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An *ex vivo* study on the hypercoagulability of brain cancer patients at an academic hospital by studying the morphological and viscoelastic properties of platelet-poor plasma

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ABSTRACT

Cancer and its associated outcomes rank among the primary contributors to global mortality. Tumour cells are not only able to alter their cellular physiology to promote their own growth but can also disrupt the coagulation system by interfering with natural signals and pathways in the body, particularly the inflammatory and coagulation pathways. As a result, about 20% of brain cancer patients suffer complications associated with coagulopathies.

Extensive research is required to understand the coagulation potential in brain cancer patients and identify the factors that trigger hypercoagulation in these patients. This study aimed to investigate the potential hypercoagulable state in brain cancer patients at an academic hospital, comparing them to healthy individuals by studying the morphological and viscoelastic properties of platelet-poor plasma (PPP), specifically focusing on fibrin formation.

This study measured the viscoelastic properties of PPP during clot formation using Thromboelastography®. Additionally, scanning electron microscopy was employed to analyse and compare the clot ultrastructural morphology of fibrin networks between patients and healthy individuals. The fibrin fibre thicknesses of both groups were then measured and compared using the ImageJ software. Branching of the fibrin fibres was measured by determining the fractal dimensions from the scanning electron microscope images with Fractalyse software.

Clinical tests—including the international normalised ratio (INR), C-reactive protein (CRP), and procalcitonin tests (PCT)—were obtained from patient records and used to create a clinical profile of the patient population. These values were compared to healthy reference ranges to identify any potential abnormalities in the patient group. The INR was used to determine the time it took for the patients' blood to clot, whereas the CRP and PCT were used to evaluate their inflammatory status.

The clinical tests showed normal INR and PCT values but elevated CRP values when compared to normal ranges. This indicated that the brain cancer patients in this study exhibited normal clotting times and no signs of bacterial infection. The elevated CRP values could be indicative of elevated inflammation caused by the brain cancer.

The viscoelastic and ultrastructural results showed that there were no significant differences in any of the analyses between the healthy individuals and those with brain cancer, except with regards to the fibre thickness. Specifically, this study found that brain cancer patients have thinner fibrin fibres than healthy individuals. Thinner fibres exhibit a reduced rate of dissolution compared to thicker fibres, resulting in the persistence of clots and, consequently, increasing the patient's susceptibility to thrombotic events.

The results from this study open avenues to further study the impact of brain cancer on the formation of fibrin fibres during clot formation. While the tests used in this study might not have been sensitive enough to identify subclinical changes, future tests measuring fibrinogen levels, coagulation factors, and clot lysis could provide valuable insights into how coagulation is affected in these patients. Such insights might reveal potential targets for more effective patient management.

Keywords: Brain cancer; Coagulation; Thromboelastography®; Scanning electron microscopy; ImageJ software

DECLARATION

I, Lilu Rademeyer, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University.

Signed: ..... 

Date: 17 April 2024.....

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LIST OF ABBREVIATIONS

α	Alpha angle
BMI	Body mass index
C/EBPβ	CCAAT/enhancer-binding protein beta
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
G	Elastic modulus strength
HMDS	Hexamethyldisilazane
IL-1β/ R	Interleukin-1β/ receptor
IL-4	Interleukin-4
IL-6/ R	Interleukin-6/ receptor
IL-8	Interleukin-8
IL-10	Interleukin-10
INR	Internationalised normalised ratio
K	Kinetics
MIF	Macrophage Migration Inhibitory Factor
MA	Maximal amplitude
MRTG	Maximum rate of thrombus generation
NFκB	NF-kappaB
OD	Optical density
OsO_4	Osmium tetroxide
PBS	Phosphate buffered saline
PCT	Procalcitonin test
PPP	Platelet-poor plasma
PS	Phosphatidylserine
R	Reaction time
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SBAH	Steve Biko Academic Hospital
STAT3	Signal transducer and activator of transcription 3
SP	Streptavidin-peroxidase

TEG	Thromboelastography
TF	Tissue factor
TMRTG	Time to the maximum rate of thrombus generation
TTG	Total thrombus generation
TGF-β	Transforming Growth Factor-β
TNF-α	Tumour necrosis factor-alpha

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Dissertation layout

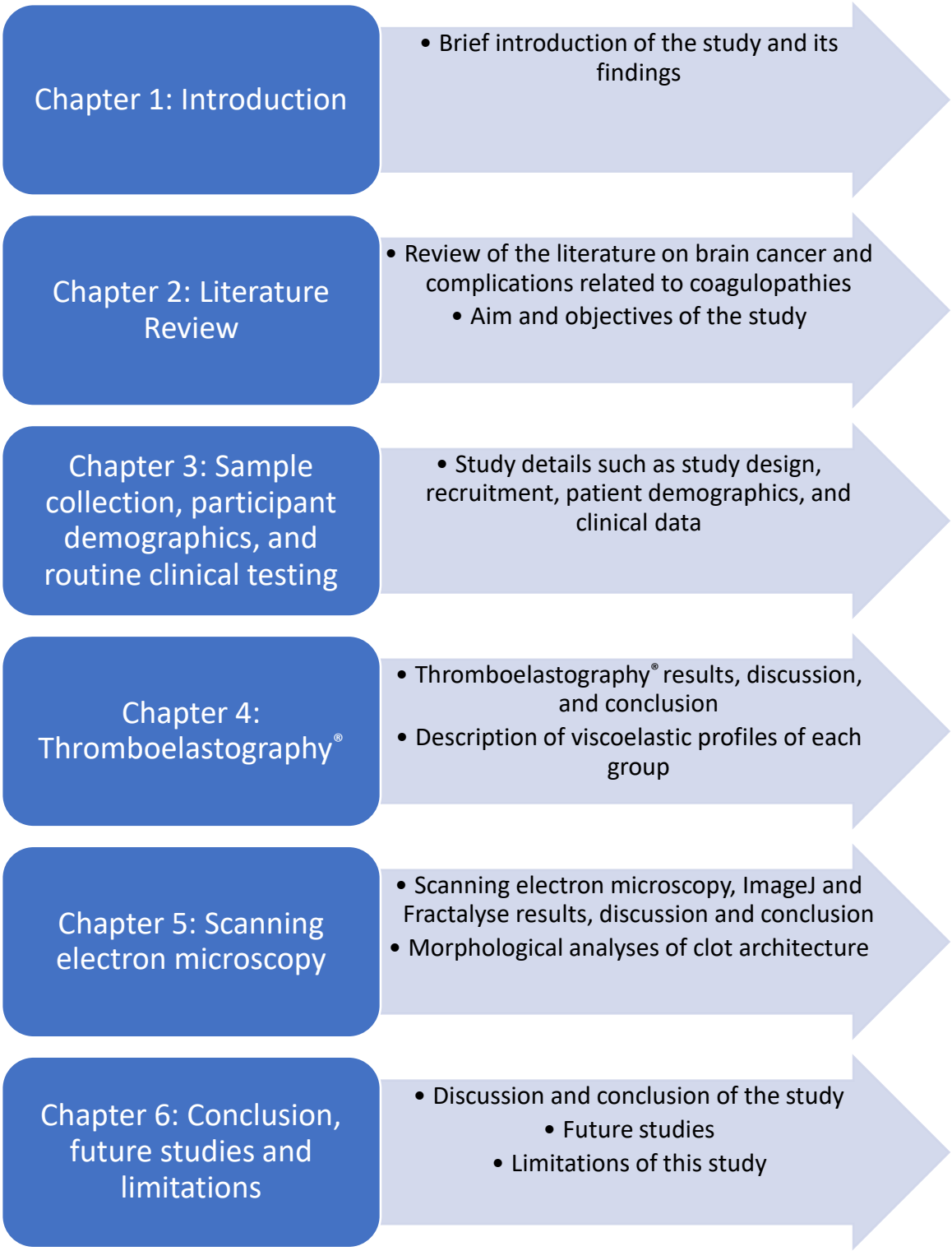

Figure 1. An overview summarizing the contents of this dissertation
A brief description of each chapter illustrates what is to be expected in the dissertation.

Chapter 1: Introduction

Coagulation, a common physiological mechanism aimed at minimizing blood loss, utilises the formation of clots to restrict blood flow at injury sites.[1] Brain cancer patients often experience coagulation complications, leading to coagulopathies such as thrombosis or haemorrhage,[2, 3] with nearly 20% of these patients developing complications that can lead to increased mortality rates if not managed properly during treatment.[4, 5] Research has established a link between inflammation and the development and sustainability of cancer,[6, 7] with studies indicating that brain cancer can directly affect coagulation through tissue factors (TF), platelets, thrombin, plasmin, and fibrin.[3, 8-12] However, it's important to note that existing research on brain cancer and coagulation predominantly addresses metastatic cancers, while studies on non-metastatic cases are largely confined to the context of peri-operative care.[7, 13]

There is a clear absence of research regarding coagulopathies in brain cancer, particularly in non-metastatic cases, that needs to be addressed. Therefore, it is important to study all contributing factors in the development of coagulopathies in brain cancer patients. Studies have shown that non-metastatic brain cancer patients exhibit increased levels of the PAI-1 protein, which inhibits the activity of plasminogen activators. This inhibition leads to reduced plasminogen levels and subsequently less lysis of fibrin.[3, 14, 15] Fibrinolysis (decreased fibrin lysis) can lead to clots that lyse at a decreased rate, thereby increasing the risk of thromboembolisms.[16, 17] Therefore, this study aims to investigate hypercoagulability in non-metastatic brain cancer patients compared to healthy individuals by studying the morphological and viscoelastic properties of platelet-poor plasma (PPP), specifically focussing on fibrin. The study of cellular components, including plasma, were covered in a separate study. This means this study could use PPP instead of platelet-rich plasma to focus on fibrinogen.

Thromboelastography® (TEG®) was used to measure viscoelastic properties during clot formation in both groups. The TEG® is a quantitative method used to measure changes in clot initiation time, clot strength, elasticity, and stability.

Scanning electron microscopy (SEM) was used to study the ultrastructural morphology of fibrin networks in all the participants in order to determine whether visible differences existed between the fibres of healthy individuals and those with brain cancer. This qualitative technique was used to study specific characteristics such as

the size, matting, and fusion of fibres. The SEM images were also analysed with software, including ImageJ and Fractalyse, to measure both the thickness and branching of fibrin fibres. Clinical test results—including the international normalised ratio (INR), C-reactive protein (CRP), and procalcitonin (PCT)—were collected from patient files to describe the relevant clinical profile of the patient population. These values were compared with healthy reference ranges to determine the clotting potential and levels of inflammation present in the patients.[7]

The clinical tests revealed normal INR and PCT values, but elevated CRP levels compared to typical ranges. This suggests that the brain cancer patients in this study exhibited normal clotting parameters and were devoid of bacterial infections. The heightened CRP levels could imply heightened inflammation attributable to the presence of brain cancer. C-reactive protein has also been shown to promote plasminogen activator inhibitor-1 (PAI-1), which leads to the reduction of plasminogen production and is also associated with thinner fibrin fibres.[18]

The results found that there was no significant difference between the viscoelastic properties of healthy individuals and those with brain cancer, implying similar clotting times and clot strength. However, the analysis did reveal that the brain cancer patients possessed significantly thinner fibres than healthy individuals, which may have an impact on the clot lysis of the patients.[16] Specifically, it could cause both a decrease in clot lysis due to thinner fibres that reduce the trigger for plasminogen production, and an increase in bond densities that reduce the rate of fibrin degradation and, therefore, clot lysis.[16, 17] There were no other differences detected in the morphology of the two groups.

These results can be used in future research to further study the link between coagulopathies and cancer and add value to improving patient management strategies.

Chapter 2: Literature review

2.1 Chapter objective

In this chapter, the literature pertaining to brain cancer, its associated inflammation, and the complications arising from coagulopathies will be reviewed.

