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Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

By

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Abstract

Intracerebral haemorrhage (ICH) is a major neurological condition that affects individuals of all age groups, gender, economic status and geographical location. Compared to healthy individuals, patients with ICH have an increased risk of coagulation complications. Previous studies have conducted research on the effects of ICH on the haemorheological, morphological and inflammatory properties of whole blood (WB) in patients with ICH. In contrast, there has been limited research on the properties of platelet-poor plasma (PPP) changes in ICH patients. Therefore, this study aims to investigate the ultrastructural and viscoelastic properties in PPP of ICH patients to identify any coagulation changes that might be linked to the complications in ICH. This will allow for a better understanding of thrombosis management and risk assessment. The study compared the PPP of spontaneous ICH (S-ICH) and traumatic ICH (T-ICH) patients to healthy controls. The methods used include scanning electron microscopy (SEM), which provided morphological data on the fibrin fibres, focusing on the fibres' thickness, network and branching. The kinetics of clot formation were investigated using thromboelastography® (TEG®). The C-reactive protein (CRP) and procalcitonin (PCT) markers from ICH patients were used to determine inflammation status in these patient groups. Both ICH patient groups demonstrated a tendency towards inflammation based on the clinical data. Clot strength and rigidity increase revealed a hypercoagulable state in both ICH patient groups based on the TEG® results. Based on the SEM results the S-ICH group displayed thinner fibrin fibres, with a tighter network. While the T-ICH group displayed thicker fibrin fibres and looser networks. Overall, these results revealed that there are differences in the blood components and coagulation in ICH patients compared to healthy controls. The findings, which are based on viscoelastic techniques, help to understand the clot dynamics that may increase the risk of thrombotic events. The results reinforce the necessity of conducting additional research into the role of ICH in relation to the characteristics of PPP among patients with ICH. The overall results of this study could assist in preserving the lives of patients suffering from ICH and also ensure the maintenance of their quality of life.

Keywords: Coagulation, Fibrin, Intracerebral haemorrhage, Scanning Electron Microscopy, Thromboelastography®

Declaration

I, Andrea Lenting, hereby declare that this research dissertation, “Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma” is my original work and where other people’s work has been used, I have properly acknowledged and referenced in accordance with the requirements as stated in the University's plagiarism prevention policy.

I have not used another student’s past written work to hand in as my own.

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Herewith I submit this thesis to the University of Pretoria for the degree Master’s in Science in Physiology.

Signature: 

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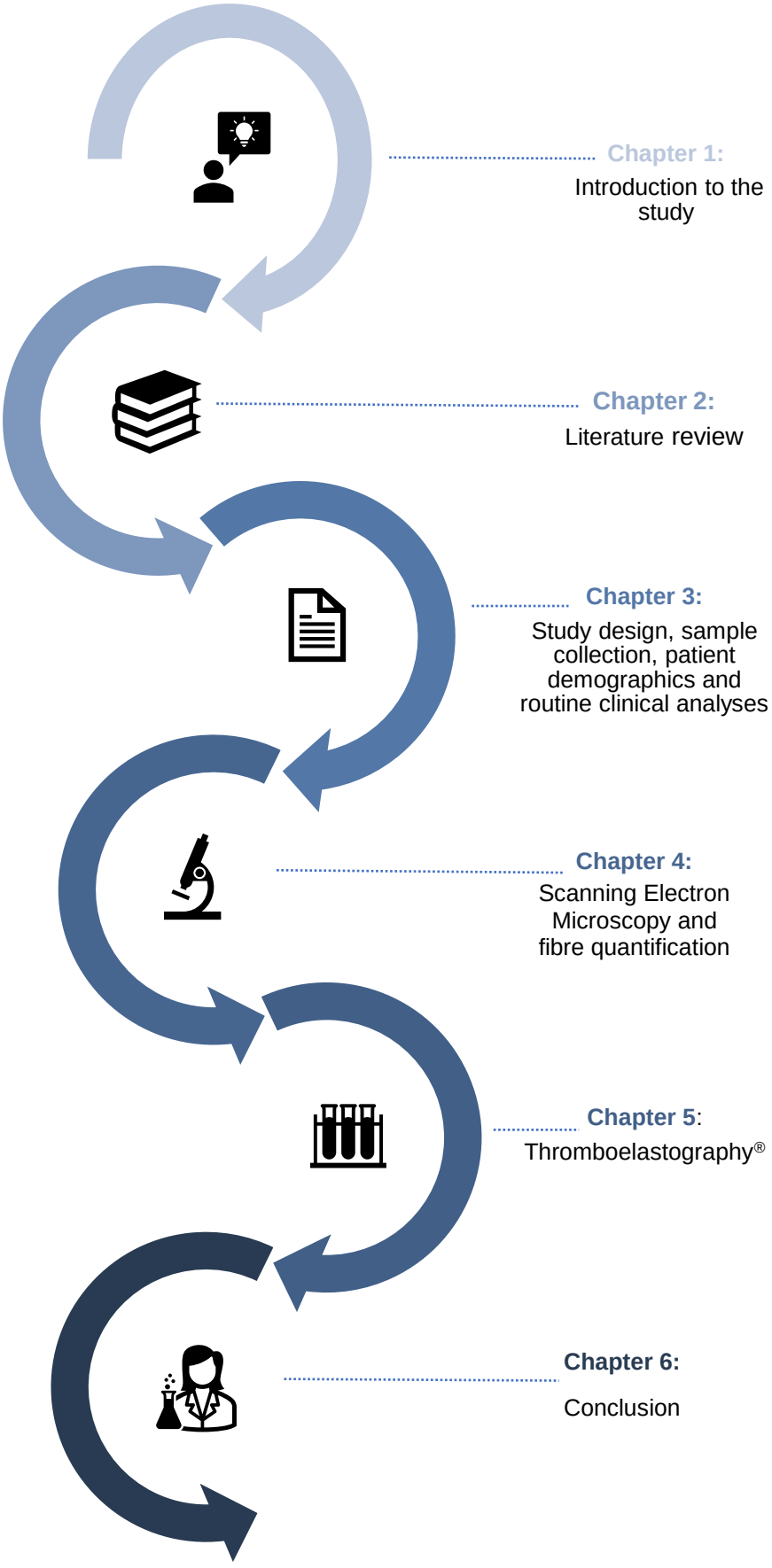
List of Abbreviations

