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Investigating hypercoagulability in brain cancer patients by studying the viscoelastic and ultrastructural properties of whole blood

For the degree

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By

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ABSTRACT

Brain cancer is a condition with a relatively high rate of loss of life, regardless of patient demographics. The location and malignancy of the tumour are both determinants of this mortality rate, however there are complications associated with the disease that also contribute to this mortality rate. One of these complications are coagulopathies which may lead to thrombotic events. Thrombosis is a reality for many brain cancer patients which may contribute to a poor prognosis. This study analysed the contribution of different components that may contribute to the overactivation of the coagulation pathway in this patient group.

Brain cancer in South Africa has an incidence rate of about 1.5/100000. There is also a strong association of coagulopathy in cancer patients that may be attributed to morphological changes in red blood cells, inflammation, as well as the influence of inflammation on the release of certain procoagulants.

This study aimed to investigate the morphological and viscoelastic changes during coagulation in patients with brain cancer by studying the components involved in coagulation and their contribution to hypercoagulability in these patients. This was done using ultrastructural and viscoelastic techniques. Light microscopy was used to determine the deformability of red blood cells by calculating the axial ratios. Scanning electron microscopy was used to study the ultrastructural properties of clots as well as red blood cells, and platelets. Lastly, the viscoelastic properties of whole blood were quantitatively analysed using thromboelastography®. This provided insight into the contributing factors to coagulopathy in brain cancer patients.

When these factors are analysed and understood, insights into the clot formation in brain cancer patients may contribute to understanding the thrombotic risk in these patients and possible interventions based on the effects of the contributing factors.

From the results it was established that the red blood cell deformability, ultrastructural properties of fibrin fibres, and viscoelastic profiles during clot formation of this patient group are changed to develop denser and faster forming clot types. This research therefore contributed to the field by providing information that can guide understanding about the relationship between primary brain cancer and coagulation of whole blood.

By using this research alongside current knowledge, targeted monitoring of the contributing coagulation factors, such as changes in fibrin formation, and subsequent intervention can be applied to treat brain cancer-associated thrombotic risk.

Keywords

coagulopathy, whole blood, brain cancer, inflammation, red blood cell deformability, viscoelasticity, thrombotic risk

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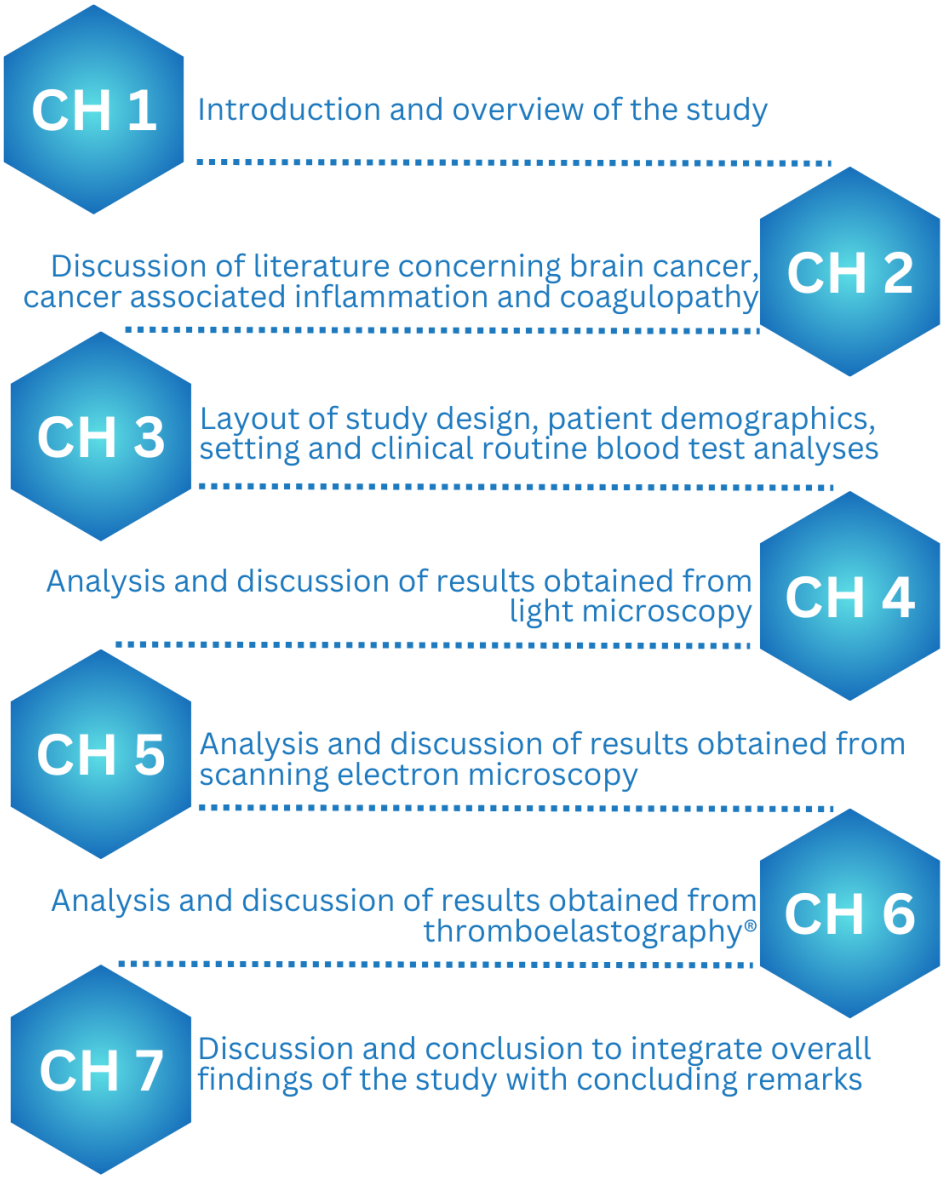
LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AFM	Atomic force microscopy
ATP	Adenosine triphosphate
aPC	Activated protein C
APS	Antiphospholipid syndrome
BCa	Brain cancer
CNS	Central nervous system
CP	Cancer procoagulants
CRC	Colorectal cancer
CRP	C-reactive protein
DVT	Deep vein thrombosis
FBC	Full blood count
GP	Glycoprotein
GBM	Glioblastoma multiforme
Hb	Haemoglobin
Hct	Haematocrit
HMDS	Hexamethyldisilane
IL	Interleukin
INR	International normalised ratio
IR	Ionising radiation
LM	Light microscopy
MA	Maximum amplitude
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MMP	Metalloproteinase
MPV	Mean platelet volume
MP	Microparticle
MRI	Magnetic resonance imaging
MV	Micro vesicle
NHRD	National health research database
NMR	Nuclear magnetic resonance
NO	Nitric oxide
OsO4	Osmium tetroxide
PARs	Protease-activated receptors
PAR2	Protease-activated receptor 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCT	Platelet haematocrit percentage
PE	Pulmonary embolism
PLT/s	Platelet/s
PT	Prothrombin
PS	Phosphatidylserine
RBC/s	Red blood cell/s
RDW	Red blood cell distribution width
SANBS	South African National Blood Service
SBAH	Steve Biko Academic Hospital
SEM	Scanning electron microscopy
SES	Socioeconomic status
suPAR	Soluble urokinase plasminogen activator

TAT	Thrombin-antithrombin complex
TE	Thromboembolism
TEG®	Thromboelastography®
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombin generation
TME	Tumour microenvironment
UP	University of Pretoria
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VET	Viscoelastic test
vWF	Von Willebrand factor
VTE	Venous thromboembolism
WB	Whole blood
WBC/s	White blood cell/s
WHO	World Health Organisation

Dissertation overview



CHAPTER ONE

INTRODUCTION

Neoplasms that stem from the meninges and intracranial tissues are collectively termed brain tumours. Primary malignant brain cancer (BCa) makes up about 2% of all adult cancers.[8] The most common BCa in adults are glioblastomas, meningiomas, and pituitary adenomas.[1, 9, 10] Glioblastomas are more commonly found in males[11] whereas meningiomas and pituitary adenomas are more prevalent in females.[12, 13] The general peak age of incidence of central nervous system (CNS) tumours is in mid-adulthood but varies between patients in their 30s and 60s.[11]

There is an established link between BCa tumours and the upregulation of inflammation.[14, 15] Cancer progression and prognosis is often dependent on cancer-associated inflammation that occurs in the tumour microenvironment (TME) as well as in systemic circulation.[16] Lymphocytes, neutrophils, platelets (PLTs), C-reactive protein (CRP) and interleukins (e.g., IL-6) are all associated with inflammatory responses that directly contribute to cancer progression.[16] By blocking immune checkpoints, the TME is able to offset and evade the innate immune system.[16]

This elevated inflammatory state could explain the high risk of thromboembolisms observed in primary BCa patients.[15] Cancer has now been established as a major independent risk factor for the leading cause of cancer patient death, venous thromboembolisms (VTEs).[17] Arterial thrombosis is proportional to the stage of cancer with 2.3% incidence at 6 months when the patient is in stage I of cancer, compared to 7.7% when the patient is in stage IV.[18] It is important to study cancer related coagulopathies in order to eventually improve patient outcomes by understanding the underlying mechanisms involved in coagulation in this patient group and using this information to develop and enhance more efficient disease management.

Therefore, the aim of this study was to investigate the morphological and viscoelastic changes during coagulation in BCa patients. Here, the routine blood tests of BCa patients were analysed to establish the haematological profile of the patient group. Their inflammation status was also assessed by measuring the CRP levels in order to establish whether or not this patient group had an increased inflammatory profile

compared to healthy norms. Light microscopy (LM) was used in this study to quantify red blood cell (RBC) deformation using axial ratios. Scanning electron microscopy (SEM) was used to study the ultrastructural morphology of whole blood (WB) clots, RBCs and their interactions with each other and PLTs in each of the groups. Lastly, thromboelastography® (TEG®) was used to investigate the viscoelastic properties of WB during clot formation in BCa patients compared to healthy individuals. The results obtained from this analysis was used to develop an overall viscoelastic profile of BCa patients.

CHAPTER TWO

LITERATURE REVIEW

2.1 CHAPTER OBJECTIVE

In this chapter, the literature involving BCa, cancer associated inflammation and coagulopathy is discussed.

2.2 DISEASE AND PREVALENCE

2.2.1 Brain Cancer

Butowski defines a brain tumour as the vast collection of cancers that originate from different cell types: either systemic tumours that have metastasised to the brain or primary tumours which originated from brain tissue.[19, 20] Central nervous system tumour incidence in Southern Africa is approximately 1.5/100000.[21] Brain tumour incidence has also been found to increase with age[22]. Kelly et al. found that the average age of South African patients with CNS tumours is 48 (± 14.56) and that males present with a higher rate of incidence of malignant tumours, compared to a higher incidence of non-malignant tumours in females.[22, 23]

Brain tumours can be of benign or malignant nature. Benign tumours are often not life threatening however, they may be in certain situations. For example when the benign tumour is located on the pons or medulla, where it causes increased intracranial pressure, cerebral oedema or herniation.[20, 24] Malignant tumours, however, are potentially life threatening. The World Health Organisation (WHO) introduced a classification system of tumours, rated on a scale of increasing malignancy.[1, 25] The classification system was developed based on the characteristics and behaviours of tumours to establish the types and locations as seen in Table 1. Some of these characteristics include vascularity, presence of central necrotic cells, the similarity between tumour cells and normal cells, rate of tumour growth, and the presence of tumour margins.[26] Tumours are also classified based on their rate of growth and proliferation.

Tumours are typically subdivided based on a scale of malignancy ranging from I to IV.[25] Grade I tumours are slow growing and are likely removable with surgery alone,

therefore being the least malignant. Grade II tumours are also slow growing however, they may invade surrounding non-tumour tissue. Grade III tumours have abnormal cells that actively reproduce and invade surrounding healthy cells and tissue. Finally, Grade IV tumours are the most malignant, showing quick proliferation and invasion of adjacent healthy tissue.[25] These tumours are actively growing due to the presence of new blood vessels in the necrotic tissue that can maintain their proliferation[26] additionally, they have a central area of necrosis.[25]

2.2.2 Types of brain cancer

Table 1: Adapted from the World Health Organisation classification of CNS tumours.[1] A broad overview.

Location	Type
Gliomas, glioneuronal tumours, and neuronal tumours	Adult-type diffuse gliomas
	Paediatric type diffuse low-grade gliomas
	Paediatric type diffuse high-grade gliomas
	Circumscribed astrocytic gliomas
	Glioneuronal and neuronal tumours
	Ependymomas
Choroid plexus tumours	
Embryonal tumours	Medulloblastoma
	Other CNS embryonal tumours
Pineal tumours	
Cranial and paraspinal nerve tumours	
Meningiomas	
Mesenchymal, non-meningothelial tumours	Soft-tissue tumours
	Vascular tumours
	Skeletal muscle tumours
	Uncertain differentiation
Chondro-osseous tumours	Chondrogenic tumours
	Notochordal tumours
Melanocytic tumours	Diffuse meningeal melanocytic neoplasms
	Circumscribed meningeal melanocytic neoplasms
Haematolymphoid tumours	Lymphomas
	Miscellaneous rare lymphomas in the CNS
	Histiocytic tumours
Germ cell tumours	
Tumours of the sellar region	
Metastases to the CNS	

CNS: central nervous system

Some brain tumours are more common. The three most common tumours tend to be meningiomas, gliomas, and pituitary adenomas. Gliomas are the most common primary CNS tumours in adults.[1, 27] Frequently, the lethal form in adults being a grade IV glioblastoma multiforme (GBM)[10], and is mostly found in individuals during their fifth and sixth decades of life.[20] This type of tumour is known to present very aggressively and despite optimal therapy, it is a cancer with high morbidity.[10] Glioblastomas are reported to be found almost 60% more commonly in males.[22]

There are currently fifteen recognised subtypes of meningiomas according to the WHO.[1] Meningiomas are highly vascularised tumours[28] and may have latency periods of up to 20-30 yeas and even then may only be discovered accidentally with magnetic resonance imaging.[29, 30] These tumours are generally considered to be benign and encapsulated. However, due to the intracranial locations where they often appear, the complications can be detrimental. Meningiomas account for 33.8% of all central nervous system tumours and present with 69% survival rate after five years.[13, 29] The incidence of these tumours have been noted to increase with age and are often reported in women with a 2:1 female to male ratio.[13]

Pituitary adenomas account for around 15-22% of primary brain lesions, which makes it the third most common type of BCa after glioblastomas and meningiomas.[9, 31] Pituitary adenomas are associated with higher morbidity and mortality rates and an increase in health problems. This BCa is more commonly found in females.[12, 32] Similar to meningiomas, some pituitary adenomas can go years without symptoms and these tumours are incidentally detected.[33]

2.2.3 Diagnosis

There are currently many technologies which may assist in the diagnosis of tumours. This includes histological analysis of tumour tissue[34], light microscopy, molecular genetics, histochemical stains, and a variety of molecular profiling approaches.[1] Imaging techniques such as computed tomography, magnetic resonance imaging, and angiography are more frequently used to assist in the diagnosis of tumours.[20] One of the main sources taken into consideration when a diagnosis is made is the WHO's classification of CNS tumours (Table 1). Often more than one of these diagnostic tools are used in order to reach one integrated diagnosis that can be made by a qualified medical doctor.

2.2.4 Clinical presentation

Symptoms vary among patients based on the tumour location. Most patients present with symptoms of three categories namely, motor, sensory/perceptual, and/or cognitive/affective.[20] The symptoms differ according to the location of the tumour or location affected by an increased intracranial pressure as previously mentioned.

2.2.5 Treatment

Most tumours are commonly treated with surgery and radiotherapy, irrespective of grade.[35, 36] There is some controversy around the optimum treatment strategies for tumours. However, each case is patient-specific and the best choice for treatment differs among medical professionals.

2.2.6 Aetiology

The causes of tumours are still a field that demands research investment. Prolonged exposure to ionising radiation (IR) is frequently reported as a risk factor of most meningiomas. It has been shown that the risks of developing a meningioma can be increased six to ten-fold when exposed to IR.[37-40] Other risk factors could include head trauma and cell phone use. However, the results are inconsistent and inconclusive with the current data.[41-46] Socioeconomic status (SES) has been studied for a long time as a potential risk factor of malignant BCa incidence. Studies show that individuals of higher SES are at greater risk of developing BCa than individuals of lower SES.[47-51] A possible explanation for these findings could be the fact that the former have better access to screening and health care.[52-56]

Genetic risk factors are mostly due to loss of function mutations in tumour suppressor genes which can be inherited or arise *de novo*.[57, 58] Roughly 5% of gliomas are hereditary.[59] Specifically, approximately 4% of paediatric and 1-2% of adult gliomas are reported to originate from Mendelian disorders or inherited syndromes.[60, 61] Hormones also play a possible role in the risk of developing meningiomas. During peak reproductive years, meningioma incidence is at a female to male ratio of 3.15:1.[29] Other studies have also shown the impact of some sex hormone receptors in meningiomas, increased incidence in post-pubertal disease in females, an association between breast cancer and meningiomas, and a change in size of tumour during the menstrual cycle's luteal phase and pregnancy.[62, 63] Atopic conditions such as hay fever, asthma, allergies, and eczema have been consistently shown to reduce the risk

of glioma development. In some cases, the risk was shown to be reduced by up to 30%[64] which may be attributed to these atopic conditions. It has also been shown that early life exposure to infections may lead to the body maintaining a heightened state of immune-surveillance which decreases the risk of abnormal cell growth which may lead to tumour development.[65-68]

2.2.7 Cancer related inflammation status and clotting potential

C-reactive protein tests are commonly used clinical tests to determine inflammation status. In breast cancer, the CRP of patients is elevated proportionally to the grade of malignancy.[69] Interleukin-6 (IL-6) is a pro-inflammatory cytokine that has an important part in breast cancer inflammation.[69-71] Bester et al. showed that IL-6 is responsible for coagulation caused by inflammation through tissue factor (TF) expression as well as abnormal fibrin production. However, the effects on the physical and viscoelastic properties of the blood cells were not significant.[72]

The degree of inflammation can be an important indicator of the progression of tumour metastasis and patient survival.[73-75] Inflammatory molecules are generally overexpressed by tumour cells.[76] According to Dagistan et al., two inflammatory markers in patients with brain tumours were found to be altered. These were increased RBC distribution width (RDW) and decreased mean PLT volume (MPV).[77] Platelets have a significant role in homeostasis[77] and the inflammatory reaction because they secrete proinflammatory molecules.[78]

Platelets associated with tumour cells evade the host cells' immune responses by promoting metastasis formation.[79] When tumour cells damage the endothelium, PLTs are activated which drives neuroinflammation.[80] Subsequently, these tumour cells express PLT receptors (known as PLT mimicry).[81] Central nervous system tumour cells can secrete PLT agonists (ADP and thrombin) which cleave the PLT PAR1 and PAR4 receptors, activating mitogenic signals in the TME.[81] When activated, PLTs also secrete proteinases and growth factors, thereby regulating tumour growth and invasion.[82] Platelets are able to form a coating around tumour cells and directly shield them from stressors circulating in the blood, thereby promoting immune system evasion, ultimately protecting the tumour cells from the immune system.[83]

Fibrin formation and clotting by-products have integral roles in both tumour progression and the development of blood clots.[3] Cancer cells have been shown to

release inflammatory cytokines, as well as fibrinolytic and procoagulant proteins. The cancer cells are also able to attach to the host cells and initiate the stimulation of the host effector cells' prothrombotic properties.[3] Changes in the haemostatic system in cancer patients are usually attributed to changed levels of the thrombin-antithrombin (TAT) complex, fibrinopeptide A and B, D-Dimer, plasmin-antiplasmin complex, prothrombin fragment F1 + 2, coagulation factor IX and X activation peptides.[84] These changes can occur without the patient exhibiting any symptoms of thrombosis or haemorrhage.[3] Tumour cells are capable of stimulating these responses in the haemostatic system by expressing the following procoagulant factors: TF, cancer procoagulant (CP), procoagulant proteins, microparticles (MP), cytokines, and adhesion molecules (Figure 1).

