulting in a prothrombotic state. [305,307]

Platelet function is therefore amplified by the interaction of activated platelets with intact erythrocytes [119,308]

Interactions between erythrocytes and platelets may be one of the reasons for an increased hypercoagulability during a healthy pregnancy and this state continues for a while post-partum. Thus, this erythrocyte/platelet interaction could possibly contribute to gestational thrombocytopenia.

164

CHAPTER 10: CONCLUSION

Different blood components were analysed by electron microscopy and flow cytometry to determine any differences found in different phases of pregnancy compared to healthy control individuals.

10.1 Flow cytometric method development

In Chapter 4 the flow cytometric comparison of platelets from a whole blood and finger-prick sample was performed as well as the impact of 24 hours storage. The importance of gating strategy for a unfixated sample was established. A platelet specific marker should be employed to take activated and non-activated into consideration. No difference was found between the unfixated whole blood and a finger-prick sample with regards to platelet function. This is advantageous for research where a sample population needs to be examined over consecutive days or an extending time interval since a finger-prick is more practical form of repeated measurements than regular blood draws. Samples can be stored for 24 hours after sampling. Unfixated whole blood and finger-prick samples can be stored in sheath fluid at 6°C for 24 hours, provided that a sufficient amount of 10µl citrate is added to the sheath fluid for a finger-prick sample. This is advantageous for studies where analysis can't be performed immediately due to either time constraints or travelling distance from the flow cytometer.

10.2 Fibrin networks

The fibrin networks from different phases of the menstrual cycle as well as different phases of pregnancy were investigated. Table 1 and 2 are summaries of the ultrastructural changes observed.

Table 1. Summary of ultrastructural changes during the menstrual cycle.

	Menstrual phase	Pre-ovulatory phase	Luteal phase
Ultrastructural changes of fibrin networks during the menstrual cycle			
Texture Configuration	Smooth Normal network	Less smooth More tightly packed	Less smooth More tightly packed Sticky appearance
Major thick fibers	Prominent	Less prominent	Less prominent
Minor thin fibers	Less prominent	More prominent	More prominent

During the menstrual cycle, the fibrin fiber morphology differs. Estrogen causes the changes in the external and internal structure of the fibrin networks identified during the menstrual cycle. The granular formation seen in the SEM analysis during the pre-ovulatory and luteal phases of the menstrual cycle was confirmed with a fibrinogen model, where the addition of estrogen altered the fibrin network morphology in the same manner as seen in whole blood. TEM investigation confirmed that the changed formation of the fibers is influenced by estrogen, not only the final structure. This research therefore is the first to show ultrastructural changes in fibrin fiber morphology resulting from the estrogen changes during the menstrual cycle.

166

Table 2. Summary of ultrastructural changes of fibrin networks during pregnancy.

	Control	Pregnancy
Ultrastructural changes of fibrin networks during pregnancy		
Major thick fibers	Form the greater part of network	Less pronounced
Minor thin fibers		Notable increase
Granular globules	Less pronounced	Large quantities of granular globules are present
	Absent during the menstrual phase of the menstrual cycle	
	Present during the pre-ovulatory and luteal phase of the menstrual cycle, coinciding with the estrogen peaks.	

The coagulation as well as the fibrinolytic system is activated during pregnancy. The increase in estrogen associated with pregnancy may cause the increase in coagulation factors and ultimately the pro-thrombotic state characteristic of pregnancy. The results suggest that activation of the coagulation system commences with pregnancy and that this pro-thrombotic state continues till at least 8 weeks after birth.

10.3 Platelets

Platelet internal and external structures were investigated for changes during pregnancy. Table 3 shows a summary of changes observed in platelet external and internal ultrastructure during pregnancy.

167

Table 3. **Summary of ultrastructural changes of platelets during pregnancy.**

	Control	Pregnancy
Ultrastructural changes of platelets during pregnancy		
External structure		
OCS pores	Few small pores	Increased and enlarged
Pseudopodia formation	Not pronounced	Several pseudopodia extend from platelet body
		Interaction of pseudopodia with adjacent platelets clearly visible
Platelet spreading	Not evident	Prominent
Membrane blebbing	Not evident	Visible
Internal structure		
Arrangement	Platelets are dispersed	Platelets are closely packed together
Alpha granules	Normal quantities	Significantly increased
Dense granules	Normal quantities	Significantly decreased
Lysosomal granules	Normal quantities	Significantly increased
Mitochondria	Normal quantities	Significantly decreased

These alterations may point to a pregnancy-specific modification. The increased alpha granule numbers might promote the pro-thrombotic state associated with pregnancy, while the decreased dense granule count along with increased numbers of lysosomal granules act as countermeasures to ensure coagulation is not pathologically activated. Mitochondrial decrease may be caused by the increase in platelet activation. All ultrastructural alterations associated with pregnancy persist for at least two months after birth.

The platelet architecture was also investigated. Table 4 shows a summary of the flow cytometric analysis of platelets during pregnancy.

Table 4. Summary of flow cytometric analysis of platelets during pregnancy.

	Control	Pregnancy
Flow cytometric analysis of platelets during pregnancy		
CD41	Normal values	Downregulation
CD42	Normal values	Upregulation
CD62	Normal values	Downregulation
CD62 Activation	Normal values	Unchanged
CD63	Normal values	Unchanged
CD63 Activation	Normal values	Unchanged

Downregulation of CD41 and CD62P along with the upregulation of CD42b may play a role in the pregnancy-specific modifications discussed in the previous chapter. The unchanged CD63 MCFI and activation percentages of CD62P and CD63 may also attribute to the preservation of female health during pregnancy.

10.4 <u>Erythrocytes</u>

Whole blood smears were investigated with SEM to determine any erythrocyte/platelet interaction. Table 5 is a summary of the ultrastructural changes of erythrocyte interaction with platelets during pregnancy.

Table 5. Summary of ultrastructural changes of erythrocyte interaction with platelets during pregnancy.

	Control	Pregnancy
Ultrastructural analysis of erythrocyte interaction with platelets during pregnancy		
Erythrocyte morphology	Normal	Normal
Platelet interaction	No interaction	Erythrocytes are closely associated with platelets Platelet spreading and pseudopodia closely associated with the erythrocyte membranes A single erythrocyte is typically involved with a single platelet, however a single platelet may have multiple interactions with erythrocytes

Interactions between erythrocytes and platelets may be one of the reasons for an increased hypercoagulability during a healthy pregnancy and this state continues for a while post-partum.

170

Thus, this erythrocyte/platelet interaction could possibly contribute to gestational thrombocytopenia.

The ultrastructural and flow cytometric analysis of fibrin networks and platelets during the menstrual cycle and pregnancy revealed some interesting results. Elevated estrogen is implicated in the hypercoagulable state associated with pregnancy; however it already influences the fibrin networks during the menstrual cycle. This shows the delicate nature of the haemostatic system to hormonal changes. The female body is equipped to handle alterations in the coagulation system as was seen from the pregnancy-specific alterations seen in the blood components.

In this study blood coagulation at three phases of normal pregnancy was investigated. This is the first morphological investigation of fibrin networks and platelet internal and external ultrastructure during normal pregnancy. The physiological changes in fibrin networks and platelets observed in this study are therefore novel findings. These alterations observed during normal pregnancy can be used as a standard for comparison to other diseased states found during pregnancy such as pre-eclampsia or HELLP syndrome. These results may shed light on possible pathological mechanisms employed in the development of abnormal or ailing pregnancy.

CHAPTER 11: REFERENCES

1. Speroff L., Glass R., Kase N. Oral contraception. Clinical Gynecologic Endocrinology and Infertility. 5th ed. Baltimore: Williams & Wilkins; 1994:715-64.

2. Kuperminc M.J. Thrombophilia and pregnancy. Reproductive Biology and Endocrinology 2003;1.

3. Eldor A. Thrombophilia, thrombosis and pregnancy. Journal of Thrombosis and Haemostasis 2001;86(1):104-11.

4. Torres P.J., Escolar G., Palacio M., Gratacos E., Alonso P.L., Ordinas A. Platelet sensitivity to prostaglandin E1 inhibition is reduced in pre-eclampsia but not in nonproteinuric gestational hypertension. British Journal of Obstetrics and Gynaecology 1996;103(1):19-24.

5. Robb A.O., Din J.N., Mills N.L., Smith I.B., Blomberg A., Zikry M.N., et al. The influence of the menstrual cycle, normal pregnancy and pre-eclampsia on platelet activation. Journal of Thrombosis and Haemostasis 2010;103(2):372-8.

6. Clark P., Brennand J., Conkie J.A., McCall F., Greer I.A., Walker I.D. Activated protein C sensitivity, protein C, protein S and coagulation in normal pregnancy. Journal of Thrombosis and Haemostasis 1998;79(6):1166-70.