A	α-angle
AVM's	Arteriovenous malformation
BBB	Blood-brain barrier
BMP	Bitmap
BP	Blood pressure
CO	Carbon monoxide
Cont	Controls
CRP	C-reactive protein
CT	Computed tomography
CVP	Central venous pressure
DVT	Deep vein thrombosis
EDH	Epidural haemorrhage
F	Female
Fe	Iron
FVII/FVIIa	Plasma factor VII/VIIa
FVIII	Factor VIII
G	Shear elastic modulus
GCS	Glasgow Coma Scale
HIV	Human immunodeficiency virus
HMDS	Hexamethyldisilazane
ICH	Intracerebral haemorrhage
ICU	Intensive care unit
INR	International normalised ratio
IPH	Intraparenchymal haemorrhage
IVH	Intraventricular haemorrhage
K	Kinetics
M	Male
MA	Maximum amplitude
Max	Maximum
Min	Minimum
MRI	Magnetic resonance imaging

MRTG	Maximum rate of thrombus generation
mTBI	Mild TBI
NHLS	National Health Laboratory Services
NHRD	National Health Research Database
NIHSS	National Institutes of Health Stroke Scale
ns	Not significant
NSAIDs	Nonsteroidal anti-inflammatory drug
OsO4	Osmium tetroxide
PAR's	protease-activated receptor
PBS	phosphate-buffered saline
PCT	Procalcitonin
POC	Point of care
PPE	Personal Protective Equipment
PPP	Platelet-poor plasma
R	Reaction time
ROS	Reactive oxygen species
SAH	Subarachnoid haemorrhage
SAP	Systolic arterial pressure
SBAH	Steve Biko Academic Hospital
SDH	Subdural haemorrhage
SDH	Standard deviation
SEM	Scanning electron microscopy
S-ICH	Spontaneous intracerebral haemorrhage
TBI	Traumatic Brain Injury
TEG®	Thromboelastography®
TF	Tissue factor
TIC	Trauma-Induced Coagulopathy
T-ICH	Traumatic intracerebral haemorrhage
TMRTG	Time to maximum rate of thrombus generation
TNF-α	Tumour necrosis factor-alpha
TTG	Total thrombus generation
UP	University of Pretoria

VKA	Vitamin K antagonist
VTE	Venous thromboembolism
VWD	Von Willebrand disease
WB	Whole blood

Dissertation site map



Chapter 1: Introduction

Intracerebral haemorrhage (ICH) is a neurological disease due to ruptures of blood vessels in the brain.[1-3] The cerebral blood vessels are susceptible to damage from spontaneous vessel rupture and traumatic events. The distinction between S-ICH and T-ICH is crucial because of the variations in the sources of bleeding.[4] Traumatic brain injury (TBI) is described as brain damage brought on by an external impact, such as a blast wave or an object penetrating the brain. Vehicle accidents, blast exposures, falls, and sports-related injuries are the leading causes of TBI.[5] There are two causes for S-ICH: primary and secondary. Amyloid angiopathy and the spontaneous rupture of intracranial small arteries harmed by hypertension are the leading causes of ICH.[6] Underlying coagulopathies, vasculitis, iatrogenic, substance abuse, cancers, and vascular anomalies are associated with secondary ICH.[6] Primary factors account for the majority of S-ICH cases.[6]

The incidence and case fatality rates of S-ICH, which makes up 10% to 40% of all cerebrovascular disorders globally, are higher in low- and middle-income nations in Asia and Africa.[7, 8] It has been reported by Kamolafe et al.[7], that the 30-day mortality rate for ICH is 40%, and that 55% of survivors of ICH recovered with functional dependence.[7] The clinical manifestation of ICH can have severe consequences. Risk stratification scores based on the Glasgow Coma Scale (GCS), bleed volume, and hydrocephalus have been associated with mortality.[7] Assessments of the functional outcome of ICH patients in various populations outside of Africa have been conducted.[7]

It can be challenging to differentiate ICH from acute ischaemic stroke, although some characteristics point to the diagnosis: headache, vomiting, seizures, rapidly worsening neurological symptoms and decreased consciousness that is frequently out of proportion to focal deficits.[9] Therefore, neuroimaging is essential for diagnosing a patient and considering the underlying aetiology.[9, 10] Although it is also useful for ischemic stroke, the National Institutes of Health Stroke Scale (NIHSS) may not be as valuable in ICH due to the higher prevalence of depressed consciousness in ICH. Due to its prognostic value being similar to that of the NIHSS, the GCS score is the most useful tool for initial evaluation.[11] Non-contrast Computed tomography (CT) is a

valuable technique for verifying the clinical manifestation of acute stroke and determining the extent and location of all kinds of ICH.[9]