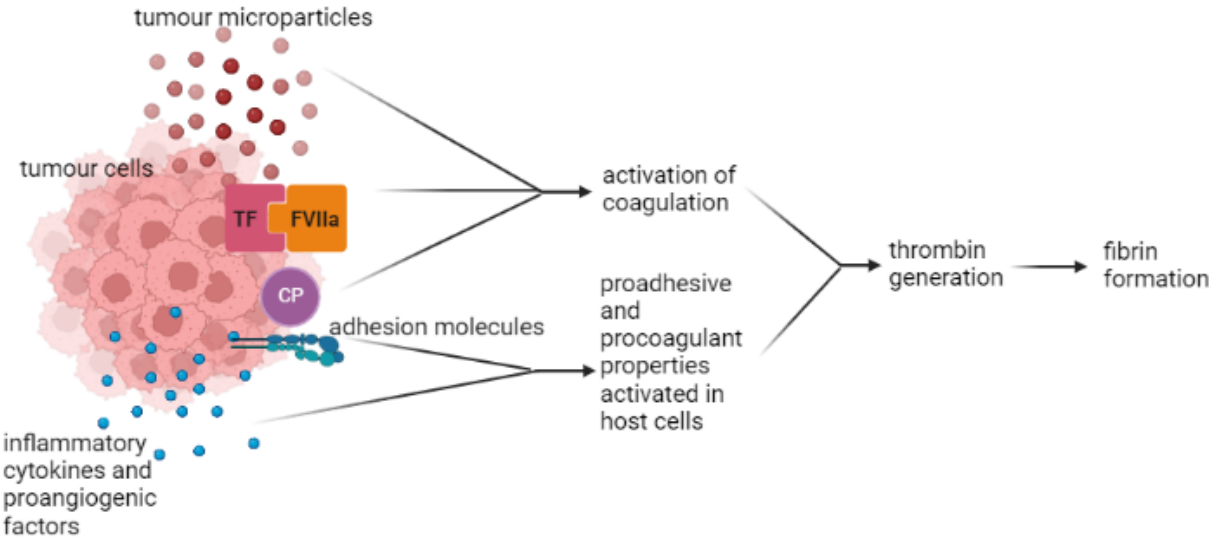


Figure 1: The mechanisms involved in thrombosis as seen in cancer. Adapted from Falanga et al.[3] CP = cancer procoagulant; TF = tissue factor;

Subendothelial cell surfaces as well as some extravascular tissues naturally express the glycoprotein TF.[85] Under normal physiological conditions, TF is not expressed by endothelial cells but is constantly produced by subendothelial and malignant cells and is naturally antagonised by TF pathway inhibitor (TFPI).[86] Tissue factor production by subendothelial cells ensures the activation of coagulation if vessel damage occurs and is released by malignant cells caused by thrombotic events.[87] Tissue factor can travel through the membrane, and subsequently forms complexes with FVIIa.[87] This binding

marks the start of the coagulation cascade and induces proteolytic activation of coagulation proenzymes (prothrombin, FIX, and FX) which in turn results in fibrin clot formation.[6]

The MPs released by tumour cells consist mostly of nucleic acids, lipids, and proteins and are strongly associated with the hypercoagulability of cancer patients.[88, 89] These MPs facilitate thrombin generation within veins when exposed to TF.[90] Surface adhesion molecules allow the tumour cells to directly associate with the host cells (leukocytes, endothelial cells, and PLTs), which leads to localised clotting activation on the vessel wall, promoting the onset of blood clot formation.[3]

Protease-activated receptors (PARs) are responsible for the strong association between inflammation and coagulation.[91, 92] Protease-activated receptors are typically overexpressed in a range of cancers found in humans and studies have shown that their expression correlates strongly with the aggressiveness of tumour cell behaviour.[93-97] The PARs pathway may be considered as the central pathway in the coagulation cascade as it affects the pathophysiology of the CNS.[81] Protease-activated receptors 1,3, and 4 are activated by either thrombin[6, 98] or protein C (aPC) respectively.[81] Coagulation proteases (FXa and FVIIa) can activate PAR 2[99-101] which in turn increases angiogenesis.[102]

Thrombin stimulates chemokine-, extracellular protein-, and growth factor release that increase the proliferation and migration of tumour cells. Thrombin activates PAR 1 which facilitates the upregulation of angiogenesis genes. This includes the upregulation of the production and signalling of metalloproteinase (MMP)-2 and vascular endothelial growth factor (VEGF) expression.[103-105] Balkanov et al. showed that during the preoperative period of BCa development, this patient group presented with an increased coagulation profile and therefore a higher thrombotic tendency.[106]

2.3 BLOOD COMPONENTS INVOLVED IN COAGULATION

2.3.1 Morphology of red blood cells, platelets, and clot architecture

Red blood cell morphology

Biophysical properties of blood are determined by elasticity and viscosity. Red blood cells make up about 45% of the cell concentration in blood and has a noteworthy effect on the flow and behaviour of WB.[107] The elasticity of RBCs is essential for travel in

microcirculation since the capillary diameter cannot be smaller than that of the RBCs. The normal RBC is very elastic and can therefore bend and change its shape with ease to ensure flow through smaller capillaries.[108] Red blood cells are mostly able to keep and uphold a high surface-to-volume ratio as well as a natural biconcave shape which arises due to the balance between water, ions, and their cytoskeletal structure.[109] The reason for the biconcave shape is to maximize the total surface area, being increased 45% as compared to a spherical shaped cell.[4] Red blood cells are 7-8 µm in size and the biconcave shape is temporarily altered, by approximately 1-3 µm every time a RBC has to move through a blood vessel.[4] The biconcave shape, elasticity and viscosity ensure that the RBCs can effectively move through small blood vessels and deliver sufficient amounts of oxygen to all tissues.

The elasticity of RBCs is dependent on several factors: deformability, aggregation, plasma viscosity, and haematocrit.[110-115] The chemical and biological functions of RBCs can introduce changes to the viscosity of blood.[108] When viscosity is increased, there is an increase in haematocrit (Hct) which comprises a mechanism for thrombosis.[4] There are multiple prothrombic alterations that RBCs undergo in contribution to coagulation. This is summarised in Figure 2.

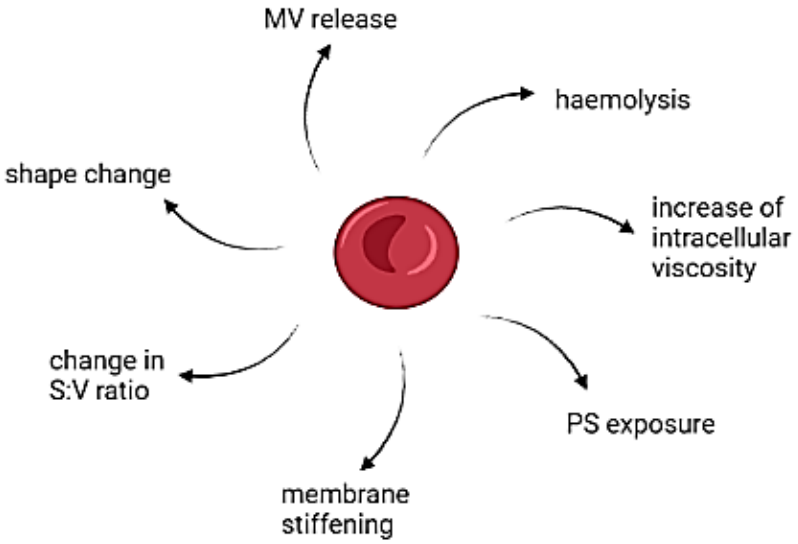


Figure 2: Prothrombic alterations of RBCs. Adapted from Weisel et al. MV = micro vesicles; S:V = surface to volume; PS = phosphatidylserine [4]

Red blood cell-platelet interaction during normal coagulation

Red blood cells contribute to haemostasis and thrombosis and do so by being prothrombic or procoagulant.[4] When activated PLTs release adenosine diphosphate (ADP), adenosine triphosphate (ATP), and thromboxane, RBCs increase the adhesion and aggregation of PLTs.[116, 117] Red blood cells can interact with the PLTs themselves through adhesive molecules including fibrinogen which contain αIIbβ3, and ICAM-4.[118, 119] At venous shear rates, RBCs are able to directly bind to PLTs[118, 120] which is important in pathological thrombotic events.[119] Under conditions of low pH and oxygen pressure, as well as in response to mechanical deformation, ADP and ATP are released by the RBCs that subsequently activate PLTs.[117] Nitric oxide (NO) is responsible for suppressing PLTs activation and is typically scavenged by haemoglobin in order to maintain homeostasis.[121] However, when RBCs are damaged, they release haemoglobin which also amplifies PLT activation by decreasing NO availability.[122] For these reasons, it has been shown that the RBCs contribute both to haemostasis and thrombosis.

Red blood cell-fibrin interaction during normal coagulation

Red blood cells contribute to microthrombosis by aggregating in rouleaux stacks.[123] There is a strong association between the tendency of RBCs to aggregate and fibrinogen plasma concentration under the influence of inflammatory conditions.[124] Binding between RBC membranes and fibrinogen may occur through receptors and/or proteins associated with integrin.[125-127] Fibrin and fibrinogen share binding sites to integrin αIIbβ3.[128] Therefore, it is possible that the mechanism of RBC-fibrinogen interaction is similar to RBC-fibrin interaction in the formation of blood clots.[4] Clot permeability is decreased by RBCs.[129-131]

The presence of RBCs interferes with the fibrin network directly.[132] With the increase of RBC concentration in a clot, the fibrin strands are larger and the clot becomes less permeable.[4] Through these changes to the fibrin network as well as through the impairment of plasminogen activation, RBC presence enhances the clot's resistance to lyse.[129] In addition, after formation of the clot, the PLTs initiate contraction.[4] During clot contraction, fibrinogen and fibrin bind to αIIbβ3 and forms a PLT-fibrin meshwork by binding outside a PLT to link other PLTs. This meshwork undergoes mechanical shortening which causes RBC compression in the clot.[128]

Red blood cells in thrombi

In response to oxidative stress, apoptosis, complement attack, or high sheer stress, phosphatidylserine (PS) is exposed on RBCs.[133-140] Red blood cells release procoagulant micro vesicles (MVs) derived from RBC membranes which then carry PS.[141-146] They directly activate Factor IX (FIX) by an elastase-like enzyme carried on the membrane[4] and FXIIIa-mediated retention of RBCs increases clot size.[147-149] The clot mechanics of blood coagulation is affected by the different ways in which RBCs deform.[4] When a clot is formed, RBC agglutination ensures an impermeable seal[7, 128] and deviate from the natural biconcave shape to a polyhedral shape (known as polyhedrocytes), which are also found in the intermediate forms.[150-152] Figure 3 below is an example of polyhedrocytes within a WB clot. They are redistributed during clot formation, being pulled to the centre of the clot.[7]

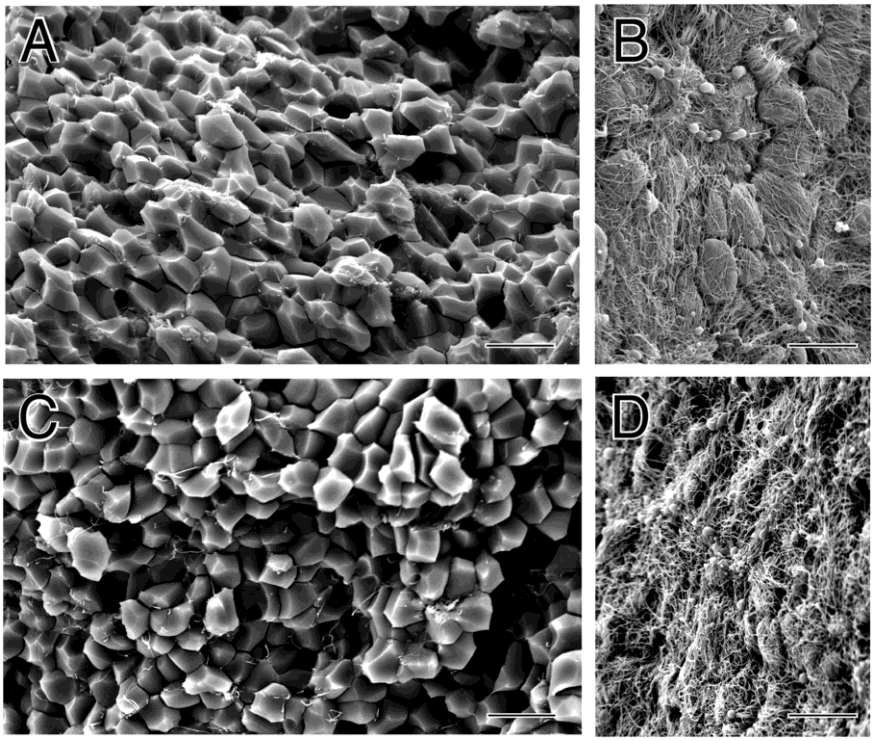


Figure 3: Scanning electron micrographs of the structure of contracted WB clots activated by thrombin followed by recalcification. A, C: inside contracted clots, showing closely packed polyhedrocytes. B, D: outside of contracted clots, revealing platelet aggregates and a thick fibrin meshwork. Scale bar = 10 µm.[7]

A decrease in RBC membrane flexibility results in a stiffening of the cells which is associated with thrombotic potential.[4] Red blood cell rigidity joined with their prothrombic properties have been reported in deep vein thrombosis (DVT) which is

often a clinical manifestation in cancer patients.[4] When the RBCs are subjected to shear stress, their membranes may be damaged by the difference in pressure within the vascular system. During an inflammatory response caused by the presence of a tumour, RBCs could be produced in different sizes. It is hypothesised that inflammatory molecules signal an alteration of iron use in the bone marrow and thereby increase the width of the RBCs.[77] This is because iron is diverted from circulation sites which results in iron-restricted RBC formation[153], altering the shape in this way.

2.3.3 Normal coagulation vs hypercoagulation

Coagulation in haemostasis is an essential response to bleeding and includes stable clot formation. Hypercoagulability on the other hand, is a pathologic state of increased tendency of the blood to coagulate even when there is no bleeding.[87] In homeostatic conditions, balance is maintained between pro-coagulant and anti-thrombotic factors. Therefore, when hypercoagulation is present, the cause is either a deficiency in anti-coagulants or the overproduction of pro-coagulants.[87]

Normal homeostasis requires the body to have balanced interactions between PLTs, coagulation proteins, and vessel walls to avoid acute bleeding.[81, 154, 155] According to Rodgers et al., "normal homeostasis"[2] includes primary homeostasis and secondary homeostasis, which involves PLT adhesion, aggregation, and generation of thrombin, as well as a fibrin mesh formation respectively. Secondary homeostasis results in the reinforcement of a PLT thrombus.[2] Defects in PLT number and function (primary homeostasis) or coagulation protein deficiency (secondary homeostasis) may cause excessive bleeding or fibrinolysis.[2]

Normal vessel walls express anticoagulant and anti-PLT activity which ensures the fluid state of blood and prevent coagulation mechanism and PLT activation.[156] The mechanism of coagulation takes place in three phases namely initiation, amplification, and propagation after which fibrinolysis takes place, while tissue repairs itself.[81] In contrast, when there is damage to the blood vessel, collagen contained on the subendothelial tissue is exposed. These fibrils include the von Willebrand factor (vWF) binding to PLTs via the glycoprotein (GP) Ib receptor. The activation of the PLTs causes aggregation mediated by fibrinogen binding to the PLT GP IbIIIa receptor.[2]

Defects in primary homeostasis which lead to bleeding are therefore rooted in GP Ib or GP IbIIIa PLT, vWF deficiencies, PLT dysfunction, or thrombocytopenia.[156]

Successful clotting depends on the reinforcement of fibrin in the initial PLT thrombus. Without fibrin reinforcement of the initial PLT thrombus, clotting will not be successful and might result in delayed bleeding.[2] For the coagulation pathway to be activated by inflammation, the TF pathway is set in motion, as seen in Figure 4.[98] This occurs when blood in the circulatory system encounters TF bearing cells in the subendothelial matrix and fibroblasts. A response is warranted if there is damage to the vascular system and/or when bacteria or proinflammatory cytokines stimulate TF expression on mononuclear and endothelial cell surfaces.[98] When the TF is exposed to coagulation factor VII (produced due to vessel damage), initiation of the coagulation pathway as well as thrombin and fibrin formation take place .[2, 98, 154] Therefore, deficiency of antibody or prothrombin, factors V, VII, VIII, IX, X, XI, and XIII, and fibrinogen are all possible reasons for the failure of maintaining secondary homeostasis.[2]

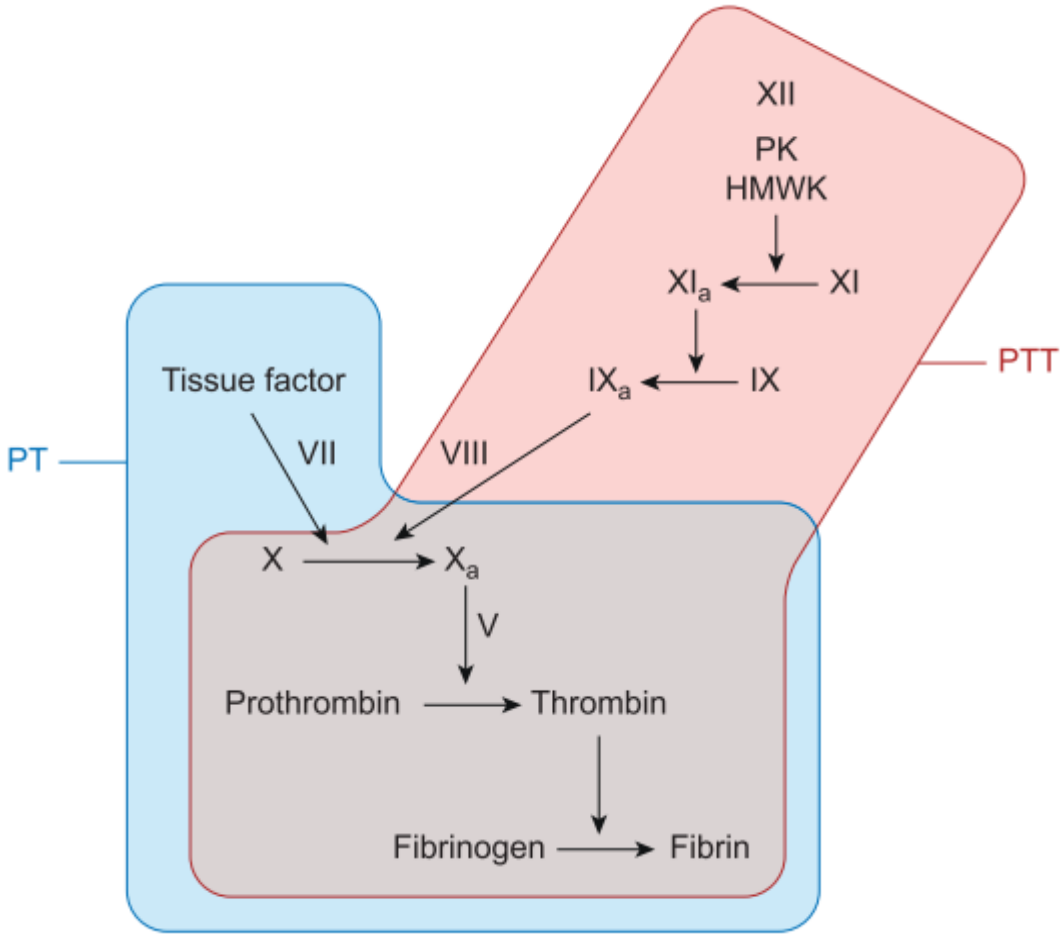


Figure 4: The coagulation pathway as summarised by Rodgers.[2] Precursor coagulation zymogens are converted to protease by enzymes. This conversion leads to the conversion of prothrombin to thrombin which in turn activates platelets and ensures the conversion of fibrinogen from soluble to an insoluble fibrin clot. PT: prothrombin time; PTT: partial thromboplastin time; HMWK: high molecular weight kininogen; PK: prekallikrein

2.3.4 Coagulopathy in cancer

It has been found that there is an increased risk of thrombosis in BCa patients as a result of circulating thrombotic markers.[3] Thrombotic risk can be ascribed to accompanying influences such as age, bed-rest, history of venous thromboembolisms (VTEs), comorbid conditions, and cancer-typical factors such as stage of tumour malignancy and cancer treatments.[157, 158]

Second to antiphospholipid syndrome (APS), cancer is a major cause of acquired hypercoagulability.[87] Often, the first diagnosis of cancer patients is thrombosis as a clinical manifestation of a tumour.[6] Approximately 90% of *in vitro* coagulation tests of cancer patients show abnormalities.[159] Production of procoagulant factors (cancer

procoagulant and TF) as well as the interaction between the tumour cells, vascular endothelium and blood, lead to a prothrombotic state.[87] Cancer procoagulant is elevated in 85% of cancer patients and activates factor X which causes the hypercoagulation.[160] Clinical data show that there is a definite association between tumour progression and a prothrombotic state.[6] In the TME, there is fibrin deposition and PLT aggregation which is indicative of local coagulation activation.[161] Zwicker et al. found elevated coagulation protein (Factor V, FV, FIX, FX, and fibrinogen) levels in 60-100% of the blood of patients diagnosed with malignant neoplasias.[162]

De Waal et al. showed that colorectal cancer (CRC) patients presented with increased risk of clotting.[163] They showed that there are morphological and pathological structural changes in the blood components of these patients which suggests a hypercoagulable state. These changes include morphological changes to RBCs, PLT activation, and a denser fibrin clot structure.[163]

Tumour cells have certain procoagulant properties. These cells can cause activation of the coagulation system, establishing a prothrombotic state or hypercoagulability that may lead to an increased risk of mortality and morbidity.[160] Procoagulant properties of tumour cells include the overexpression of TF[164] (which, as discussed, is a major catalyst of the coagulation cascade), increased phospholipid PS surface exposure,[165] and procoagulant MV shedding.[6]