7. Hellgren M., Blömback M. Studies on blood coagulation and fibrinolysis in pregnancy, during delivery and in the puerperium. I. Normal condition. Gynecologic and Obstetric Investigation 1981;12(3):141-54.

8. Kjellberg U., Andersson N.E., Rosén S., Tengborn L., Hellgren M. APC resistance and other haemostatic variables during pregnancy and puerperium. Journal of Thrombosis and Haemostasis 1999;81(4):527-31.

9. Cerneca F., Ricci G., Simeone R., Malisano M., Alberico S., Guaschino S. Coagulation and fibrinolysis changes in normal pregnancy increased levels of procoagulants and reduced levels of inhibitors during pregnancy induce a hypercoagulable state, combined with a reactive fibrinolysis. European Journal of Obstetrics Gynecology and Reproductive Biology 1997;73(1):31-6.

10. Sattar N., Greer I.A., Rumley A., Stewart G., Shepherd J., Packard C.J., et al. A longitudinal study of the relationships between haemostatic, lipid, and oestradiol changes during normal human pregnancy. Journal of Thrombosis and Haemostasis 1999;81(1):71-5.

11. Almagor M., Gorten D., Yaffe H. Decreased levels of fibrinogen in very high parity women. European Journal of Obstetrics & Gynecology and Reproductive Biology 2003;106(2):148-50.

12. Colman-Brochu S. Deep vein thrombosis in pregnancy. The American Journal of Maternal/Child Nursing 2004;29(3):186-92.

13. Dunlop W. Normal Pregnancy: Physiology and Endocrinology. In: Edmonds D.K., Dewhurst J., editors. Dewhurst's textbook of obstetrics and gynaecology for postgraduates. 6th ed. Oxford: Blackwell Science Ltd; 1999:76-90.

14. O'Riordan M.N., Higgins J.R. Haemostasis in normal and abnormal pregnancy. Best Practice and Research: Clinical Obstetrics and Gynaecology 2003;17(3):385-96.

15. Mosesson M.W. Fibrinogen and fibrin structure and functions. Journal of Thrombosis and Haemostasis 2005;3(8):1894-904.

16. Weisel J.W. Fibrinogen and fibrin. In: Parry D.A.D., Squire J., editors. Coiled-Coils, Collagen & Elstomers.San Diego: Elsevier; 2005:247-99.

17. Medved L., Ugarova T., Veklich Y., Lukinova N., Weisel J. Electron microscope investigation of the early stages of fibrin assembly. Twisted protofibrils and fibers. Journal of Molecular Biology 1990;216(3):503-9.

173

18. Weisel J.W., Nagaswami C., Makowski L. Twisting of fibrin fibers limits their radial growth. Proceedings of the National Academy of Sciences of the United States of America 1987;84(24):8991-5.

19. Weisel J.W., 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):111-28.

20. Ryan E.A., Mockros L.F., Weisel J.W., Lorand L. Structural origins of fibrin clot rheology. Biophysical Journal 1999;77(5):2813-26.

21. Weisel J.W. The mechanical properties of fibrin for basic scientists and clinicians. Biophysical Chemistry 2004;112(2-3 SPEC. ISS.):267-76.

22. Weisel J.W. Structure of fibrin: Impact on clot stability. Journal of Thrombosis and Haemostasis 2007;5(SUPPL. 1):116-24.

23. Mustard J.F., Packham M.A. Factors influencing platelet function: adhesion, release, and aggregation. Pharmacological Reviews 1970;22(2):97-187.

24. Holmsen H. Prostaglandin endoperoxide - thromboxane synthesis and dense granule secretion as positive feedback loops in the propagation of platelet responses during 'The basic platelet reaction'. Journal of Thrombosis and Haemostasis 1977;38(4):1030-41.

25. Mills D.C.B., Macfarlane D.E. Platelet receptors. In: Gordon J.L., editor. Platelets in Biology and Pathology.New York: North-Holland; 1976. p. 159-93.

26. Holmsen H., Day H.J., Setkowsky C.A. Secretory mechanisms. Behaviour of adenine nucleotides during the platelet release reaction induced by adenosine diphosphate and adrenaline. Biochemical Journal 1972;129(1):67-82.

174

27. Smith J.B., Ingerman C., Kocsis J.J., Silver M.J. Formation of prostaglandins during the aggregation of human blood platelets. Journal of Clinical Investigation 1973;52(4):965-9.

28. Stedman's Medical Dictionary. 28th ed. Philadelphia: Lippincott Williams & Wilkins; 2006. p. 477.

29. Silverthorn D.U. Human Physiology: an integrated approach. 4th ed. San Fracisco: Pearson Benjamin Cummings; 2007.

30. Espey L.L. Ovulation. In: Knobil E., Neill J.D., editors. Encyclopedia of Reproduction.San Diego: Academic Press; 1999: 605-14.

31. Robker R.L., Russell D.L., Yoshioka S., Sharma S.C., Lydon J.P., O'Malley B.W., et al. Ovulation: A multi-gene, multi-step process. Steroids 2000;65(10-11):559-70.

32. Richards J.S. Hormonal control of gene expression in the ovary. Endocrine Reviews 1994;15(6):725-51.

33. Richards J.S., Fitzpatrick S.L., Clemens J.W., Morris J.K., Alliston T., Sirois J. Ovarian cell differentiation: A cascade of multiple hormones, cellular signals, and regulated genes. Recent Progress in Hormone Research 1995;50(1):223-54.

34. Richards J.S., Russell D.L., Robker R.L., Dajee M., Alliston T.N. Molecular mechanisms of ovulation and luteinization. Molecular and Cellular Endocrinology 1998;145(1-2):47-54.

35. Espey L.L., Bellinger A., Healy J.A. Ovulation: an inflammatory cascade of gene expression. In: Leung P.C.K., Adashi E.Y., editors. The Ovary.New York: Academic Press; 2004. p. 145-65.

36. Espey L.L., Lipner H. Ovulation. In: Knobil E., Neill J.D., editors. The Physiology of Reproduction.New York: Raven Press; 1994. p. 725-80.

175

37. Cole L.A., Ladner D.G., Byrn F.W. The normal variabilities of the menstrual cycle. Fertility and Sterility 2009;91(2):522-7.

38. Gentry P.A., Plante L., Schroeder M.O.B., Lamarre J., Young J.E., Dodds W.G. Human ovarian follicular fluid has functional systems for the generation and modulation of thrombin. Fertility and Sterility 2000;73(4):848-54.

39. Suchanek E., Mujkic-Klaric A., Grizelj V., Simunic V., Kopjar B. Protein concentration in pre-ovulatory follicular fluid related to ovarian stimulation. International Journal of Gynecology and Obstetrics 1990;32(1):53-9.

40. Shalgi R., Kraicer P., Rimon A. Proteins of human follicular fluid: the blood follicle barrier. Fertility and Sterility 1973;24(6):429-34.

41. Gentry P.A., Downie H.G. Blood coagulation and hemostasis. In: Swenson M.J., Reece W.O., editors. Duke's physiology of domestic animals. 11th ed. Ithaca: Cornell University Press; 1993:49-63.

42. Davie E.W., Fujikawa K., Kisiel W. The coagulation cascade: Initiation, maintenance, and regulation. Biochemistry 1991;30(43):10363-70.

43. Yamada M., Gentry P.A. The hemostatic profile of equine ovarian follicular fluid. Thrombosis Research 1995;77(1):45-54.

44. Gentry P.A., Zareie M., Liptrap R.M. Fibronectin concentrations correlate with ovarian follicular size and estradiol values in equine follicular fluid. Animal Reproduction Science 1996;45(1-2):91-102.

45. Wang J.S., Jen C.J., Lee H.L., Chen H.I. Effects of short-term exercise on female platelet function during different phases of the menstrual cycle. Arteriosclerosis, Thrombosis, and Vascular Biology 1997;17(9):1682-6.

176

46. Markham S.M., Dubin N.H., Rock J.A. The effect of the menstrual cycle and of decompression stress on arachidonic acid-induced platelet aggregation and on intrinsic platelet thromboxane production in women compared with men. Am J Obstet Gynecol 1991;165(6 I):1821-9.

47. Sarchielli P., Tognoloni M., Russo S., Vulcano M.R., Feleppa M., Malá M., et al. Variations in the platelet arginine/nitric oxide pathway during the ovarian cycle in females affected by menstrual migraine. Cephalalgia 1996;16(7):468-75.

48. Teran E., Escudero C., Vivero S. Physiological changes in platelet aggregation and nitric oxide levels during menstrual cycle in healthy women. Nitric Oxide - Biology and Chemistry 2002;7(3):217-20.

49. Motomiya T., Yamazaki H. Plasma β-thromboglobulin and platelet factor 4 during the normal menstrual cycle. Acta Haematologica Japonica 1981;44(1):193-5.