During the first few hours, ICH-related brain damage first manifests as a mass effect brought on by haemorrhage formation.[12] An inflammatory response is frequently observed at the site of an intracerebral hematoma after ICH.[13] Systemic inflammatory response syndrome may be an indication that the inflammatory processes that cause tissue damage around the hematoma have been activated; this damage could have an impact on the hematoma's growth.[14] Local inflammation caused by ICH causes the blood-brain barrier (BBB) to break down, allowing circulatory inflammatory cells to enter.[14]

Both hypercoagulability and hypocoagulability result from haemostasis dysregulation brought on by traumatic brain injury.[15] A hypercoagulable TEG® profile has been linked to all subtypes of acute stroke, including ischemic stroke, ICH, and subarachnoid haemorrhage, when compared to controls.[16] Research indicates a late propensity to clot formation follows an early tendency to bleed.[15] To better understand this intricate coagulation disturbance and its implications for the treatment of patients with ICH, further research is recommended.[16]

The aim of the study was to investigate clot formation in ICH patients by examining both the morphological and viscoelastic features of PPP in these patients. The results of CRP and PCT tests results were taken from hospital records to evaluate each patient's inflammatory status. To assess their profiles, normal reference ranges were used to compare the results. Scanning electron microscopy was used to compare the fine ultrastructural characteristics of fibrin fibres in ICH patients to those in healthy controls. ImageJ was utilised to measure the fibrin fibre thickness of ICH patients compared to healthy controls. Compared to healthy controls, the branching of fibrin fibres in ICH patients was examined using the Fractal analysis system. The clot kinetics in ICH patients and controls were investigated using TEG®. The results from the inflammatory markers revealed that the CRP and PCT parameters were not within the normal reference ranges. This indicated an increased inflammatory status in ICH patients. The SEM results of the S-ICH group revealed modest changes in the fibrin fibres; some areas had thinner fibres that were fused, and tightly packed, and other areas were standard in structure. In the T-ICH group, SEM results displayed standard

fibrin networks in some areas and thick, fused, and loosely packed structures in others. Significance was observed in the T-ICH patient group, compared to the control group for the branching of the fibrin fibres. However, no significance was observed in the S-ICH patient group compared to the control group for the branching. The TEG® technique was used to analyse dynamic changes in the patient groups to identify a clot profile and it was used to confirm the hypercoagulable state in ICH patients. The maximum amplitude (MA), shear elastic modulus (G), maximum rate of thrombus generation (MRTG), and total thrombus generation (TTG) parameters were all significantly different and increased in the S-ICH patient group. In the T-ICH patient group, an increase and significant difference was observed in the MA, MRTG and TTG parameters. When combined, the results of these methods have improved our understanding of clot dynamics and changes in its components in the hypercoagulable state, particularly in patients with ICH.

Using PPP, the study's findings were used to identify and describe some of the behaviours of the important parameters in the advancement of hypercoagulability in ICH patients. The objective is to gain insights into the coagulation process and potentially aid medical professionals in formulating management strategies and treatment protocols to improve the quality of life for patients with ICH. Most studies done on ICH patients use only WB to identify coagulation abnormalities. Therefore, this study contributed to the current body of knowledge by deepening our knowledge on the morphological and viscoelastic properties of PPP in ICH patients. Further research should incorporate studies on PPP and the biochemical quantitative and qualitative analysis in ICH patients.

Chapter 2: Literature Review

2.1 Chapter objectives

To provide context for the current topic, a review of recent literature was included in this chapter.

2.2 Intracerebral haemorrhage

2.2.1 Introduction to Intracerebral haemorrhage

Intracerebral haemorrhage is among the most prevalent and destructive forms of acute stroke.[17, 18] A localised haemorrhage in the brain parenchyma and related compression of brain tissue are the outcomes of several pathophysiological processes that cause bleeding within the cranial vault due to blood vessel rupture.[17, 18] The two leading causes of spontaneous ICH are chronic hypertension and cerebral amyloid angiopathy.[17] Examples of secondary aetiologies include haemorrhagic transformation from ischemic stroke or venous thrombosis, and bleeding from visualised vasculopathy or tumours.[17] Illicit drug use, coagulopathy, and platelet dysfunction can all exacerbate ICH. Neurological function is lost as a result of a haemorrhagic stroke brought on by a vessel rupture.[19]

In sub-Saharan Africa, the burden of neurological diseases has increased over the years.[20] According to estimates, stroke has a 316 per 100,000 annual incidence, a 315 per 100,000 prevalence, and an 84% three-year fatality rate in Africa.[20] Interpersonal violence accounts for a large portion of the persistently high rate of trauma in South Africa, a low- to middle-income nation with notable disparities in wealth and access to healthcare.[21] According to estimates of the global burden of disease, with representation from Africa, roughly 50% of ICH cases occurred in people under the age of 65.[22] Based on studies done by Sarfo et al.[22], nearly 75% of patients with ICH were younger than 65 years. Based on research done by Maina et al.[23], it is anticipated that the number of TBI cases in Africa will increase to between 6 and 14 million cases per year by 2050. Similarly, it is anticipated that the prevalence of stroke will rise in Africa in comparison to the majority of non-African nations.[23, 24]

Acute ICH can present with both focal and nonfocal neurological signs and symptoms.[25] Decreased level of consciousness, headache, nausea and vomiting are nonfocal symptoms. Focal symptoms are dependent on the location of the ICH.