High levels of thrombin in the TME of gliomas is common, which activates the PAR 2 signal downstream[166] which would suggest an increased rate of coagulation in these patients in contrast to their age-equivalent control counterparts. Tissue factor is also upregulated in the cells within the TME[167] in addition to tumour free tissue[168] which causes thrombin production and neurotoxic effects.[81] Blood coagulation is activated locally at the tumour site as well as in the systemic environment through the release of TF by TF-expressing MPs which are released.[169-171] In addition, enzymes in the coagulation cascade are activated by tumour cells which causes an increase in aggregated PLTs and fibrin deposition.[6, 98, 172] Therefore, it is possible for tumour cells to maintain an elevated active state of the coagulation system.[169]

The surface of phospholipid PS possesses a negative charge which is used in the assembly of catalytic active coagulation complexes by binding to essential proteins in the cascade.[6, 173, 174] The MVs are vesicular structures that are found on normal and malignant cells and are formed from the zeiosis (extensive formation of protrusions) of their plasma membranes .[175] The effect of these vesicles on tumour cells also have an effect on coagulation activation *in vitro* and *in vivo*[176] because of TF presence on their surfaces as illustrated in Figure 5.[177-180] They are able to bind to vascular injury sites through interacting with P-selectin which is present in activated PLTs.[181, 182] Furthermore, they may transfer the TF that they carry to the membrane of PS-exposing active PLTs which leads to a surface more conducive to coagulation, ultimately enhancing the formation of thrombin.[183, 184] Micro vesicles which add to cancer-associated thrombosis mostly originate from cancer cells, although they may also originate from the endothelium, monocytes, and PLTs.[88, 177, 182, 185-187]

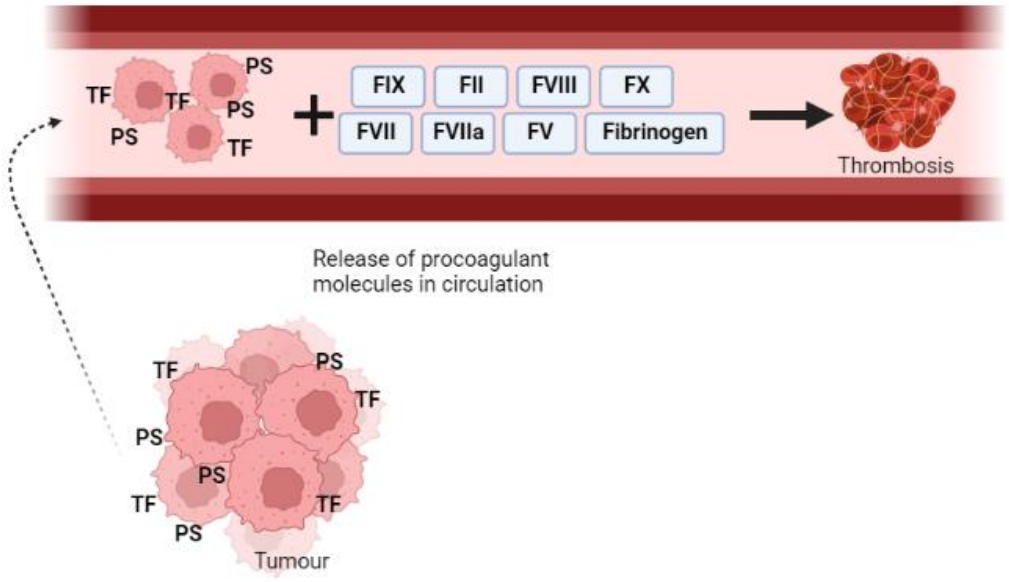


Figure 5: Tumour cells secrete TF-bearing MVs that expose PS on their surface. Procoagulant MVs can reach circulation and activate coagulation, thereby contributing to thrombus formation within the intravascular environment. Adapted from Glima et al.[6] TF: tissue factor; PS: phosphatidylserine; MV: microvesicle.

Double check the reference for above image as reference 6 is not Glima et al. (Luize, Robson. Activation of blood coagulation in cancer: Implications for tumour progression. Bioscience Reports. 2013;33(5):701-10.10.1042/bsr20130057)

In 1865, Trousseau's syndrome was first described. This syndrome shows that the blood of people with cancer tends to clot on its own, even when there are no noticeable signs of inflammation.[188] Trousseau's syndrome is specifically associated with lung

and pancreatic cancers and is characterised as a VTE variant that involves recurring thrombosis, migrating in superficial veins and less common locations for instance, chest wall and arms.[189]

Studies show that patients with cancer in the lungs, pancreas, ovaries, and gastrointestinal tract reported with an increased rate of thromboembolic deaths.[190-193] In another study, approximately 20% of patients diagnosed with DVT or pulmonary embolism (PE) are cancer patients.[87] Some cancers (for example gastrointestinal, hematologic, and lung cancer)[194] could be associated with an increased risk of a prothrombotic state. This may also be influenced by certain concomitant factors such as the stage of malignancy, therapeutic intervention, and bedrest.[160] It is twice as likely for thromboembolism (TE) recurrence to be identified in cancer patients, even when oral anticoagulant therapy is applied.[195, 196] Additionally, cancer patients are also more likely to respond to anticoagulant therapy and require longer hospitalisation.[160]

When studying cancer patients with brain metastases, Walter et al.[168] concluded that coagulation factor activity in patients with metastatic cerebral carcinomas is increased in the peripheral blood, which is in agreement with the hypothesis that the coagulation factors and receptors could control the malignant behaviour of some cancers. After the surgical removal of tumours, there was a decrease in coagulation factor activity in these patients compared to the control group. However, it was still elevated in the cancer patients. Hypercoagulability can cause thromboembolic complications[168] such as DVT or PEs which could be fatal. In up to 30% GBM patients, VTEs are developed throughout the course of the cancer.[197-200] Patients with meningiomas are also generally more prone to developing VTEs, however it is not as common in these patients.[201-205]

Platelets are involved in inflammation, coagulation, tumour growth and survival, and metastasis.[171, 206] Some studies show that the TME starts releasing and expressing coagulation factors only once the metastatic phase has been reached. These factors cause activation of PLTs, and enable the tumour cells to mimic PLT receptors that allow them to attach to endothelial cells, merging with the immune system.[171] It is known that PLT activation also has a cascade event where more PLT activation leads to an increased risk of thrombotic events.[207] Because these changes may only be

detected with clinical assessments (for example C-reactive protein test to determine inflammation), the effects of the TME may go unnoticed but still be active on a subclinical level. Mucins that are atypically glycosylated in carcinomas are secreted into the bloodstream and also contribute to thrombus occurrence *in vivo*.[208, 209] Through bidirectional signalling with PLTs and neutrophils, PLT-rich microthrombi formation is promoted by these carcinoma mucins.[210, 211]

Finally, experimental models of brain tumours have shown that repressor gene and oncogene-mediated neoplastic transformations activate coagulation.[212] In addition, a mutation in the EGFR gene causes cancer cells to be hypersensitive to coagulation protein (e.g. TF) stimulation resulting in a tumour-induced growth-promoting microenvironment.[213] This informs the field of the reciprocal connection between cancer and blood clot formation, which is facilitated by oncogenic cells promoting clot generation and clotting proteins promoting cancer proliferation and distribution. Considering the literature reviewed, it is suggested that treatment options for anticancer therapy should be influenced by the targeting of the haemostatic system.[6, 159, 214]

It is clear from the literature that BCa patients have a procoagulant tendency. Thus, this study aimed to identify and describe these procoagulant tendencies as well as investigate the mechanism involved, specifically during clot formation. It is essential to know how these changes contribute to the thrombosis risk of BCa patients and if the potential triggers behind these changes in WB can be identified. Insights provided by the study findings could offer possible treatments or management strategies for BCa patients. This study made use of ultrastructural and viscoelastic techniques to investigate coagulopathy in patients with BCa.

2.4 AIMS AND OBJECTIVES

With a better understanding of the effects and mechanisms involved in coagulopathy and with the understanding that the coagulation systems of primary BCa patients are likely at risk of being over activated due to the increased inflammatory status of these patients, the following questions are addressed in this dissertation:

1. Can BCa-associated thrombotic risk be better understood by identifying specific contributing factors?
2. Can the ultrastructural and viscoelastic changes of WB during clot formation provide a better understanding of hypercoagulation in BCa patients?

To address these questions, the following aim and objectives were set:

Aim: To study the ultrastructural and viscoelastic changes in the blood of BCa patients from Steve Biko Academic Hospital compared to healthy individuals to investigate factors that contribute to hypercoagulation.

Objectives:

The following objectives were used to achieve the study aim.

- To obtain a full blood count (FBC) from patient records for each BCa patient for a haematological profile compared to healthy reference ranges.
- To obtain CRP from patient records to establish patient inflammation status compared to that of healthy reference ranges.
- To determine RBC deformability in BCa patients compared to healthy individuals by calculating axial ratios using light microscopy (LM) and CellAnalyzer.
- To study the morphological changes of RBCs, PLTs, and clot architecture in BCa patients compared to healthy individuals, using SEM.
- To measure the viscoelastic properties of WB in BCa patients compared to healthy individuals using TEG®.

CHAPTER THREE

STUDY DESIGN, PATIENT DEMOGRAPHICS, SETTING AND CLINICAL ROUTINE BLOOD TESTS

3.1 STUDY DESIGN AND PATIENT DEMOGRAPHICS

This study was an analytical, patient-centred, and laboratory-based study. The samples were analysed *ex vivo* which was obtained from a control group of 35 healthy individuals and a patient group of 24 individuals with different primary (non-metastatic), brain tumours at Steve Biko Academic Hospital (SBAH), Pretoria, South Africa. The primary cancers included in this study were gliomas, meningiomas and pituitary adenomas which are the most common types of BCa in adults.[13, 29]

3.2 ETHICAL CONSIDERATIONS

Ethical approval was obtained for this study from the Faculty of Health Sciences Research Ethics committee, University of Pretoria (UP) (232/2022). See Appendix A for details. Informed consent was done and obtained for both groups of participants before any blood was drawn. After collection of the blood samples, all the methods conducted were in line with the requirements of the ethics committee and according to the Declaration of Helsinki. Approval was also granted by the National Health Research Database (NHRD) (GP_202207_019) for permission to work in SBAH and to access patient records. See Appendix B for details.

3.3 SAMPLING CRITERIA AND PROCEDURES OF SAMPLE COLLECTION: CONTROL AND PATIENT

3.3.1 Control group

The control group consisted of healthy individuals. Eligible participants identified from family, friends, colleagues and associates were recruited. Eligibility was determined by a set inclusion and exclusion criteria which was designed to match the patient group except a diagnosis of BCa. Healthy participants were matched by age and biological sex to the patient group in order to best imitate the group. Prospective participants were informed about the study and their participation. All willing participants filled out a consent form (see Appendix C). In addition, the participants were asked to fill out a questionnaire, the details of which can be found in Appendix D. Only once the

questionnaire was filled out and the consent form signed, one 4.5 mL citrate tube (3.8% sodium citrate) of blood was drawn from each participant by a qualified phlebotomist. All participant information was dealt with anonymously by assigning each participant a unique ID, e.g., C01. More information on the control group demographics can be found in Table 2.

The inclusion criteria for the control group was as follows:

- any biological sex
- Ages between 25 and 75

The exclusion criteria the control group was as follows:

- HIV positive (Only participants with a known HIV status were included)
- Smokes tobacco or uses any tobacco related product
- Use of chronic medication (including oral contraceptives)
- Has any condition which could present with chronic inflammation (including hypertension)
- Has a history of an immune-compromised status
- Use of the following: herbal supplements, or anti-coagulative (such as warfarin) medication within two weeks prior to the sample collection date
- Diagnosed with metastases
- Diagnosed with hypophyseal tumour(s)
- Diagnosed with tumours of ciliated squamous or columnar ependymal cells of the ventricles
- Excessive alcohol consumption (drinking five or more alcoholic drinks on the same occasion at least one day in the past 30 days)[215]

3.3.2 Brain cancer patients

This study was done in collaboration with the staff at SBAH who assisted in identifying eligible patients to participate in this study. Potentially eligible patients were identified based on information obtained from patient files. These records were reviewed for exclusion criteria and the eligible patients were approached to participate in this study. All patients willing to participate were informed about the study and the nature of their participation. Thereafter, they filled out a consent form. See Appendix E. In addition, the patients were asked to fill out a questionnaire, the details of which can be found in

Appendix F. Only once the questionnaire was filled out and the consent form signed, one 4.5 mL citrate tube (3.8% sodium citrate) of blood was drawn from each patient by a qualified phlebotomist. All patient information was dealt with anonymously. This was done by assigning each patient a unique ID, e.g., P01. More information on the patient group demographics can be found in Table 2.

The inclusion criteria for the patient group was as follows:

- Any biological sex
- Age range: 25-75 years[23]
- Diagnosed with a brain cancer
- Primary tumour
- Treatment naïve

The exclusion criteria for the patient group was as follows:

- Patients already partaking in treatment
- HIV positive (Only participants with a known HIV status were included)
- Smokes tobacco or uses any tobacco related product
- Use of oral contraceptives
- Has any condition which could present with chronic inflammation (including hypertension)
- Has a history of an immune-compromised status
- Use of the following: herbal supplements, or anti-coagulative (such as warfarin) medication within two weeks prior to the sample collection date
- Excessive alcohol consumption (drinking five or more alcoholic drinks on the same occasion at least one day in the past 30 days)[215]

Table 2: Participant demographic details.

Mean age (Standard deviation)	Male: Female	P value
Control group		0.0001
37,46 (±10,02)	22:13	
Patient group		
48.52 (±13.20)	16:9	

The criteria for recruitment included that the patients had to be treatment naïve. However, since the patients were admitted to the Neurosurgery ward at SBAH, it is expected that some patients were placed on a treatment plan which could not be prohibited due to their participation in the study. The aim was therefore to include patients using medication that did not have a negative impact on the coagulation system. A list of the medications that the patients received are listed in Table 3. If there were any medications that could alter the coagulation system, it was taken into consideration when analysing and interpreting the results. All collections occurred before any surgery took place.

Table 3: Patient medication details

Medication	Number of patients	Effects on coagulation
Paracetamol	15	No significant effect on blood clotting[216]
Tramadol	7	↑ clotting time; ↑ clot formation time; no effect on clot firmness[217]
Epilim	7	No effect on hemostasis[218]
Phenytoin	2	↑ thrombin generation[219]
Clexane	2	↓ thrombin activity[220]
Prednisone	1	↑ thrombin generation[221]
Citalopram	1	↓ PLT adhesion to fibrinogen; however no significant effect on plasma coagulation[222]
Ridaq	1	No significant effect on blood clotting[223]
Enalapril	1	No confirmed significant effect on blood clotting (potential ↓ PLT activity)[224]
Amlodipine	1	↓ expression of genes involved in coagulation activation[225]
Largactil	1	No significant effect on blood clotting pre-surgery[226]
Metoclopramide	1	↓ thrombosis when taken in conjunction with aspirin[227]
Simvastatin	1	↑ fibrin clot lysis; ↑ clot permeability; ↑ clot structure [228-232]
Folic acid	1	No significant effect on blood clotting[233]
Hydrochlorothiazide	1	No significant effect on blood clotting[234]
Decadron	1	↓ coagulation[220]
Lansoloc	1	Possible ↓ PLT count[235]

3.4 ROUTINE CLINICAL BLOOD TESTS

As a standard in SBAH, there is a set of routine blood testing that is done while a patient is admitted. For this study, two of these tests have been selected to better inform the researcher of the inflammatory status and the haematological profile of the patients. These tests, their significance, unit of measurement as well as reference ranges will be briefly discussed.

Full blood count

A FBC is used to measure the amount of blood components such as the number of RBCs, white blood cells (WBCs), and PLTs. It also determines the mean platelet

volume (MPV), platelet haematocrit percentage (PCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), haematocrit percentage (Hct) and haemoglobin concentration (Hb). These parameters, their descriptions, and formulas (where applicable) are summarised in Table 4.

Table 4: Haematological parameters obtained from the full blood count patient records.[236]

Parameter	Unit	Description	Formula
RBC	x 10^{12} cells/L	Number of RBCs	
WBC	cells/L	Number of WBCs	
PLT	cells/L	Number of PLTs	
MPV	fl	Mean PLT volume (average volume of individual PLTs)	
MCV	fl	Mean corpuscular volume (average volume of individual RBCs)	
PCT	%	PLT haematocrit percentage	PCT = PLT count × MPV / 10,000
MCH	pg	Mean corpuscular haemoglobin (average haemoglobin content of RBCs)	MCH = Hb/RBC
HCT	%	Haematocrit percentage	HCT = RBCxMCVx100 Absolute HCT =RBC x MCV
MCHC	g/dL	Mean corpuscular haemoglobin concentration (average concentration of haemoglobin in RBCs)	MCHC = (Hb/HCT) x 100
Hb	g/dL	Concentration of haemoglobin in the blood (photometrically measured at 540nm)	HCT= RBC x MCV
RCDWc	%	RBC distribution width (measure of the extent of variation in RBC volume and size)	(SD of MCV/MCV) x 100

All these parameters were assessed against healthy reference ranges to identify any abnormalities in the haematological profiles of the patients. See Table 5 for these results.

Table 5: Full blood count results of the patient group with healthy reference ranges obtained from laboratory reports.

	Full blood count results								
	WBC count ($x10^9$/L)	RBC count ($x10^{12}$/L)	Hb (g/dL)	Hct (%)	MCV (fl)	MCH (Pg)	MCHC (g/Dl)	RCDW (%)	PLT count ($x10^9$/L)
Reference range (lower-upper limits)	3.92-10.4	4.19-5.85	13.4-17.5	39-51	83.1-101.6	27.8-34.8	33.0-35.0	12.1-16.3	171-388
Patient									
P01	**18.96 H**	**3.34 L**	**10.60 L**	**31.10 L**	93.10	31.70	34.10	13.30	203.00
P02	10.18	4.81	13.50	42.30	87.90	28.10	**31.90 L**	13.90	**117.00 L**
P03	8.79	4.70	13.80	39.00	**83.00 L**	29.40	**35.40 H**	12.30	267.00
P04	**11.16 H**	3.82	**12.60 L**	**37.20 L**	97.40	33.00	33.90	12.30	274.00
P05	9.57	**4.16 L**	**12.20 L**	**36.20 L**	87.00	29.30	33.70	12.80	172.00
P06	4.58	4.96	**12.30 L**	**38.40 L**	**77.40 L**	**24.80 L**	**32.00 L**	14.60	256.00
P07	9.9	4.93	13.20	42.20	85.60	**26.80 L**	**31.30 L**	15.10	235.00
P08	10.35	5.09	15.50	47.30	92.90	30.50	**32.80 L**	13.40	265.00
P09	9.02	5.02	15.60	45.90	91.40	31.10	34.00	13.50	203.00

P10		**17.04 H**	4.66	15.50	44.40	**95.30**	33.30	34.90	14.20	**112.00 L**
P11		10.34	4.84	14.30	44.40	91.70	29.50	**32.20 L**	13.20	**112.00 L**
P13		8.55	5.13	16.60	48.70	94.90	32.40	34.10	12.90	203.00
P14		5.49	5.27	14.60	45.10	85.60	**27.70 L**	**32.40 L**	13.10	250.00
P15		**13.5 H**	4.88	15.00	44.60	91.40	30.80	33.60	13.50	227.00
P16		5.64	**4.12 L**	**12.20 L**	**36.80 L**	89.30	29.60	33.20	13.60	268.00
P17		**13.16 H**	5.10	14.70	46.30	90.80	28.80	**31.70 L**	15.10	220.00
P18		6.64	4.36	**12.70 L**	**38.80 L**	89.00	29.10	**32.70 L**	12.80	370.00
P19		5.88	4.29	**12.90 L**	39.30	91.60	30.00	**32.80 L**	16.10	338.00
P20		**1.83 L**	**5.**35	16.60	48.30	90.30	31.00	34.40	13.20	233.00
P21		**26.57 H**	**4.15 L**	**11.70 L**	**37.30 L**	89.90	28.20	**31.40 L**	**20.00 H**	**424.00 H**
P22		10.36	**6.13 H**	**18.50 H**	**51.40 H**	83.80	30.20	**36.00 H**	**12.30**	**390.00 H**
P23		7.40	**4.18 L**	**12.30 L**	**38.90 L**	93.10	29.40	**31.60 L**	13.20	313.00
P24		**1.43 L**	4.64	**12.50 L**	39.80	85.80	**26.90 L**	**31.40 L**	13.50	309.00
P25		6.32	5.23	**17.80 H**	**51.18 H**	99.00	34.00	34.40	14.40	263.00
Mean (SD)		10.53 (±3.87)	4.69 (±0.55)	13.95 (±1.66)	41.93 (±4.88)	89.61 (±4.47)	29.89 (±2.45)	33.31 (±1.21)	13.44 (±0.80)	206.86 (±58.38)

WBC: white blood cell; RBC: red blood cell; Hb: haemoglobin; Hct: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; RCDW: red cell distribution width; PLT: platelet. L indicates a value lower than the healthy reference range. H indicates a value higher than the healthy reference range.