50. Yamazaki H., Motomiya T., Sonoda M., Miyagawa N. Changes in platelet aggregability after ovariectomy. Journal of Thrombosis and Haemostasis1979;42(4):1332-46.

51. Ersöza G., Zergeroðlub A.M., Yakaryilmaz A. The effect of submaximal exercise on platelet aggregation during late follicular and midluteal phases in women. Thrombosis Research 2002;108(2-3):147-50.

52. Stedman's Medical Dictionary. 28th ed. Philadelphia: Lippincott Williams & Wilkins; 2006:1553.

53. Warrell D.A., Cox T.M., Firth J.D., Benz E.J.Jr. Oxford Textbook of Medicine. 4th ed. Oxford: Oxford University Press; 2005.

54. Sherwood L. Human Physiology - From Cells to Systems. Seventh ed. Belmont: Brooks Cole; 2008.

55. Hellgren M. Hemostasis during normal pregnancy and puerperlum. Seminars in Thrombosis and Hemostasis 2003;29(2):125-30.

177

56. Holmes V.A., Wallace J.M.W. Haemostasis in normal pregnancy: A balancing act? Biochemical Society Transactions 2005;33(2):428-32.

57. McColl M.D., Ramsay J.E., Tait R.C., Walker I.D., McCall F., Conkie J.A., et al. Risk factors for pregnancy associated venous thromboembolism. Journal of Thrombosis and Haemostasis 1997;78(4):1183-8.

58. Greer I.A. Haemostasis and thrombosis in pregnancy. In: Bloom A.L., Forbes C.D., Thomas D.P., Tuddenham E.G.D., editors. Haemostasis and Thrombosis. 3rd ed. Edinburg: Churchhill Livingstone; 1994. p. 987-1015.

59. Brenner B. Haemostatic changes in pregnancy. Thrombosis Research 2004;114(5-6 SPEC. ISS.):409-14.

60. Dahlman T., Hellgren M., Blömback M. Changes in blood coagulation and fibrinolysis in the normal puerperium. Gynecologic and Obstetric Investigation 1985;20(1):37-44.

61. Liu X., Jiang Y., Shi H., Yue X., Wang Y., Yang H. Prospective, sequential, longitudinal study of coagulation changes during pregnancy in Chinese women. International Journal of Gynecology and Obstetrics 2009;105(3):240-3.

62. Drouet L., Bal Dit Sollier C. Fibrinogen: Factor and marker of cardiovascular risk. Journal des Maladies Vasculaires 2002;27(3):143-56.

63. Nakashima A., Kobayashi T., Terao T. Fibrinolysis during normal pregnancy and severe preeclampsia relationships between plasma levels of plasminogen activators and inhibitors. Gynecologic and Obstetric Investigation 1996;42(2):95-101.

64. Bellart J., Gilabert R., Fontcuberta J., Borrell M., Miralles R.M., Cabero L. Fibrinolysis changes in normal pregnancy. Journal of Perinatal Medicine 1997;25(4):368-72.

178

65. Ayhan A., Akkok E., Urman B., Yarali H., Dundar S., Kirazh S. Beta-thromboglobulin and platelet factor 4 levels in pregnancy and preeclampsia. Gynecologic and Obstetric Investigation 1990;30(1):12-4.

66. Andersson T., Lorentzen B., Hogdahl H., Clausen T., Mowinckel M.C., Abildgaard U. Thrombin-inhibitor complexes in the blood during and after delivery. Thrombosis Research 1996;82(2):109-17.

67. Louden K.A., Broughton Pipkin F., Heptinstall S., Fox S.C., Mitchell J.R.A., Symonds E.M. A longitudinal study of platelet behaviour and thromboxane production in whole blood in normal pregnancy and the puerperium. British Journal of Obstetrics and Gynaecology 1990;97(12):1108-14.

68. Boehlen F., Hohlfeld P., Extermann P., Perneger T.V., De Moerloose P. Platelet count at term pregnancy: A reappraisal of the threshold. Obstetrics and Gynecology 2000;95(1):29-33.

69. Karalis I., Nadar S.K., Al Yemeni E., Blann A.D., Lip G.Y.H. Platelet activation in pregnancy-induced hypertension. Thrombosis Research 2005;116(5):377-83.

70. Sejeny S.A., Eastham R.D., Baker S.R. Platelet counts during normal pregnancy. Journal of Clinical Pathology 1975;28(10):812-3.

71. Fay R.A., Hughes A.O., Farron N.T. Platelets in pregnancy: Hyperdestruction in pregnancy. Obstetrics and Gynecology 1983;61(2):238-40.

72. Flood K., Peace A., Kent E., Tedesco T., Dicker P., Geary M., et al. 209: Platelet function in the first trimester. Am J Obstet Gynecol 2009;201(6, Supplement 1):S89-S90.

73. Wallenburg H.C.S., Van Kessel P.H. Platelet lifespan in normal pregnancy as determined by a nonradioisotopic technique. British Journal of Obstetrics and Gynaecology 1978;85(1):33-6.

179

74. Danon D. Aging of the erythrocyte. In: Cristafolo V., editor. Handbook of cell biology of aging. Florida: CRC Press; 1985. p. 317-40.

75. Piomelli S., Lurinsky G., Wasserman L.R. The mechanism of red cell aging. I. Relationship between cell age and specific gravity evaluated by ultracentrifugation in a discontinuous density gradient. The Journal of Laboratory and Clinical Medicine 1967;69(4):659-74.

76. Peck T.M., Arias F. Hematologic changes associated with pregnancy. Clinical Obstetrics and Gynecology 1979;22(4):785-98.

77. Harrison K.A. Blood volume changes in normal pregnant Nigerian women. The Journal of obstetrics and gynaecology of the British Commonwealth 1966;73(5):717-23.

78. Lund C.J., Donovan J.C. Blood volume during pregnancy. Significance of plasma and red cell volumes. Am J Obstet Gynecol 1967;98(3):393-403.

79. McFee J.G. Anemia in pregnancy - a reappraisal. Obstetrical and Gynecological Survey 1973;28(11):769-93.

80. Huisman A., Aarnoudse J.G., Krans M., Huisjes H.J., Fidler V., Zijlstra W.G. Red cell aggregation during normal pregnancy. British Journal of Haematology 1988;68(1):121-4.

81. Bollini A., Hernández G., Luna M.B., Cinara L., Rasia M. Study of intrinsic flow properties at the normal pregnancy second trimester. Clinical Hemorheology and Microcirculation 2005;33(2):155-61.

82. van den Broek N.R., Letsky E.A. Pregnancy and the erythrocyte sedimentation rate. BJOG: An International Journal of Obstetrics & Gynaecology 2001;108(11):1164-7.

83. Lukacín S., Rychnavský J., Mojzis J., Mirossay L., Jurcová E., Nicák A. Changes of erythrocyte microrheology during normal pregnancy and after delivery. European Journal of Obstetrics & Gynecology and Reproductive Biology 1996;66(2):125-8.

180

84. Kaibara M., Marumoto Y., Kobayashi T. Erythrocyte filterability and fetal development in normal pregnancy. Am J Obstet Gynecol 1985;152(6 I):719-20.

85. Mandelli B., Polatti F., Bolis P.F. Study of erythrocyte deformability in physiological pregnancy. Clinical and Experimental Obstetrics and Gynecology 1985;12(1-2):16-20.

86. Rodgers B.D., Hreshchyshyn M.M., Lee R.V., Rodgers D., Ambrus C.M. Erythrocyte filterability in normal and high-risk pregnancy. Obstetrics and Gynecology 1988;71(2):192-7.

87. Boulot P., Brun J.F., Lagoueyte C., Viala J.L., Orsetti A. Red cell filterability in preeclamptic versus normal pregnant women and in their newborns. Revista Portuguesa de Hemorreologia 1989;3(1):49-56.

88. Huisman A., Aarnoudse J.G., Heuvelmans J.H.A., Goslinga H., Fidler V., Huisjes H.J., et al. Whole blood viscosity during normal pregnancy. British Journal of Obstetrics and Gynaecology 1987;94(12):1143-9.

89. Oosterhof H., Wichers G., Fidler V., Aarnoudse J.G. Blood viscosity and uterine artery flow velocity waveforms in pregnancy: A longitudinal study. Placenta 1993;14(5):555-61.

90. Heilmann L., Hojnacki B., Tempelhoff v. G.-F. Red cell deformability in gestational hypertension. Gynecologic and Obstetric Investigation 1994;37(3):155-9.

91. Mitsui Y., Eguchi K., Hiramatsu Y., Noji S. Changes in erythrocyte deformability in normal pregnancy and pregnancy-induced hypertension, as revealed by electron spin resonance. Acta Medica Okayama 1994;48(1):1-5.