Headaches and vomiting are more common with ICH than with strokes.[26] Identifying the macrovascular causes in ICH is critical because they may have immediate therapeutic and prognostic effects.[27] Table 2.1 illustrates the risk factors for S-ICH. Given the link between ICH and previous stroke or transient ischemic attack, managing these risk factors, as well as other ischemic stroke risk factors, should be crucial to ICH prevention.[17]

Table 2.1: Risk Factors for Spontaneous ICH.[17]

ICH risk factors	
Modifiable	**Nonmodifiable**
Hypertension	Prior ICH
Coagulopathy (medication-related, acquired)	Advanced age
Current smoking	Male sex
Overconsumption of alcohol	Non-White ethnicity
Diabetes	Cerebral amyloid angiopathy
Illicit drugs	Chronic kidney disease
	Coagulopathy (congenital)
	Tumours
	Vascular lesions (both spontaneous and hereditary): aneurysms, AVM's, cavernous angiomas, Moyamoya disease or syndrome
Arteriovenous malformation (AVM's), intracerebral haemorrhage (ICH)	

Spontaneous ICH and T-ICH have different causes.[17] Therefore, it is important to differentiate between the two forms of ICH. A rupture of arteries in the brain causes non-traumatic ICH, also known as S-ICH.[22] Primary non-traumatic ICH and secondary non-traumatic ICH are the two categories of non-traumatic intracerebral haemorrhage.[4] Primary non-traumatic ICH occurs without a coagulation issue or vascular malformation. In contrast, Secondary non-traumatic ICH is brought on by vascular malformation.[4]

Trauma is one of the primary healthcare issues in modern societies.[28] Direct harm to the cerebral vasculature is one of the causative agents of TBI. Patients frequently experience haemorrhage, oedema, abnormal blood flow, and disruption of the BBB as early, immediate events.[5] There are three levels of TBI severity: mild, moderate, and severe. The most prevalent type of TBI, known as mild TBI (mTBI), causes no obvious changes in the brain. After mTBI, skull fractures, haemorrhage, and altered brain structures are uncommonly seen on CT scans and standard magnetic resonance

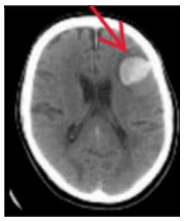
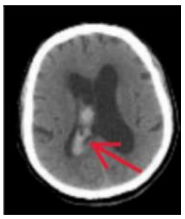
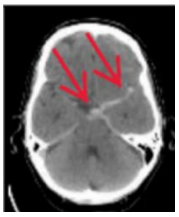
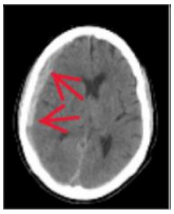
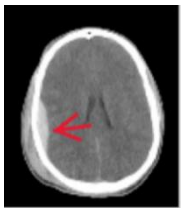
imaging (MRIs). Mild TBI frequently causes the patient to experience transient symptoms.[5] On the other hand, moderate and severe TBIs cause oedema and haemorrhage, among other obvious neuroimaging abnormalities. Haemorrhage is more common in moderate and severe injuries and accounts for 46% of all TBIs.[5]

2.2.2 Types of haemorrhages

In a study performed by Sarfo et al.[22], CT scan was used to confirm the diagnosis of all cases of ICH. Based on the epicentre of the bleed, the anatomic location was classified as non-lobar (basal ganglia, brainstem, cerebellum, thalamus) or lobar (frontal, occipital, parietal, temporal).[22]

Intraparenchymal haemorrhage (IPH), intraventricular haemorrhage (IVH), epidural haemorrhage (EDH), subdural haemorrhage (SDH), and subarachnoid haemorrhage (SAH) are the five main forms of bleeding.[29] The location of the bleeding, the mechanism causing the bleeding, the source and mode of the bleeding, and lastly, the symptoms connected to the bleeding all influence how different each type of bleeding is from the others.[30] A summary of the different types of haemorrhages and their peculiarities are shown in Table 2.2.

Table 2.2: Different types of haemorrhages and their peculiarities.[30]

	Intraparenchymal	Intraventricular	Subarachnoid	Subdural	Epidural
Location	Inside of the brain	Inside of the ventricle	Between the arachnoid and the pia mater	Between the dura and the arachnoid	Between the dura and the skull
Imaging					
Mechanism	High blood pressure, trauma, arteriovenous malformation, tumour, etc	Can be associated with both intraparenchymal and subarachnoid haemorrhages	Aneurysm rupture, trauma, or arteriovenous malformations	Trauma	Trauma or after surgery
Source	Arterial or venous	Arterial or venous	Predominantly arterial	Venous (bridging veins)	Arterial
Shape	Typically rounded	Conforms to ventricular shape	Tracks along the sulci and fissures	Crescent	Lentiform
Presentation	Acute (sudden onset of nausea, headache, vomiting)	Acute (sudden onset of nausea, headache, vomiting)	Acute (worst headache of life)	May be insidious (worsening headache)	Acute (altered mental status and skull fracture)

The primary focus of our research will continue to be intracerebral haemorrhages. The amount of the damage can be ascertained once the bleeding spot has been located. An underlying aetiology of ICH is frequently thought to be vascular risk factors.[9] However, the underlying pathological cause, like vascular malformation, amyloid angiopathy, or small vessel disease, is either poorly understood or goes unnoticed.[9] Table 2.3 summarises the underlying causes of ICH. The attending clinician must look into the cause of S-ICH, which is a heterogeneous condition. The most common causes are sporadic cerebral amyloid angiogenesis and deep perforating vasculopathy.[9]

Table 2.3: Indications of the underlying causes of intracerebral hemorrhage[9]

Aetiology	Main Feature
Deep perforating vasculopathy	Haematoma in the basal ganglia or brainstem; old intracerebral haemorrhage or microbleeds in the basal ganglia or brainstem; lacunes; white matter lesions