C-reactive protein test

The CRP test is used to measure the amount of CRP in the blood as milligrams of CRP per litre of blood (mg/L). The liver produces CRP in response to inflammation and a high level of CRP in the blood therefore acts as a marker for inflammation. The summary statistics of the results of the patient group CRP tests is shown in Table 6.

Table 6: CRP routine blood test results with the healthy reference range.

Patient ID n=25	**CRP result (Reference range: < 10 mg/L)**
P01	34.00
P02	247.00
P03	4.00
P04	387.00
P05	1.00
P06	26.00
P07	2.00
P08	14.00
P09	5.00
P10	3.00
P11	174.00
P12	-
P13	11.00
P14	<1
P15	<1
P16	1.00
P17	11.00
P18	-
P19	2.00
P20	0.99
P21	<1
P22	1.00
P23	2.00
P24	15.00
P25	2.00
Mean (SD)	**102.38** (±61.47)

3.5 DISCUSSION

The FBC results showed no abnormality in the patient group profiles as compared to the healthy reference ranges. Individually, some parameters were outside of the healthy reference ranges (WBC count: 24%, RBC count: 24%, Hb: 48%, HCT: 40%, MCV: 16%, MCHC: 56%, RCDW: 8%, PLT count: 20%). However, when calculated for each parameter, the means of all the parameters were within their respective healthy reference ranges. This only shows that generally primary, non-metastatic BCa did not have a major impact on the clinically tested haematological profile of the patients. However, the measured parameters of a FBC are not primarily used to identify coagulopathy in patients.

The mean CRP showed an abnormal CRP value for the patient group (106.97 mg/L) when assessed against the healthy reference range of < 10 mg/L. Not all patients presented with abnormal CRP levels. Only 36% of patients had an abnormal CRP, which confirms that there is a presence of an increased state of inflammation in the patient group. There were two patients on anti-inflammatory medication (Patient 10 was given Ridaq, and Patient 23 was given Decadron). Neither of these patients had an elevated CRP value.

It is however important to take into account that routine test results may differ over the course of time, and during the stages of BCa. Primary, non-metastatic cancer does not always present with as high a state of inflammation as in metastatic cancers.[237] This would be an important consideration for future related studies.

3.7 CONCLUSION

There was a total of 60 participants that agreed to participate in this study. The control group consisted of 35 individuals (22 males and 13 females) with an average age of 35.81. The patient group consisted of 25 individuals (16 males and 9 females) with an average age of 49.29. There was a significant difference between the ages of the two groups with a P-value of ≤ 0.0001.

Routine blood test results were consulted in order to gain a clearer indication about the overall health of the patient group and give insight into their haematological components. From the results obtained it is clear that there were no abnormalities in

the haematological profiles of the patients as indicated by the FBC results. However, some patients did have increased CRP counts.

Prothrombin time activated partial thromboplastin time, thrombin time, D-dimer and international normalised ratio (INR) did not form part of the routine clinical work-up and was therefore not included in the routine test results. These routine tests are used in standard practice to identify any thrombotic risk in patients; however, the aim was to study the viscoelastic properties during clot formation and the structural properties of the blood components to better understand the changes that occur in these patients. As discussed in Chapter 2, inflammation has an impact on the initiation of the coagulation cascade and the presence of cancer contributes to inflammation that leads to the overactivation of the coagulation system. Though these tests prove important in assisting with diagnoses and treatment plans, they do not give detailed insight into the coagulopathy of this study population, therefore further investigation is required into the specific parameters of coagulopathy. To do this, LM, SEM and TEG® were used.

CHAPTER FOUR

LIGHT MICROSCOPY

4.1 CHAPTER OBJECTIVES:

The objectives that directed the research in this chapter were:

- To determine the axial ratios of RBCs which will be indicative of their shape
- To compare the axial ratios of the control group and patient group

4.2 INTRODUCTION

Medical investigation using the light microscope was first published by Petrus Borellus in 1653, whereafter LM has become a popular technique used for the analysis of cell morphology and pathology.[238] Pertaining to this study, RBCs were stained and viewed with a LM in order to measure the variation in cell shape. The LM can also be used to study cell morphology; however, this was not the main aim in the chapter.

Light microscopy has been used to study structural changes in RBCs and gave insight into the behaviour of RBCs in terms of maintaining their natural biconcave shape when introduced to different inflammatory conditions such as diabetes, haemochromatosis, arthritis, Parkinson's, or Alzheimer's disease.[239-245] To study the morphological changes of RBCs under diabetic conditions, Pretorius et al. employed the combination of LM and axial ratio measurement of the RBCs in order to establish any trends of deformability in the study population.[246] Since BCa also results in widespread inflammation, using LM to view the RBCs of these patients may prove just as useful. The degree of deformation of RBCs can be compared between individuals with pathologies such as these and healthy individuals using LM[247] to study the direct impact, if any, of these conditions on RBCs.

Haematologists have been using LM for decades to classify conditions and diagnose patients by viewing RBC shapes.[248] Although LM only gives a general view of RBCs, it is still an important technique that can provide valuable information with regards to the morphological changes of these cells when they are subjected to inflammatory conditions. For example, the axial ratios of RBCs can be measured in order to identify any deformability.[249] As mentioned previously, healthy RBCs generally stay true to

their natural biconcave, discoid shape. They have a central area of pallor and have no cellular inclusions. The pathophysiology of RBCs may manifest as deviations from the typical shape, size, colour, presence of inclusions, and distribution of cells. This is termed poikilocytosis which is when at least 10% of the RBC population show pathology in some way. Therefore, if it is seen with the LM that there are extensive misshapen RBCs in a blood smear, it may contribute to abnormal blood rheology. Common RBC morphological findings are named and explained in Table 7.

Table 7: Commonly observed RBC morphologies. Adapted from Ford.[250]

RBC morphology	Morphological definition
Acanthocyte (Spur cell)	Irregular distributed, variably sized, and pointy projections on cell surface
Anisochromia	Amount of central pallor variation
Anisocytosis	Size variation among the same population
Basophilic stippling: coarse	Variably sized basophilic granular discolorations across cytoplasm (Wright-stained film)
Basophilic stippling: fine	Small, uniform, punctuate basophilic dots across cytoplasm (Wright-stained film)
Degmacyte (Blister/bite cell)	Semi-circular indentation in cytoplasmic border. Some may show 'roof' of indentation (blister cell) or no 'roof' (bite cell)
Dimorphism	Two distinct RBC populations
Echinocyte (burr cell)	Regularly distributed, equally sized, rounded projections on cell surface
Elliptocyte	Oval shaped RBCs
Heinz body	Submembranous/epimembranous small round masses in RBCs (Heinz body stained)
Howell-Jolly body	Solitary, relatively large mass in haemoglobinised portion of RBC (Wright-stained film)
Hypochromia	Central pallor zone is greater than the normal 1/3 of RBC diameter
Irregularly contracted cell	Small, dark RBC, lacking zone of central pallor
Pappenheimer body	Multiple small dark blue/purple granular inclusions within haemoglobinised portion seen in one region of RBC
Polychromasia	Colour variability with some bluish cells amongst normal red coloured cells
RBC agglutination	RBC aggregation into multicellular masses
Rouleaux	RBC aggregation into linear patterns
Schistocyte	Fragmented RBCs, triangular/angulated morphology, often missing zone of central pallor
Sickle cell	Crescentic with two sharply pointed ends or linear with two tapered, slightly rounded ends
Spherocyte	Smaller and darker than normal, lacking zone of central pallor, perfectly (or almost perfect) round edge
Stomatocyte	Linear zone of central pallor, usually parallel to long axis. In some cases, line of pallor may be bi- or trifurcated
Target cell	Central red area within zone of central pallor
Dacrocyte (Teardrop cell)	Tapered to a point at one end of RBC

4.3 MATERIALS AND METHODS:

The WB samples collected were analysed within four hours of collection and prepared for LM. A blood smear was made for each participant from the citrated blood sample. Ten µL of blood was pipetted onto a microscope slide. Another slide was then held onto the blood drop at an angle of 30° which ensured the spread of the blood drop along the breadth of the slide. To create the smear, the angled microscope slide was then gently dragged along the length of the other slide. The blood was then left to dry at room temperature.

The slide was then briefly submerged in methanol for five minutes. This was done to fix the blood cells to the microscope slide. The slide was once again left to air dry after being removed from the methanol. Thereafter, the slide was submerged in Haematoxylin stain for five minutes. After staining, the slide was washed under running water until the water ran clear off the slide. The slide was again left to air dry at room temperature. After drying, the slide was submerged in Eosin stain for 1 minute. After staining with Eosin, the slide was washed under running water until the water ran clear off the slide. The slide was then left to air dry at room temperature whereafter a cover glass slip was mounted using Entallan for viewing. A Zeiss AXIO Imager.M2 light microscope was used to view and image the RBCs on a 100x magnification. A list of the reagents used in LM preparation can be found in Appendix G.

To measure the cells accurately, five representative images were taken per sample. CellAnalyzer software was used to measure 50 randomly chosen RBCs per sample. The measurements were exported into Microsoft Excel where the largest diameters were divided by the smaller, giving the axial ratio as described in Figure 6. These measurements were compared between participants in the same group to establish any trends within the group whereafter the trends within the respective groups were compared to one another. Normally, RBCs have an axial ratio equal to or very close to one since they are ideally round which means that the length and diameter of the cell would be equal. Deviations in RBC shape will result in axial ratios that are greater

than one. The increase in axial ratio is invertedly proportional to the degree of deformation of the cell.

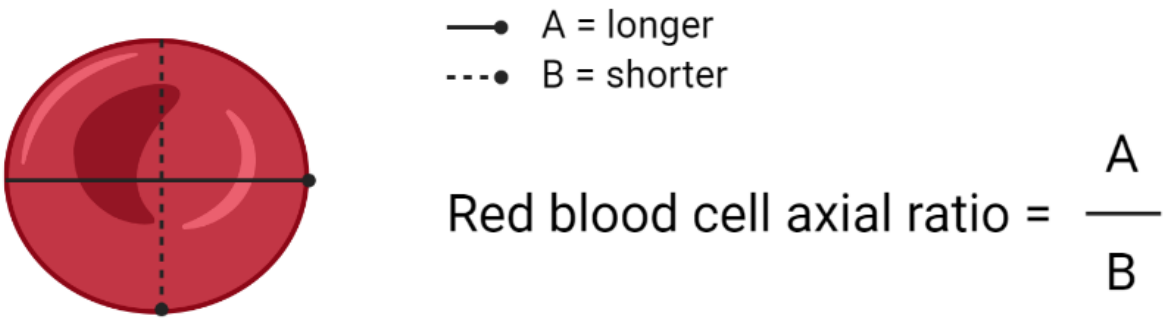


Figure 6: Image showing the process of measurement and calculation of red blood cell axial ratios.

4.4 STATISTICAL ANALYSIS

GraphPad was used to perform a Shapiro-Wilk normality test on both the control and patient group data. The test showed the data was non-parametric therefore, a Mann-Whitney-Wilcoxon two-sided test was performed to compare the axial ratios of the control group to the axial ratios of the patient group. The results were presented as a median with minimum and maximum values. A P-value ≤0.05 was regarded as significant. See the data summarised in Tables 8 and 9.

4.5 RESULTS

Tables 8 and 9 provide the average of 50 axial ratio measurements for each participant in the control and patient group respectively. The average axial ratio obtained for the control group was 1.10 and the average for the patient group was 1.14 with a significant P-value of ≤0.0001 when comparing the data of both groups.

Table 8: Summary statistics of axial ratio measurements of the control group.

Control group (n= 1700 cells)	
Control ID	**Average axial ratio per 50 cells**
C01	1,15
C02	1,19
C03	1,13
C04	1,10
C05	1,09
C06	1,07
C07	1,06
C08	1,09
C09	1,06
C10	1,08
C11	1,06
C12	1,06
C13	1,06
C14	1,09
C15	1,08
C16	1,12
C17	1,06
C18	1,06
C19	1,05
C20	1,05
C21	1,07
C22	1,10
C24	1,09
C25	1,06
C26	1,09
C27	1,11
C28	1,07
C29	1,16
C30	1,08
C31	1,06
C32	1,07
C33	1,06
C34	1,06
C35	1,07
Median (min; max)	1.10 (1.00; 1.84)

Table 9: Summary statistics of axial ratio measurements of the patient group.

Patient group (n= 1100 cells)	
Patient ID	**Average axial ratio per 50 cells**
P01	1.08
P02	1.18
P03	1.23
P04	1.56
P05	1.17
P06	1.31
P07	1.14
P08	1.13
P09	1.09
P10	1.11
P11	1.13
P12	1.26
P13	1.09
P14	1.12
P15	1.21
P16	1.13
P17	1.21
P18	1.15
P19	1.14
P20	1.11
P21	1.24
P22	1.10
Median (min; max)	1.14 (1.00; 2.61)

4.5.1 Red blood cell smears

Micrographs are provided to briefly show the smears as they appear with LM. However, these images were not used to study the morphology of the RBCs and was only used for the determination of the RBC axial ratios. The following images were chosen as representative of both the control (Figure 7) and patient (Figure 8) group.

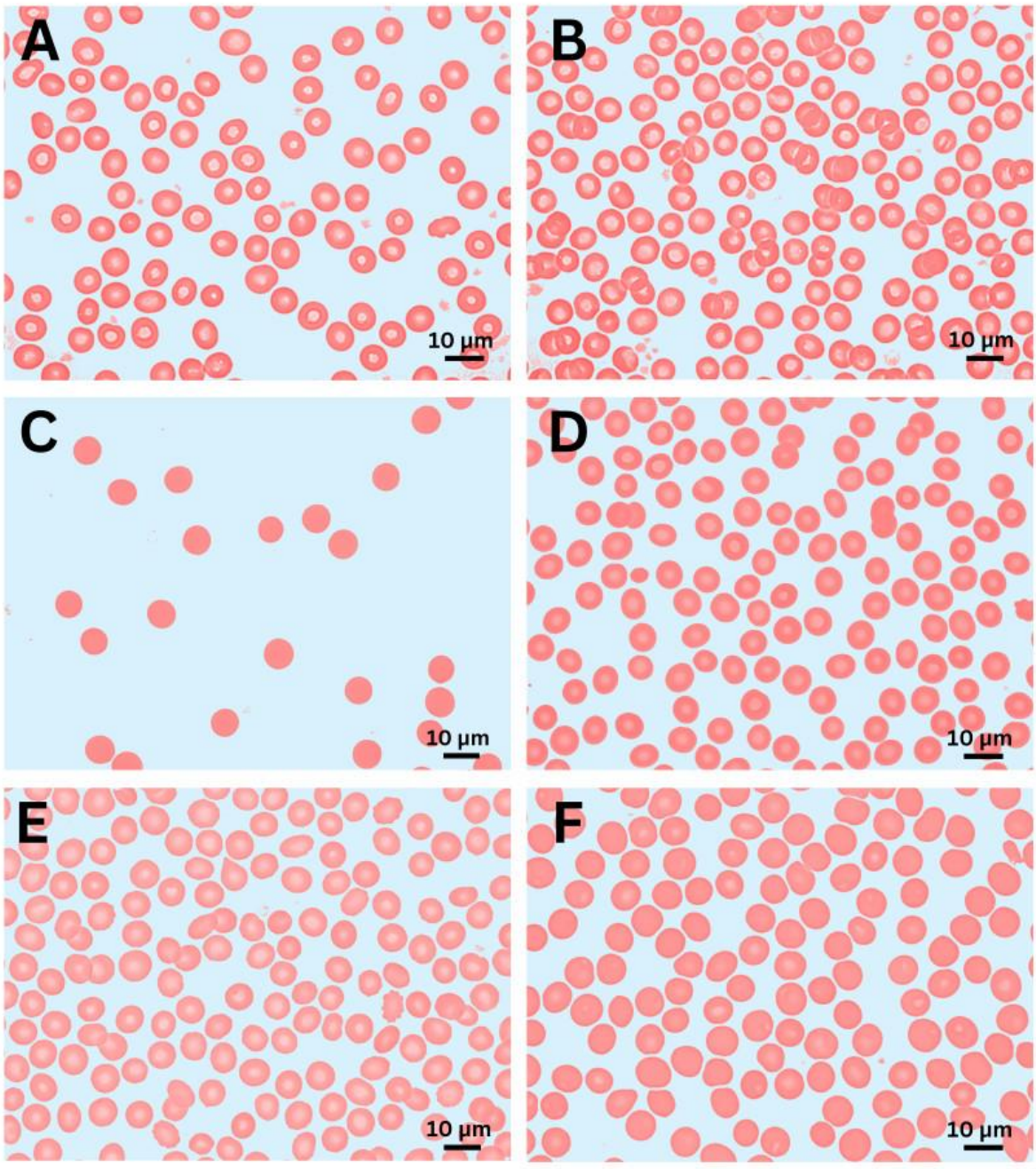


Figure 7: Micrographs A-F represent the WB smears of the control group. Minimal variation in the size of the RBCs can be seen.

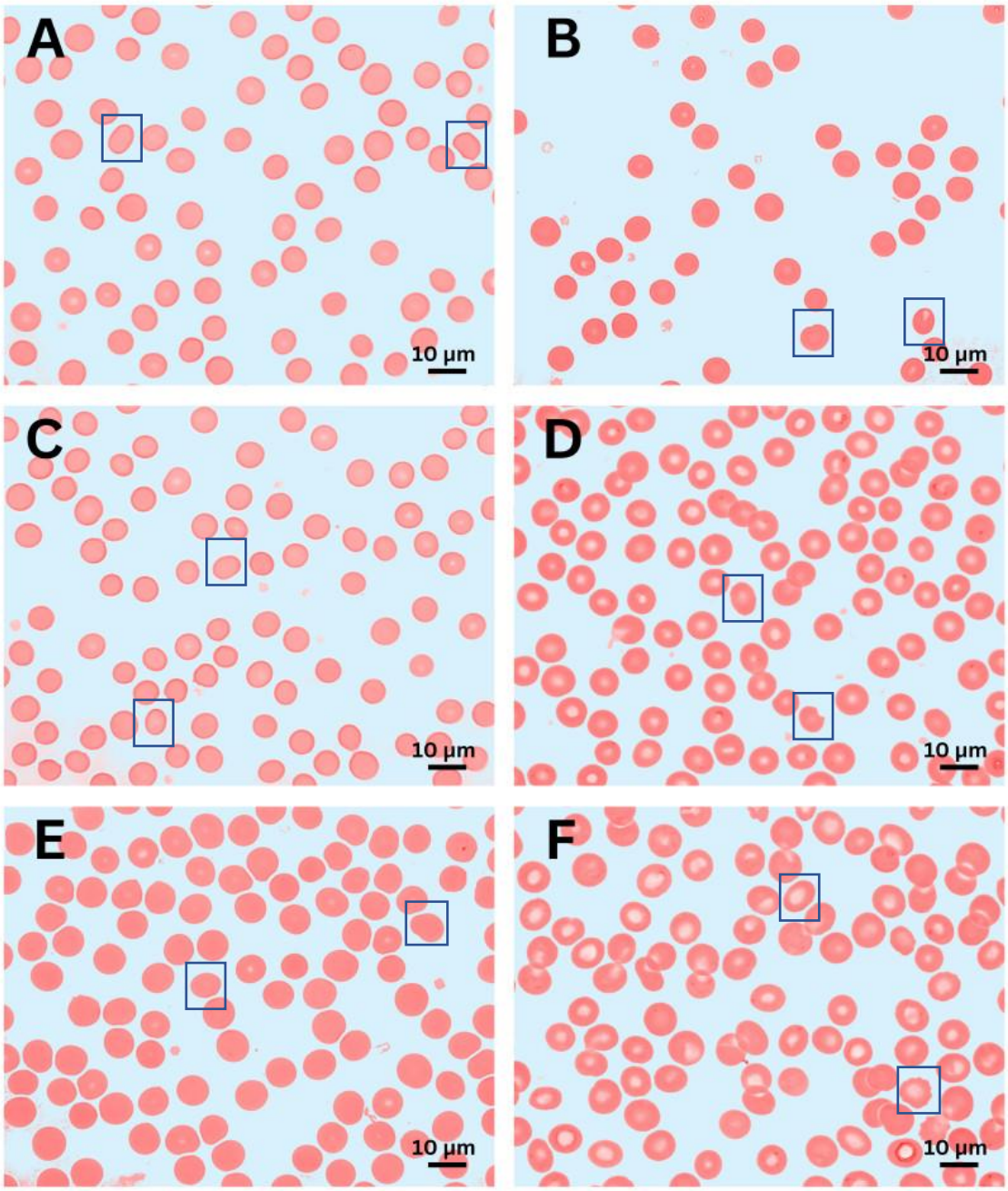


Figure 8: Micrographs A-F represent the WB smears of the patient group. Some RBCs varied in size and shape. RBCs that deviated from the normal size and shape are indicated by the blue squares.

Analysis of the WB smears of the control group revealed a slight variation in the size of some RBCs and there appears to be limited variety in RBC shape. The cells seem to generally adhere to the typical circular shape of a healthy RBC. The micrographs of the patient group showed the presence of oval or misshapen RBCs. This is in support of the disparity in the calculated axial ratios between the control and patient groups.