92. Weed R.I. The importance of erythrocyte deformability. The American Journal of Medicine 1970;49(2):147-50.

181

93. Mohandas N., Chasis J.A., Shohet S.B. The influence of membrane skeleton on red cell deformability, membrane material properties, and shape. Seminars in Hematology 1983;20(3):225-42.

94. Bennett V. Spectrin-based membrane skeleton: A multipotential adaptor between plasma membrane and cytoplasm. Physiological Reviews 1990;70(4):1029-65.

95. Pinder J.C. Red cell membrane cytoskeleton and the control of membrane properties. Biochemical Society Transactions 1991;19(4):1039-41.

96. Lurie S. Age distribution of erythrocyte population in late pregnancy. Gynecologic and Obstetric Investigation 1990;30(3):147-9.

97. Lurie S. Changes in age distribution of erythrocytes during pregnancy: A longitudinal study. Gynecologic and Obstetric Investigation 1993;36(3):141-4.

98. Lurie S., Danon D. Life span of erythrocytes in late pregnancy. Obstetrics and Gynecology 1992;80(1):123-6.

99. Lurie S., Danon D. The effect of erythropoietin on age distribution of erythrocytes in pregnant mice. Journal of Maternal-Fetal Investigation 1996;6(2):107-9.

100. Bolton F.G., Street M.J., Pace A.J. Changes in erythrocyte volume and shape in pregnancy. British Journal of Obstetrics and Gynaecology 1982;89(12):1018-20.

101. Eguchi K., Sawai T., Mizutani Y., Yonezawa M. Comparative study of erythrocyte deformability in maternal and cord blood. American Journal of Perinatology 1995;12(1):39-43.

102. Beutler E. The relationship of red cell enzymes to red cell life-span. Blood Cells 1988;14(1):69-75.

103. Landaw S.A. Factors that accelerate or retard red blood cell senescence. Blood Cells 1988;14(1):47-59.

104. Lurie S., Mamet Y. Red blood cell survival and kinetics during pregnancy. European Journal of Obstetrics & Gynecology and Reproductive Biology 2000;93(2):185-92.

105. Brecher G., Stohlman J.F. Reticulocyte size and erythropoietic stimulation. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, N Y) 1961;107:887-91.

106. Spivak J.L. Serum immunoreactive erythropoietin in health and disease. Journal of Perinatal Medicine 1995;23(1-2):13-7.

107. Moccia G., Miller M.E., Garcia J.F., Cronkite E.P. The effect of plethora on erythropoietin levels. Proceedings of the Society for Experimental Biology and Medicine 1980;163(1):36-8.

108. Stohlman J.F. Humoral regulation of erythropoiesis. VII. Shortened survival of erythrocytes produced by erythropoietine or severe anemia. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, N Y) 1961;107:884-7.

109. Seno S., Miyahara M., Asakura H., Ochi O., Matsuoka K., Toyama T. Macrocytosis resulting from early denucleation of erythroid precursors. Blood 1964;24:582-93.

110. Howells M.R., Jones S.E., Napier J.A.F. Erythropoeisis in pregnancy. British Journal of Haematology 1986;64(3):595-9.

111. Zivny J., Kobilkova J., Neuwirt J., Andrasova V. Regulation of erythropoiesis in fetus and mother during normal pregnancy. Obstetrics and Gynecology 1982;60(1):77-81.

112. Jepson J.H. Endocrine control of maternal and fetal erythropoiesis. Canadian Medical Association journal 1968;98(18):844-7.

113. Cotes P.M., Canning C.E., Lind T. Changes in serum immunoreactive erythropoietin during the menstrual cycle and normal pregnancy. British Journal of Obstetrics and Gynaecology 1983;90(4):304-11.

114. Landaw S.A., Bristol S.K., Van Dyke D.C. Prolongation of RBC survival in the hypophysectomized rat. Proceedings of the Society for Experimental Biology and Medicine 1971;138(1):152-6.

115. Heilmann L. Blood rheology and pregnancy. Bailliere's Clinical Haematology 1987;1(3):777-99.

116. Letsky E.A. Erythropoiesis in pregnancy. Journal of Perinatal Medicine 1995;23(1-2):39-45.

117. Cavill I. Iron and erythropoiesis in normal subjects and in pregnancy. Journal of Perinatal Medicine 1995;23(1-2):47-50.

118. Michelson A.D., Linden M.D., Barnard M.R., Furman M.I., Frelinger A.L. Flow cytometry. In: Michelson A.D., editor. Platelets. Second ed. San Diego: Academic Press; 2007:545-63.

119. Santos M.T., Valles J., Marcus A.J., Safier L.B., Broekman M.J., Islam N., et al. Enhancement of platelet reactivity and modulation of eicosanoid production by intact erythrocytes. A new approach to platelet activation and recruitment. Journal of Clinical Investigation 1991;87(2):571-80.

120. LaRosa C.A., Rohrer M.J., Benoit S.E., Rodino L.J., Barnard M.R., Michelson A.D., et al. Human neutrophil cathepsin G is a potent platelet activator. Journal of Vascular Surgery 1994;19(2):306-19.

121. Shattil S.J., Cunningham M., Hoxie J.A. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 1987;70(1):307-15.

184

122. Michelson A.D., Ellis P.A., Barnard M.R., Matic G.B., Viles A.F., Kestin A.S. Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, adenosine diphosphate, or an in vivo wound. Blood 1991;77(4):770-9.

123. Abrams C., Shattil S.J. Immunological detection of activated platelets in clinical disorders. Journal of Thrombosis and Haemostasis1991;65(5):467-73.

124. Michelson A.D. Platelet activation by thrombin can be directly measured in whole blood through the use of the peptide GPRP and flow cytometry: Methods and clinical applications. Blood Coagulation and Fibrinolysis 1994;5(1):121-31.

125. Schmitz G., Rothe G., Ruf A., Barlage S., Tschope D., Clemetson K.J., et al. European Working Group on Clinical Cell Analysis: Consensus protocol for the flow cytometric characterisation of platelet function. Journal of Thrombosis and Haemostasis 1998;79(5):885-96.

126. Fijnheer R., Modderman P.W., Veldman H., Ouwehand W.H., Nieuwenhuis H.K., Roos D., et al. Detection of platelet activation with monoclonal antibodies and flow cytometry. Changes during platelet storage. Transfusion 1990;30(1):20-5.

127. Gawaz M., Bogner C. Changes in platelet membrane glycoproteins and platelet-leukocyte interaction during hemodialysis. Clinical Investigator 1994;72(6):424-9.

128. Wun T., Paglieroni T., Sazama K., Holland P. Detection of plasmapheresis-induced platelet activation using monoclonal antibodies. Transfusion 1992;32(6):534-40.

129. Triulzi D.J., Kickler T.S., Braine H.G. Detection and significance of alpha granule membrane protein 140 expression on platelets collected by apheresis. Transfusion 1992;32(6):529-33.

130. Gutensohn K., Bartsch N., Kuehnl P. Flow cytometric analysis of platelet membrane antigens during and after continuous-flow plateletpheresis. Transfusion 1997;37(8):809-15.

185

131. Phillips D.R., Charo I.F., Parise L.V., Fitzgerald L.A. The platelet membrane glycoprotein IIb-IIIa complex. Blood 1988;71(4):831-43.

132. McMichael A.J., Beverley P.C.L., Cobbold S., Crumpton M.J., Gilks F.M., Gotch N., et al. Leukocyte Typing III. Oxford: Oxford University Press; 1987.

133. Du X., Ginsberg M.H. Integrin alpha IIb beta 3 and platelet function. Journal of Thrombosis and Haemostasis 1997;78(1):96-100.

134. Wang C., Mody M., Herst R., Sher G., Freedman J. Flow cytometric analysis of platelet function in stored platelet concentrates. Transfusion Science 1999;20(2):129-39.

135. George J.N. Changes in platelet membrane glycoproteins during blood bank storage. Blood Cells 1992;18(3):501-11.

136. Isenberg W.M., McEver R.P., Phillips D.R. The platelet fibrinogen receptor: An immunogold-surface replica study of agonist-induced ligand binding and receptor clustering. Journal of Cell Biology 1987;104(6):1655-63.

137. Woods J., Wolff L.E., Keller D.M. Resting platelets contain a substantial centrally located pool of glycoprotein IIb-IIIa complex which may be accessible to some but not other extracellular proteins. Journal of Biological Chemistry 1986;261(32):15242-51.

138. Wencel-Drake J.D., Plow E.F., Kunicki T.J. Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. American Journal of Pathology 1986;124(2):324-34.

139. Sjöbring U., Ringdahl U., Ruggeri Z.M. Induction of platelet thrombi by bacteria and antibodies. Blood 2002;100(13):4470-7.