2.2 Brain cancer

2.2.1 Overview and incidence

Brain tumours are an abnormal growth of cells within the brain and its surrounding tissues, varying widely from benign to malignant [19], with both being potentially deadly.[20, 21] Benign cancers can be dangerous due to their location and their ability to infiltrate locally.[20] This means some benign tumours are life threatening, while others can be treated, depending on these factors.[20] While benign brain cancers were once a leading cause of cancer-related mortality, documented as among the top ten in 1998,[22] they have since become less prevalent but remain a significant concern in adult mortality.[23]

Besides degrees of malignancy, brain cancer can also be classified according to where it originates within the central nervous system. Primary, non-metastatic brain cancers are those that originate from the tissues in and surrounding the brain.[20]

Among the various cancer types, brain cancer is comparatively rare. The occurrence and survival rates of the various cancers differs from region to region geographically.[24-27] Globally, the incidence of malignant brain cancer is estimated at 5.57 per 100 000 individuals.[27] In Southeast Africa, this incidence decreases to 0.71 per 100 000 for brain-specific cancers and 0.13 per 100 000 for cancers that extend into the meninges.[27]

The majority of brain cancers are gliomas, which originate from the supportive glial cells in the brain, including astrocytomas, glioblastomas, and oligodendroblastomas.[20, 27, 28] Meningiomas also represent a significant proportion of brain cancers.[29]

The typical life expectancy for brain cancer patients is around 5 years, which varies based on the type and histological properties of the cancer.[20, 24] Meningiomas, for instance, tend to have a better prognosis than gliomas.[20] Brain cancer is more

prevalent in the elderly than in children, with the average diagnoses being at 48 years of age in South Africa.[24-26, 30] Specifically, gliomas are most common among individuals aged 40 to 70 years, whereas meningiomas frequently occur in those aged 50 to 60 years.[28, 30]

Generally, the incidence of brain cancer is higher in men than in women.[24-26, 30] However, a few studies in Africa (Sudan and South Africa) showed a higher prevalence of brain cancer among women.[31, 32] Although men have a higher incidence of brain cancer, women are more likely to develop meningiomas than men.[20, 28, 29]

Some established risk factors for the development of gliomas include high-dose radiation, gender, and increasing age.[28] Risk factors for meningioma development include high-dose radiation, gender (possibly due to hormonal differences), and genetic susceptibility.[29] These risk factors are important considerations during the diagnosis of brain cancer.

2.2.2 Diagnostics and symptoms of brain cancer

As brain cancers are located within and interfere with the normal functioning of the brain, they can cause many health complications, including headaches, epilepsy, sensory loss, cognitive loss, dysphasia, personality changes, thrombosis, and fatigue.[4, 33]

Currently methods for cancer diagnosis include routine check-ups and screenings for both focal or generalised symptoms through physical examinations and analysis of blood and urine samples.[34, 35] Body scanning technology such as imaging—mostly magnetic resonance imaging (MRI)—spectroscopy, and endoscopy are also used as diagnostic tools.[21, 34-37] Additionally, maladaptive chronic inflammation, which is associated with cancers, offers a potential diagnostic indicator.[38] One of the greatest barriers to brain cancer diagnosis is getting contrasting agents through the blood-brain barrier,[39] leading to potential delays in diagnosis as patients' conditions may only be identified at a later stage.[34]

Diagnostics are essential for developing more precise treatment plans.[34] The earlier brain cancer is diagnosed, the easier it is to treat; thus significantly improving the patient's prognosis.[34]

2.2.3 Complications of treating brain cancer

Primarily, treatment for brain cancer involves surgical excision, chemotherapy, or radiotherapy.[40] Coagulation complications, such as thromboembolisms, are common in most surgical procedures.[41] Specifically, peri-operative studies of non-metastatic brain cancer have shown hypercoagulability and thrombosis.[3, 13] This prevalence is attributed to surgery's role in increasing the activation of coagulation pathways and factors, which may be further exacerbated if the cancer itself has already impacted those pathways and factors. These interactions are discussed in more detail in section 2.4.2.[42]

Certain chemotherapy treatments are known to be thrombogenic, thereby increasing the risk of thromboembolic events in brain cancer patients.[4] These chemotherapeutic drugs can activate the coagulation system on multiple fronts. They damage the vascular endothelium, exposing tissue factor (TF)—a transmembrane protein—and thereby stimulating the coagulation pathway; stimulate procoagulants; inhibit anticoagulants, such as proteins C and S; and they elevate TF expression through released cytokines from chemotherapeutic tumour cell apoptosis.[43, 44] It is also established that coagulopathies can be induced from radiation, and therefore radiotherapy, through similar mechanisms.[45]

Cancer induces chronic inflammatory responses in the body, which may explain its association with coagulopathies.[7] From literature it is clear that there is a link between cancer and coagulation,[2, 3] further connected by cancer-related inflammation. Inflammatory cytokines influence the coagulation pathway by inducing TF to over activate the coagulation system, thus increasing the responsiveness of platelets and elevating fibrinogen synthesis.[46]

2.3 Cancer-related inflammation

2.3.1 Overview

Inflammation is generally associated with immune responses to infections, diseases, and injuries.[6, 47] The main function of inflammation in the body is to restore homeostasis after an infection or injury.[6] Inflammation entails a sequential series of

physiological responses aimed at eliminating damaged tissue and facilitating tissue repair.[47]

Some reactions involved in the process of inflammation include: increased micro-vessel permeability, attachment of leukocytes and platelets to injured vascular endothelium, migration of serum proteins, deposition of fibrin, movement of leukocytes and red blood cells, cell apoptosis, and the growth of new tissues and blood vessels.[47, 48]

Pro-inflammatory cytokines, including tumour necrosis factor-alpha (TNF-alpha), interleukin-1β (IL-1β), and interleukin-6 (IL-6), released by monocytes, mediate the liver cells' secretion of acute phase proteins, namely CRP and fibrinogen, in response to the presence of foreign bodies, such as brain cancer.[6, 49] These acute phase proteins not only contribute to the formation of the brain's endothelial cells but also promote the synthesis of prostaglandins. Prostaglandins, in turn, predominantly trigger inflammatory symptoms by affecting the central nervous system.[6]

Inflammation represents a major concern for brain cancer patients, given the strong association between cancers and chronic inflammation.[7] Consequently, the relationship between cancer and inflammation has become a pivotal focus point for both clinical and translational research endeavours.[50]

2.3.2 Inflammation in brain cancer patients

In the event that inflammation does not cease, usually due to persistent infections or chronic cellular injury, chronic inflammation occurs.[6] In this case, the inflammatory mediators themselves can damage the host.[6] Some chronic inflammatory conditions include atherosclerosis, obesity, asthma, neurodegenerative diseases, rheumatoid arthritis, and cancer.[6]

In all cancer cases there is an increase in tissue generation and a reduction in cell apoptosis, which contributes to a state of chronic inflammation—known in this case as maladaptive chronic inflammation.[7] Increased tissue generation is induced by cancer cells' innate ability to both stimulate growth factors circulating in the body and increase the expression of growth factor receptors on their cell surfaces, the combination of which allows cancers to proliferate more than healthy tissue would.[7] Apoptosis of

cancer cells is usually caused by tumour suppressor genes and inflammatory cytokines.[7] However, a cancer that manages to deactivate these pathways can avoid apoptosis and proliferate unchecked.[7]

Tumours frequently involve immune-associated cellular proteins, like signal transducer and activator of transcription 3 (STAT3), and inflammatory microenvironments.[6, 51] The initiation of this microenvironment is set on by damage caused by cancer itself and is exacerbated by the pro-inflammatory factors controlled by the cancer.[52] The damage caused by cancer pushing on blood vessels exposes TF in the endothelium of these vessels, initiating both inflammatory and coagulation responses.[48, 52] Hypoxia resulting from compressed vessels leads to cytokine production and angiogenic growth factors, which stimulate the growth of new blood vessels and recruit macrophages.[53] These inflammatory responses persist for as long as the tumour is present and do no not alleviate the way inflammation usually does after injury.[53]

Brain tumour cells themselves can express various inflammatory cytokines, which are all listed in Table 1 along with their respective functions.[54]

Table 1. Inflammatory cytokines in brain cancer [54]:

This table illustrates the common cytokines found in brain cancer inflammation and how they participate in its pathogenesis.

Cytokines	Normal function	Brain cancer pathogenesis
Pro-inflammatory		
Interleukin-1β (IL-1β)	Induction of other pro-inflammatory cytokine release Production of CRP and PCT	Association with metastasis Involvement in anti-apoptotic signalling
Interleukin-6 (IL-6)	Maturation of B-cells Involvement in neurogenesis Production of CRP, PCT, and fibrinogen	Cancer cell proliferation and invasiveness Promotion of angiogenesis and resistance to apoptosis
Interleukin-8 (IL-8)	Chemo-attraction of white blood cells Inhibition of endothelial cell apoptosis	Promotion of angiogenesis and metastasis
Macrophage Migration Inhibitory Factor (MIF)	Regulation of p53 function	Influence on cancer cell proliferation and apoptosis
Tumour Necrosis Factor-alpha (TNF-α)	Stimulation of T-cell growth Production of PCT Influences dendritic cell maturation	Promotion of glioma cell invasion and angiogenesis Association with cachexia
Anti-inflammatory		
Interleukin-4 (IL-4)	Regulation of allergy response Maturation and proliferation of B-cells, mast-cells, and T-cells	Immune-evasion mechanisms of glioma microenvironment Inhibition of micro-glial production of pro-inflammatory cytokines
Interleukin-10 (IL-10)	Downregulation of type 1 T helper cell cytokines	Inhibition of T-cell proliferation Enhancement of cancer progression
Transforming Growth Factor-β (TGF-β)	Inhibition of B- and T-cell proliferation Prevention of dendritic cell maturation Interference with cytotoxic T cell development	Immunosuppression Stimulation of cancer cell migration and angiogenesis

The connection between brain cancer and inflammation is clear. Research indicating the association between brain cancer and coagulopathies underscores the intrinsic capability of cancer to affect key coagulation components, including TF, platelets, thrombin, plasmin, and fibrin.[3, 8-12] Moreover, studies have indicated a propensity for coagulopathy development in patients with brain cancer.[3, 13]

2.4 Coagulation

2.4.1 Overview of coagulation

Normally, when blood vessels are damaged, haemostasis is activated to seal the injury site and stop blood loss through clotting and vasoconstriction.[55, 56] Haemostasis has three main functions, namely: sealing damaged blood vessels, maintaining the fluidity of the blood, and removing blood clots after restoration of vascular integrity.[57]

Normal endothelium in blood vessels have a negatively charged surface that repels platelets. These cells also secrete prostacyclin, adenosine diphosphate, and nitric oxide to dilate blood vessels and reduce platelet function.[55]

Upon blood vessel damage, a platelet plug forms through the adherence of platelets to the damaged vessel wall and each other.[57] Additionally, tissue factor (TF), a transmembrane protein released from the damaged tissue, leads to the activation of coagulation factors that culminate in the production of thrombin. Thrombin acts as a pro-coagulant, increases platelet activation, and assists in the formation of fibrin fibres.[55, 57] Fibrin plays a vital role in stabilizing the platelet plug, which is attached to the collagen of the damaged endothelium, and together they form the clot.[55, 57] This fibrin clot is later removed by fibrinolysis.[56] The process is described in more detail in Figure 2.

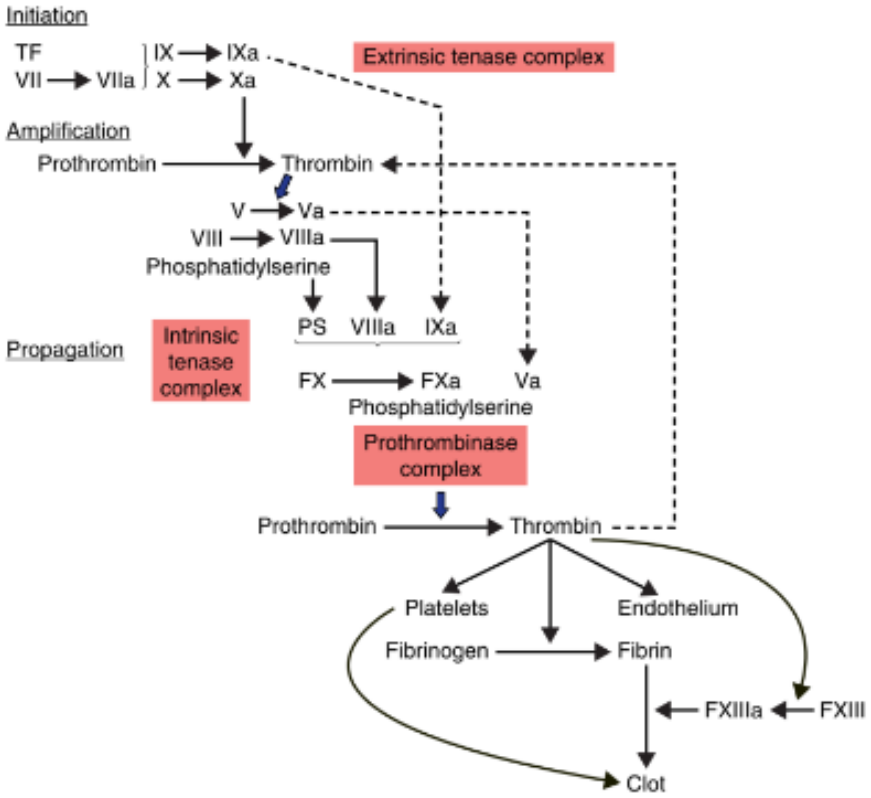

Figure 2. Coagulation pathway [58] A diagrammatic view of the coagulation pathway, specifying the three phases: initiation, amplification, and propagation. Abbreviations: Tissue factor (TF), Phosphatidylserine (PS). All other abbreviations are coagulation factors named after the roman numeral used in the abbreviation, e.g: Factor seven (VII)