4.6 DISCUSSION

When comparing the axial ratios of the control and patient groups, a significant difference between the average axial ratios of the two groups was obtained. The average axial ratio of the patient group was significantly higher than that of the control group, suggesting that there are more deformed RBCs in the patient group, even if the change appears minimal.

Although it is very important for RBCs to be able to temporarily deform to allow passage through microvasculature,[114] some studies have found that certain diseases decrease deformability of RBCs. Faustina et al. found that when RBCs are in contact with tumour cells, that the deformability of the RBCs decrease.[251] This would mean that the cells are not returning to the natural round, biconcave shape as they should. The biconcave shape normally increases the surface area for diffusive exchange by about 43%.[252] Without this essential ability to deform, oxygen and carbon dioxide exchange between tissues and blood is negatively affected.

A possible explanation for the observed increase in deformed cells in this study may be that the cells were deformed due to being more rigid. Pathological conditions such as inflammation can cause rigidification of RBCs.[253] Increased rigidity of RBCs would cause a decrease in their deformability. If this decreased deformability is widespread in the blood, there might be more RBCs that are deformed and unable to efficiently move through smaller vasculature and contribute to an increased thrombotic risk. Rigidity of the RBCs may cause direct obstruction of the microvasculature and ineffective clearance from circulation due to the inability of the cells to temporarily change shape in order to successfully pass through microvasculature. [254,255] Red blood cell stiffness increases the generation of thrombin, which would contribute to thrombosis.[4] This is because inflammatory conditions cause RBCs to lose membrane asymmetry and causes the exposure of PS.[136] This exposure causes thrombin generation through coagulation complexes (intrinsic tenase and prothrombinase) assembly on the PS matrix.[256] However, no tests for RBC rigidity were conducted and therefore would require further investigation by using methods such as atomic force microscopy (AFM).

4.7 CONCLUSION

From the finding it is evident that the patient group had more deformed RBCs compared to the control group. This poses a risk of ineffective transport of oxygen and carbon dioxide, and clearance of deformed RBCs.[257] If there is obstruction of the microvasculature, cerebral complications can occur due to ineffective oxygen transport to tissues.[258] In addition, abnormal RBC deformability (more deformed RBCs) increases inflammation and the risk of thrombotic events by means of microvascular occlusion.[259]. In order to gain deeper understanding of distinct morphological changes it is necessary to utilise techniques that offer higher magnification such as SEM[248] which will be discussed in Chapter 5.

CHAPTER FIVE:

SCANNING ELECTRON MICROSCOPY

5.1 CHAPTER OBJECTIVE

The objective that directed the research in this chapter was:

- To investigate the ultrastructural changes of RBCs in WB and in clots, and their interactions with PLTs in BCa patients using SEM

5.2 INTRODUCTION

Scanning electron microscopy is used to study cells and tissue at a higher magnification, allowing the researcher to study the surface morphology of a sample.[260] It can therefore be used to clarify and confirm the findings of LM in greater detail. Scanning electron microscopy is an ultrastructural, single-cell level method for studying special characteristics of RBCs.[261, 262] Highly detailed resolution of RBCs is made possible by assessing these cells with a SEM. Membrane morphologies, surface topographic inspection, shape, as well as mutual interactions of RBCs and PLTs can be studied using this ultrastructural technique.[263-265] Additionally, SEM can be used to look at clot ultrastructure by studying individual cells and fibrin packaging at high resolution to determine how these components behave during coagulation.[248, 261, 265-274]

Mereuta et al. also used SEM to study the ultrastructural features of blood. The researchers showed in detail the interactions between RBCs, PLTs and clots.[275] The researchers deemed SEM a good technique since they were able to study the clots in detail but more importantly, they could identify three distinct areas within clots. These were RBC-rich areas which mainly composed of tightly packed RBCs, mixed areas with bundles of PLTs, WBCs, RBCs, and fibrin fibres and lastly fibrin-rich areas which consisted of dense fibrin masses and a few RBCs.[275]

In a study investigating RBC membrane changes in patients with Type 2 diabetes conducted by Buys et al., WB smears were studied using SEM. The researchers noted a change in the morphology of RBCs from diabetic patients with extended projections from the membranes as well as twisting of these cells around spontaneously formed

fibrin fibres when compared to healthy controls.[276] The researchers suggested that this twisting may lead to stretching of the fibres, causing them to be more resistant to lyse, reinforcing clot stability.[277]

In a study carried out by Avsievich et al., RBCs were viewed with SEM in order to establish the interaction and adhesion of these cells.[278] The study produced high resolution SEM images of RBC aggregates and showed the extent of interaction between the cells as well as membrane changes when RBCs aggregate. This aggregation can increase the viscosity of blood, leading to ineffective microcirculation, resulting in the potential exacerbation of diseased states.[279]

Bester et al. conducted a study of the effects of inflammatory cytokines (IL1β, IL-6, and IL-8) on RBC structure.[280] To do so, the researchers used SEM to investigate these effects on RBCs and PLTs. They found that all three cytokines caused pathophysiology of RBCs and PLTs. The researchers saw hyperactivation and spreading of PLTs and the RBC structure resembled that of eryptosis, thereby showing that RBCs and PLTs are very sensitive to cytokine presence.[280]

Based on the findings of the abovementioned studies, SEM has proven to be a technique that offers high resolution imaging of the ultrastructural characteristics of RBCs, fibres, PLTs as well as the interactions between these components in different diseased conditions. It is therefore considered to be a useful technique in this study since the aim was to shed light on the behaviour of these blood components in BCa patients.

5.3 MATERIALS AND METHODS

Scanning electron microscopy preparation for whole blood samples

The citrated WB samples were analysed within 4 hours of collection and prepared for SEM. For the SEM, a standard protocol was followed.[281] A 10 mm glass coverslip was prepared with 10 µL of WB. For each participant, two coverslips were prepared. One with only 10 µL of WB and the other with 10 µL of WB and added 5 µL of thrombin (3.33 IU/mL) which was donated by the South African National Blood Services (SANBS). The coverslips were left to dry after which they were washed with a 0.01 M phosphate-buffered saline (PBS) for 15 minutes. A solution of 4% formaldehyde was

used for primary fixation for 30 minutes. After fixation, the coverslips were washed again using PBS, three consecutive times for 3 minutes each.

Osmium tetroxide (OsO_4) was then used for secondary fixation for 15 minutes. Following secondary fixation, the coverslips were washed with PBS another three times for 3 minutes each with PBS. Ethanol was used for serial dehydration at increasing concentrations of 30%, 50%, 70%, 90%, and 100%. Each step of the serial dehydration lasted 3 minutes, repeating the 100% ethanol dehydration step three times. Hexamethyldisilane (HMDS) was then used to dry the coverslips for 30 minutes. Once dry, the coverslips were mounted onto aluminium stubs with carbon tape after which they were carbon coated.

The first carbon coat took place at a 90° angle and the samples were mounted accordingly. The second and third coats covered the samples at a 120° angle, and this was done by mounting the sample and then tilting it to the left and then to the right using a Quorum Q150T ES carbon coater. A Zeiss ULTRA plus FEG-SEM was used at the microscopy and microanalysis unit at UP to visualise the RBCs, formed clots, and PLTs. The samples were viewed using the in-lens detector at an accelerating voltage of 2 kV. All the reagents used for SEM during this study are listed and detailed in Appendix G.

The investigator prepared the samples after which the supervisor and investigator viewed and analysed the samples. The data obtained from the SEM was qualitative data and the following properties were studied:

1. The overall shape of the RBCs
2. The topography of the RBC membranes
3. The presence of microparticles
4. Platelet presence and pseudopodia formation
5. The structure and density of fibrin networks or matted deposits

The data obtained from SEM was captured and analysed as follows. The sample viewings took place following a minimum of five representative images in low machine magnification (5 000x). Representative features of interest were identified and imaged at increasing machine magnifications (10 000x to 20 000x). The representative images were labelled according to their magnification and sample of origin.

5.4 RESULTS

The following images were chosen as representative of the control group and the patient group. Observations were made and compared between the participants within the same group to establish trends whereafter the trends within the respective groups were compared to one another.

Figure 9 shows the WB smears of the control group. Here, the RBCs appear to have a normal round, biconcave shape with limited interaction between the cells (blue square in Figure 9 A). This is evident in all the micrographs presented in Figure 9. This interaction can also be seen in Figure 9 G and H. Figures 9 G and H were taken at a higher magnification and show that the membranes of the cells are smooth. There are limited PLTs present as seen in all the micrographs. When PLTs were present, there was limited spreading and interaction with the RBCs as indicated by the white square in micrograph E. Even when there is spreading of PLT pseudopodia, there is still no to limited interactions with the RBCs, as observed in micrograph E.

Referring to Figure 10, there appears to be slight to no changes to the overall shape of the cells in the patient group. The shapes of the cells are consistent with that of the control group with little variation. For example, some oval RBCs can be seen in Figure 10 C and E, indicated by the blue squares. In the micrographs taken at higher magnification (E and F), the surfaces of the cells appear relatively smooth. As seen in B, F, and H, there were some areas where the cells were aggregated.

When considering the PLT presence in the patient group, there appears to be increased PLT presence. This is seen in Figure 10 A, B, D, and G. These PLTs showed more spreading, more branching of pseudopodia and interaction with the RBCs. This interaction can be observed in micrograph A and D as indicated by the blue squares.

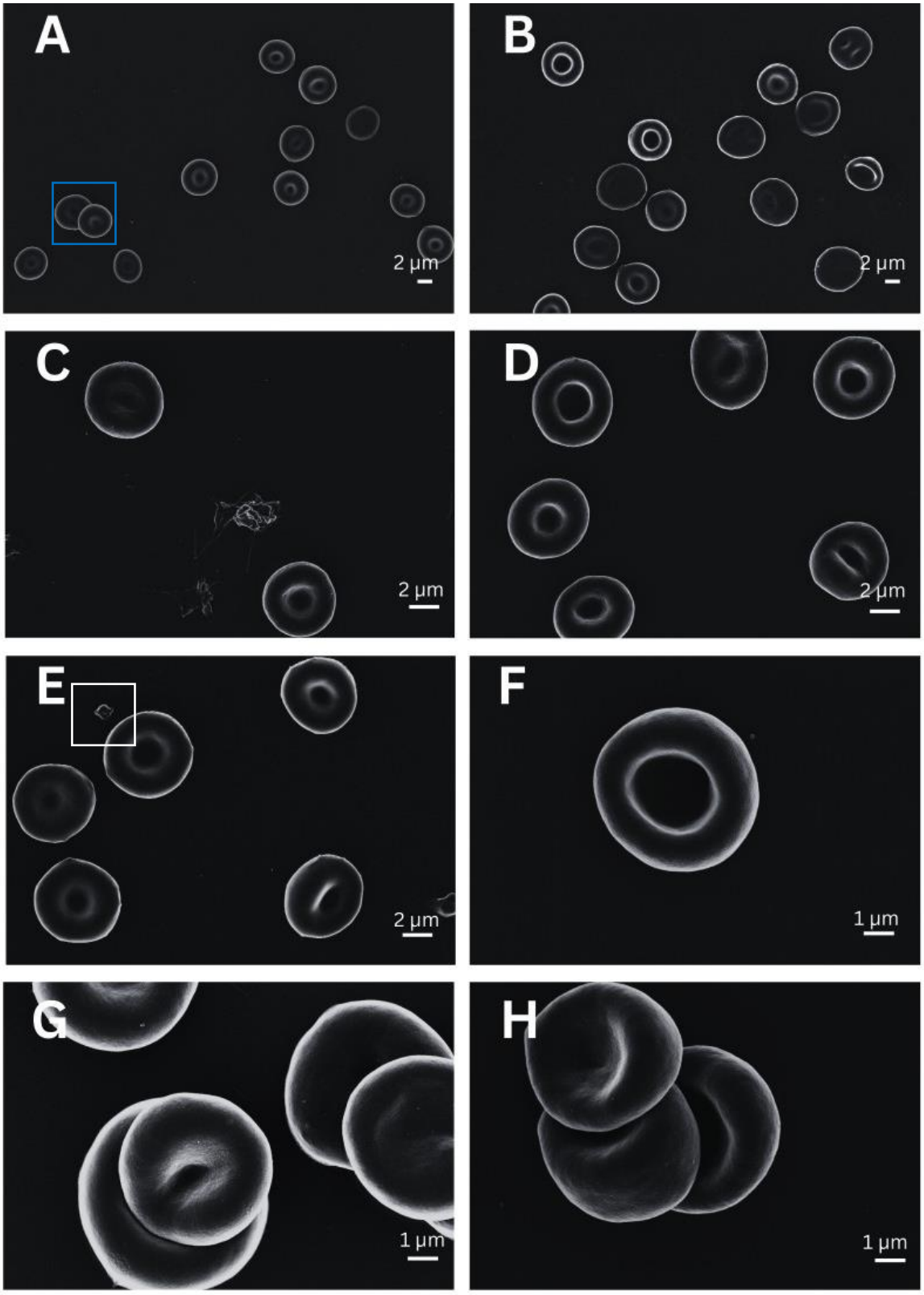


Figure 9: WB smears of the control group viewed with SEM. Showing the RBCs with a normal, round, biconcave shape. Smooth cell surfaces of the RBCs (G-H). Limited interaction between the cells (A, G, H), but generally distributed evenly (A-F). Platelet presence with no interaction with the cells (C, E). Blue square indicates RBC interaction. White square indicates RBC-PLT interaction. A, B magnification: 5000; C, D, E magnification: 10000; F, G, H magnification: 20000.

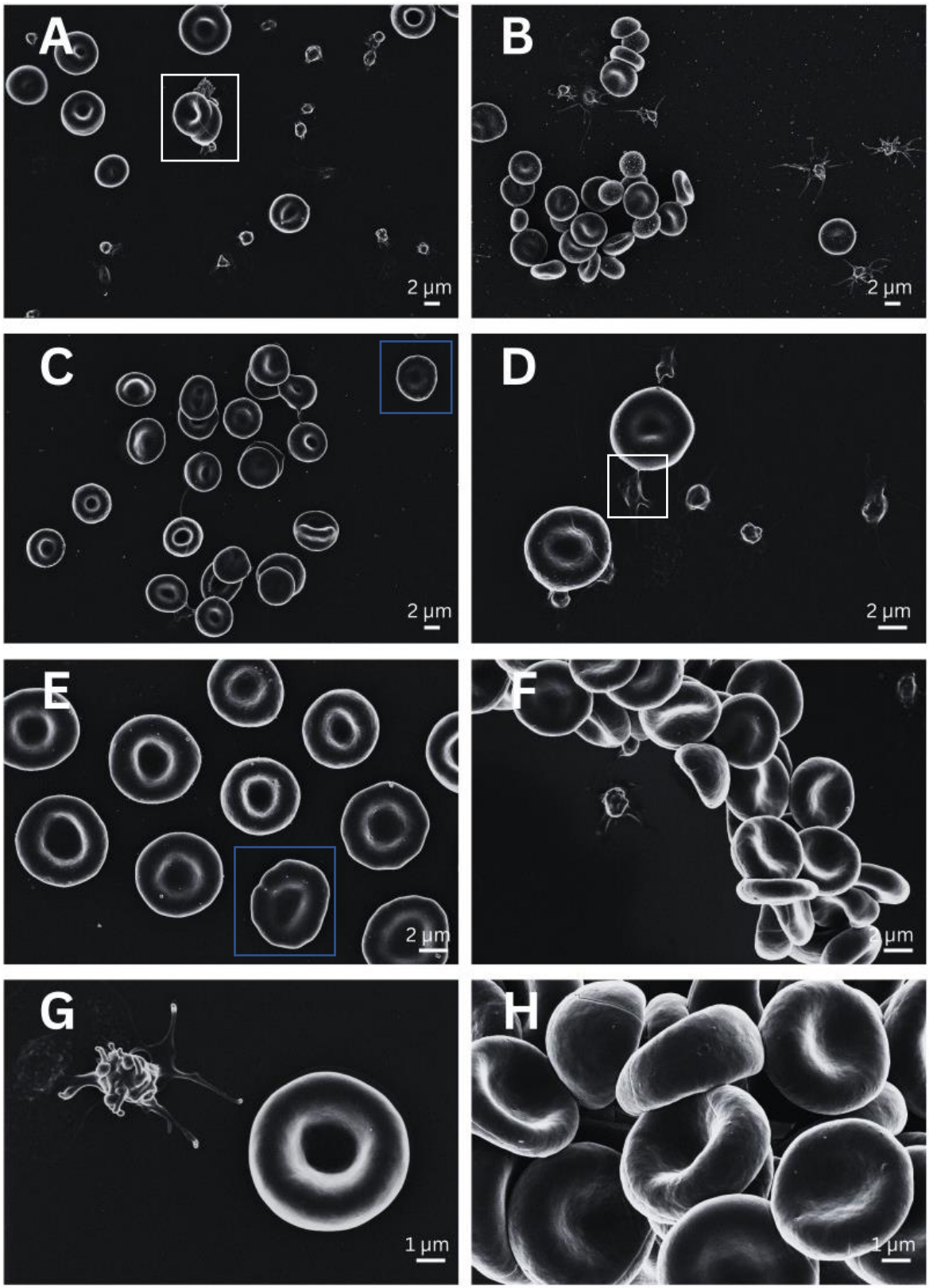


Figure 10: Whole blood smears of the patient group viewed with SEM. Some variation in size and shape are shown (B, C, E). Some RBCs aggregate as seen in (B, F, H). Also showing PLT presence and interaction with the RBCs (A, B, D, G). White squares indicate RBC-PLT interaction. Blue squares indicate RBCs with shape changes. A, B, C magnification: 5000; D, E, F magnification: 10000; G, H magnification: 20000.

The WB clot structures of the control group are presented in Figure 11 A-H. The clots of the control group showed normally shaped RBCs and a uniform distribution of fibrin fibres. The force exerted by the fibres does not seem to induce significant alterations in the shape of the RBCs. There is limited fibre tangling and fusion present in the control smears. The single fibrin fibres can be observed in all the micrographs of Figure 11.

The patient group WB clots are shown in Figure 12 A-H. The patient group showed minimal RBC shapes changes within the clots. RBCs were trapped within the fibrin fibres however the shape of the RBCs appear to be unaffected by the force exerted by the fibres. Some clots showed areas with more thinner fibres visible between the thicker fibres. There were observed areas of disproportionate clumping as indicated by the white squares in Figure 12 A, C, and D. Furthermore, tightly woven fibre masses were also observed in micrographs E, F, and G indicated by the blue squares. Some areas of the clots appeared denser and as shown in micrograph H a fusion and tangling of the fibrin fibres were also visible (purple square).

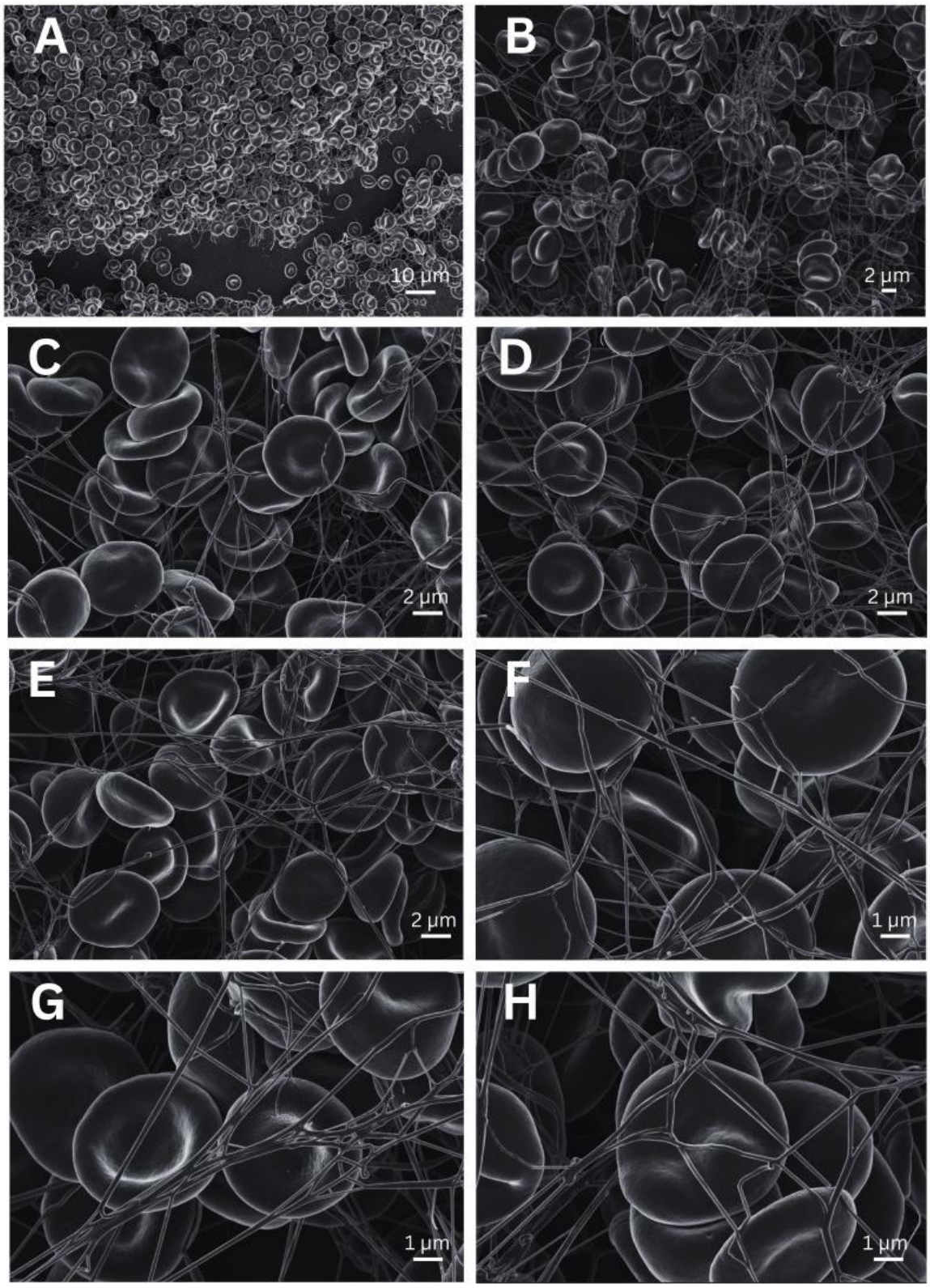


Figure 11: WB clots of the control group viewed with SEM (A-H). Showing normal cell shape, and evenly distributed fibres. A magnification: 2000; B magnification: 5000; C, D, E magnification: 10000; F, G, H magnification: 20000.