140. Ware J., Ruggeri Z.M. Platelet adhesion receptors and their participation in hemostasis and thrombosis. Drugs of Today 2001;37(4):265-74.

141. André P., Srinivasa Prasad K.S., Denis C.V., He M., Papalia J.M., Hynes R.O., et al. CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. Nature Medicine 2002;8(3):247-52.

142. Clementson K.J. A short history of platelet glycoprotein Ib complex. Journal of Thrombosis and Haemostasis2007;98(1):63-8.

143. Luo S.Z., Li R. Specific Heteromeric Association of Four Transmembrane Peptides Derived from Platelet Glycoprotein Ib-IX Complex. Journal of Molecular Biology 2008;382(2):448-57.

144. Ruan C., Du X., Xi X. A murine antiglycoprotein Ib complex monoclonal antibody, SZ 2, inhibits platelet aggregation induced by both ristocetin and collagen. Blood 1987;69(2):570-7.

145. Du X., Beutler L., Ruan C. Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. Blood 1987;69(5):1524-7.

146. Rivera J., Lozano M.L., Corral J., Gonza¦ülez-Conejero R., Marti¦ünez C., Vicente V. Platelet GP Ib/IX/V complex: Physiological role. Journal of Physiology and Biochemistry 2000;56(4):355-66.

147. Michelson A.D. CD42 cluster workshop report. In: Schlossman S.F.e.al., editor. Leucocyte Typing V, White Cell Differentiation Antigens. Oxford University Press; 1995. p. 1309-10.

148. Blanchard D., Borche L., Petit-Frioux Y., Muller J.Y. Cell expression and biochemical characterization of platelet antigens recognized by workshop platelet panel mAb. In: Schlossman S.F.e.al., editor. Leucocyte Typing V, White Cell Differentiation Antigens. Oxford University Press; 1995. p. 1225-9.

149. Berndt M.C., Shen Y., Dopheide S.M., Gardiner E.E., Andrews R.K. The vascular biology of the glycoprotein Ib-IX-V complex. Journal of Thrombosis and Haemostasis2001;86(1):178-88.

187

150. Kansas G.S. Selectins and their ligands: Current concepts and controversies. Blood 1996;88(9):3259-87.

151. McEver R.P. Properties of GMP-140, an inducible granule membrane protein of platelets and endothelium. Blood Cells 1990;16(1):73-83.

152. De Bruijne-Admiraal L.G., Modderman P.W., Von dem Borne A.E.G.K., Sonnenberg A. P-selectin mediates Ca2+-dependent adhesion of activated platelets to many different types of leukocytes: Detection by flow cytometry. Blood 1992;80(1):134-42.

153. Holme S., Sweeney J.D., Sawyer S., Elfath M.D. The expression of p-selectin during collection, processing, and storage of platelet concentrates: Relationship to loss of in vivo viability. Transfusion 1997;37(1):12-7.

154. Divers S.G., Kannan K., Stewart R.M., Betzing K.W., Dempsey D., Fukuda M., et al. Quantitation of CD62, soluble CD62, and lysosome-associated membrane proteins 1 and 2 for evaluation of the quality of stored platelet concentrates. Transfusion 1995;35(4):292-7.

155. Maecker H.T., Todd S.C., Levy S. The tetraspanin superfamily: Molecular facilitators. FASEB Journal 1997;11(6):428-42.

156. Nieuwenhuis H.K., Van Oosterhout J.J.G., Rozemuller E., Van Iwaarden F., Sixma J.J. Studies with a monoclonal antibody against activated platelets: Evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. Blood 1987;70(3):838-45.

157. Metzelaar M.J., Korteweg J., Sixma J.J., Nieuwenhuis H.K. Comparison of platelet membrane markers for the detection of platelet activation in vitro and during platelet storage and cardiopulmonary bypass surgery. Journal of Laboratory and Clinical Medicine 1993;121(4):579-87.

158. Pretorius E. The role of platelet and fibrin ultrastructure in identifying disease patterns. Pathophysiology of Haemostasis and Thrombosis 2008;36(5):251-8.

159. Pretorius E. Quantifying changes in fibrin fiber network morphology. Ultrastructural Pathology 2011;35(4):150-4.

160. Pretorius E., Briedenhann S., Marx J., Smit E., Van Der Merwe C., Pieters M., et al. Ultrastructural comparison of the morphology of three different platelet and fibrin fiber preparations. Anatomical Record 2007;290(2):188-98.

161. Pretorius E., Humphries P., Ekpo O.E., Smit E., Van Der Merwe C.F. Comparative ultrastructural analyses of mouse, rabbit, and human platelets and fibrin networks. Microscopy Research and Technique 2007;70(9):823-7.

162. Marti G.E., Stetler-Stevenson M., Bleesing J.J.H., Fleisher T.A. Introduction to flow cytometry. Seminars in Hematology 2001;38(2):93-9.

163. Ault KA, Mitchell J. Chapter 17 Analysis of Platelets by Flow Cytometry. 42[C], 275-294. 1994.

164. Lazarus A.H., Wright J.F., Blanchette V., Freedman J. Analysis of platelets by flow cytometry. Transfusion Science 1995;16(4):353-61.

165. Michelson A.D. Flow cytometry: A clinical test of platelet function. Blood 1996;87(12):4925-36.

166. Coller B.S., Peerschke E.I., Scudder L.E., Sullivan C.A. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: Additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood 1983;61(1):99-110.

167. Montgomery R.R., Kunicki T.J., Taves C. Diagnosis of Bernard-Soulier syndrome and Glanzmann's thrombasthenia with a monoclonal assay on whole blood. Journal of Clinical Investigation 1983;71(2):385-9.

189

168. Wall J.E., Buijs-Wilts M., Arnold J.T., Wang W., White M.M., Jennings L.K., et al. A flow cytometric assay using mepacrine for study of uptake and release of platelet dense granule contents. British Journal of Haematology 1995;89(2):380-5.

169. Michelson A.D., Barnard M.R., Krueger L.A., Frelinger III A.L., Furman M.I. Evaluation of Platelet Function by Flow Cytometry. Methods 2000;21(3):259-70.

170. van Velzen JF, Laros-van Gorkom BAP, Pop GAM, van Heerde WL. Multicolor flow cytometry for evaluation of platelet surface antigens and activation markers. Thrombosis Research 2012;130(1):92-8.

171. Goodall A.H., Appleby J. Flow-cytometric analysis of platelet-membrane glycoprotein expression and platelet activation. Methods in molecular biology (Clifton, N J) 2004;272:225-53.

172. Pham A., Wang J. Bernard-Soulier syndrome: An inherited platelet disorder. Archives of Pathology and Laboratory Medicine 2007;131(12):1834-6.

173. Robert S., Poncelet P., Lacroix R., Arnaud L., Giraudo L., Hauchard A., et al. Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: A first step towards multicenter studies? Journal of Thrombosis and Haemostasis 2009;7(1):190-7.

174. Aurich K., Spoerl M.C., Fürll B., Sietmann R., Greinacher A., Hosten N., et al. Development of a method for magnetic labeling of platelets. Nanomedicine : Nanotechnology , Biology and Medicine 2012;8(5)537-44.

175. Chow S., Hedley D., Grom P., Magari R., Jacobberger J.W., Shankey T.V. Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. Cytometry Part A 2005;67(1):4-17.

176. Prabhala R.H., Neri P., Bae J.E., Tassone P., Shammas M.A., Allam C.K., et al. Dysfunctional T regulatory cells in multiple myeloma. Blood 2006;107(1):301-4.

190

177. Hess D.A., Wirthlin L., Craft T.P., Herrbrich P.E., Hohm S.A., Lahey R., et al. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. Blood 2006;107(5):2162-9.

178. Weil R. Sodium citrate in the transfusion of blood. Journal of the American Medical Association 1983;250(14):1901-4.

179. Ejim O.S., Powling M.J., Dandona P., Kernoff P.B.A., Goodall A.H. A flow cytometric analysis of fibronectin binding to platelets from patients with peripheral vascular disease. Thrombosis Research 1990;58(5):519-24.

180. Wehmeier A., Tschope D., Esser J., Menzel C., Nieuwenhuis H.K., Schneider W. Circulating activated platelets in myeloproliferative disorders. Thrombosis Research 1991;61(3):271-8.

181. Ault K.A., Rinder H.M., Mitchell J.G., Rinder C.S., Lambrew C.T., Hillman R.S. Correlated measurement of platelet release and aggregation in whole blood. Cytometry 1989;10(4):448-55.

182. Matzdorff A.C., Kühnel G., Kemkes-Matthes B., Pralle H. Quantitative assessment of platelets, platelet microparticles, and platelet aggregates with flow cytometry. Journal of Laboratory and Clinical Medicine 1998;131(6):507-17.