Coagulation occurs through the intrinsic and extrinsic pathways. The extrinsic pathway, considered the main initiator of coagulation, is activated through TF exposed during injury, while the intrinsic pathway sustains the clotting process afterwards.[55] Coagulation factors are mainly produced in the liver in inactive forms and spread throughout the body in that state until they are activated.[55] The production of many of these factors is dependent on the activity of vitamin K in the liver.[55] Inflammatory cytokines, as well as thrombin, can stimulate hepatocytes to convert fibrinogen into fibrin as part of this pathway.[58, 59]

Fibrinogen is converted to fibrin, catalysed by thrombin, to form a network of fibres that constitutes a fibrin clot.[55] Fibrinogen consists of three polypeptide chains linked together by 29 disulphide bonds, which are altogether 45 nanometres long.[60, 61] The fibrinogen structure is illustrated in Figure 3. Thrombin cleaves soluble fibrinogen into insoluble fibrin polymers. These polymers undergo many interactions with factor XIIIa

and calcium to lengthen into protofibrils, which aggregate laterally to form the fibre networks of the fibrin clot.[60, 61] Additionally, zymogen plasminogen binds to its activator and fibrin to turn into plasmin. Plasmin then lyses the fibrin monomers and cross-linked fibrin to prevent excess clotting.[60] This process is shown in Figure 4.

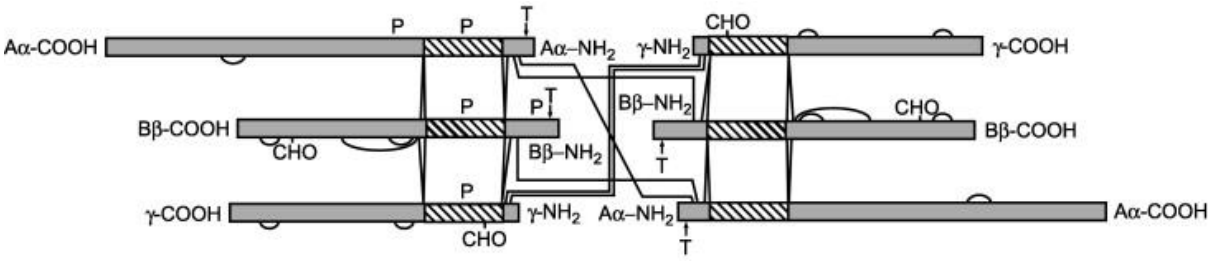

Figure 3. Fibrinogen structure.[60] A diagrammatic view of fibrinogen, consisting of three polypeptide chains linked together by 29 disulphide bonds, which are altogether 45 nanometres long. The different chains are represented by bars proportional to their number of amino acids. The diagonally striped boxes represent the coiled-coil regions. Thrombin and plasmin cleavage sites are labelled as T and P, respectively.

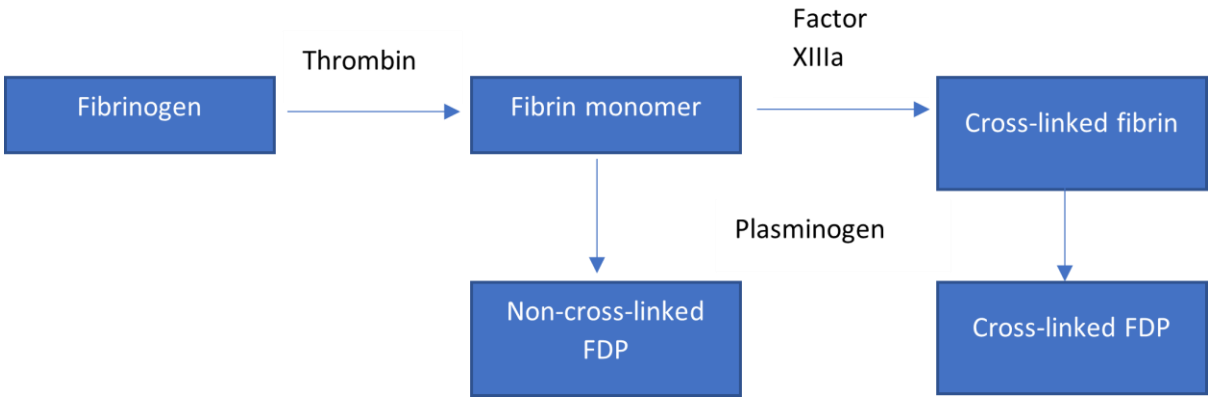

Figure 4. Formation of fibrin and fibrinogen degradation products[61] Process of thrombin and plasminogen activity to produce fibrin and cross-linked and non-cross-linked FDP. (Created using Microsoft Office Word software)

The body has natural anticoagulant abilities to prevent excessive clot formation and to keep clots localised to the damaged area.[55] The primary component of this system is antithrombin, produced by the liver, which inhibits certain coagulation factors.[55] Other inhibitors include the TF pathway inhibitor and proteins C and S.[55] In certain chronic inflammatory conditions, a pathological disorder termed coagulopathy may arise, characterized by abnormal haemostatic function that either reduces or enhances clotting capacity.[56, 62] Coagulopathy can result from primary (defective blood vessels

or platelets) and secondary (defective clotting and fibrinolytic factors) causes.[55, 56] Brain cancer is often associated with the development of both primary and secondary coagulopathies.[3, 4, 8]

2.4.2 Coagulation in brain cancer patients

Cancer-related coagulopathies are the second most likely cause of death in cancer patients.[4, 5] Cancer-related coagulopathies are often referred to as Trousseau's syndrome or venous thromboembolism.[4]

Tumour cells have hallmarks that alter cell physiology to promote their own growth and have shown to impact the coagulation system.[2, 7] These hallmarks include self-sufficiency in growth signals, insensitivity to growth inhibitors (inhibition of tumour suppressor genes), limitless replication potential, reduced apoptosis, changed cellular metabolism (deactivation of the mitochondria and use of glycolysis in its stead), sustained angiogenesis, tissue invasion abilities, and the evasion of host immune responses.[7]

These cells have an innate ability to interfere with the natural signals and pathways of the body, including the haemostatic system.[7] Cancer cells not only block mechanisms that could lead to their destruction or inhibition but also activate angiogenesis, ensuring a continuous supply of oxygen and nutrients is provided for the tumour's growth.[7] Coagulation can be an important factor for cancer growth and metastasis, especially through the actions of platelets.[8, 9]

Cancer cells release TF that not only acts as an inhibitor for the proteins and enzymes involved in anticoagulation pathways but also serves as an activator for the coagulation pathway, leading to hypercoagulation.[10] Additionally, these cancer cells can release factors that mimic the way thrombin and plasmin convert fibrinogen into fibrin to form fibrin clots. They can also cause an immune response and inflammation, which increases platelet recruitment and hypercoagulation in general.[11, 12] Due to the link between inflammation and hypercoagulability, and the link between brain cancer and inflammation, one can surmise a natural inclination for hypercoagulability in brain cancer patients.

Intrinsic tumour factors, cancer size, and location could all significantly disrupt the coagulation process.[5] Evidence suggests that certain oncoprotein coding genes and tumour suppressors also play a role in regulating many coagulation factors and proteins.[63] When tumour factors inhibit these proteins, they are also inhibiting the coagulation abilities of the body. The brain is the richest source of thromboplastins in the body, indicating that injuries to the brain—whether through surgery or another form—could cause a high release of thromboplastin into the circulation, causing coagulopathies to develop.[5]

Tumour growth and survival are also closely linked to fibrin formation and lysis.[3] This influences the body's natural ability to form and lyse clots.[64] Observations conducted on individuals afflicted with non-metastatic brain cancer demonstrated heightened levels of the PAI-1 protein, an inhibitor of plasminogen activators.[2, 13, 14] This phenomenon precipitates diminished levels of plasminogen, consequently attenuating fibrinolytic activity.[2, 13, 14] Reduced fibrinolysis leads to a decrease in the breakdown of fibrin, which can engender clots with reduced rates of dissolution, consequently heightening the susceptibility to thromboembolic occurrences.[15, 16]

Risk factors identified as indicators of an increased likelihood for developing coagulopathies in cancer patients include cancer-sites, haemoglobin levels, pre-chemotherapy status, platelet and leukocyte counts, as well as body mass index (BMI).[4]

Investigations into the relationship between brain cancer and coagulation have predominantly focused on metastatic cancer.[7] What's more, among studies involving non-metastatic cancer, the emphasis has largely been on perioperative patients.[13] This highlights a notable research gap concerning understanding coagulopathies in brain cancer, underscoring the need for a detailed examination of all contributing factors implicated in the onset of coagulopathies among brain cancer patients.

The findings from this study may provide a foundation for future investigations aimed at elucidating the association between coagulopathies and cancer, thereby enhancing the development of patient management strategies.

2.5 Aim and objectives

2.5.1 Aim

This study aimed to investigate hypercoagulability in benign brain cancer patients at an academic hospital and compare it to that of healthy individuals by studying the morphological and viscoelastic properties of PPP, specifically focusing on fibrin.

2.5.2 Objectives

The following objectives were used to achieve the aim:

1. To determine the clotting time by using the international normalised ratio (INR) from patient records of brain cancer patients and compare these to healthy reference ranges.
2. To assess the inflammation status of brain cancer patients by using the C-reactive protein (CRP) and procalcitonin tests (PCT) taken from patient records and comparing these to healthy reference ranges.
3. To measure the viscoelastic properties during clot formation of brain cancer patients compared to healthy individuals by using Thromboelastography®.
4. To study the ultrastructural morphology of fibrin networks in brain cancer patients compared to healthy individuals by using scanning electron microscopy.
5. To measure the thickness and branching of fibrin fibres in brain cancer patients compared to healthy individuals by using the ImageJ software.

Chapter 3: Sample collection, participant demographics, and routine clinical testing

3.1 Study design

This study is a patient-based analytical, cross-sectional study. Sample analysis using the methods outlined in chapters four and five was done on blood samples *ex vivo*, obtained from a control group of 27 healthy individuals, and a patient group of 26 non-metastatic (benign) brain cancer patients. The patient group consisted of patients diagnosed with non-metastatic brain cancer such as glioma, meningioma, schwannoma, or pituitary adenoma by a qualified medical doctor.

3.2 Study setting

Patient recruitment and blood sample collection took place at Steve Biko Academic Hospital (SBAH), Department of Neurosurgery, whereas the sample analysis was done at the University of Pretoria, Prinshof campus, Basic Medical Sciences building, lab 6-20.

In order to ensure a homogenous sample population, background information was gathered through a questionnaire, capturing the demographics of participants. Patient demographics were used to ensure an even distribution of patients across age and/or biological sex.[30, 65] This information was also used to describe the study's population.

This study formed part of a larger research project. The other segment of this project, led by Zenobia Seyfert, focused on investigating the ultrastructural, viscoelastic, and biochemical changes in the whole blood of brain cancer patients compared to healthy individuals, aiming to identify clotting abnormalities. This includes the study of the platelets. The control group samples were shared between the two studies.

3.3 Participant information

3.3.1 Control participants

Willing participants who met the inclusion criteria were recruited from the healthy family members, friends, and colleagues of the research team to serve as a control group. The control group consisted exclusively of healthy individuals.

Recruitment of a healthy control group from the same population as the patient group ensured homogeneity between the groups to limit confounding factors. The inclusion and exclusion criteria were carefully chosen to closely imitate the patient group in all respects aside from the presence of brain cancer, as described in the selection criteria.