Cerebral amyloid angiopathy	Apolipoprotein E (ε4); cortico-subcortical microbleeds; cognitive decline; cortical superficial siderosis; lobar intracerebral haemorrhage; transient focal neurological episodes
Brain arteriovenous malformation	Calcification; flow voids; extension to other brain compartments;
Intracranial arterial aneurysm	Disproportionate subarachnoid extension
Cavernous malformation	Minor, homogeneous intracerebral haemorrhage with no extension to other brain compartments
Intracranial venous thrombosis	Headaches before the onset of intracerebral haemorrhage; high relative oedema volume; intracerebral haemorrhage near sinuses or veins; onset in pregnancy or postpartum
Dural arteriovenous fistula	Abnormal dilated cortical vessels; subarachnoid or subdural extension
Haemorrhagic transformation of cerebral infarction	Diffuse acute ischaemic lesions in other arterial territories or substantial areas of acute ischaemic lesions adjacent to the intracerebral haemorrhage
Severe clotting factor deficiency, such as haemophilia	Abnormal coagulation tests
Tumour (primary/metastasis)	Large perihematomal oedema
Vasculitis	Focal diffuse arterial stenosis; headaches; minor acute ischaemic lesions in numerous arterial territories
Infective endocarditis	Acute ischaemic lesions in different arterial territories; diffuse brain microbleeds; small irregular arterial aneurysms
Posterior reversible encephalopathy syndrome	Parietal and occipital asymmetrical oedematous lesions; thunderclap headaches

2.2.3 Diagnosis of intracerebral haemorrhage

When bleeding is detected early, the doctor can determine immediate treatment plans for the patient.[30] Magnetic resonance imaging or non-contrasted CT scans are recommended for the initial imaging test for ICH.[10] A CT scan's quick performance allows for prompt decision-making about possible thrombolysis and continuing care.[31] For acute blood disorders, non-contrast CT is extremely sensitive and specific. Magnetic resonance is more sensitive at identifying prior haemorrhages and is just as sensitive at detecting acute blood than non-contrast CT.[10] By measuring the exact amounts of perihematomal oedema and herniation, MRI can identify underlying

structural lesions.[26, 32] Cost and availability are the primary disadvantages of using MRI in an acute situation.[10]

Medical imaging technologies like CT and MRI are widely used to verify the presence and extent of injuries.[5] The GCS, which assesses a patient's motor, verbal, and eye-opening responses, is used first to score the extent of the injury.[5] A predictive tool is crucial for clinical decision-making due to the high fatality rate of ICH. A grading system called the ICH score assesses survival outcomes 30 days following nontraumatic ICH.[33] The ICH score was obtained by retrospectively analysing 152 spontaneous ICH patients who visited the University of California, San Francisco.[10] Five independent factors, each stratified and ordered based on the strength of correlation, form the basis of the six-point rating system.[33] These factors include GCS, the patient's age (≥80 years), ICH volume, IVH and ICH origin. Table 2.4 summarises the ICH score and how it is calculated. However, in the very early phases of ICH patients' care, clinical grading scales such as the ICH score should never be used alone to restrict interventions.[10] Figure 2.1 illustrates how the ICH volume (ml) is calculated as part of the ICH score calculation.

Table 2.4: Initial ICH score and predicted 30-day mortality according to total score.[10]

Component	Points	Total ICH score	30-day mortality (%)
Glasgow Coma Scale			
3-4	2	0	0-10
5-12	1		
13-15	0		
Age (years)			
≥ 80	1	1	7-13
< 80	0		
ICH volume (ml)			
≥ 30	1	2	30-44
< 30	0		
Presence of intraventricular haemorrhage			
Yes	1	3	56-78
No	0		
Infra-tentorial origin of ICH			
Yes	1	4	70-100
No	0		
Total ICH score	**0-6**	**5-6**	**100**

The five independent predictors of 30-day mortality according to the original ICH scores are displayed in the first column (Glasgow Coma Scale, age, ICH volume, intraventricular haemorrhage, and infra-tentorial location of ICH). The total score is the sum of the five components, varying from 0 to 6 points (column 3). The higher the total score (column 3), the higher the predicted 30-day mortality (column 4). Intracerebral haemorrhage (ICH)