183. Hübl W., Assadian A., Lax J., Meixner U., Fang I.F., Hagmüller G., et al. Assessing aspirin-induced attenuation of platelet reactivity by flow cytometry. Thrombosis Research 2007;121(1):135-43.

184. Rand M.L., Kuhle S. Platelets and platelet function testing in children. Progress in Pediatric Cardiology 2005;21(1):63-9.

185. Rubens F.D., Labow R.S., Waghray G., Robblee J. The importance of sampling site in the measurements of whole-blood platelet flow cytometry. Journal of Cardiothoracic and Vascular Anesthesia 1998;12(3):309-13.

191

186. Reed G.L. Platelet secretory mechanisms. Seminars in Thrombosis and Hemostasis 2004;30(4):441-50.

187. Fox J.E.B., Lipfert L., Clark E.A., Reynolds C.C., Austin C.D., Brugge J.S. On the role of the platelet membrane skeleton in mediating signal transduction. Journal of Biological Chemistry 1993;268(34):25973-84.

188. Becker R.C., Tracy R.P., Bovill E.G., Mann K.G., Ault K. The clinical use of flow cytometry for assessing platelet activation in acute coronary syndromes. Coronary Artery Disease 1994;5(4):339-45.

189. Yano Y., Kambayashi J., Kawasaki T., Sakon M. Quantitative determination of circulating platelet microparticles by flow cytometry. International Journal of Cardiology 1994;47(SUPPL.):S13-S20.

190. Schlossman S.F., Boumsell L., Gilks W., Harlan J.M., Kishimoto T., Morimoto C., et al. CD antigens 1993. Blood 1994;83(4):879-80.

191. Hsu-Lin S.C., Berman C.L., Furie B.C. A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. Journal of Biological Chemistry 1984;259(14):9121-6.

192. Stenberg P.E., McEver R.P., Shuman M.A. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. Journal of Cell Biology 1985;101(3):880-6.

193. Jennings L.K., Phillips D.R. Purification of glycoproteins IIb and III from human platelet plasma membranes and characterization of a calcium-dependent glycoprotein IIb-III complex. Journal of Biological Chemistry 1982;257(17):10458-66.

194. Peerschke E.I.B. Platelet membrane glycoproteins: Functional characterization and clinical applications. American Journal of Clinical Pathology 1992;98(4):455-63.

192

195. Ruggeri Z.M. The platelet glycoprotein Ib-IX complex. Progress in hemostasis and thrombosis 1991;10:35-68.

196. Michelson A.D. Thrombin-induced down-regulation of the platelet membrane glycoprotein Ib-IX complex. Semin Thromb Hemost 1992;18(1):18-27.

197. Michelson A.D., Barnard M.R. Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb-IIIa complex. Blood 1987;70(5):1673-8.

198. Michelson A.D., Benoit S.E., Furman M.I., Barnard M.R., Nurden P., Nurden A.T. The platelet surface expression of glycoprotein V is regulated by two independent mechanisms: Proteolysis and a reversible cytoskeletal-mediated redistribution to the surface-connected canalicular system. Blood 1996;87(4):1396-408.

199. Hourdille P., Heilmann E., Combrie R., Winckler J., Clemetson K.J., Nurden A.T. Thrombin induces a rapid redistribution of glycoprotein Ib-IX complexes within the membrane systems of activated human platelets. Blood 1990;76(8):1503-13.

200. Herd C.M., Page C.P. Pulmonary immune cells in health and disease: Platelets. European Respiratory Journal 1994;7(6):1145-60.

201. Lowe G.D.O., Rumley A., Mackie I.J. Plasma fibrinogen. Annals of Clinical Biochemistry 2004;41(6):430-40.

202. Bailey A.L., Scantlebury D.C., Smyth S.S. Thrombosis and antithrombotic therapy in women. Arteriosclerosis, Thrombosis, and Vascular Biology 2009;29(3):284-8.

203. Kadir R.A., Economides D.L., Sabin C.A., Owens D., Lee C.A. Variations in coagulation factors in women: Effects of age, ethnicity, menstrual cycle and combined oral contraceptive. Journal of Thrombosis and Haemostasis1999;82(5):1456-61.

204. Butenas S., Mann K.G. Blood coagulation. Biochemistry (Moscow) 2002;67(1):3-12.

193

205. Leng X.H., Hong S.Y., Larrucea S., Zhang W., Li T.T., López J.A., et al. Platelets of Female Mice Are Intrinsically More Sensitive to Agonists Than Are Platelets of Males. Arteriosclerosis, Thrombosis, and Vascular Biology 2004;24(2):376-81.

206. Liao S.L., Chen W.Y., Kuo J.S., Chen C.J. Association of serum estrogen level and ischemic neuroprotection in female rats. Neuroscience Letters 2001;297(3):159-62.

207. Suzuki S., Brown C.M., Wise P.M. Neuroprotective effects of estrogens following ischemic stroke. Frontiers in Neuroendocrinology 2009;30(2):201-11.

208. Wong J.H., Dukes J., Levy R.E., Sos B., Mason S.E., Fong T.S., et al. Sex differences in thrombosis in mice are mediated by sex-specific growth hormone secretion patterns. Journal of Clinical Investigation 2008;118(8):2969-78.

209. van der Spuy W.J., Pretorius E. The Interrelation between Inflammation, Thrombosis and Neuroprotection in Cerebral Ischaemia. Reviews in the Neurosciences 2012;23(3):269-78.

210. Wroblewski Lissin L., Cooke J.P. Phytoestrogens and cardiovascular health. Journal of the American College of Cardiology 2000;35(6):1403-10.

211. Gebara O.C.E., Mittleman M.A., Sutherland P., Lipinska I., Matheney T., Xu P., et al. Association Between Increased Estrogen Status and Increased Fibrinolytic Potential in the Framingham Offspring Study. Circulation 1995;91(7):1952-8.

212. Niswender G.D., Nett T.M. The corpus luteum and its control in infraprimate species. In: Knobil E., Neill J.D., editors. The Physiology of Reproduction.New York: Raven; 1994:781.

213. Ferin M., Jewelewicz R., Warren M. The Menstrual Cycle: Physiology, Reproductive Disorders, and Infertility. Oxford: Oxford University Press; 1993.

194

214. Fritz M.A., Speroff L. Clinical gyneacologic endocrinology and infertility. 8th ed. Philadelphia: Lippincott Williams & Wilkins; 2010.

215. Dighe A.S., Moy J.M., Hayes F.J., Sluss P.M. High-resolution reference ranges for estradiol, luteinizing hormone, and follicle-stimulating hormone in men and women using the AxSYM assay system. Clinical Biochemistry 2005;38(2):175-9.

216. Giardina E.G.V., Chen H.J., Sciacca R.R., Rabbani L.E. Dynamic variability of hemostatic and fibrinolytic factors in young women. Journal of Clinical Endocrinology and Metabolism 2004;89(12):6179-84.

217. Marieke Knol H., Kemperman R.F.J., Kluin-Nelemans H.C., Mulder A.B., Meijer K. Haemostatic variables during normal menstrual cycle: A systematic review. Journal of Thrombosis and Haemostasis 2012;107(1):22-9.

218. Trigg D.E., Wood M.G., Kouides P.A., Kadir R.A. Hormonal influences on hemostasis in women. Seminars in Thrombosis and Hemostasis 2011;37(1):77-86.

219. Eyre L., Gamlin F. Haemostasis, blood platelets and coagulation. Anaesthesia & Intensive Care Medicine 2010;11(6):244-6.

220. Austin S.K. Haemostasis. Medicine 2009;37(3):133-6.

221. Nash M.J., Cohen H. Disorders of Thrombosis and Haemostasis. Surgery (Oxford) 2002;20(9):203-8.

222. Monagle P., Massicotte P. Developmental haemostasis: Secondary haemostasis. Seminars in Fetal and Neonatal Medicine 2011;16(6):294-300.

223. van Geffen M., van Heerde W.L. Global haemostasis assays, from bench to bedside. Thrombosis Research 2012;129(6):681-7.

224. Minors D.S. Haemostasis, blood platelets and coagulation. Anaesthesia & Intensive Care Medicine 2007;8(5):214-6.

195

225. Batty P., Smith J.G. Haemostasis. Surgery (Oxford) 2010;28(11):530-5.

226. Mathias M., Liesner R. Understanding haemostasis. Paediatrics and Child Health 2007;17(8):317-21.

227. Gladner J.A. The action of thrombin on fibrinogen. In: Laki K., editor. Fibrinogen.New York: Marcel Dekker; 1968:87-115.

228. Allford S.L., Machin S.J. Haemostasis. Surgery (Oxford) 2007;25(6):241-4.

229. Gentry P.A. Comparative aspects of blood coagulation. The Veterinary Journal 2004;168(3):238-51.

230. Bombeli T., Spahn D.R. Updates in perioperative coagulation: Physiology and management of thromboembolism and haemorrhage. British Journal of Anaesthesia 2004;93(2):275-87.