Inclusion criteria:

- Able to provide informed consent
- Any biological sex
- Ages between 20 and 80[30]

Exclusion criteria:

- Smokes or uses any tobacco or related products
- Use of chronic medication
- Has any condition which could present with chronic inflammation
- Has a history of an immune-compromised status
- Has used herbal supplements, corticosteroids, anti-inflammatory, anti-platelet, or anti-coagulative medication within two weeks prior to the blood sample collection date
- HIV positive status (If the status is known)
- Excessive alcohol consumption defined as consuming five or more drinks on the same occasion on at least one day in the past 30 days
- Diagnosed with having non-metastatic brain cancer or metastases, sarcoma of the skull, or cancers of ciliated squamous or columnar ependymal cells of the ventricles
- Has autoimmune or viral diseases
- Has any disease affecting the blood circulation or the coagulation system

3.3.2 Brain cancer participants

Prof Padayachy and Dr Grobbelaar, under the supervision of Prof Padayachy, facilitated consultations between patients and the primary investigator. During patient consultations, the primary investigator explained the purpose of this study, secured informed consent, and

filled in the questionnaire. The blood samples were collected by qualified medical personnel in 4 mL citrated tubes.

All participants were required to sign an informed consent form and complete a questionnaire prior to blood sample collection, preventing those who did not meet the study's inclusion criteria from undergoing unnecessary blood collection.

Only patients that met the inclusion criteria were recruited for the study. No patient was required to stop medication to participate in the study. Furthermore, patients whose medication did not negatively impact blood coagulation, and therefore did not present as confounding factors, were also recruited. A list of these medications is provided in Table 3, accompanied by references confirming their neutral impact on the coagulation system.

Inclusion criteria:

- Able to provide informed consent
- Any biological sex
- Ages between 20 and 80
- Diagnosed with having **non-metastatic** brain cancer (specifically gliomas, adenomas, and meningiomas)

Exclusion criteria:

- Smokes or uses any tobacco or related products
- Has any comorbidities which could present with chronic inflammation
- Has a history of an immune-compromised status
- Has used herbal supplements within two weeks prior to blood sample collection date
- HIV positive status (determined from patient files)
- Excessive consumption of alcohol defined as consuming five or more drinks on the same occasion on at least one day in the past 30 days
- Diagnosed with metastases, sarcoma of the skull, or cancers of ciliated squamous or columnar ependymal cells of the ventricles
- Has an autoimmune or viral diseases
- Using any chronic medication that negatively impacts the coagulation system

3.3.3 Participant demographics

Participant demographics can be seen in Table 2, while patient medications can be seen in Table 3. There were no significant differences for age between the groups, therefore the controls were age matched.

Table 2: Participant demographics

Demographic		Control (n=27)	Brain cancer (n=26)
Age	Median (min, max)	41 (25, 61)	44 (29, 73)
	P-value	0.0754	
	Significant difference	No	
Biological sex	Number (Male/Female)	14/13	16/10
Cancer type	Number (Glioma/ meningioma/ schwannoma/ pituitary adenoma)	-	10/8/2/6

Table 3: Patient medications

Medication	Number of patients	Effects on coagulation
Paracetamol	15	No significant effect on blood clotting[66]
Tramadol	7	No effect on clot firmness [67]
Epilim	7	No effect on haemostasis[68]
Phenytoin	2	↑ collagen maturation[69]
Clexane	2	↓ thrombin activity[70]
Prednisone	1	↑ thrombin generation[71]
Citalopram	1	No significant effect on plasma coagulation[72]
Ridaq	1	No significant effect on blood clotting[73]
Enalapril	1	No confirmed significant effect on blood clotting[74]
Amlodipine	1	↓ expression of genes involved in coagulation activation[75]
Largactil	1	No significant effect on blood clotting pre-surgery[76]
Metoclopramide	1	↓ thrombosis when taken in conjunction with aspirin[77]
Simvastatin	1	↑ fibrin clot lysis; ↑ clot permeability; ↑ clot structure [78-82]
Folic acid	1	No significant effect on blood clotting[83]
Hydrochlorothiazide	1	No significant effect on blood clotting[84]
Decadron	1	↓ coagulation[70]
Lansoloc	1	Possible ↓ platelet count[85]

3.4 Ethical considerations

This study was approved by the MSc and research ethics committee (ethics number: 253/2022, see appendix B) of the Faculty of Health Sciences, University of Pretoria. The proposal was also approved by the national health research database (NHRD reference number: *GP_202208_041*) for permission to work with patients in SBAH as well as to access patient records.

The following ethical concerns were considered:

- Participants were not obligated to participate
- Participants were not treated differently if they decided not to participate
- All contact sessions with the participants were conducted privately
- All the results were kept confidential and were made available to the participant on request
- If any serious abnormalities were detected, the recruiting doctor was informed
- There were no costs involved for any of the participants that participated
- The participants could withdraw from participating at any time during the course of the study without explanation and prejudice
- Simple language was used to ensure that the participants fully understood the purpose of the study and what their participation entailed
- The blood sample collections coincided with routine blood tests to minimize inconvenience, requiring only one additional tube of blood

3.5 Clinical information obtained from patient records

3.5.1 International normalised ratio

The International normalised ratio is used to evaluate the extrinsic pathway of coagulation by acting as an indicator of clotting time.[86] It is calculated by dividing the tested prothrombin time by the normal prothrombin time and raising it to the power of the international sensitivity index.[87] To ascertain the tested prothrombin time, a mixture of phospholipid membranes embedded with TF, calcium, and a citrated plasma sample is added to a tube to activate it.[86] The duration from the addition of these factors until the onset of clot formation is recorded.[86] The INR values for each patient were obtained from their patient records at the hospital. The INR mean and standard deviation (SD) are represented in Table 4.

3.5.2 C-reactive protein and procalcitonin tests

Inflammation within the body can be identified by measuring acute phase reactants, which can be done with tests like the C-reactive protein (CRP) and a procalcitonin (PCT).[47, 59]

Testing the inflammatory status of patients can help determine the link between inflammation and coagulation. The CRP and PCT values for each patient were obtained from their patient records at the hospital. It was necessary to differentiate between inflammation resulting from bacterial infection (indicated by PCT) and inflammation associated with brain cancer (likely indicated by CRP). The CRP and PCT results can be seen in Table 4.

3.5.3 Clinical test results

During patient management, routine tests are conducted to monitor patient progress and the disease state. Results for the INR, CRP, and PCT were recorded if available in the patient files. As a result, there are some missing data, and not all of the patients had results for all the tests. Datasets were first tested for normality using the Shapiro-Wilk test. Parameters obtained from INR, CRP, and PCT measurements were described using the mean and standard deviation (SD) at a 95% confidence interval. All tests were conducted at a 5% significance level using GraphPad version 8. The results were then compared to normal reference ranges, as shown in Table 4. Values that were outside the normal range are highlighted in bold.

Table 4: Clinical test results

Clinical test	Unit	Mean±SD	Normal range
International normalised ratio n=16		0.99±0.08	<2
C-reactive protein n=25	Mg/L	**40.12±74.66**	<10
Procalcitonin tests n=15	Ug/L	0.14±0.24	<0.5

As indicated in Table 4, the INR and PCT values fell within normal ranges, whereas the CRP levels were generally increased. The increased inflammatory status may be due to the presence of cancer.

3.6 Discussion and conclusion

The demographics of this study contain age-matched controls with a median age of 41 and a patient group with a median age of 44.

During inflammation, CRP is released by the liver and forms part of the natural anti-coagulation system of the body.[55] The CRP coats the foreign body (most likely bacteria) and signals it for phagocytosis, causing that foreign body to go through apoptosis.[88] C-reactive Protein has also been shown to promote plasminogen activator inhibitor-1 (PAI-1) and platelet activation.[18] This whole process is initiated by inflammatory cytokines such as IL-6 and IL-1β, as illustrated in Figure 5.[88] Just as with the CRP, the cytokine IL-6 stimulates the hepatocytes in the liver to produce more fibrinogen in response to inflammation.[59] In cases with brain cancer, cytokine levels are elevated, resulting in the heightened synthesis of CRP.[89] A progressive increase of the CRP protein can be seen in infections within the first 48 hours.[90, 91]

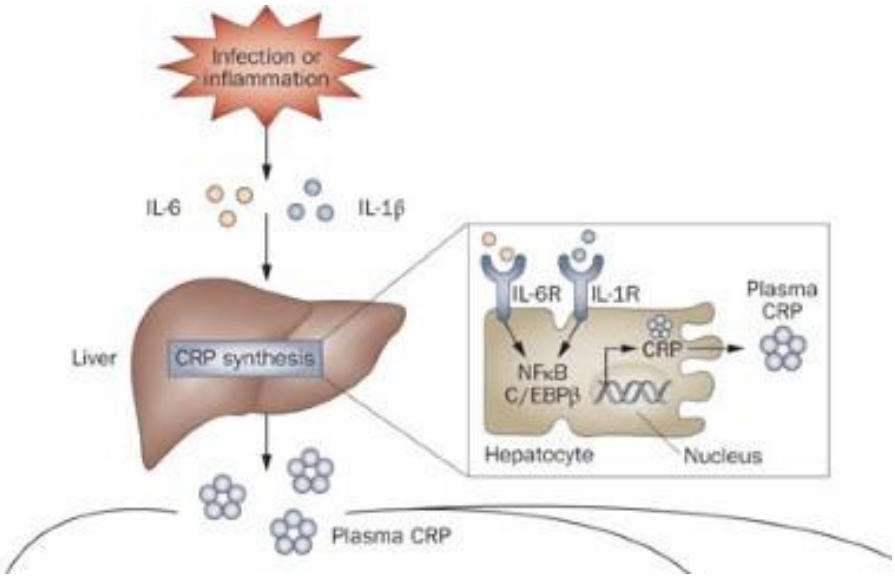

Figure 5. C-Reactive Protein synthesis[88] The effect of the inflammation system on CRP synthesis. Abbreviations: Receptor (R) for Interleukin cytokines, NF-kappaB (NFκB), CCAAT/enhancer-binding protein beta (C/EBPβ).

A PCT measures the levels of procalcitonin in the blood, which are elevated during infections and, consequently, inflammation.[47] The gene for the procalcitonin protein is activated by cytokines, such as IL-6, IL-1β, and TNF-α.[92] Procalcitonin is the pre-hormone for calcitonin, and its presence is indicative of infectious diseases or sepsis.[93]

The patient group in this study exhibited a general increase in inflammatory status, which is likely due to the presence of the cancer. This inference is supported by elevated CRP levels, rather than PCT levels, as PCT is strictly elevated during bacterial infections and does not reflect chronic, non-bacterial, or neoplastic diseases.[94] This further confirms the likelihood of cancer being the cause of the high CRP values.

This chapter confirmed that the patient group had an increased inflammatory profile. In the next chapter, the viscoelastic properties of both groups will be analysed using Thromboelastography® (TEG®).

Chapter 4: Thromboelastography®

4.1 Chapter objective

In this chapter, the following objective will direct the research:

To measure the viscoelastic properties of PPP during clot formation in brain cancer patients compared to healthy individuals using TEG®.

4.2 Introduction

Thromboelastography® is an analytical method used to measure the viscoelastic changes that occur during coagulation and fibrinolysis. Fibrin, with its viscous and elastic properties, possesses high elasticity and stability.[95] As a filamentous protein polymer, fibrin exhibits increased stiffness when subjected to tension or shear forces, which would cause resistance during a TEG® analysis.[95]

Thromboelastography® is used to detect and quantify dynamic changes in blood during clotting, thus providing an overview of the coagulation process's functionality and allowing it to be used to assess the risks of bleeding in a clinical setting.[62-64] The TEG® generates results from the blood samples through a rotating pin that constantly measures the resistance encountered by the forming fibrin clot against the pin. These results are then depicted as a graph, offering detailed measurements of various parameters, which are listed in Table 5.

Previous studies have utilized TEG® testing to examine blood clotting in cancer patients.[96-98] Some of these studies aimed to demonstrate TEG®'s superiority (holistic) as an analysis tool over routine coagulation tests—such as prothrombin time—since it measures the entire haemostatic process.[96, 98] Quan, et al. showed that TEG® has a higher detection rate for hypercoagulability than other standard coagulation tests.[98] There is a general consensus among most studies about the value of TEG® for providing a holistic overview of the coagulation process, from clot formation to fibrinolysis.[96-98] A study analogous to this dissertation's research, focusing on whole blood in non-metastatic breast cancer patients, endeavoured to enhance comprehension of coagulopathies utilizing TEG®.[97] The results of these studies have shown hypercoagulability in many metastatic cancer patients when compared to the results of healthy individuals, but not for non-metastatic patients.[96, 97] Research

involving the use of TEG® with brain cancer patients has predominantly been conducted in a perioperative setting, suggesting a gap in pre-operative research.[13, 99] Thus, Thromboelastography® is poised to be an effective tool for detecting hypercoagulability and viscoelastic changes during coagulation.