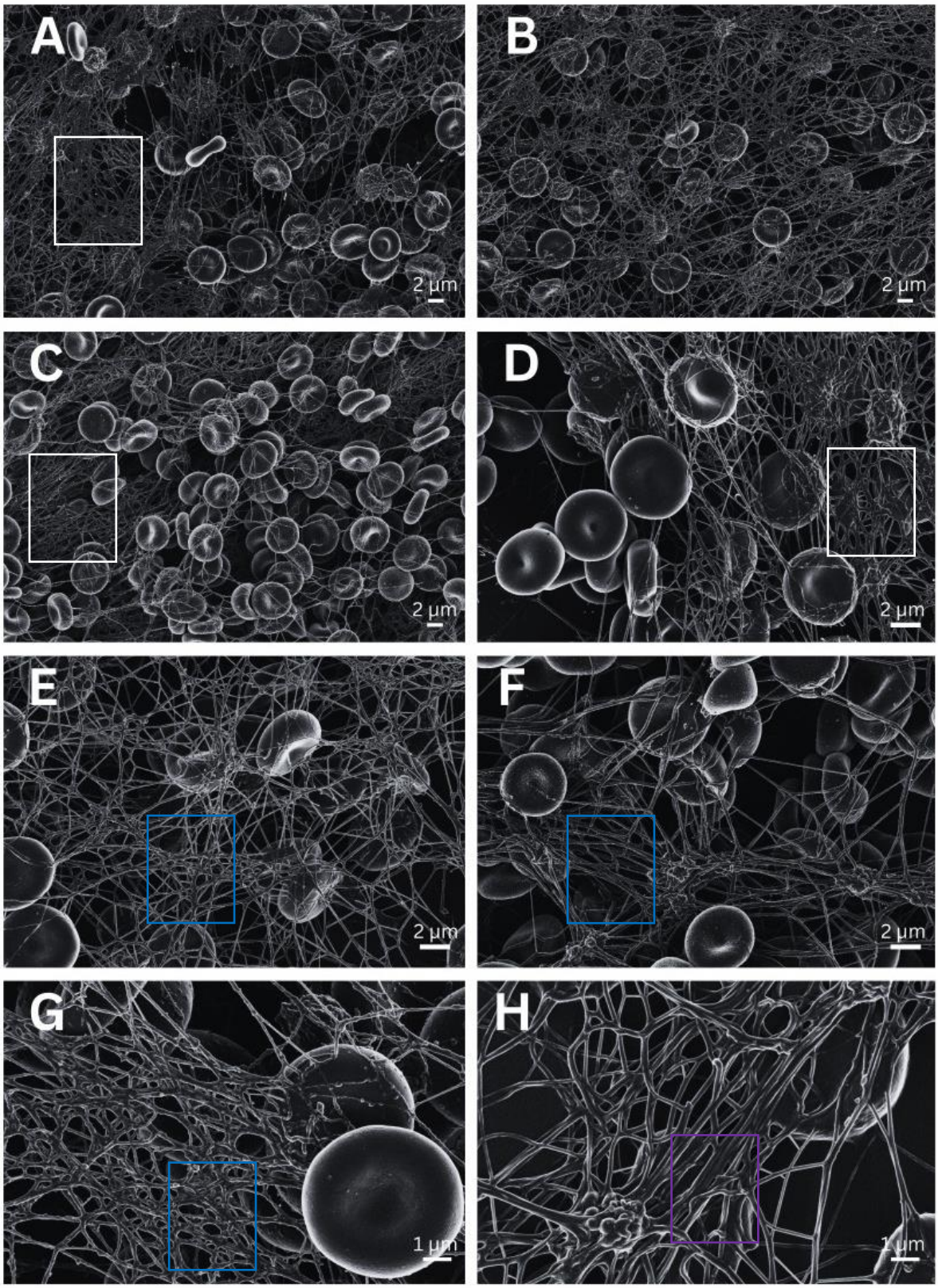


Figure 12: WB clots of the patient group viewed with SEM. Normal RBC shapes with an uneven distribution of the fibres (A-D). Some fibres are tightly woven (E-G) and there is fusion and tangling of the fibres (H) observed in the patient group. White squares indicate disproportionate clumping of fibres. Blue squares show tightly woven fibres. Purple square indicates fusion and tangling of fibres. A, B. C magnification: 5000; D, E, F magnification: 10000; G, H magnification: 20000.

5.5 DISCUSSION

This technique was used to identify any morphological differences between the two groups upon closer investigation with higher magnification.

During clot formation, it is expected that RBCs associate closely with one another as well as with fibrin fibres. Based on existing research on the blood of cancer patients, it was expected to see more widespread PLT presence, thicker clots and increased RBC interaction in the blood of BCa patients as compared to the healthy individuals.[282, 283] Platelets are easily activated through preparation and/or contact to the surface of glass,[284] however, because the same procedure of sample preparation was followed for both groups, the PLT presence in the two groups can be compared. As proposed by Suzuki et al., increased PLT activity may be caused by the expression of podoplanin by cancer cells. Podoplanin induces PLT aggregation.[283] Subeikshanan et al. found the PLT count of glioma patients to be significantly higher than that of healthy controls.[285] In addition, Reynes et al. found that there was a higher rate of endogenous thrombin generation in patients with glioblastomas as compared to healthy controls.[286] This could serve as an explanation for the generally high incidence of hypercoagulability in glioma patients.[287]

Nunes et al. found that these changes may not be as pronounced in non-metastatic cancer patients but rather that the main effects can be seen in the fibrin networks that were observed to be more tightly woven in these patients as compared to healthy controls.[206] Consistent with these findings, the micrographs obtained in this study highlighted that the most significant difference between the control and patient groups was the arrangement of the fibrin fibres within clots. A possible contributor to these observations may be α-defensins which are released by activated neutrophils (due to inflammation).[288] This protein is responsible for the acceleration of fibrin polymerisation and causes fibres to branch more and become more tightly packed. α-Defensins also combine forming clots and reduce fibrinolysis.[288]

A possible explanation for the denser appearance of the clots in the patient group, could be that the increased inflammatory state of the patient group causes over-activation of the coagulation system by means of increased pro-coagulants, i.e., fibrinogen. When IL-1β, IL-6, and IL-8 are elevated, clot stiffness can increase due to

these interleukin ability to stiffen clots through fibre crosslinking and compaction.[289, 290]

However, some studies show that there are no elevated levels of fibrinogen in glioma patients when comparing the blood of these patients with controls.[291, 292] Yet, when fibrinogen levels are elevated, there is a significant correlation with increased mortality.[293-295] Two studies found a hypofibrinolyisis state in patients with gliomas. This conclusion was reached by comparing the PAI-1 activity and clot lysis time between controls and adult glioma patients. This comparison revealed prolonged clot lysis time as well as increased PAI-1 activity in glioma patients.[282, 296]

5.6 CONCLUSION

Scanning electron microscopy is an important technique that provides valuable insight about the individual blood components in BCa patients. The SEM of the BCa patient blood samples provided information on the ultrastructure of clots and their components as well as RBC structure individually and as part of the clots, as well as their interactions with PLTs. These interactions and behaviours noted with SEM could explain some of the physiological hypercoagulable tendencies seen in these patients such as DVTs or VETs. This may prove useful to clinicians when considering thrombolytic treatments for BCa patients. There is evidence of RBCs promoting thrombus formation and enhancing clot stability.[297]

When WB is prepared for SEM by adding thrombin, healthy individuals present with a WB clot network showing typical biconcave RBCs entangled within a network of individual branching elongated fibres. There may also be a rare occurrence of platelet remnants with pseudopodia merging into the network of fibrin. Candido et al. showed that this structure is consistent with clot strength and stiffness findings obtained from TEG®.[261] To further assess the clotting kinetics of BCa patients the TEG® was used.

CHAPTER SIX

THROMBOELASTOGRAPHY®

6.1 CHAPTER OBJECTIVE

The objective that directed the research in this chapter was:

- To analyse the viscoelastic properties of WB during clot formation in BCa patients compared to the WB of healthy individuals using TEG®

6.2 INTRODUCTION

Thromboelastography® is a viscoelastic test (VET) that was established in 1948 by Hartert[298] and is a technique used for the rapid analysis of the rate and strength of clot formation and WB clot lysis.[299] Some researchers favour this technique above traditional coagulation tests when analysing the hypercoagulable state as well as haemorrhage risk of prostate cancer patients, haemophilia, postpartum haemorrhage, and patients undergoing cardiac or brain trauma surgery.[300-303]

TEG® is primarily sensitive to three blood components: levels of fibrinogen in WB and plasma, Hct, and MP levels in plasma.[304] This technique proves specifically useful since it continuously measures clot formation, clot strength, PLT function and clot lysis whereas other coagulation tests typically only provide readings until the phase of first fibrin strand formation.[305]

A TEG® activates the coagulation system of a small sample of blood *ex vivo* and monitors active changes of clot formation and lysis.[306] By inducing coagulation, TEG® analysis records the viscoelastic changes that take place during coagulation.[298] The parameters measure viscoelastic changes in WB without separating plasma.[5] Thromboelastographs can be used to identify hyper- or hypocoagulation as well as the severity of these coagulopathies using WB and PLT poor plasma.[261, 299, 307-312]

This technique may also serve as an indirect measure of thrombin generation (TG).[308] Tripodi et al. found that the WB TEG® profiles of β-thalassemia patients showed hypercoagulability but not in the PLT-poor plasma TG profiles, thereby suggesting blood cell components and/or PLTs to be the primary determinants of thrombotic risk.[313]

A typical presentation of TEG® results is shown in Figure 13. The data is presented in a graph with time on the horizontal axis in minutes, and clot firmness on the vertical axis. There are several factors of coagulation analysed by the TEG®. The maximum amplitude (MA) is measured in millimetres and measures the overall clot stiffness and is used to evaluate primary and secondary haemostasis.[299] It is also useful in guiding PLT transfusion.[314] An MA of < 50-55 mm indicates ineffective PLT involvement in coagulation which signals PLT transfusion. A lower MA specifies lower PLT numbers as well as PLT dysfunction.[299]

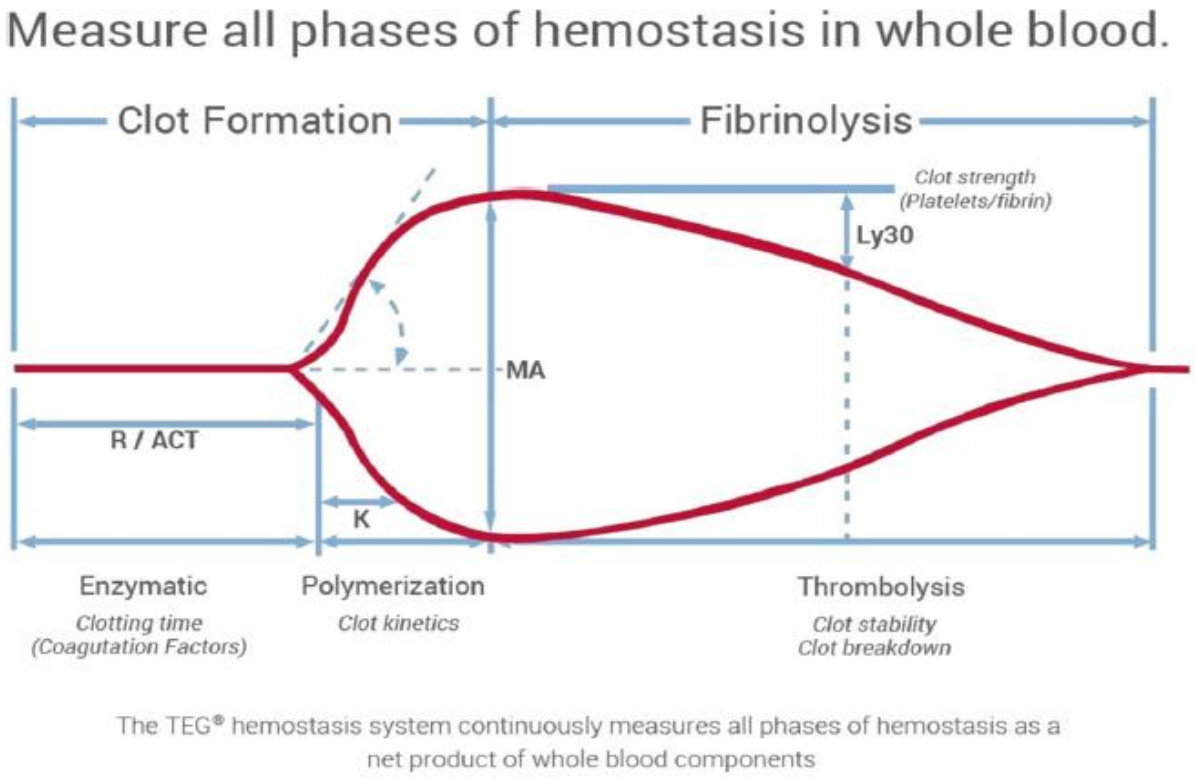


Figure 13: A typical presentation of a thromboelastographic tracing curve. Clot formation and lysis are illustrated by the respective expansion and contraction of the curve.[5]

The R-time is a measure of the time it takes from the start of measurement to the start of clot formation. A higher R time is associated with lower concentration of clotting factors which indicates the need for factor replacement.[299] The k-time indicates the time taken from the start of clot formation until the clot firmness reaches an amplitude 20 mm. An α-angle of < 45° indicates low of dysfunctional fibrinogen which calls for fibrinogen replacement.[299] It is important to keep in mind that overall coagulation is different between males and females due to the differences in haematocrit and plasma content in the respective sexes.[304]

Furthermore, secretion of sex-specific growth hormones (particularly oestrogen) mediate differences in thrombosis due to changing the expression of coagulation inhibitor genes found in the liver.[315, 316] The outcome hereof being that men are more susceptible to thrombosis than women.[315]

6.3 MATERIALS AND METHODS

Thromboelastography® for the quantitative analysis of the viscoelastic properties of whole blood

Thromboelastography® is sensitive to the time from venepuncture to analysis. Citrated blood can be safely analysed within 8 hours of drawing the blood.[317] The TEG® assays were conducted using a TEG® 5000 computer-controlled device (Haemoscope Corp., Niles, IL, USA) and by using WB. The TEG® assays were conducted as indicated by manufacturer-guidelines using TEG® kits. The standard TEG® reaction should have 340 µL of WB from a sodium citrate tube. To a loaded TEG® cup, 20 µL of 0.2 M calcium chloride was added. After adding the calcium chloride, 340 µL of WB was then added and the assay was left to run. All the reagents used for TEG® during this study are listed and detailed in Appendix G.

Coagulation and clot kinetics were then monitored by the specialised TEG® computer program. This was measured based on pre-programmed parameters. Coagulation ended once the parameters were met, and the results were reported both numerically and as a graphical tracing. As only clot formation is relevant to this study, the test was terminated once MA was reached. Table 10 presents a summary of different effects on coagulation depending on the increase or decrease of each TEG® parameter. Table 11 explains the standard TEG® tracing and viscoelastic parameters that were used in this study.

Table 10: Summary of the viscoelastic parameters, their units and descriptions that was studied using TEG®. Adapted from Pretorius et al.[261]

Code	Parameter	Reference ranges [305, 318-320]	Units	Description
R	Reaction time	9-27	Minutes	Rate of initial clot formation with an amplitude of 2 mm.

α	Angle	2-58	Degrees	Rate by which clot amplitude increases (i.e., rate of clot formation). Reflects the speed of fibrin build up and cross linking. Represents the slope between the representative traces by R and K.
K	Clot kinetics	2-9	Minutes	The time it takes from initial clot formation to a clot reaching an amplitude of 20 mm. Reflects the speed of clot polymerisation
MA	Maximum amplitude	44-64	mm	The maximum stiffness of the developed clot. Reflects fibrin clot strength (i.e., overall clot stability).
G	Shear elastic modulus strength	3.6-8.9	Dynes/cm^2	Resistance of clot to deformation
TMRTGG	Time to the maximum rate of thrombus generation	5-23	Minutes	Time interval before maximum speed of clot growth
MRTGG	Maximum rate of thrombus generation	0-10	dynes/cm^{2}s	The maximum velocity of clot growth or maximum rate of thrombus generation using G.
TGG	Total thrombus generation	251-1014	Dynes/cm^2	Total clot strength. Indicates the amount of total resistance generated during clot formation. Total area under the velocity curve during growth of the clot, representing the amount of clot strength generated during clot growth.

Table 11: Interpreting TEG® results in terms of coagulopathy.[261]

Parameter	Hypocoagulable		Hypercoagulable	
R	↑	Clots form slower	↓	Clots form faster
K	↑	Clot reaches 20 mm strength slower	↓	Clot reaches 20 mm strength quicker
A	↓	Decreased thrombin burst resulting in less fibrin fibre crosslinking	↑	Increased thrombin burst resulting in more fibrin fibre crosslinking
MA	↓	Decreased PLT and/or fibrin interaction which results in less dense, less rigid clot	↑	Increased PLT and/or fibrin interaction which results in a more dense, more rigid clot
MRTGG	↓	Decreased clot growth	↑	Increased clot growth
TMRTGG	↑	Increased time from the initiation of clot formation to maximum clot formation	↓	Decreased time from initiation of clot formation to maximum clot formation
TGG	↓	Decreased clot strength	↑	Increased clot strength

6.4 STATISTICAL ANALYSIS

All participants' TEG® parameters that were measured are presented in Table 12 and 13 for the control and patient groups respectively. The results from the statistical

analysis are summarised in Table 14. The normality test, Shapiro-Wilk test, was performed. For non-parametric data the Mann-Whitney Wilcoxon two-sided statistical test was performed for the analysis of all eight parameters. The data is presented as a median with minimum and maximum values.

6.5 RESULTS

The results obtained from the TEG® analyses for the control and patient groups are tabulated in Tables 12 and 13 respectively. These results show the TEG® parameters that make up the coagulation profiles of each individual.

Table 12: TEG® raw data obtained from the TEG® analyses of the control group.