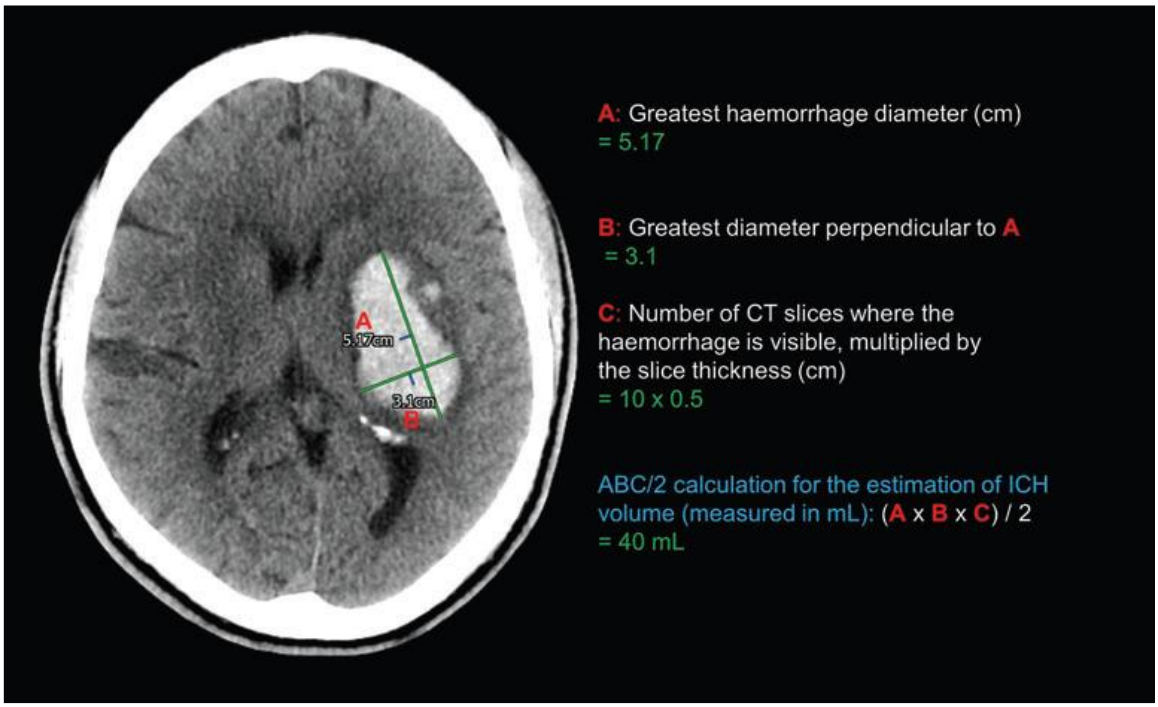


Figure 2.1: Illustration of ICH volume calculation.[11]

2.2.4 Mechanisms of injury due to intracerebral haemorrhage

Injury from ICH is divided into two categories: primary injury, which results from the actual haemorrhage, and secondary injury, which is caused by downstream pathways that are activated when intraparenchymal blood is present.[17] Within the first several days following the haemorrhage, primary injury occurs and is associated with the mass effect of the initial hematoma, hydrocephalus, and hematoma expansion.[17] The activation of harmful pathways, such as oxidative stress, iron and blood-related toxicity, and inflammation, results in secondary injury, which develops over the course of days to weeks.[17]

The onset of the inflammatory cascade is one of several complex and multidimensional cellular and molecular processes that follow the formation of a hematoma. Cytokine release and oxidative stress induction are indicators of secondary brain injury and lead to neuronal degeneration.[34] Figure 2.2 illustrates the molecular and cellular processes that characterise secondary brain injury.

Furthermore, it has been determined that proinflammatory cell presence—specifically, the early elevation of neutrophils—contributes to early neurological decline. Another bystander or frequent possible cause of hematoma growth and worse outcomes is fever, which worsens brain metabolism and speeds up excitotoxicity, which leads to neuronal death.[34]

The location of ICH significantly impacts decision-making. Due to the increased risk of herniation and brainstem compression symptoms in the limited area of the posterior fossa, neurosurgical surgery is often advised for infratentorial bleeding.[11] Perihematomal oedema, hydrocephalus, or the mass effect of the bleed could all contribute to elevated intracranial pressure. External ventricular drainage as a standalone treatment for hydrocephalus is contraindicated in this context, particularly when basal cistern compression is present, as this approach could lead to adverse outcomes.[11]