4.3 Materials and methods

Platelet-poor plasma (PPP) was extracted from whole blood samples using the single-step method described by Rikkert et al. This involved centrifuging the blood for 15 minutes at 2000 g at room temperature. Thereafter, the supernatant was obtained for the PPP samples, leaving 1 cm of plasma above the pellet.[100] Fresh PPP was used for the TEG® and SEM preparation in order to study the effect of fibrinogen without the influence of cellular components. Any residual samples were stored long term at -80 ºC for possible future studies.

Platelet-poor plasma (340 µL) was placed in a TEG® cup together with 20 µL of 0.2 M calcium chloride ($CaCl_2$) (Barker Medical, 7003) to activate the coagulation process. The samples were then analysed using the TEG® 5000 computer-controlled device (Haemoscope Crop., Niles, IL, USA). The process was allowed to run until maximal amplitude (MA) was reached, since only clot formation was relevant to this study.

Parameters obtained during the TEG® analysis were recorded for each sample in an Excel spreadsheet. During analysis, patient values were compared to control values as there are no normal reference ranges for PPP samples.

Table 5: Thromboelastography® parameters[101]

Parameters	Units	Description
Reaction time (R)	Minutes	Time from the start of the test to initial fibrin formation.
Kinetics (K)	Minutes	Time taken to achieve a certain level of clot strength
Alpha angle (α)	Degrees	The angle measured between reaction time and kinetics, which represents the speed at which fibrin build up and cross-linking takes place (rate of clot formation).
Maximal amplitude (MA)	Millimetres	Maximum strength/stability of clot.
Maximum rate of thrombus generation (MRTG)	Dynes/cm^{-2}	The maximum velocity of clot growth observed.
Time to the maximum rate of thrombus generation (TMRTG)	Minutes	The time interval observed before the maximum speed of the clot growth.
Total thrombus generation (TTG)	Dynes/cm^{-2}	The amount of total resistance generated during clot formation (total area under the velocity curve).
Elastic modulus strength (G)	Dynes/cm^{-2} per thousand	A log derivation of maximum amplitude that represents clot strength.

4.4 Statistical analysis

Datasets were first tested for normality with the Shapiro-Wilk test. Parameters obtained from TEG® analysis were not normally distributed (non-parametric); therefore, summary statistics were used to describe the minimum, median, and maximum values. A Mann-Whitney test was used to compare the two groups using a 95% confidence interval. All tests were conducted at a 5% level of significance using GraphPad version 8.

4.5 Results

The TEG® was used to quantify the viscoelastic properties of PPP samples during clot formation in brain cancer patients in comparison with healthy individuals. The results obtained from the TEG® analysis are displayed in Table 6.

Table 6: Thromboelastography® results

Parameters		Control (n=20)	Brain cancer (n=24)
R	Median (min, max)	15.6 (5.8, 35.2)	15.2 (4.5, 86.4)
	P-value	0.9396	
	Significant difference	No	
K	Median (min, max)	3.05 (0.8, 7.2)	3.25 (0.9, 12.9)
	P-value	0.5121	
	Significant difference	No	
α	Median (min, max)	49.1 (28.2, 87.8)	49.9 (4.5, 76.8)
	P-value	0.2528	
	Significant difference	No	
MA	Median (min, max)	44.9 (27.2, 93)	53 (4.2, 75)
	P-value	0.2251	
	Significant difference	No	
MRTG	Median (min, max)	4.41 (2.1, 10.1)	4.86 (0.16, 16.5)
	P-value	0.7485	
	Significant difference	No	
TMRTG	Median (min, max)	20.1 (6.5, 79.5)	19.5 (5.33, 93.6)
	P-value	0.9861	
	Significant difference	No	
TTG	Median (min, max)	408 (187, 3834)	566 (22.5, 1411)
	P-value	0.2366	
	Significant difference	No	
G	Median (min, max)	4.05 (1.9, 66.5)	5.6 (0.2, 15)
	P-value	0.2249	
	Significant difference	No	
Abbreviations: Reaction time (R), Kinetics (K), Alpha angle (α), Maximal amplitude (MA), Maximum rate of thrombus generation (MRTG), Time to the maximum rate of thrombus generation (TMRTG), Total thrombus generation (TTG), Elastic modulus strength (G)			

The data presented in Table 6 reveal that the statistical analysis, as indicated by the p-values, demonstrated no significant differences between the control and brain cancer patient groups.

4.6 Discussion

The findings showed no significant difference between the groups, which indicates that fibrinogen rich blood samples from brain cancer patients did not have abnormal clot kinetics. These results align with research done on breast and colon cancers, where it was found that viscoelastic properties only differed in individuals with metastatic cancer but not in those with non-metastatic cancer.[96, 97] Similarly, a study examining both non-metastatic and metastatic ovarian masses showed that TEG® could significantly differentiate between the two states of cancers.[102]

Goncalves, et al. suggested that in non-metastatic patients, most of the coagulation and inflammatory changes predominantly occur within the tumour microenvironment

and to a lower degree than in metastatic patients.[97] This observation aligns with the literature review, which indicated that coagulation and inflammation are mechanisms leveraged to support cancer proliferation—a process less aggressive in benign tumours.[7-9] This may indicate that non-metastatic patients have a lower risk of developing coagulopathies.

Due to the nature of non-metastatic cancer, Goncalves, et al. highlighted that point-of-care tests such as the TEG® may not be able to detect abnormal viscoelastic changes in these patients, suggesting the need for alternative diagnostic methods in these cases.[97] Despite the limitations of TEG® in detecting viscoelastic differences, both the Goncalves study and the present study observed a difference in the fibrinogen morphology between the patient group and the control group, indicating a difference in coagulation that is not detectable by the TEG®.[97] This may make malignancy itself a greater risk factor for thrombotic events.[4, 10]

Despite the elevated inflammatory status in the patient group, the TEG® did not yield significant results. It's well documented that a state of inflammation can lead to increased platelet activation, which might be possible in brain cancer patients.[18] However, the centrifugation process used to prepare samples removes platelets from the plasma, thus making it impossible to show the platelet-driven effects of inflammation in the TEG® analysis.

There is also an increased thrombotic risk that comes with brain cancer treatment.[4] However, in this study we only included patients with no cancer treatment history, which may explain the lack of clotting abnormalities.

4.7 Conclusion

The viscoelasticity of fibrinogen-rich blood samples in brain cancer patients was found to be similar to that of healthy individuals. This outcome indicates the need for employing alternative techniques that might be more sensitive to coagulation changes, such as quartz crystal microbalance or clot lysis time assays, in future studies. Furthermore, ultrastructural studies may be able to identify changes in the formed clots. Specifically, the use of scanning electron microscopy to study the ultrastructural morphology of the PPP samples can provide further insight.

Chapter 5: Scanning electron microscopy

5.1 Chapter objective

In this chapter, the following objectives will direct the research:

- To study the ultrastructural morphology of fibrin networks in brain cancer patients compared to healthy individuals using SEM.
- To measure the thickness and branching of fibrin fibres in brain cancer patients compared to healthy individuals using the ImageJ software and Fractalyse.

5.2 Introduction

Thrombin and fibrinogen interact with each other during the process of blood clotting, wherein thrombin breaks down the fibrinogen molecule into two fibrinopeptides, subsequently forming fibrin monomers.[103] These fibrin monomers aggregate to construct a branched fibrin network of double-stranded protofibrils that are laterally linked, which can be analysed through ultrastructural techniques such as SEM.[103, 104] Scanning electron microscopy can be used to analyse morphological changes in whole blood or PPP during clotting.[101, 105] When used on PPP samples, clear images of fibrin fibre networks can be taken.[101]

Studies on the morphological properties of fibrin fibres have been conducted both with healthy individuals and patients with inflammatory conditions, including diabetes, thrombo-embolic ischemic stroke, Alzheimer's type dementia, and Parkinson's disease to determine fibrin's role in the coagulation complications associated with these conditions. [101, 106, 107]

In these studies, SEM's highly sensitive ultrastructural technique identified the specific changes to fibrin fibres that were involved in clotting.[101] In healthy individuals, they found clear branching and individual, granulated, elongated fibres.[101, 106] In hyper-coagulable states—such as diabetes, ischemic stroke, and Alzheimer's—the fibres appeared more as matted masses or were fused together, showing fewer individual fibres overall.[101, 106, 107] Conversely, hypo-coagulable conditions were characterised by short, branching fibres or no fibres at all.[101]

Scott et al. identified a relationship between the structure of a fibrin clot and the fibrinolysis rate of its fibres.[108] Certain SEM studies confirmed that clots composed of thicker fibres are more likely to elongate and subsequently lyse faster than thinner fibres, which exhibited slower rates of clot lysis.[108, 109] This difference in lysis rate is attributed to the diameter of the fibrin fibres.[109] Thinner fibres diminish the stimulus for plasminogen production and have higher bond densities and tightly knit cross-linked structures.[16, 17, 108] Consequently, these characteristics reduce the rate of fibrin degradation and, subsequently, clot lysis.

Scanning electron microscopy is a useful tool for studying the ultrastructural morphology of fibrin networks. Furthermore, images obtained through SEM can be quantitatively analysed using ImageJ software, allowing for the measurement of fibre thickness and branching. This analysis assesses the effectiveness of fibrin clot formation and lysis.[110, 111]

5.3 Materials and methods

Platelet-poor plasma (10 µL) was smeared onto a glass coverslip, followed by the addition of 5 µL of thrombin (donated by the South African National Blood Service) to activate the fibrin networks. The coverslip was left to dry at room temperature for 3 minutes before being placed into its own well in a 24-well plate. It was then washed by gently immersing it in 0.01 M phosphate buffered saline (PBS) solution (Sigma, P5493-1L) (pH 7.4) for 15 minutes. After washing, the smear was fixed with 4% formaldehyde (Sigma, F8775-500ML) for 30 minutes and then rinsed three times with PBS, for 3-minute intervals each. The smears were then further fixed by covering them in 1% osmium tetroxide (OsO4) (Sigma, 75632-10ML) for 15 minutes. Once again, the smears were washed three times with PBS for 3-minute intervals each. The smears were then gradually dehydrated by first covering them for 3 minutes in progressively higher concentrations of ethanol (Sigma, 24102-4x2.5L), i.e., 30%, 50%, 70%, 90%, and then finally three times with 100% ethanol. Following the dehydration step, the smears were immersed in hexamethyldisilazane (HMDS) (Sigma, 440191-1L) for 30 minutes, after which the HMDS was removed and replaced with a single drop of HMDS. The smears were then left to dry overnight. Next, the smears were mounted

onto metal plates with carbon tape and coated with carbon to provide conduction for the smears in the SEM.

The Zeiss Crossbeam 540 FEG-SEM or Zeiss Ultra Plus FEG-SEM (Carl Zeiss Microscopy, Munich, Germany) at the microscopy and microanalysis unit (UP, Hatfield Campus) was used to study the ultrastructural morphology of the samples at 1 kV, using the InLens detector. Fibrin networks were the objects of interest during the investigation.

For each individual sample, the following properties were noted:

1. Smooth or globular fibres
2. Presence of dense, matted fibrin deposits
3. Branching of the fibrin networks (quantified with Fractalyse)
4. Fibre thickness (quantified with ImageJ)
5. Distribution of fibres

Sample analysis involved capturing a series of micrographs to highlight the study's features of interest. A minimum of two representative images per participant were taken at a low magnification (approximately 10 000 x machine magnification) to show a general overview of the sample. Features of interest were then identified, and an additional series of images were captured at higher magnifications (approximately 20 000 x machine magnification) to detail these features. The images were stored on the Google Drive account of the supervisor, Prof J Bester, and were also uploaded to the University of Pretoria's research data repository platform, where they will be retained for a minimum of 15 years.

Fibrin fibre thickness was measured using ImageJ software on the micrographs taken using the SEM. The micrographs were imported into ImageJ (ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health: http://rsbweb.nih.gov/ij/) and 50 fibres per individual were measured. The average thickness of each participant's 50 fibres was calculated and recorded in an Excel spreadsheet.

The branching of fibrin networks was measured using Fractalyse software on the same SEM micrographs that were assessed with the ImageJ software. The micrographs

were imported into Fractalyse, where the fractal dimensions of the networks were analysed. The results of this analysis were recorded into an Excel spreadsheet.

5.4 Statistical analysis

Datasets were first tested for normality with the Shapiro-Wilk test. Parameters obtained from SEM analysis were not normally distributed (non-parametric); accordingly, summary statistics were used to describe the minimum, median, and maximum values. Furthermore, a Mann-Whitney test was used to compare the two groups using a 95% confidence interval. All tests were conducted at a 5% level of significance, and all tests were done using GraphPad version 8.