231. Curry A.N.G., Pierce J.M.T. Conventional and near-patient tests of coagulation. Continuing Education in Anaesthesia, Critical Care and Pain 2007;7(2):45-50.

232. Hellgren M. Hemostasis during normal pregnancy and puerperium. Seminars in Thrombosis and Hemostasis 2003;29(2):125-30.

233. Davis G.L. Hemostatic changes associated with normal and abnormal pregnancies. Clinical laboratory science: journal of the American Society for Medical Technology 2000;13(4):223-8.

234. Lockwood C.J. Heritable coagulopathies in pregnancy. Obstetrical and Gynecological Survey 1999;54(12):754-65.

235. Bremme K.A. Haemostatic changes in pregnancy. Best Practice & Research Clinical Haematology 2003;16(2):153-68.

236. Dalaker K., Prydz H. The coagulation factor VII in pregnancy. British Journal of Haematology 1984;56(2):233-41.

196

237. De Boer K., Ten Cate J.W., Sturk A., Borm J.J.J., Treffers P.E. Enhanced thrombin generation in normal and hypertensive pregnancy. Am J Obstet Gynecol 1989;160(1):95-100.

238. Girling J.C. Physiology of pregnancy. Anaesthesia & Intensive Care Medicine 2004 1;5(7):215-8.

239. Uchikova E.H., Ledjev I.I. Changes in haemostasis during normal pregnancy. European Journal of Obstetrics & Gynecology and Reproductive Biology 2005 1;119(2):185-8.

240. Thornton P., Douglas J. Coagulation in pregnancy. Best Practice & Research Clinical Obstetrics & Gynaecology 2010;24(3):339-52.

241. Knijff S.C.M. Summary of contraindications to oral contraceptives. New York: Parthenon Publishing Group; 2000.

242. Pabinger I., Grafenhofer H. Pregnancy-associated thrombosis. Wiener Klinische Wochenschrift 2003;115(13-14):482-4.

243. Torshin I.Y. Physiology and medicine. New York: Nova Science Publishers; 2007.

244. La Corte ALC, Philippou H, Arins RAS. Role of fibrin structure in thrombosis and vascular disease 2011;83:75-127.

245. Wolberg A.S. Determinants of fibrin formation, structure, and function. Current Opinion in Hematology 2012;19(5):349-56.

246. Wolberg A.S., Campbell R.A. Thrombin generation, fibrin clot formation and hemostasis. Transfusion and Apheresis Science 2008;38(1):15-23.

247. Wolberg A.S. Plasma and cellular contributions to fibrin network formation, structure and stability. Haemophilia 2010;16:7-12.

248. Mosesson M.W. Fibrinogen functions and fibrin assembly. Fibrinolysis and Proteolysis 2000;14(2-3):182-6.

249. Wolberg A.S., Aleman M.M., Leiderman K., Machlus K.R. Procoagulant Activity in Hemostasis and Thrombosis: Virchow's Triad Revisited. Anesthesia & Analgesia 2012;114(2):275-85.

250. Wolberg A.S. Thrombin generation and fibrin clot structure. Blood Reviews 2007;21(3):131-42.

251. Hamik A., Lin Z., Kumar A., Balcells M., Sinha S., Katz J., et al. Kruppel-like factor 4 regulates endothelial inflammation. Journal of Biological Chemistry 2007;282(18):13769-79.

252. Blombäck B., Carlsson K., Fatah K., Hessel B., Procyk R. Fibrin in human plasma: Gel architectures governed by rate and nature of fibrinogen activation. Thrombosis Research 1994;75(5):521-38.

253. Collet J.P., Park D., Lesty C., Soria J., Soria C., Montalescot G., et al. Influence of fibrin network conformation and fibrin diameter on fibrinolysis speed: Dynamic and structural approaches by confocal microscopy. Arteriosclerosis, Thrombosis, and Vascular Biology 2000;20(5):1354-61.

254. Collet J.P., Lesty C., Montalescot G., Weisel J.W. Dynamic changes of fibrin architecture during fibrin formation and intrinsic fibrinolysis of fibrin-rich clots. Journal of Biological Chemistry 2003;278(24):21331-5.

255. Poort S.R., Rosendaal F.R., Reitsma P.H., Bertina R.M. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996;88(10):3698-703.

256. Pretorius E., Bronkhorst P., Briedenhann S., Smit E., Franz R.C. Comparisons of the fibrin networks during pregnancy, nonpregnancy and pregnancy during dysfibrinogenaemia using the scanning electron microscope. Blood Coagulation and Fibrinolysis 2009;20(1):12-6.

257. Weisel J.W. Fibrinogen and Fibrin. In: David A.D.P., editor. Advances in Protein Chemistry Fibrous Proteins: Coiled-Coils, Collagen and Elastomers. Volume 70 ed. Academic Press; 2005:247-99.

258. Rendu F., Brohard-Bohn B. The platelet release reaction: Granules' constituents, secretion and functions. Platelets 2001;12(5):261-73.

259. Rendu F., Brohard-Bohn B. Platelet organelles. In: Gresede P., editor. Platelets in thrombotic and non-thrombotic disorders: pathophysiology, pharmacology and therapeutics.Cambridge: Cambridge University Press; 2002. p. 104-12.

260. Zarbock A., Polanowska-Grabowska R.K., Ley K. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. Blood Reviews 2007;21(2):99-111.

261. Kenny L., Baker P.N., Cunningham F.G. Chapter 18 - Platelets, Coagulation, and the Liver. In: Marshall D.L., MD, James M.R., MD, Cunningham F.G., editors. Chesley's Hypertensive Disorders in Pregnancy (Third Edition).San Diego: Academic Press; 2009:335-51.

262. White J.G. Platelet structure. In: Michelson A.D., editor. Platelets. 2nd ed. Burlington: Academic Press; 2007:45-74.

263. Calverley D.C., Thiehelt C.D. Platelet structure and function in hemostasis and thrombosis. In: Greer J.P., editor. Wintrobe's clinical hematology.Philadelphia: Lippincott Williams & Wilkens; 2009:490-527.

264. White J.G., Escolar G. The blood platelet open canalicular system: A two-way street. European Journal of Cell Biology 1991;56(2):233-42.

265. Sheu J.R., Hsiao G., Shen M.Y., Lin W.Y., Tzeng C.R. The hyperaggregability of platelets from normal pregnancy is mediated through thromboxane A 2 and cyclic AMP pathways. Clinical and Laboratory Haematology 2002;24(2):121-9.

266. Janes S.L., Goodall A.H. Flow cytometric detection of circulating activated platelets and platelet hyper-responsiveness in pre-eclampsia and pregnancy. Clinical Science 1994;86(6):731-9.

267. Fitzgerald D.J., Mayo G., Catella F., Entman S.S., FitzGerald G.A. Increased thromboxane biosynthesis in normal pregnancy is mainly derived from platelets. Am J Obstet Gynecol 1987;157(2):325-30.

268. Dadhich S., Agrawal S., Soni M., Choudhary R., Jain R., Sharma S., et al. Predictive value of platelet indices in development of preeclampsia. Journal of SAFOG 2012;4(1):17-21.

269. Zangeneh M., Ebrahimi B., Khosravi S.M., Veisi F., Rezavand N., Ghadami M.R. The comparison of indices and number of platelet in normal and preeclamptic pregnant women. Iranian Journal of Obstetrics, Gynecology and Infertility 2012;15(25):8-13.

270. Konijnenberg A., Stokkers E.W., van der Post J.A.M., Schaapb M.C.L., Boer K., Bleker O.P., et al. Extensive platelet activation in preeclampsia compared with normal pregnancy: Enhanced expression of cell adhesion molecules. Am J Obstet Gynecol 1997;176(2):461-9.

271. Abildgaard U., Heimdal K. Pathogenesis of the syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP): A review. European Journal of Obstetrics Gynecology and Reproductive Biology 2013;166(2):117-23.

272. Cecati M., Sartini D., Pozzi V., Giannubilo S.R., Ferretti F., Stortoni P., et al. Clues to apoptosis pathway involvement in hemolysis, elevated liver enzyme, and low

200

platelet (HELLP) syndrome and intrauterine growth restriction (IUGR). Journal of Maternal-Fetal and Neonatal Medicine 2013;26(1):26-31.

273. Nityanand S., Pande I., Bajpai V.K., Singh L., Chandra M., Singh B.N. Platelets in essential hypertension. Thrombosis Research 1993 1;72(5):447-54.

274. Flaumenhaft R. Molecular basis of platelet granule secretion. Arteriosclerosis, Thrombosis, and Vascular Biology 2003;23(7):1152-60.