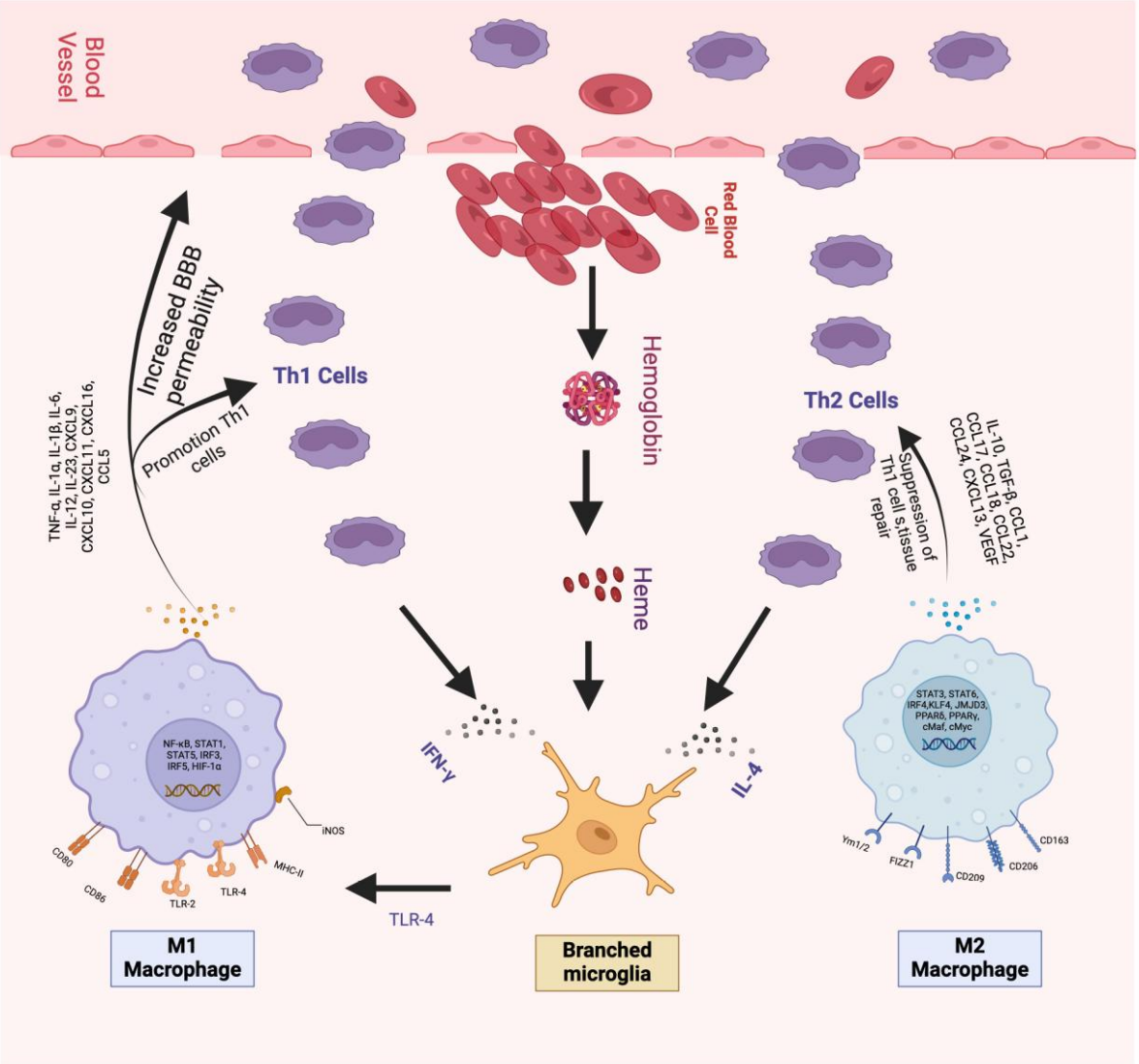


Figure 2.2: Illustration of the beginning of the cellular inflammation process.[34] On the left side, the pro-inflammatory component is illustrated, commencing with the activation of M1 cells. This activation leads to the secretion of several cytokines, notably IL-1, TNF-alpha, and interferon-gamma, which intensify cellular injury and compromise the integrity of the blood-brain barrier. Conversely, the right side of the figure depicts the anti-inflammatory aspect, characterized by M2 cells. These M2 cells produce cytokines such as IL-4, IL-10, and TGF-beta, among others indicated in the figure, which reduce the expression of Th1 cells and facilitate cellular repair.[34]

Figure 2.3 summarises the pathological mechanisms of brain injury after ICH. After ICH, a variety of factors influence secondary brain injury, which is the primary cause of irreversible neurological deficits and brain death.[35] Thus, it is essential to concentrate as soon as possible on the intricate pathological relationship influencing secondary brain injury following ICH. At the same time, identifying the commonalities among pathological factors aids in identifying the primary causes of secondary brain injury formation following ICH.[35]