Control	TEG® results							
	R	K	A	MA	G	MRTGG	TMRTGG	TGG
C1	13,80	3,30	55,90	67,50	10,40	4,24	21,17	1061,26
C2	13,30	4,20	50,70	66,50	9,90	3,99	19,08	1011,00
C3	9,60	5,20	49,60	49,50	4,90	2,39	15,58	497,76
C4	9,80	4,80	52,20	65,00	9,30	2,94	19,33	944,76
C5	10,40	6,80	45,00	55,00	6,10	1,82	19,50	622,15
C6	11,20	4,20	51,40	68,50	10,90	4,58	17,67	1109,86
C7	9,00	2,80	61,90	65,50	9,50	4,24	14,00	958,54
C8	8,30	3,10	60,20	54,50	6,00	3,62	13,33	598,74
C9	9,90	4,90	50,80	55,50	6,20	2,43	15,42	626,48
C10	8,50	3,20	57,90	67,50	10,40	4,41	14,58	1048,67
C11	8,90	3,10	59,90	57,00	6,60	5,42	12,08	664,52
C12	13,20	2,30	57,90	61,60	8,00	6,15	16,08	802,89
C13	9,80	2,60	54,00	64,60	9,10	6,84	13,75	934,31
C14	11,80	3,70	43,80	59,40	7,30	4,14	16,17	734,62
C15	16,20	5,70	35,60	58,70	7,10	3,07	23,42	717,05
C16	12,00	2,40	58,20	62,70	8,40	5,69	15,17	844,27
C17	5,30	1,60	64,70	63,40	8,60	7,69	7,33	867,8
C18	9,90	3,20	47,30	52,20	5,50	3,87	12,83	552,9
C19	13,20	4,10	41,70	61,00	7,80	3,33	19,42	786,58
C20	11,80	3,70	25,70	50,10	5,00	3,50	14,75	503,01
C21	7,20	3,30	33,30	56,80	6,60	3,58	9,08	660,15
C22	11,40	4,80	50,80	69,00	11,10	2,52	15,42	1135,40
C23	7,90	6,10	40,50	63,50	8,70	4,44	17,92	884,71
C24	6,10	2,80	63,40	51,50	5,30	3,75	9,83	533,35

C25	11,80	3,60	54,50	59,50	7,40	4,32	16,58	732,93
C26	21,50	7,50	40,00	45,50	4,20	1,88	29,67	422,86
C27	15,00	5,80	42,70	63,10	8,50	3,91	23,80	871,26
C28	12,20	3,60	55,60	62,00	8,20	4,65	16,33	825,28
C29	14,10	2,80	61,50	62,00	8,20	5,50	17,50	821,39
C30	18,20	5,80	43,20	59,00	7,20	3,33	27,08	727.44
C31	23,30	7,00	48,10	50,50	5,10	2,19	26,75	512,76
C32	13,90	3,00	60,80	54,00	5,90	4,33	17,58	596,02
C33	16,60	9,20	40,00	46,00	4,30	1,49	27,58	433,31
C34	11,60	2,40	66,30	68,00	10,60	13,01	14,50	1060,80
C35	8,80	2,20	71,20	73,30	13,70	11,38	12,70	1254,6

R: reaction time; K: kinetics; A: angle; MA: maximum amplitude; G: shear elastic modulus strength; MRTGG: maximum rate of thrombus generation; TMRTGG: time to maximum rate of thrombus generation; TGG: total thrombus generation.

Table 13: TEG® raw data obtained from the TEG® analyses of the patient group.

Patient	**TEG® results**							
	R	**K**	**A**	**MA**	**G**	**MRTGG**	**TMRTGG**	**TGG**
P01	5,00	1,40	53,50	70,20	11,80	9,51	7,08	1183,91
P02	8,20	2,80	53,80	51,60	5,30	4,09	10,33	535,68
P03	6,60	1,80	48,40	70,70	12,10	8,08	9,92	1215,70
P04	4,70	1,60	68,50	73,60	13,90	10,53	7,67	1402,19
P05	7,80	1,60	66,70	60,70	7,70	7,28	9,25	773,10
P06	9,70	2,20	54,20	65,00	9,30	6,32	12,5	931,00
P07	10,30	2,80	49.00	57,90	6,90	5,88	18,08	690,90
P08	5,80	1,90	62,00	58,20	6,90	5,64	7,08	702,90
P09	3,30	8,80	39,70	29,30	2,10	1,87	3,92	206,93
P11	20,10	5,80	40,40	74,10	14,30	3,68	24,00	912,63
P12	11,10	2,60	53,30	66,60	10,00	6,59	15,33	1001,88
P13	15,80	5,80	33,80	61,20	7,90	2,97	24,17	792,97
P14	19,90	5,20	38,00	59,00	7,20	3,48	28,25	721,24
P15	13,20	7,20	28,60	60,00	7,50	2,68	21,50	752,76
P16	14,20	3,20	50,80	60,90	7,80	5,33	18,83	779,28

P17	7,70	2,10	61,80	72,50	13,20	7,32	11,58	1325,06
P18	6,80	1,60	57,90	68,00	10,60	8,59	9,33	1066,80
P19	7,80	1,90	62,80	70,40	11,90	7,81	6,70	1191,60
P20	5.80	2.60	41.30	68.70	11.00	5.21	9.83	1106.10
P21	3,20	1,20	72,80	67,50	-	9,16	4,83	1045,80
P22	13.20	3,90	47,90	67,30	10,30	6,46	20,25	1033,10
P23	8,80	4,20	36,20	67,80	-	2.56	24,00	623,01
P24	15,60	6,00	30,40	55,40	-	4,09	16,08	1059,77
P25	12,00	5,70	32,10	52,60	-	2,31	17,92	557,35

R: reaction time; K: kinetics; A: angle; MA: maximum amplitude; G: shear elastic modulus strength; MRTGG: maximum rate of thrombus generation; TMRTGG: time to maximum rate of thrombus generation; TGG: total thrombus generation.

Table 14: The TEG® data comparing the summary statistics of the control and patient groups. The median, minimum value, maximum value, as well as P-value is showed. A p-value of ≤0.05 indicates significance.

Parameter	Median (min; max)		P-value
	Control group n = 35	Patient group n =25	
R	11.60 (5.30;23.30)	8.00 (3.20;20.10)	0.01 *
K	3.60 (1.60;9.20)	2.60 (1.20;8.80)	0.02 *
Ang	51.40 (25.70;71.20)	49.90 (28.60;72.80)	0.58
MA	61.00 (45.50;73.30)	66.10 (29.30;74.10)	0.02 *
G	7.80 (4.20;13.70)	9.50 (2.10;14.30)	0.03 *
MRTGG	3.99 (1.49;13.01)	5.86 (1.87;10.53)	0.01 *
TMRTGG	16.17 (7.33;29.67)	10.96 (3.92;28.25)	0.01 *
TGG	786.60 (422.90;1255.00)	945.10 (206.90;1402.00)	0.02 *

R: reaction time; K: kinetics; A: angle; MA: maximum amplitude; G: shear elastic modulus strength; MRTGG: maximum rate of thrombus generation; TMRTGG: time to maximum rate of thrombus generation; TGG: total thrombus generation; ***:** significant difference

Figures 14 and 15 show representative graphs of the control and patient groups that showcased the most representative TEG® runs of the respective groups. The graphs of the patient group showed more variation in the TEG® values than that of the control group.

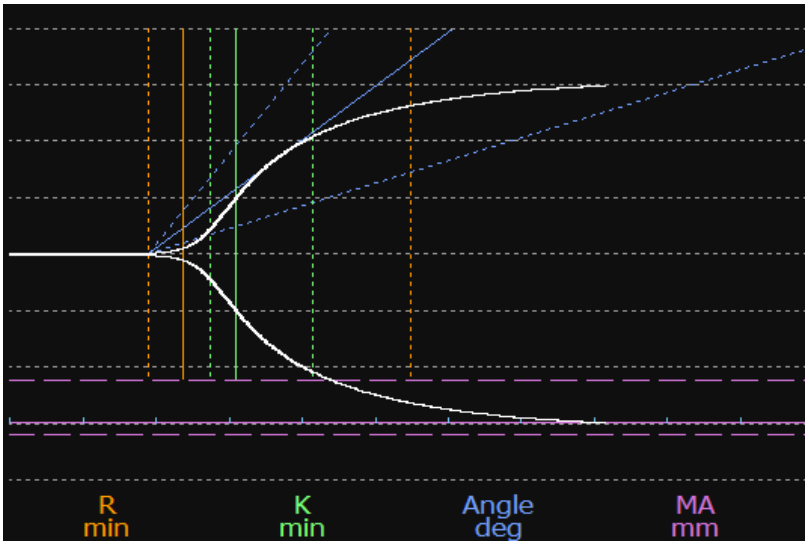


Figure 14: Representative graph of the control group TEG®. Showing all parameters within the reference ranges. R: reaction time; K: kinetics; MA: maximum amplitude.

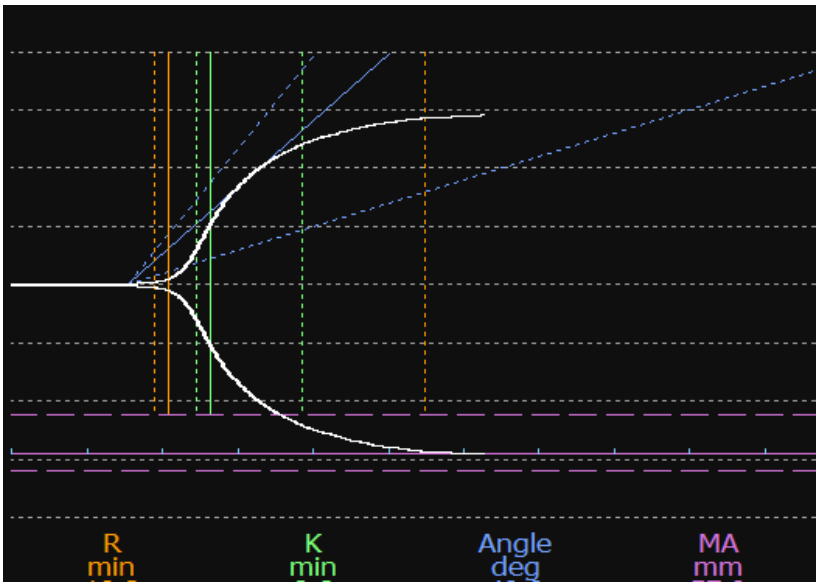


Figure 15: Representative graph of the patient group TEG®. Showing faster R and K times, normal angle, and increased MA. R: reaction time; K: kinetics; MA: maximum amplitude.

6.6 DISCUSSION

This method was chosen in the study to quantify the viscoelastic properties of clot formation in primary BCa patients. In a hypercoagulable state, there is a typical profile seen such as an increase in α-angle, MA, MRTGG, and TGG as well as a decrease in the R-time, K-time, and TMRTGG.[261] In the recorded TEG® results, there was no significant difference between the α-angle measurements of the control and patient groups. However, MA, G, MRTGG, and TGG were increased in the patient group. Additionally, R-time, K-time, and TMRTGG were decreased in the patient group. When compared to healthy reference ranges obtained from literature, most of the patient group values were still within the healthy reference ranges even though it was different from the control group values obtained in this study, indicating rather a tendency to hypercoagulate. Only the significant differences will be discussed here by using the summarised information presented in Tables 10 and 11 for the interpretation of the results.

The observed decreased R-time is indicative of faster clot formation which aligns with hypercoagulability in the patient group. An increased G value is also indicative of a hypercoagulability since the clots formed in the patient group were more rigid and therefore more resistant to change. A decreased K measurement indicates that the clot strength is reached quicker and further corresponds to hypercoagulability in the patient group. Since the MA is higher in the patient group than in the control group, it can be concluded that there is increased fibrin and/or PLT interaction in the patient group which results in more dense and rigid clots.[261] This too is consistent with hypercoagulability. This increased MA is confirmed by the SEM micrographs discussed in Chapter 5 that show increased fibre interaction, tangling, and denser areas of fibres when compared to the control group micrographs.

The MRTGG is a measure of the maximum velocity of clot growth (or maximum rate of thrombus generation), Since the MRTGG was higher in the patient group, it can be concluded that the clot growth is increased in the patient group, indicating hypercoagulability. Since TMRTGG represents the time before the maximum rate of clot growth, a decreased TMRTGG value as observed in the patient group, suggests that the maximum rate of clot growth was reached faster in the patient group. This decreased value

represents a hypercoagulable parameter. The TGG measurement is an indication of the amount of total resistance generated during clot formation, i.e., total clot strength. The TGG being higher in the patient group therefore confirms that this parameter is also consistent with hypercoagulability.

Panigada et al. used TEG® to investigate the effects of COVID-19 on the haemostasis of infected patients. The results showed that there was a significant increase in the hypercoagulability of patients that tested positive for COVID-19.[309] The TEG® results showed a specific decrease in the K values and increase in the MA values which was indicative of a faster clotting time as well as fibrin clot strength respectively.[309] This study suggests a possible reason for the hypercoagulability may be an increase in circulating MVs. These particles may stem from monocytes and PLTs that are carriers of procoagulants.[309] This is a plausible explanation because circulating MVs may trigger a hypercoagulable state, and are shown to be associated with increased VTE risk.[321]

Using the results of TEGs® on the WB of prostate cancer patients, Toukh et al. found evidence of hypercoagulability in this patient group. This finding was most significant in metastatic cancer patients. The parameters most affected by this type of cancer was the R-time, which was significantly reduced and the angle, and MA which showed significant increases when compared to the controls in this study.[322] The researchers also highlighted the TF expressing MVs and their role in coagulation initiation.[322]

The increase in MA can be supported by the changes in PLT interaction with the fibrin fibres also observed with SEM. The denser appearance of the fibres would allow for more interaction and a closer association between the fibres, resulting in a denser clot as reflected by the increased MA values. Since there was also increased PLT presence and interaction observed in the micrographs of the SEM, this further supports the likelihood that the PLTs might be more active in BCa due to the increased inflammatory state, and then interacts with fibrin, resulting in stronger, more rigid clots.[323] The decrease in K time shows that these clots are not only stronger, but also forms quite quickly. The increase in MRTGG may be due to the increased inflammatory state resulting in activation of the coagulation cascade which causes TF and FXII activation, resulting in an increase in thrombin

generation.[155, 324] This increase results in the increased MRTGG observed and contributes to the clots forming faster than in the control group.

Thrombotic events in cancer patients, specifically those that include denser, stiffer clots that are more resistant to lysis are associated with increased inflammation and oxidative stress, and mainly fibrin-related mechanisms.[325] This is due to cancer cells producing and releasing inflammatory cytokines (IL-6), prothrombic MPs, procoagulant and fibrinolytic proteins (MMPs, VEGF), all contributing to hypercoagulability which can result in thrombotic events.[326]

6.7 CONCLUSION

Based on the results obtained from the TEG®, it is evident that there is hypercoagulability present in the patient group based on seven out of the eight measured parameters matching a hypercoagulable profile. Though not all the samples of the patient group held to a hypercoagulable profile, the group as a whole did present as hypercoagulable. It thereby confirms that clot formation in these patients is changed and could hold a potential risk for the development of clotting complications in these patients. If the BCa status is combined with treatment or other procoagulant co-morbidities it could only elevate the risk to thrombosis in these patients. The increase in MA could possibly be a result of pro-clotting factors that are activated because of the increased inflammatory state. The pro-clotting factor may be fibrinogen as there was also tightly woven fibres seen in Chapter 5. Therefore, future studies could include the measurement of fibrinogen to determine if fibrinogen may be the pro-clotting factor that is responsible for the denser, faster forming clots observed through the decreased K values, increased MA values, as well as the SEM images discussed in Chapter 5.

CHAPTER SEVEN

CONCLUSION AND FUTURE DIRECTION

DISCUSSION AND CONCLUSION

Cancer has been and remains one of the most studied diseases of life. Specifically, BCa, which has maintained the attention of researchers all over the world. Brain cancer is known for having a generally poor prognosis and a high mortality rate. There is also a known association between cancer and thrombotic events. This poses a dilemma that still lacks a sure solution. To reduce and manage the number of thrombotic events that occur in cancer patients, there must be more research dedicated to informing therapeutic strategies that treats the underlying cause. Research on metastatic BCa is on the rise, however, to assess the risk that patients with primary, non-metastatic BCa face is not widely researched. Therefore, this study was aimed at investigating the haematological and clotting properties of these patients to investigate and identify the prominent factors that could contribute to the potential risk for hypercoagulability associated with primary, non-metastatic BCa. Hypercoagulability poses a serious risk for contributing to PE and/or DVT development in the lower limbs of patients[309] and should therefore be taken into consideration when treatment is established for this patient group. Figure 16 summarises the main findings of each of the research chapters (Chapters 3 - 6).

The combined chapters in this dissertation showcase an overall biophysical coagulation profile in a population of BCa patients. This was then compared to healthy controls and a number of alterations were identified in the patient group. In Chapter 4, the average axial ratio measurements of the patient group were significantly higher than that of the control group. This established that the patient group had more deformed RBCs present per sample taken compared to the control group. When the shapes of RBCs change, there is a potential risk created due to its possible impact on the viscosity and sheer stress of blood[327] moving especially within the smaller venules, arterioles, and capillaries.

The most prominent findings from the SEM analyses were the altered fibres within the WB clots. The clots showed a more tightly woven network of fibres, more fibrin folding, fusing,

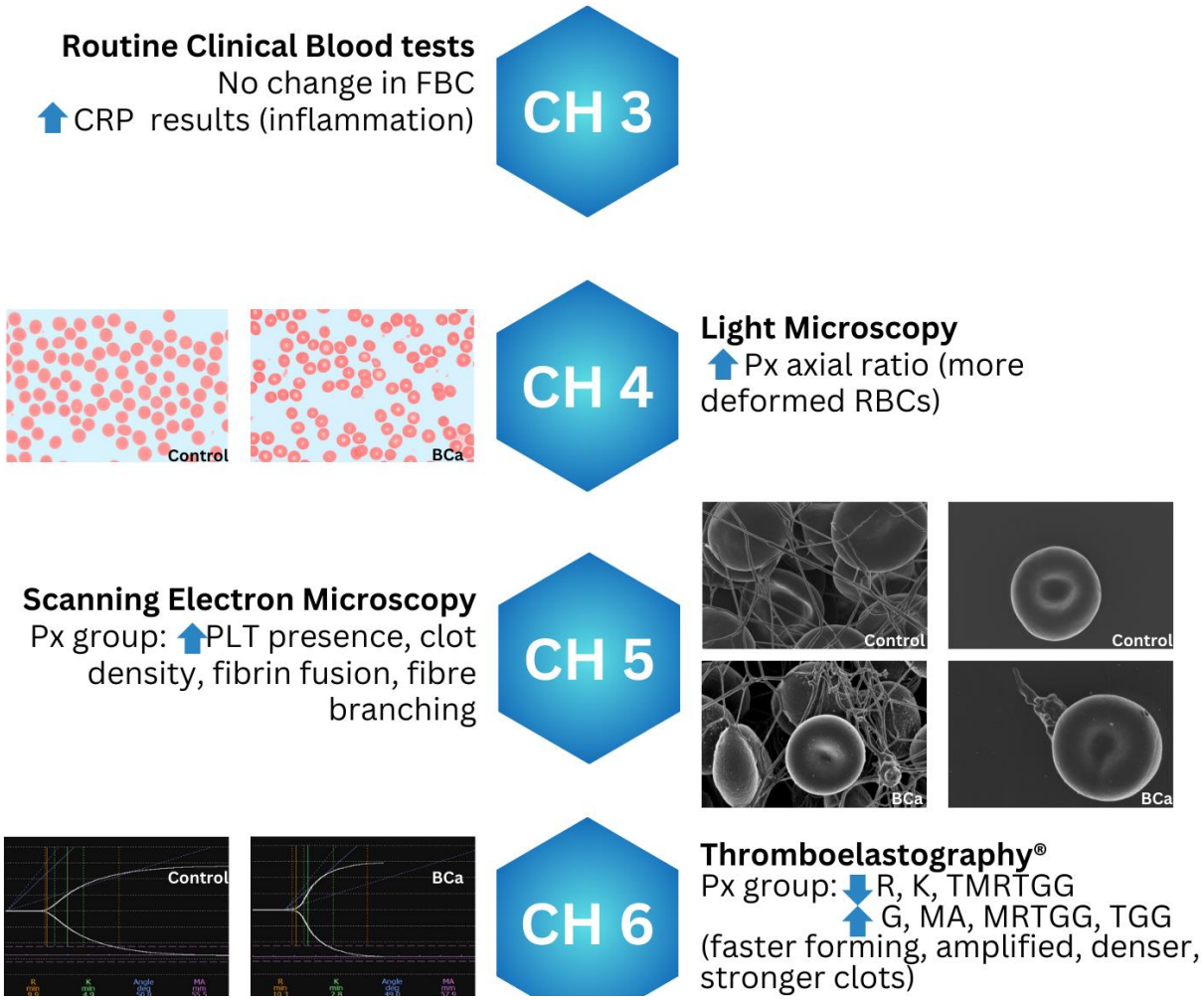


Figure 16: Brief overview of research chapters and main findings. FBC: full blood count; CRP: C-reactive protein; RBC: red blood cell; PLT: platelet; R: reaction time; K: kinetics; TMRTGG: Time to the maximum rate of thrombus generation; G: shear elastic modulus strength; MA: maximum amplitude; MRTGG: maximum rate of thrombus generation; TGG: total thrombus generation; Px: patient.

and cross-linking. These changes in the fibres of the BCa patient group were confirmed with the findings of the TEG®. Specifically related to the increased MA values which indicates increased clot rigidity and strength. Even though there was not any clinical presentation of these changes, it must be noted that this patient group did present with a changed clotting profile that may still have adverse subclinical effects for the patient long-term.

The SEM images revealed notable alterations in the fibres of the BCa group, suggesting a potential formation of micro clots. While these micro clots may not pose the same level of fatality as a thromboembolism, they have the capacity to obstruct microvasculature, thereby contributing to reduced tissue perfusion. Denser networks of fibrin strands are associated with resistance to clot lysis.[328, 329] Common issues that this could lead to would be decreased tissue perfusion, prolonged healing time for wounds, vascular injury, or gradual progression of organ damage.[328, 329]

Taking these findings into consideration, it can be concluded that there is a trend of hypercoagulability associated with this patient group. Furthermore, this presence/potential of hypercoagulability can be augmented when combined with other inflammatory conditions such as high blood pressure, smoking, diabetes, if the cancer becomes metastatic or when patients are put on different treatment plans. This is because these conditions can all have adverse effects on the coagulation system. Diabetic patients often present with a prothrombotic state due to PLT hypersensitivity and hypofibrinolysis.[330] High blood pressure increases coagulation by decreasing fibrinolysis.[331] Since hypertension and inflammation is also proven to be interlinked,[332] the increase in TF expression, decrease in fibrinolytic activity, [333] and PARs-mediated coagulation[98] as seen in inflammatory conditions can also influence and enhance these effects when paired with high blood pressure.