5.5 Results

5.5.1 Scanning electron microscopy

The images obtained from the SEM analysis are displayed in Figures 6-9. Figures 6 and 7 depict the PPP smears of the control group under both low (Figure 6- ten thousand times) and high (Figure 7- twenty thousand times) magnification. The brain cancer patient's PPP smears are presented in Figures 8 and 9.

Under low magnification, as illustrated in Figures 6 and 8, the control group's PPP smears reveal an organised network of fibres consisting of distinct fibres. Conversely, the patient group displays a more disorganised network of fibres, characterized by visible tangles and fusion of fibres.

In Figure 9, arrows pinpoint the major features found in the collective images of the brain cancer patient's PPP smears. These arrows are used to highlight any abnormalities in the samples, such as tangles in the fibres, fusion of fibres, and thinner fibres connecting to thicker fibre branches.

With the higher magnification, as shown in Figures 7 and 9, there is a discernible difference in fibrin network cross-linking. The control group exhibits wider connections between fibres, showing larger gaps in between. Conversely, the patient group exhibits tighter cross-linking, where the fibres form more connections.

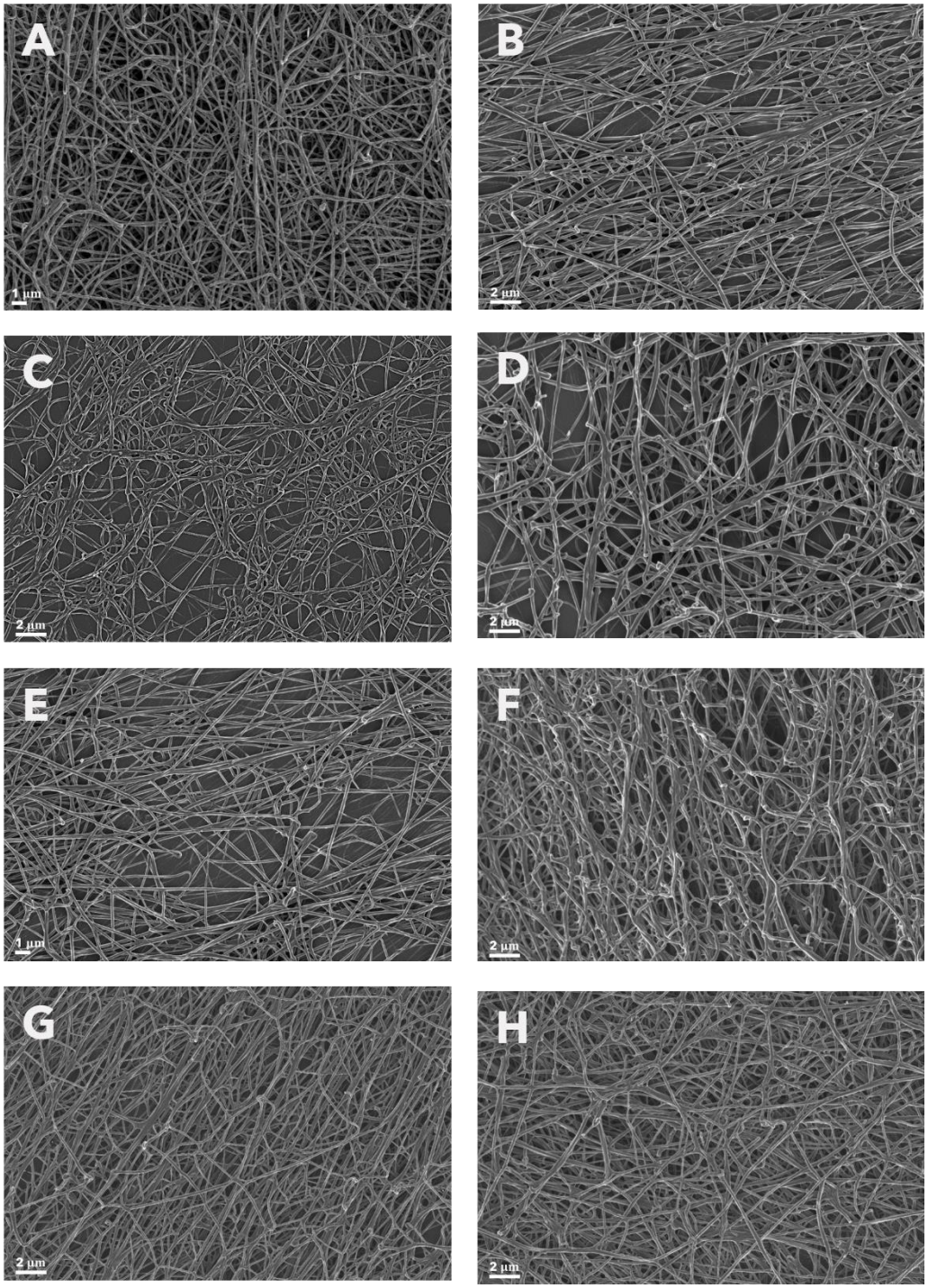

Figure 6: Scanning electron microscope images of controls on low magnification: Images A-H show the control group's fibrin with an organised network of fibres at 10k magnification.

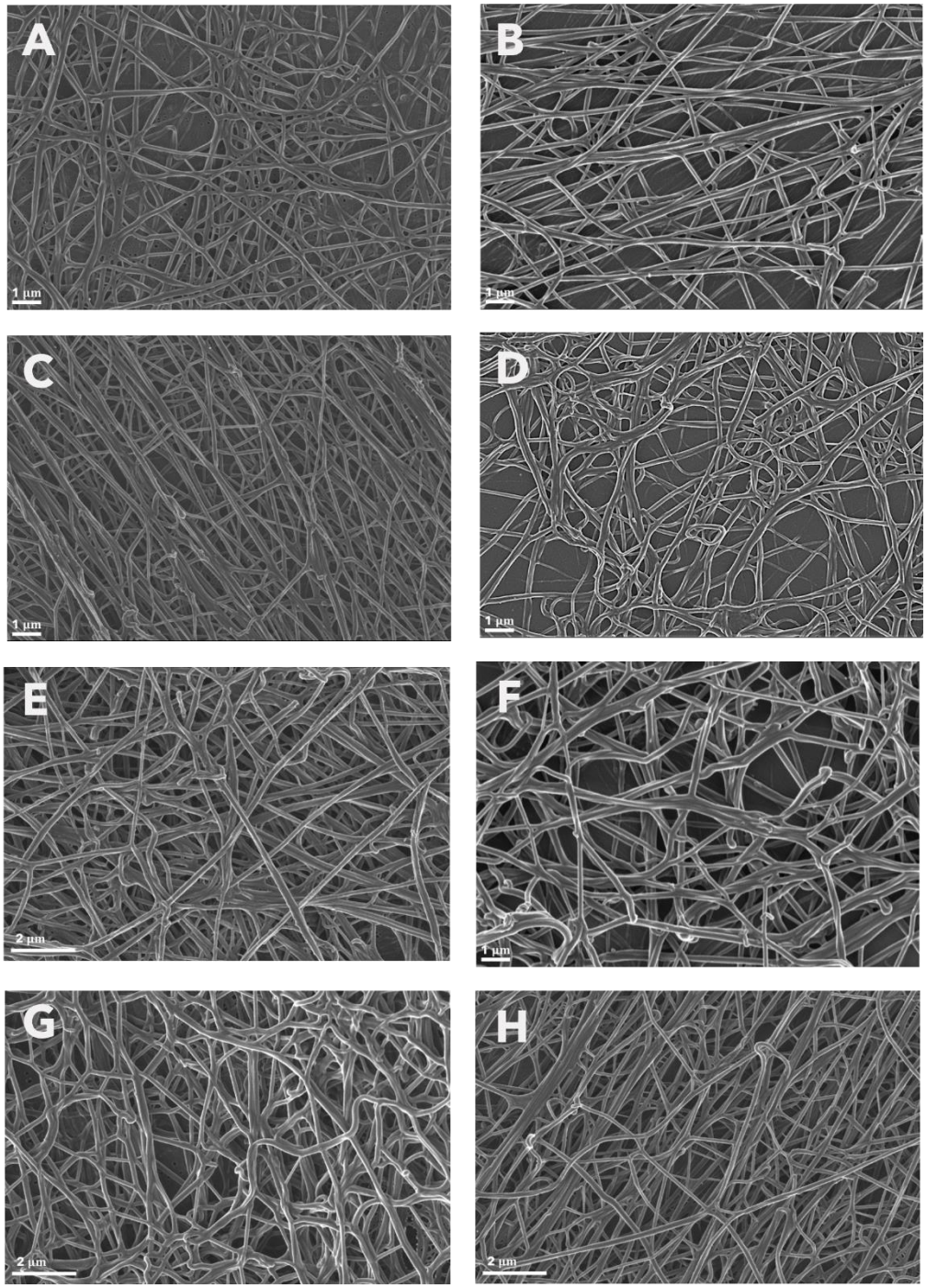

Figure 7: Scanning electron microscope images of controls on high magnification: Images A-H show the control group's fibrin fibres with normal branching at 20k magnification.

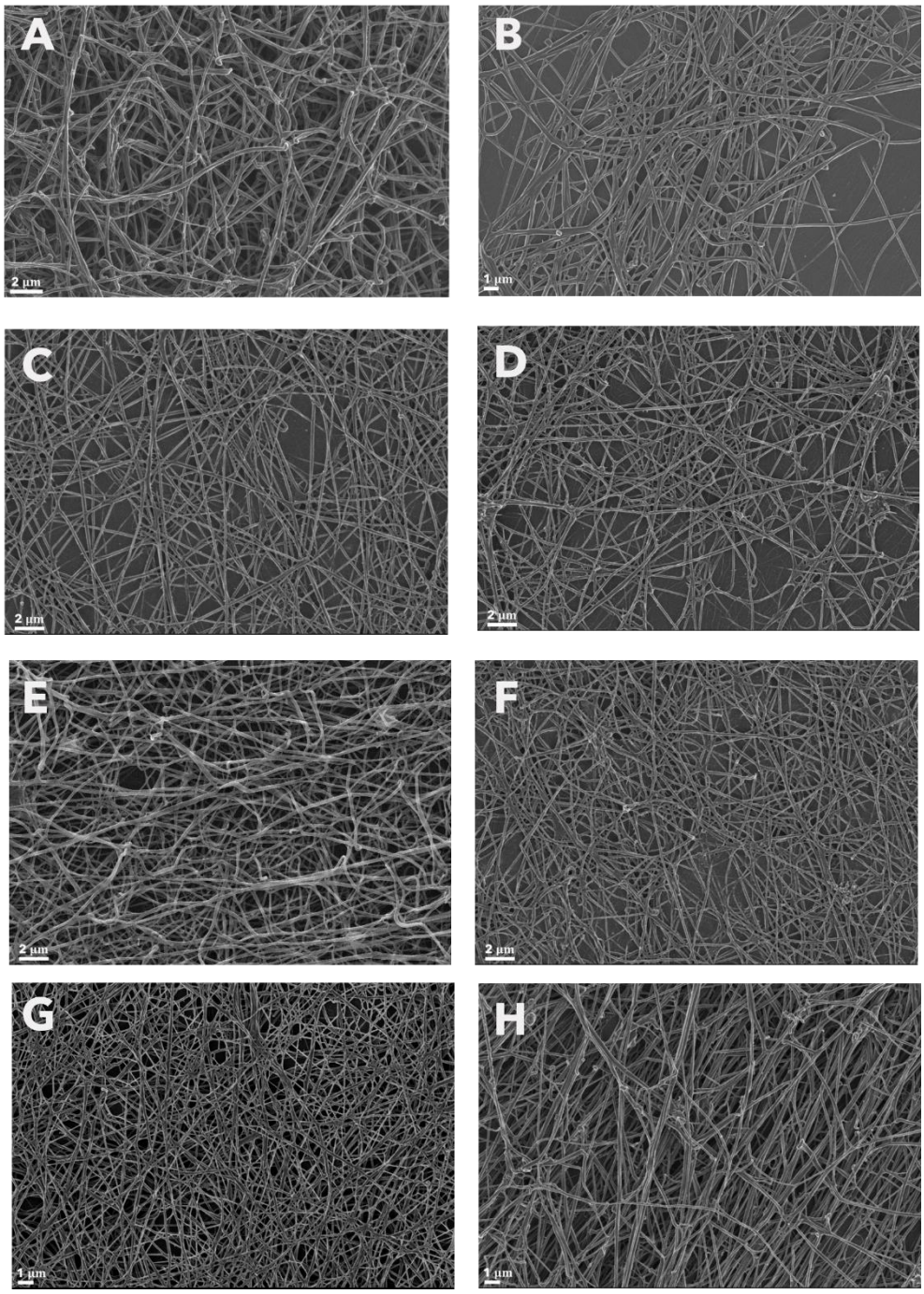

Figure 8: Scanning electron microscope images of brain cancer patient group on low magnification: Images A-H show the fibrin fibres of brain cancer patients at 10k magnification. The fibrin fibres show some disorganised fibres in the network of fibres with noticeable tangling and fusion of fibres.