275. Blair P., Flaumenhaft R. Platelet α-granules: Basic biology and clinical correlates. Blood Reviews 2009;23(4):177-89.

276. Warren B.A. The ultrastructure of platelet pseudopodia and the adhesion of homologous platelets to tumour cells. British Journal of Experimental Pathology 1970;51(6):570-80.

277. Warren B.A., Vales O. The adhesive dendritic pseudopodium of the platelet and the release reaction. Microvascular Research 1972;4(2):159-78.

278. Lee D., Fong K.P., King M.R., Brass L.F., Hammer D.A. Differential dynamics of platelet contact and spreading. Biophysical Journal 2012;102(3):472-82.

279. Gottlieb R.A. Apoptosis. In: Lichtman M.A., Kipps J., Kaushansky K., Beutler E., Seligsohn U., Prehal J.T., editors. Williams' haematology.New York: McGraw Hill; 2006.

280. Wolf B.B., Goldstein J.C., Stennicke H.R., Beere H., marante-Mendes G.P., Salvesen G.S., et al. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. Blood 1999;94(5):1683-92.

281. Novotny W.F., Girard T.J., Miletich J.P., Broze J. Platelets secrete a coagulation inhibitor functionally and antigenically similar to the lipoprotein associated coagulation inhibitor. Blood 1988;72(6):2020-5.

201

282. Holmsen H., Weiss H.J. Secretable storage pools in platelets. Annual Review of Medicine 1979;30:119-34.

283. Prada D.A., Richards M., Kettler J.G. Amine storage vesicles in platelets. In: Gordon J.L., editor. Platelets in Biology and Pathology.Amsterdam: Elsevier/North Holland; 1981:107-46.

284. Rand M.L., Perry D.W., Packham M.A., Gemmell C.H., Yeo E.L., Kinlough-Rathbone R.L. Conditions influencing release of granule contents from human platelets in citrated plasma induced by ADP or the thrombin receptor activating peptide SFLLRN: Direct measurement of percent release of β-thromboglobulin and assessment by flow cytometry of P-selectin expression. American Journal of Hematology 1996;52(4):288-94.

285. Griffiths G. WhatGÇÖs special about secretory lysosomes? Seminars in Cell & Developmental Biology 2002;13(4):279-84.

286. Joseph R., Riddle J.M., Welch K.M., D'Andrea G. Platelet ultrastructure and secretion in acute ischemic stroke. Stroke 1989;20(10):1316-9.

287. Givan A.L. Flow Cytometry. First Principles. New York: Wiley-Liss; 1992.

288. Courtney J.M., Lamba N.M.K., Sundaram S., Forbes C.D. Biomaterials for blood-contacting applications. Biomaterials 1994;15(10):737-44.

289. Haycox C.L., Ratner B.D. In vitro platelet interactions in whole human blood exposed to biomaterial surfaces: Insights on blood compatibility. Journal of Biomedical Materials Research 1993;27(9):1181-93.

290. McEver R.P., Bennett E.M., Martin M.N. Identification of two structurally and functionally distinct sites on human platelet membrane glycoprotein IIb-IIIa using monoclonal antibodies. Journal of Biological Chemistry 1983;258(8):5269-75.

291. Wolff R., Plow E.F., Ginsberg M.H. Interaction of thrombospondin with resting and stimulated human platelets. Journal of Biological Chemistry 1986;261(15):6840-6.

292. Shattil S.J., Hoxie J.A., Cunningham M., Brass L.F. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. Journal of Biological Chemistry 1985;260(20):11107-14.

293. Shattil S.J., Newman P.J. Integrins: Dynamic scaffolds for adhesion and signaling in platelets. Blood 2004;104(6):1606-15.

294. Coller B.S., Shattil S.J. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. Blood 2008;112(8):3011-25.

295. Frenette P.S., Weiss L. The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity. Blood 2000;96(7):2469-78.

296. Palabrica T., Lobb R., Furie B.C., Aronovitz M., Benjamin C., Hsu Y.M., et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. Nature 1992;359(6398):848-51.

297. Michelson A.D., Benoit S.E., Kroll M.H., Li J.M., Rohrer M.J., Kestin A.S., et al. The activation-induced decrease in the platelet surface expression of the glycoprotein Ib-IX complex is reversible. Blood 1994;83(12):3562-73.

298. Michelson A.D., Barnard M.R., Hechtman H.B., Macgregor H., Connolly R.J., Loscalzo J., et al. In vivo tracking of platelets: Circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. Proceedings of the National Academy of Sciences of the United States of America 1996;93(21):11877-82.

299. Berger G., Hartwell D.W., Wagner D.D. P-selectin and platelet clearance. Blood 1998;92(11):4446-52.

300. Nishibori M., Cham B., McNicol A., Shalev A., Jain N., Gerrard J.M. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulophysin. Journal of Clinical Investigation 1993;91(4):1775-82.

301. Star J., Rosene K., Ferland J., Dileone G., Hogan J., Kestin A. Flow cytometric analysis of platelet activation throughout normal gestation. Obstetrics and Gynecology 1997;90(4 I):562-8.

302. Bagamery K., Landau R., Kvell K., Graham J. Different platelet activation levels in non-pregnant, normotensive pregnant, pregnancy-induced hypertensive and pre-eclamptic women. A pilot study of flow cytometric analysis [1]. European Journal of Obstetrics Gynecology and Reproductive Biology 2005;121(1):117-8.

303. Harlow F.H., Brown M.A., Brighton T.A., Smith S.L., Trickett A.E., Kwan Y.L., et al. Platelet activation in the hypertensive disorders of pregnancy. American Journal of Obstetrics and Gynecology 2002;187(3):688-95.

304. Turitto V.T., Weiss H.J. Red blood cells: Their dual role in thrombus formation. Science 1980;207(4430):541-3.

305. Luthje J. Extracellular adenine compounds, red blood cells and haemostasis: Facts and hypotheses. Blut 1989;59(4):367-74.

306. Valles J., Santos M.T., Marcus A.J., Safier L.B., Broekman M.J., Islam N., et al. Downregulation of human platelet reactivity by neutrophils participation of lipoxygenase derivatives and adhesive proteins. Journal of Clinical Investigation 1993;92(3):1357-65.

307. Marcus A.J., Safier L.B., Broekman M.J., Islam N., Fliessbach J.H., Hajjar K.A., et al. Thrombosis and inflammation as multicellular processes: Significance of cell-cell interactions. Journal of Thrombosis and Haemostasis 1995;74(1):213-7.

204

308. Valles J., Santos M.T., Aznar J., Marcus A.J., Martinez-Sales V., Portoles M., et al. Erythrocytes metabolically enhance collagen-induced platelet responsiveness via increased thromboxane production, adenosine diphosphate release, and recruitment. Blood 1991;78(1):154-62.

309. Cunningham F.G., Lowe T., Guss S., Mason R. Erythrocyte morphology in women with severe preeclampsia and eclampsia. Preliminary observations with scanning electron microscopy. American Journal of Obstetrics and Gynecology 1985;153(4):358-63.

310. Born G.V.R., Bergquist D., Arfors K.E. Evidence for inhibition of platelet activation in blood by a drug effect on erythrocytes. Nature 1976;259(5540):233-5.

311. Bergqvist D., Arfors K.E. Haemostatic platelet plug formation in the isolated rabbit mesenteric preparation. An analysis of red blood cell participation. Journal of Thrombosis and Haemostasis1980;44(1):6-8.

312. Hellem A.J., Borchgrevink C.H., Ames S.B. The Role of Red Cells in Haemostasis: the Relation between Haematocrit, Bleeding Time and Platelet Adhesiveness. British Journal of Haematology 1961 Jan 1;7(1):42-50.

313. Turitto V.T., Baumgartner H.R. Platelet interaction with subendothelium in a perfusion system: physical role of red blood cells. Microvascular Research 1975;9(3):335-44.

314. Santos M.T., Valles J., Aznar J., Perez-Requejo J.L. Role of red blood cells in the early stages of platelet activation by collagen. Journal of Thrombosis and Haemostasis1986;56(3):376-81.

315. McCrae K.R., Samuels P., Schreiber A.D. Pregnancy-associated thrombocytopenia: Pathogenesis and management. Blood 1992;80(11):2697-714.

205

APPENDIX A

Albe C. Swanepoel, Andre Stander, Etheresia Pretorius. Original research paper: Flow cytometric comparison of platelets from a whole blood and finger-prick sample: impact of 24 hours storage. Hematology 2013;18(2):106-14.

APPENDIX B

Albe C. Swanepoel, Etheresia Pretorius. Red blood cell and platelet interactions in healthy females during early and late pregnancy as well as post-partum. Blood 2013; 121(19): 3788.

Imaged used for cover of Blood, 9 May 2013, Volume 121, Issue 19.