Smoking exacerbates the effects of high blood pressure on coagulation. Sivagangailakshmi et al. showed that smoking alters coagulation profiles by influencing prothrombin time, higher fibrinogen levels, and PLT count. Furthermore, chronic smoking increases PLT aggregability,[334] elevated FXIII and TF pathway inhibitor.[335] Therefore, by combining a condition like this with primary, non-metastatic BCa or if the cancer should become metastatic, it is expected that coagulation will be upregulated. This of course can have detrimental effects that stem from thrombotic events.

Ultimately, this study provided valuable insights into clotting tendencies in this patient group, and it should be emphasised that patients at risk should be monitored and that a treatment targeting fibrin should be considered.

LIMITATIONS

With 25 patients in total, this study has a relatively low sample size. This was mainly due to the strict exclusion criteria to limit any confounding factors. A small sample size may have an effect on the statistical power of the obtained results and possibly influence significance. The age range for this study was wide in order to include as many patients as possible. This led to a significant difference between the average ages of the control group compared to the patient group ($p<0.0001$). Since convenient sampling was used to recruit all the patients, the ages were dependent on the patients admitted during the recruitment period. The difference in age was not excluded as a confounding factor, however age has not been showed to contribute to major changes to blood cells.

FUTURE STUDIES

Future studies could include a larger number of patients. For the purpose of identifying the possible contributors to the observed increase in certain TEG® parameters, future studies could also focus on measuring the levels of fibrinogen, PLT activation, and/or thrombin generation. In doing so, isolating the procoagulants responsible for increasing the risk of hypercoagulation. In addition, AFM can be used to study RBC rigidity as well as measuring fibrin branching and thickness to investigate the presence of any changes to the formed fibres. This can direct the development of management strategies or targeted anticoagulation therapies. Future avenues of study on this topic might also include only metastatic BCa patients since some studies suggest that metastases might have a greater effect on coagulation as discussed in this dissertation.

The effect of different treatment options may also be worthwhile to explore in order to establish any trends or changes in haematological and clotting properties. While this study only focused on the three most common CNS tumours to limit confounders, future research could also explore the effects of different types of CNS cancers and/lesions. Future research might also include the further subdivision of results based on the age of patients and controls to establish age-related trends as well as patients that have started treatment

plans or patients with comorbidities. C-reactive protein as a biomarker of inflammation can be general, it may therefore be worthwhile exploring other biomarkers that are particularly linked to cancer. Soluble urokinase plasminogen activator (suPAR) is a biomarker that is showing to be promising correlation with inflammation and needs further investigation on its role in oncology.

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APPENDIX A: ETHICS APPROVAL

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

4 July 2022

Approval Certificate
New Application

Dear Miss ZJ Seyfert

Ethics Reference No.: 232/2022
Title: Investigating the hypercoagulability in brain cancer ptients by studying the viscoelastic and ultrastructural properties of whole blood

The **New Application** as supported by documents received between 2022-04-26 and 2022-06-29 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-06-29 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-07-04.
- Please remember to use your protocol number (232/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

20 June 2023

Approval Certificate
Annual Renewal

Dear Miss ZJ Seyfert,

Ethics Reference No.: 232/2022 – Line 1
Title: Investigating the hypercoagulability in brain cancer ptients by studying the viscoelastic and ultrastructural properties of whole blood

The **Annual Renewal** as supported by documents received between 2023-05-29 and 2023-06-14 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-06-14 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2024-06-20.
- Please remember to use your protocol number (232/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Professor C Kotzé
MBChB, DMH, MMed(Psych), FCPsych, Phd
***Acting Chairperson**: Faculty of Health Sciences Research Ethics Committee*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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APPENDIX B: NHRD APPROVAL

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

STEVE BIKO ACADEMIC HOSPITAL

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For attention: janette Bester

NHRD Ref Number: GP_202207_019

Re: REQUEST FOR PERMISION TO CONDUCT RESEARCH AT STEVE BIKO ACADEMIC HOSPITAL

TITLE: Investigating hypercoagulability in brain cancer patients by studying the viscoelastic and ultrastructural properties of whole blood

Permission is hereby granted for the above-mentioned research to be conducted at Steve Biko Academic Hospital. This is done in accordance to the "Promotion of access to information act No 2 of 2000".

Please note that in addition to receiving approval from Hospital Research Committee, the researcher is expected to seek permission from all relevant department. Furthermore, collection of data and consent for participation remain the responsibility of the researcher.

The hospital will not incur extra cost as a result of the research being conducted within the hospital.

You are also required to submit your final report or summary of your findings and recommendations to the office of the CEO.

STATUS OF APPLICATION: Approvec

Date: 2022-07-26

Dr. J S. Mangwane
Manager: Medical Service

APPENDIX C: CONTROL INFORMED CONSENT

ICD 2 c

HEALTHY PARTICIPANT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE: *Investigating the viscoelastic and ultrastructural properties of whole blood in brain cancer patients*

Principal Investigator: Zenobia Jacomine Seyfert

Supervisor: Prof Janette Bester

Institution: University of Pretoria

Daytime telephone number: 067 388 5225

DATE AND TIME OF INFORMED CONSENT DISCUSSION:

				:
dd	**month**	**year**		**Time**

Dear Prospective Research Participant

Dear Mr / Ms / Mrs ……

1) INTRODUCTION

You are invited to volunteer for a laboratory-based research study conducted by the Department of Physiology (School of Medicine, Faculty of Health Science). I am doing this research for Master's degree purposes at the University of Pretoria. The information in this document is provided to help you to decide if you would like to participate. Before you agree to take part in this study, you should fully understand what is involved. If you have any questions, which are not fully explained in this document, do not hesitate to ask the researcher. You should not agree to take part unless you are completely happy with the kind of questions that will be asked. It is advised that you inform your personal doctor of your participation in this study.

2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of this study is to study the coagulation changes in brain cancer that might indicate thrombotic risk. By doing so we wish to learn more about the clotting behavior of the blood of brain cancer patients which and how that might affect the risk of having blood clots block arteries and/or veins. This will allow us to understand if brain cancer patients in South Africa have changed blood clotting properties. To accomplish this, we will be using specialized microscopes (scanning electron microscope and light microscope) to examine the structure of red blood cells, and platelets. We will also be using equipment that tests the blood clotting properties (thromboelastography and enzyme-linked immunosorbent assay) to determine the degree to which clotting is changed in the blood. These analyses will be done on the blood of brain cancer patients and compared to that of individuals without brain cancer, such as yourself should you decide to participate.

3) EXPLANATION OF PROCEDURES AND WHAT WILL BE EXPECTED FROM PARTICIPANTS

This study involves answering some questions regarding your health and any illnesses, examination of yourself, weight and height measurements, and taking some blood samples. One tube of blood will be drawn by a qualified nurse or phlebotomist into a citrate tube, each containing 5 ml of blood (or the equivalent of one teaspoon).

Within four hours of collection, the samples will be analysed and prepared for thromboelastography, light microscopy, and scanning electron microscopy. The blood will then be frozen short term at -80 ºC until all the sample recruitment is completed. The samples will also then be used for enzyme-linked immunosorbent assay (cotinine levels).

The blood collection process will only occur once, and no follow-up tests will be required.

4) ADDITIONAL USES FOR SAMPLES

The samples that you give to this study principal investigator could one day lead to discoveries using methods and tests not included in this protocol. This may include studying epigenetic changes using quantitative polymerase chain reaction (qPCR) and/or metabolomics using nuclear magnetic resonance (NMR) spectroscopy. This will be used to further study the clotting changes in brain cancer patients compared to healthy individuals in order to identify any abnormalities. These tests may only be identified after the results from this study have been obtained. To that end, we would like to keep the samples for as long as they are deemed useful for research purposes. This research could potentially be used for purposes not specified above for up to five years of collection. However, you may specify a shorter period of time for the study principal investigator to keep the samples.

You have the right to withdraw your consent at any time and may request that the samples you give to the study principal investigator be destroyed. If you choose to do so, contact your study doctor, Zenobia Seyfert, at 067 388 5225. Although you are free to withdraw your consent, it is possible the samples may have already been used for research purposes and data derived from such research will not be destroyed. In that event, the study principal investigator will promptly destroy any remaining samples. Ethical approval will be obtained before any further testing on the residual samples.

We would like you to complete a questionnaire. It will take approximately five minutes. We will collect the questionnaire from you before you leave the ward/ study site. We will be available to help you with the questionnaire. The researcher will keep the completed questionnaires in a safe place to make sure that only people working on the study will have access to it. Please do not write your name on the questionnaire. This will ensure that your answers are kept confidential (so nobody will know what you have answered).

The questionnaire consists of three parts:

Part 1: Personal information (age and gender

Part 2: Medical information (smoking and drinking habits, chronic medications, HIV status)

Part 3: Medical history (chronic medication, contraception, medication/supplements)

5) RISK AND DISCOMFORT

The only possible risk and discomfort involved in the blood collection from a vein which could result in bruising, bleeding, and less common infection. For your protection, this procedure will be done under sterile conditions by a medical doctor .

6) POSSIBLE BENEFITS OF THIS STUDY

Although you may not benefit directly, the study results may help us to improve the understanding of brain cancer related complications in the future. Many of these tests are routine, and we will be able to treat you, should you have any problems.

7) ETHICS APPROVAL

This Protocol was submitted (232/2022) to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, Medical Campus, Tswelopele Building, Level 4-59, Telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving humans. A copy of the Declaration may be obtained from the investigator should you wish to review it.

8) INFORMATION

If you have any questions concerning this study, you may contact Miss Zenobia Seyfert. Cell: 0673885225.

9) CONFIDENTIALITY

All records from this study will be regarded as confidential. All participants partaking in this study will be provided an alphanumeric number, e.g. A01. This will ensure the confidentiality of information collected. All results will be published or presented in such a way that it is not possible to identify the participants. The hard copies of all your records will be kept in a locked facility at the Department of Physiology at the University of Pretoria.

10) YOUR RIGHTS AS A RESEARCH PARTICIPANT

Your participation in this study is entirely voluntary and you may refuse to participate or stop at any time without stating a reason. Your withdrawal will not affect the way you will be treated.

11) COMPENSATION

You will not be paid to take part in the study. You will also carry no cost for partaking in this study.

12) CONSENT TO PARTICIPATE IN THIS STUDY

- I confirm that the person requesting my consent to take part in this study has told me about the nature and process, any risks or discomforts, and the benefits of the study.
- I have also received, read and understood the above written information about the study.
- I have had adequate time to ask questions and I have no objections to participate in this study.
- I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results.
- I understand that I will not be penalised in any way should I wish to discontinue with the study and my withdrawal will not affect my employment or student status.
- I am participating willingly.

- I have received a signed copy of this informed consent agreement.

_____ _____

Participant's name (Please print) Date

_____ _____

Participant's signature Date

_____ _____

Researcher's name (Please print) Date

_____ _____

Researcher's signature Date

Participant Code: _____

AFFIRMATION OF INFORMED CONSENT BY AN ILLITERATE PARTICIPANT

(if suitable)

I, the undersigned, _____ have read and have explained fully to the participant, named _____, the participant informed consent document, which describes the nature and purpose of the study in which I have asked the participant to participate. The explanation I have given has mentioned both the possible risks and benefits of the study. The participant indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her standard care.

I hereby certify that the participant has agreed to participate in this study.

_____ _____

Participant's name (Please print) Date

_____ _____

Participant's Signature or Mark Date

_____ _____

Investigator's Name (Please print) Date

_____ _____

Investigator's Signature Date

_____ _____

Name of the person who witnessed the

informed consent (Please print) Date

_____ _____

Signature of the Witness Date

Participant Code: _____

APPENDIX D: CONTROL GROUP QUESTIONNAIRE

Date Captured (*dd/mm/yyyy*):	Allocated study ID (e.g., A01) (*Will be allocated by the investigator*)

PERSONAL INFORMATION

Sex: ◯ male ◯ female ◯ prefer not to say | Age:

MEDICAL INFORMATION AND HISTORY

Do you smoke tobacco or any related product? If yes, for how long?	○ NO ○ YES _____
In the past 30 days, have you consumed five/more alcoholic drinks on the same occasion?	○ NO ○ YES

Do you have or have you had any of the following conditions?

- ○ Diabetes
- ○ High blood pressure
- ○ Heart problems
- ○ Stroke
- ○ Arthritis
- ○ Heart attacks
- ○ Inflammatory conditions
- ○ Allergies Please Specify: _____
- ○ Other Please specify: _____

HIV Status:	◯ POSITIVE	◯ NEGATIVE	◯ UNKNOWN

MEDICAL HISTORY

Are you taking any chronic medication? If yes, please specify.	○ NO ○ YES _____
Are you taking contraceptives?	○ NO ○ YES _____

Have you taken any of the following within the last two weeks?

- ○ Vitamin supplements
- ○ Corticosteroids
- ○ Anti-inflammatories
- ○ Anti-coagulative
- ○ Other Please specify: _____

APPENDIX E: PATIENT INFORMED CONSENT

ICD 2 c

PATIENT'S INFORMATION & INFORMED CONSENT DOCUMENT

STUDY TITLE: *Investigating the viscoelastic and ultrastructural properties of whole blood in brain cancer patients*

Principal Investigator: Zenobia Jacomine Seyfert

Supervisor: Prof Janette Bester

Institution: University of Pretoria

Daytime telephone number: 067 388 5225

DATE AND TIME OF INFORMED CONSENT DISCUSSION:

				:
dd	**month**	**year**		**Time**

Dear Prospective Research Participant

Dear Mr / Ms / Mrs …….

1) INTRODUCTION

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2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of this study is to study the coagulation changes in neurosurgical conditions (such as brain cancer) that might indicate thrombotic risk. By doing so we wish to learn more about the clotting behavior of the blood of these patients and how that might affect the risk of having blood clots block arteries and/or veins. This will allow us to understand if these patients in South Africa have changed blood clotting properties. To accomplish this, we will be using specialized microscopes (scanning electron microscope and light microscope) to examine the structure of red blood cells, and platelets. We will also be using equipment that tests the blood clotting properties (thromboelastography) to determine the degree to which clotting is changed in the blood. These analyses will be done on the blood of patients with neurosurgical conditions and compared to that of healthy individuals.

3) EXPLANATION OF PROCEDURES AND WHAT WILL BE EXPECTED FROM PARTICIPANTS

This study involves answering some questions regarding your health and any illnesses, examination of yourself, and taking some blood samples. One tube of blood will be drawn by a qualified nurse or phlebotomist into a citrate tube, each containing 5 ml of blood (or the equivalent of one teaspoon).

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(qPCR) and/or metabolomics using nuclear magnetic resonance (NMR) spectroscopy. This will be used to further study the clotting changes in patients with neurosurgical conditions compared to healthy individuals in order to identify any abnormalities. These tests may only be identified after the results from this study have been obtained. To that end, we would like to keep the samples for as long as they are deemed useful for research purposes. This research could potentially be used for purposes not specified above for up to five years of collection. However, you may specify a shorter period of time for the study principal investigator to keep the samples.

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Although you may not benefit directly, the study results may help us to improve the understanding of neurosurgical conditions (such as brain cancer) related complications in the future. Many of these tests are routine, and we will be able to treat you, should you have any problems.

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Your participation in this study is entirely voluntary and you may refuse to participate or stop at any time without stating a reason. Your withdrawal will not affect the way you will be treated.

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- I have also received, read and understood the above written information about the study.
- I have had adequate time to ask questions and I have no objections to participate in this study.
- I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results.
- I understand that I will not be penalised in any way should I wish to discontinue with the study and my withdrawal will not affect my employment or student status.
- I am participating willingly.
- I have received a signed copy of this informed consent agreement.

_____ _____

Participant's name (Please print) Date

_____ _____

Participant's signature Date

_____ _____

Researcher's name (Please print) Date

_____ _____

Researcher's signature Date

Participant Code: _____

AFFIRMATION OF INFORMED CONSENT BY AN ILLITERATE PARTICIPANT

(if suitable)

I, the undersigned, _____ have read and have explained fully to the participant, named _____, the participant informed consent document, which describes the nature and purpose of the study in which I have asked the participant to participate. The explanation I have given has mentioned both the possible risks and benefits of the study. The participant indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her standard care.

I hereby certify that the participant has agreed to participate in this study.

_____ _____

Participant's name (Please print) Date

_____ _____

Participant's Signature or Mark Date

_____ _____

Investigator's Name (Please print) Date

_____	_____
Investigator's Signature	Date
_____	_____
Name of the person who witnessed the informed consent (Please print)	Date
_____	_____
Signature of the Witness	Date

Participant Code: _____

APPENDIX F: PATIENT GROUP QUESTIONNAIRE

Date Captured (*dd/mm/yyyy*):	Allocated study ID (e.g., A01) (*Will be allocated by the investigator*)

PERSONAL INFORMATION	
Hospital Number:	Diagnosis:
Date of Birth (*dd/mm/yyyy*):	Age:
Sex: ◯ male ◯ female ◯ prefer not to say	

MEDICAL INFORMATION AND HISTORY	
Do you smoke tobacco or any related product? If yes, for how long?	○ NO ○ YES _____
In the past 30 days, have you consumed five/more alcoholic drinks on the same occasion?	○ NO ○ YES

Do you have or have you had any of the following conditions?

- ○ Diabetes
- ○ High blood pressure
- ○ Heart problems
- ○ Stroke
- ○ Arthritis
- ○ Heart attacks
- ○ Inflammatory conditions
- ○ Allergies Please Specify: _____
- ○ Other Please specify: _____

HIV Status:	◯ POSITIVE ◯ NEGATIVE ◯ UNKNOWN

MEDICAL HISTORY	
Are you taking any chronic medication? If yes, please specify.	○ NO ○ YES _____
Are you taking contraceptives?	○ NO ○ YES _____

Have you taken any of the following within the last two weeks?

- ○ Vitamin supplements
- ○ Corticosteroids
- ○ Anti-inflammatories
- ○ Anti-coagulative
- ○ Other Please specify: _____

APPENDIX G: REAGENTS AND EQUIPMENT USED FOR LM, SEM, AND TEG®.

Item	Supplier
Light microscopy	
Zeiss Axio Imager.M2 use	Zeiss Laboratory for Microscopy and Microanalysis, University of Pretoria
Microscope slides	Labocare
Eppendorf single channel Pipette (2-20 µl)	Labcal Solutions cc
Pipette tips (2-20 µl)	Labocare
Methanol	Sigma Aldrich
Mayer's haematoxylin stain	Sigma Aldrich
Eosin stain	Sigma Aldrich
Entellan mounting solution	Sigma Aldrich
Microscope cover glasses (22x40 mm)	Paul Marienfield GmbH & Co. KG
Scanning electron microscopy	
Zeiss ULTRA Plus FEG-SEM	Zeiss Laboratory for Microscopy and Microanalysis, University of Pretoria
Microscope cover glasses (10 mm)	Lasec
Lyophilized human thrombin	South African National Blood Service
Eppendorf single channel pipette (2-20 µl)	Lasec
Pipette tips (2-20 µl)	Lasec
Phosphate buffered saline (0.01 m)	Sigma Aldrich
Formaldehyde (4%)	Sigma Aldrich
Glass pasteur pipettes	Lasec
Osmium tetroxide (≥99%)	Sigma Aldrich
Ethanol	Sigma Aldrich
Hexamethyldisilane	Sigma Aldrich
Well cell culture plate	Greiner bio-one
Aluminium stubs	Advanced laboratory solutions
Carbon tape	Advanced laboratory solutions
Quorum q150t es carbon coater	Quorum Technologies Ltd. Laboratory for Microscopy and Microanalysis, University of Pretoria
Thromboelastography®	
Haemoscope TEG® analyser	Haemoscope Corporation
Eppendorf single channel pipette (100–1000 µL)	Lasec
Pipette tips (100–1000 µL)	Lasec
TEG® cups	Haemonetics Corporation
Calcium chloride (0.2 M)	Haemonetics Corporation

APPENDIX H: TURNITIN PLAGIARISM REPORT

ORIGINALITY REPORT

10%	6%	7%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to University of Pretoria Student Paper	3%
2	repository.up.ac.za Internet Source	1%
3	"Posters", Journal of Thrombosis and Haemostasis, 07/2009 Publication	<1%
4	atuder.org.tr Internet Source	<1%
5	zombiedoc.com Internet Source	<1%
6	Trauma Induced Coagulopathy, 2016. Publication	<1%
7	"ECR 2019: Book of Abstracts", Insights into Imaging, 2019 Publication	<1%
8	dergipark.org.tr Internet Source	<1%
9	www.hindawi.com	