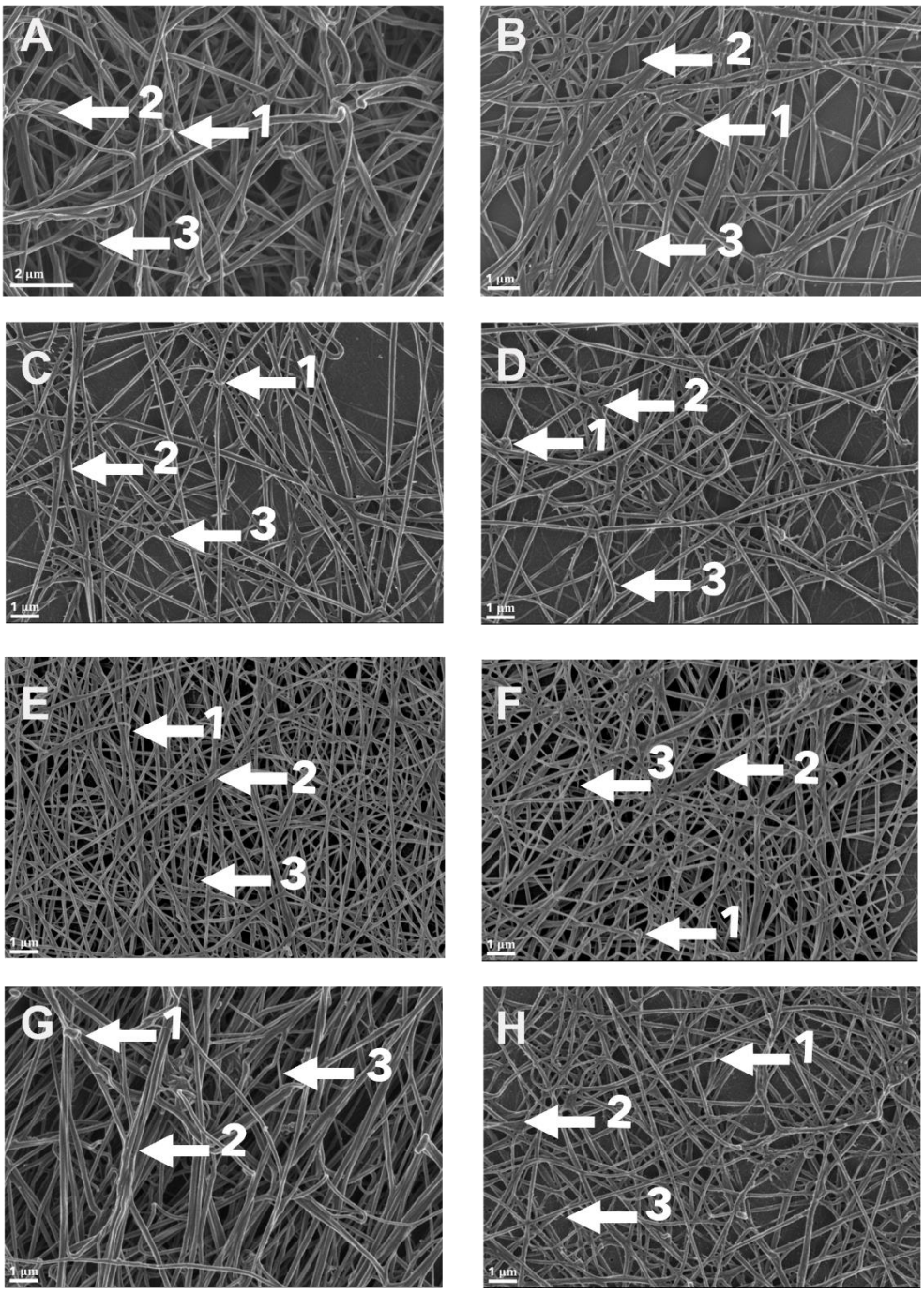

Figure 9: Scanning electron microscope images of brain cancer patient group on high magnification: Images A-H show fibrin fibres of brain cancer patients at 20k magnification. Arrows labelled as 1 show tangles in the fibres, arrows labelled as 2 show fusion of fibres, and arrows labelled as 3 show thinner fibres connecting to thicker fibre branches. The fibres here show more tightly cross-linked fibres.

5.5.2 ImageJ and Fractalyse

Table 7 shows the results of the fibre thickness and branching analysis done on the SEM images. These results reveal that brain cancer patients had thinner fibrin fibres than healthier individuals. This disparity was found to be significantly different.

The fibre branching for both groups proved similar as the p-value showed no significant difference between the healthy individuals and the patient group.

Table 7: ImageJ and Fractalyse results

Parameters		Control (n=21)	Brain cancer (n=24)
Thickness	**Median (min, max)**	144 (105, 208)	129 (92, 168)
	P-value	0.0026	
	Significant difference	**Yes**	
Fractal dimensions	**Median (min, max)**	2.6 (2.32, 2.65)	2.61 (2.51, 2.72)
	P-value	0.5076	
	Significant difference	No	

5.6 Discussion

When comparing Figures 6 and 7 with Figures 8 and 9, it became evident that there is a more noticeable fusion and tangling of the fibres in the brain cancer patients compared to the controls.

At increased magnification (refer to Figures 7 and 9), distinctions in cross-linking are apparent. In the control group, the fibres are more organised with less tight cross-linking. Conversely, in the patient group, the fibres appeared to have a tighter cross-linking.

Fibrin fibres are filamentous protein polymers that are highly resistant to shearing and tension.[95] However, on the rare occasions when these fibres deform, they congeal together, causing the fusions depicted in Figures 6-9, more noticeably in Figures 8 and 9.[112] This phenomenon is more noticeable in patients with coagulopathies.[107, 113] In the research done by Goncalves et al., the SEM analysis displayed significant fusion in fibrin fibres, indicating that these morphological changes have previously been seen in non-metastatic cancer studies.[97]

The coagulation factor XIIIa, activated by thrombin, plays an important role in stabilizing fibrin fibres.[114] The presence of this coagulation factor actually induces the random assembling of fibres, as opposed to organised bunching, resulting in a tangled affect.[114] Additionally, it has been shown that the presence of factor XIIIa reduces the average diameter of fibrin fibres. This reduction enhances resistance to fibrinolysis, thereby promoting fibrin stability.[115]

In Figures 8 and 9, thinner fibres were visible, which corresponds with the results concerning fibre thickness obtained through ImageJ software analysis. These results confirmed that the patients in this study have thinner fibrin fibres than the healthy individuals.

This data suggests that although TEG® analysis indicates clots from brain cancer patients possess a similar strength to those of healthy individuals, the increased prevalence of thinner fibres may result in slower fibrin degradation. Consequently, this structural difference could increase the risk of developing complications like thromboembolisms.[16, 17] Thinner fibres are known to diminish plasminogen activation stimuli, possess higher bond densities, and form more tightly cross-linked structures, collectively contributing to reduced fibrinolysis and slower clot lysis rates.[16, 17, 108]

Cancer is known to stimulate the coagulation system in order to promote stronger, more persistent clots.[10] This stimulation often involves altering fibrinolytic activity, usually through the alteration of plasminogen-related protein levels.[3, 10] An example of this is the action of the PAI-1 protein, which inhibits plasminogen activators, thereby inhibiting fibrinolysis.[3] Given that cancer is associated with chronic inflammation, the observed elevation in CRP levels suggests that an increase in PAI-1 levels is likely.[7, 18]

Overall, this causes hypo-fibrinolysis, a condition that may not be detected by the TEG®, which in this study was primarily focused on clot formation rather than lysis.[3] It is also possible that differences in the formation of clots remained undetected due to the use of PPP instead of whole blood, as was described in chapter 4 of this dissertation. The observation of thinner fibres could be attributed to alterations in the plasminogen pathway, a phenomenon also found in a breast cancer study.[116]

The fractal dimensions of the two groups shown to be identical, indicating that their fibres branched in similar ways. Normally, it is expected that thinner fibres would result

in denser fibre networks accompanied by additional branching.[117-119] However, the studies underpinning these expectations primarily focused on late-stage cancer patients and on patients with ischemic stroke.[118, 119] These conditions differ considerably from early stage non-metastatic brain cancer. The SEM images confirm increased tight cross-linking, but not necessarily increased branching as expected. Further research is needed to clarify this.

5.7 Conclusion

The study's morphological observations show a slight increase in tangling, fusion, and cross-linking in brain cancer patients. More significantly, they show that brain cancer patients exhibit thinner fibrin fibres, which may have implications for both clotting and the lysis of clots. Thinner fibres contribute to a decline in clot lysis time in patients.

This may contribute to hyper-coagulopathies in brain cancer patients, which needs to be considered in their treatment planning. Consequently, it is suggested that factor XIIIa and PAI-1 need to be monitored and studied in patients with non-metastatic brain cancer. Understanding their effect on coagulation could provide insights for possible treatment management.

All patients included in this study were selected based on the absence of co-morbidities that have a major impact on coagulation. Therefore, it should be taken into consideration at diagnosis that comorbidities may worsen the current state of coagulopathy.

Chapter 6: Discussion and conclusion

6.1 Conclusion

Patients with brain cancer commonly experience coagulation-related complications that predispose them to thrombotic events. Approximately one out of every five individuals with brain cancer experience such complications, which, if inadequately managed during treatment, can contribute to elevated mortality rates. This study aimed to investigate hypercoagulability in brain cancer patients in comparison to healthy individuals by studying the morphological and viscoelastic properties of PPP, with a specific focus on fibrin.

Previous literature has indicated that non-metastatic cancers show significantly less coagulopathies pre-operatively than metastatic cancers.[4, 10] Non-metastatic cancer does not invade surrounding tissues or distant organs.[28] It typically grows slowly and maintains a localized, non-invasive nature.[19, 20] As a result, it is less likely to cause vascular damage, angiogenesis, or release procoagulant substances that are often associated with metastatic cancers.

However, findings specific to non-metastatic brain cancer have shown an impact on secondary haemostasis, explicitly fibrinolysis.[3, 15] In these findings, the plasminogen activators in the coagulation pathway were inhibited by the PAI-1 proteins induced by the cancer inflammation.[3, 15] This system keeps clots from being lysed and causes a condition called hypo-fibrinolysis.

The overall findings of this study are described in Table 8.

Table 8: Results summary

Method	Control	Brain cancer
Clinical tests	-	Normal INR and PCT levels. Elevated CRP levels.
TEG®	Normal	Normal
SEM	Normal, organised network of fibres. Showed some tangling and fusion of fibres. Normal cross-linking of fibres.	More pronounced tangling, fusion, and tight cross-linking of fibres. Less organised network of fibres with thinner fibres visible, specifically between thicker fibre branches.
ImageJ	Normal thickness	Thinner fibres
Fractalyse	Normal	Normal

C-reactive Protein, an inflammatory marker that was elevated in the brain cancer patients of this study, is also known to stimulate the production of PAI-1 and activate platelets.[18] The process of centrifugation, which removes platelets and other blood components from whole blood to produce PPP, might obscure potential abnormalities in clot formation that could be influenced by inflammation. Regarding this, the findings of this study revealed no discernible differences in the viscoelastic properties of PPP between healthy individuals and those with brain cancer. This absence of differences may be due to the use of PPP, suggesting that the findings do not entirely rule out the possibility of abnormal clot formation.

On a morphological level, the patient group showed slightly more tangling and fusion of fibres, suggesting the potential presence of coagulopathy and likely an elevation in factor XIIIa levels, which have shown to increase tangling in fibres.[107, 113, 114]

Another ultrastructural and quantitative finding is that the plasma from brain cancer patients exhibits thinner fibrin fibres. Thinner fibres are associated with an increase in tight cross-linking between fibres, which was evident in the ultrastructural images taken in this study. Along with the factor XIIIa, this result could be a consequence of the elevated PAI-1 levels, both of which correlate with thinner fibres.[16, 17, 115] Furthermore, elevated PAI-1 levels result in inhibited plasmin activators, leading to reduced levels of plasmin for fibrinolysis.[3]

Thinner fibres exhibit a reduced rate of dissolution compared to thicker fibres, resulting in the persistence of clots and, consequently, increasing the patient's susceptibility to thrombotic events. All participants included in this study were selected based on the absence of co-morbidities known to substantially influence coagulation. Hence, it is crucial to recognize at the time of diagnosis that the presence of comorbidities such as diabetes, heart disease, rheumatoid arthritis, and other coagulation disorders could exacerbate the existing state of coagulopathy. This needs to be taken into consideration when treating patients with brain cancer.

Currently, studies on treatment for hypo-fibrinolysis have mostly focused on molecular-level interventions in patients with osteonecrosis, where this type of coagulopathy is prevalent.[120-122] In these studies, the treatment often centres around anti-coagulants. However, the use of anti-coagulants may not be viable for all brain cancer patients,

especially considering the surgical and other treatment requirements that their condition necessitates.[120-122]

6.2 Future studies

Future studies can:

- Repeat these methods on patients with metastatic brain cancer and other cancer types to gain a holistic view of how cancer effects and is affected by the coagulation pathway, specifically the fibrin networks.
- Conduct molecular studies on CRP, factor XIIIa, and plasminogen inhibitors (such as PAI-1) to determine which specific proteins play a part in the coagulopathies in brain cancer patients.
- Conduct molecular studies using a secondary common marker after CRP, such as IL-6 or IL-10.
- Focus on brain cancer patients who smoke in order to see whether smoking might influence the results of this study in order to help future studies determine their exclusion criteria.
- Repeat these methods with more patients to see if different results arise from a greater sample population.
- Investigate the branching of fibrin fibres in non-metastatic cancer patients in more detail using additional types of microscopies such as a transmission electron microscope or atomic force microscope.

6.3 Study limitations

This study faced several limitations, including a limited sample population, which was influenced by tight time constraints, stringent exclusion criteria, and the challenge of securing willing patients to participate in the study.

Additionally, the study encountered missing data issues, attributable to equipment errors or insufficient sample volumes for analysis.

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Appendix A

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**

10 August 2022

Approval Certificate
New Application

Dear Ms LC Rademeyer

Ethics Reference No.: 253/2022
Title: An ex vivo study on the hypercoagulability of brain cancer patients at an academic hospital by studying the morphological and viscoelastic properties of platelet-poor plasma

The **New Application** as supported by documents received between 2022-04-28 and 2022-08-10 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-08-10 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 2023-08-10.
- Please remember to use your protocol number (253/2022) 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)

Research Ethics Committee
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University of Pretoria, Private Bag x323
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

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**

13 July 2023

Approval Certificate
Annual Renewal

Dear Ms LC Rademeyer,

Ethics Reference No.: 253/2022 – Line 1
Title: An ex vivo study on the hypercoagulability of brain cancer patients at an academic hospital by studying the morphological and viscoelastic properties of platelet-poor plasma

The **Annual Renewal** as supported by documents received between 2023-06-22 and 2023-07-12 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-07-12 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 2024-07-13.
- Please remember to use your protocol number (253/2022) 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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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. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

Faculty of Health Sciences **Research Ethics Committee**

11 April 2024

Acknowledgement Certificate
Research Completed or Terminated

Dear Ms LC Rademeyer,

Ethics Reference No.: 253/2022 – Line 2
Title: An ex vivo study on the hypercoagulability of brain cancer patients at an academic hospital by studying the morphological and viscoelastic properties of platelet-poor plasma

The **Research Completed Report** as supported by documents received between 2024-03-12 and 2024-04-10 for your research, was acknowledged by the Faculty of Health Sciences Research Ethics Committee on 2024-04-10 as resolved by its quorate meeting.

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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Appendix B

Participant Questionnaire

Date Captured: DD / MM / YYYY	Allocated Study ID (e.g. C1): *(Will be allocated by Investigator)*
Personal Information	
Hospital Number:	
Age:	Gender:
Type of cancer and stage:	
Investigations still to be done (indicate dates):	
Treatment received for Brain Cancer (current and previous e.g. surgery, radiation):	
Medical Information & History	
Do you smoke tobacco or any related product?	☐ Yes ☐ No
Would you say that you have consumed 5/more drinks on the same occasion in the past 30 days?	☐ Yes ☐ No
Do you have or have you ever had any of the following conditions?	
☐ Diabetes ☐ High Blood Pressure ☐ Heart Problems ☐ Heart attacks ☐ Inflammatory conditions	☐ Stroke ☐ Arthritis ☐ Other *Specify:* _____ ☐ Allergies *Specify:* _____
HIV Status:	☐Positive ☐Negative ☐Unknown
Medication history:	
☐Yes ☐No Are you taking any chronic medication? *Specify:* _____ *Have you taken any of the following within the last 2 weeks?*	

☐Yes	☐No	Vitamin-supplements
☐Yes	☐No	Corticosteroids
☐Yes	☐No	Anti-inflammatories
☐Yes	☐No	Anti-coagulative
		Other: _____

Participant information leaflet and informed consent form

Study Title An *ex vivo* study on the hypercoagulability of patients with neurosurgical conditions (such as brain cancer) at an academic hospital by studying the morphological and viscoelastic properties of PPP

Sponsor: NRF Y-rated funding

Principal Investigator: Miss. Lilu Rademeyer

Department of Physiology, University of Pretoria

082 356 8740

Ethical clearance number: 253/2022

Date and time of first informed consent discussion: ……

Date and time

Dear prospective participant

Dear .....

You are invited to participate in a laboratory-based research study conducted by the Department of Physiology (School of Medicine, Faculty of Health Sciences) at the University of Pretoria. The information in this document is 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 investigator. You should not agree to take part unless you are completely happy about all the procedures involved. It is strongly recommended that you inform your personal doctor of your participation in this study, wherever possible.

Individuals matching the following criteria do not meet the criteria to participate in the study: Smokers or users of any tobacco or related products, users of chronic medication, anyone with a condition which could present with chronic inflammation, anyone with a history of an immune-compromised status, anyone having used herbal or vitamin supplements, corticosteroids, anti-inflammatory, anti-platelet, or anti-coagulative medication within two weeks prior to blood sample collection date (such as aspirin or warfarin), anyone with an HIV positive, anyone who excessively consumes alcohol (drinking five or more drinks on the same occasion on at least one day in the past 30 days), diagnosed with metastases, sarcoma of the skull; cancers of ciliated squamous or columnar ependymal cells of the ventricles, anyone with an autoimmune or viral disease, anyone with a disease affecting the blood circulation and the coagulation system. After informed consent has been provided a questionnaire will be done to ensure that potential participants that do not qualify for inclusion in the study do not have to undergo any clinical tests.

2) The nature and purpose of this study

The researcher is investigating increased clot formation in patients with neurosurgical conditions compared to individuals without them. This will allow us to understand if patients with neurosurgical conditions in South Africa have changed blood clotting properties. To do this research, we will use a specialized microscope (scanning electron microscope) to look at the structure of the blood components; as well as equipment that tests blood clotting properties (called a thromboelastograph and turbidimetry) to determine the degree to which clotting is changed in the blood. Fibrinogen and

cotinine levels will be measured using a specialized method. The fibrinogen levels will be used to determine the amount of clotting protein in the blood. The cotinine levels will allow us to see whether you (the participant) use tobacco-related products. If this test is positive, you will be excluded from this research study, and your blood sample will be disposed of. We will collect data from routine testing to determine the time it takes for blood to clot by determining the international normalised ratio. We will be determining inflammation status by running C-reactive protein and procalcitonin tests. We will compare the results to those of participants who do not have neurosurgical conditions.

3) Explanation of procedure and what will be expected from participants.

This study involves answering some questions with regards to your health and any illnesses and taking some blood samples.

One tube of blood will be drawn by a qualified doctor into a citrate tube, each containing 4 mL of blood or the equivalent of one teaspoon.

Samples will be analysed within four hours of collection and will be used for the Thromboelastography and Scanning electron microscopy preparation. The blood (2 mL) will then be frozen at -80ºC until all sample recruitment is completed and the samples will then be used for the turbidimetry and the ELISA assays (fibrinogen and cotinine levels). From the routine blood tests done at the hospital, we will request the international normalised ratio (INR), C-protein (CRP) and Procalcitonin tests (PCT) data. The blood collection process will only be done once, and no follow-up tests will be required.

4) Additional uses for samples

The samples that you give to this study could one day lead to discoveries using methods such as assessment of metabolomics using nuclear magnetic resonance (NMR) spectroscopy and epigenetic changes using quantitative polymerase chain reaction (qPCR). 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. This research could potentially be used for up to 5 years after collection. Ethical approval will be obtained before any further testing on the residual samples will be done.

You have the right to withdraw your consent at any time and may request that the samples you give to the study be destroyed. If you choose to do so, contact the study principal investigator, Lilu Rademeyer. 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.

5) Possible risks and discomforts involved.

The only possible risk and discomfort involved is the taking of blood from a vein which can result in bruising and bleeding and less common infection and bleeding from the puncture site. For your protection, the procedures will be done under sterile conditions by a qualified doctor.

6) Possible benefits of this study.

Although you may not benefit directly, the study results may help us to improve the treatment and understanding of neurosurgical conditions in the future. Many of these tests are done routinely on patients and we will be able to treat you, should you have any problems.

7) Compensation

You will not be paid to take part in the study. There are no costs to you to participate in this study.

8) If you do not want to participate in this study, you will still receive standard treatment for your illness.

9) Your rights as a research participant.

Your participation in this trial is entirely voluntary and you may refuse to participate or stop at any time without stating any reason. Your withdrawal will not affect your treatment.

10) Ethics approval.

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, 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 human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

11) Information

If you have any questions concerning this study, please contact Miss Lilu Rademeyer tel: 082 356 8740.

12) Confidentiality

All information obtained during the course of this study will be regarded as confidential. Each participant that is taking part will be provided with an alphanumeric coded number e.g. A001. This will ensure the confidentiality of information so collected. Only the researcher will be able to identify you as a participant. Results will be published or presented in such a fashion that participants remain unidentifiable. The hard copies of all your records will be kept in a locked facility at the Department of Physiology at The University of Pretoria**.**

13) Consent to participate in this study

- **I have received, read, or have had read to me in a language I understand and understood the above-written information about the study, before signing consent.**
- **I have had adequate time to ask questions and I have no objections to participating 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 penalized in any way should I wish to discontinue the study and that withdrawal will not affect my further treatments.**
- **I am participating willingly.**

……. ……..

Participant's name and signature Date

……. ……..

Investigators name and signature. Date

……. ……..

Witness name and signature. Date

Participant code…….

Verbal participant informed consent

(Applicable when participants cannot read or write)

I, the undersigned, ……..…have read and have explained fully to the participant, named …….. and/or his/her relative, the participant information leaflet, which has indicated 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 and the alternative treatments available for his illness. 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 treatment.

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

…….. ……..

Participant's name and signature Date

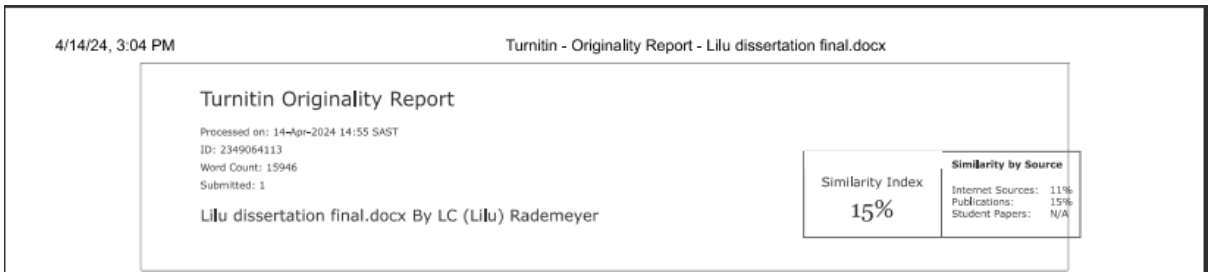
…….. ……..

Investigators name and signature. Date

…….. ……..

Doctor/-Witness name and signature. Date

Appendix C



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Appendix D

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences
Department of Immunology

Letter of Statistical Clearance

Monday, March 28, 2022

This letter is to confirm that the MSc student with the Name: **LC Rademeyer**, Student No: **17014222** studying at the University of Pretoria discussed the project with the title; ***An ex vivo study on coagulopathies in brain tumours using platelet-poor plasma***, with me.

I hereby confirm that I am aware of the project that the statistical analysis and sample size described and the data generated for the project is appropriate for achieving the research aims.

Yours sincerely

Prof Pieter WA Meyer

Ass. Professor and HoD

Prof PWA Meyer
Head of Department: Immunology
ResCom appointed Biostatistician
University Pretoria

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