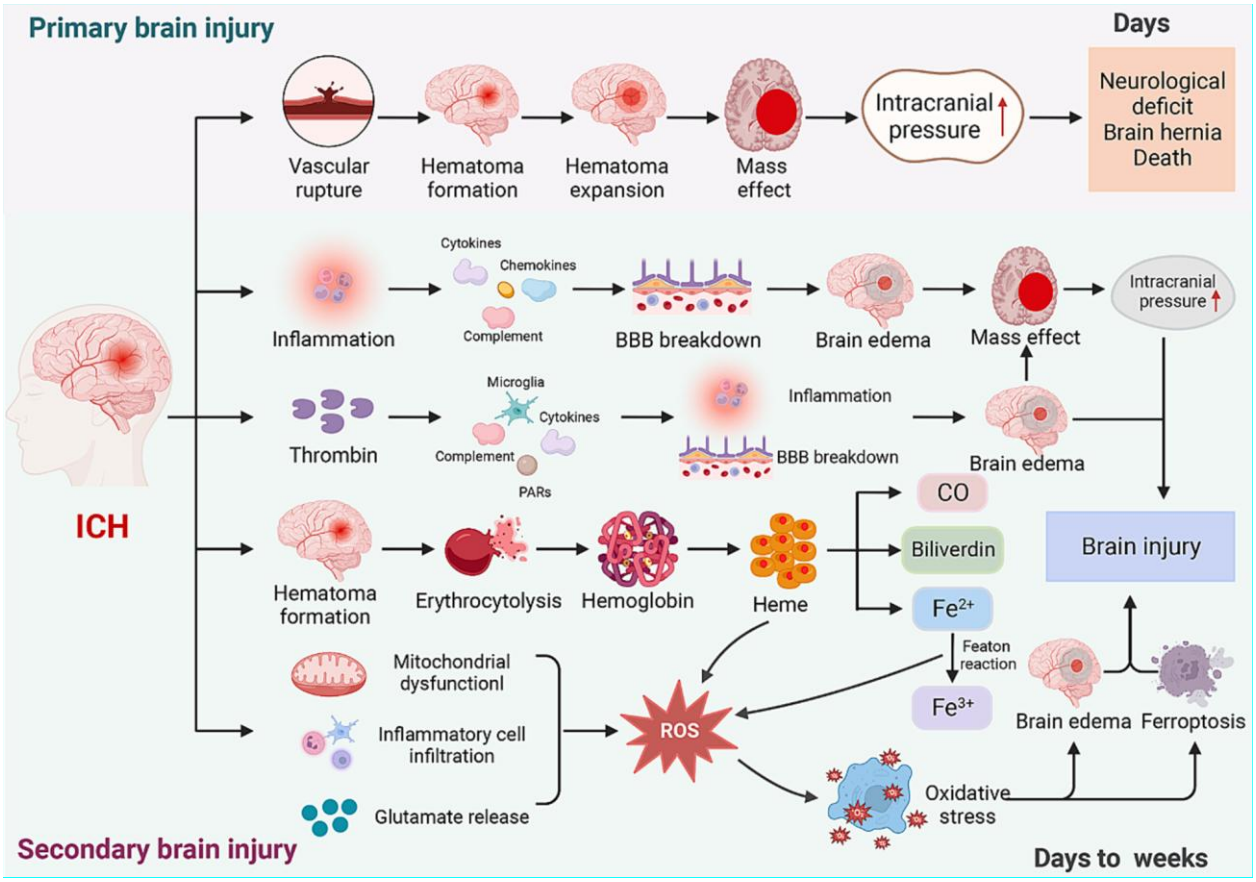


Figure 2.3: Illustration of the pathological mechanisms of brain injury after intracerebral haemorrhage (including primary and secondary brain injury).[35] Several substances reach the brain parenchyma through the compromised blood-brain barrier following the rupture of cerebral arteries. These elements cause pathogenic processes such as oxidative stress, cytotoxicity, and inflammation, which exacerbate the rupture of the blood-brain barrier, the formation of brain oedema, the growth of hematomas, and severe neurological impairments or neuronal death.[35] Intracerebral haemorrhage (ICH), Blood-brain barrier (BBB), protease-activated receptor (PAR's), carbon monoxide (CO), iron (Fe), and Reactive oxygen species (ROS).

2.3 Bleeding event

Patients who endure the initial trauma of brain injury frequently experience persistent and debilitating neurological deficits.[36] A complex series of biochemical reactions are triggered by mechanical strain and damage to brain tissue, which frequently results in hypoxia, ischemia, brain swelling, and oedema. The preservation of a healthy brain depends on the cerebral arteries. In the context of TBI, while the damage to brain tissue is of paramount concern, it is important to recognise that most substantial cases also involve some degree of vascular injury. Any impairment or dysfunction of the blood vessels consequently jeopardises the integrity of neural tissue.[36]

Although pathophysiology linked to vessel dysfunction is also frequently seen, haemorrhage is the primary clinical manifestation of vessel injury in TBI. Although clinical observations don't provide much information about more subtle damage that

might occur, the presence of bleeding indicates that vessels frequently undergo deformations that result in rupture.[36] Blood from injured vessels is typically restricted to spreading inside the area between adjacent membranes, provided that the membranes surrounding the brain remain intact due to the boundaries they define. Therefore, the compartment into which blood seeps is used to classify the type of haemorrhage.[36]

The processes underlying the development of ICH encompass several interrelated factors, including dysfunction of the BBB, cerebral oedema, and the induction of cell apoptosis.[37] Additionally, these mechanisms involve inflammatory responses, oxidative stress, and the activation of signalling pathways that govern angiogenesis, alongside the inhibition of pathways that are crucial for preserving the phenotype of vascular smooth muscle cells.[37] Numerous internal mechanisms have been found that may help to reduce the pathology linked to the BBB's permeability.[38]

It is important to remember that in cases of ICH, neuroinflammation plays a major role in brain damage in its early stages. However, the inflammatory changes that follow in neutrophils, macrophages, and astrocytes may help with recovery in later phases.[38] This entails reducing inflammation and encouraging angiogenesis, neuronal plasticity, and neurogenesis. Furthermore, the integrity of the BBB may be indirectly impacted by the complex inflammatory network created when immune cells in the central nervous system interact with peripheral immune cells.[38, 39] Brain oedema formation after ICH is caused by excessive thrombin synthesis, which exacerbates the inflammatory response and BBB degradation by activating complement, microglia, cytokines, and protease-activated receptors.[35] This is despite the protective impact of low thrombin concentrations.[35] Thrombin serves as a crucial activated element within the coagulation cascade pathway.

2.4 Haemostasis

Blood coagulation and platelet-mediated primary haemostasis are two crucial components of the body's defence mechanism against blood loss.[40] Three major steps form the haemostasis process: vasoconstriction, temporary platelet plug formation and coagulation.[41] Numerous chemical components and functions are involved in a thorough investigation of haemostasis.[41] Limited research has indicated that blood clotting disorders and disrupted haemostasis elevate the risk of ICH.[42] At vessel injury

sites, bleeding is stopped by the platelets and fibrin that form the haemostatic plug. The conventional theory of blood clotting describes two pathways working together: the intrinsic and extrinsic pathways. The intrinsic pathway enhances the initiation phase initiated by the extrinsic pathway. Transmembrane receptor tissue factor (TF) and plasma factor VII/VIIa (FVII/FVIIa) are the main components of the extrinsic route, whereas plasma factors; FXI, FIX, and FVIII are the main components of the internal system.[43]

2.4.1 The Coagulation cascade

The complicated process of blood coagulation involves several biochemical events that result in the production of a clot. This gel-like network forms at the injury site and acts as a plug to stop blood loss.[44] The coagulation process is commonly referred to as a "cascade," in which coagulation factors that come before them activate those that come after.[45] In the 1960s, the coagulation cascade model was initially presented.[46]

The physiological initiation of the coagulation cascade occurs when TF is revealed on the sub-endothelial layer following injury to the vascular wall. This exposure of TF interacts with factor VII, setting off a sequence of biochemical reactions that culminate in thrombin production. Fibrin monomers are created when thrombin enzymatically interacts with the blood's soluble protein fibrinogen. Following the formation of half-staggered oligomers, these oligomers branch out, grow laterally and longitudinally to form a three-dimensional network, lengthen to form protofibrils, and aggregate to form thicker fibres. Additionally, factor XIII creates covalent connections that further crosslink them. The amount and rate at which thrombin interacts with fibrinogen determines the physical characteristics of this network, which is a crucial sign of the clot's quality.[44] Figure 2.4 illustrates the steps of the coagulation cascade.

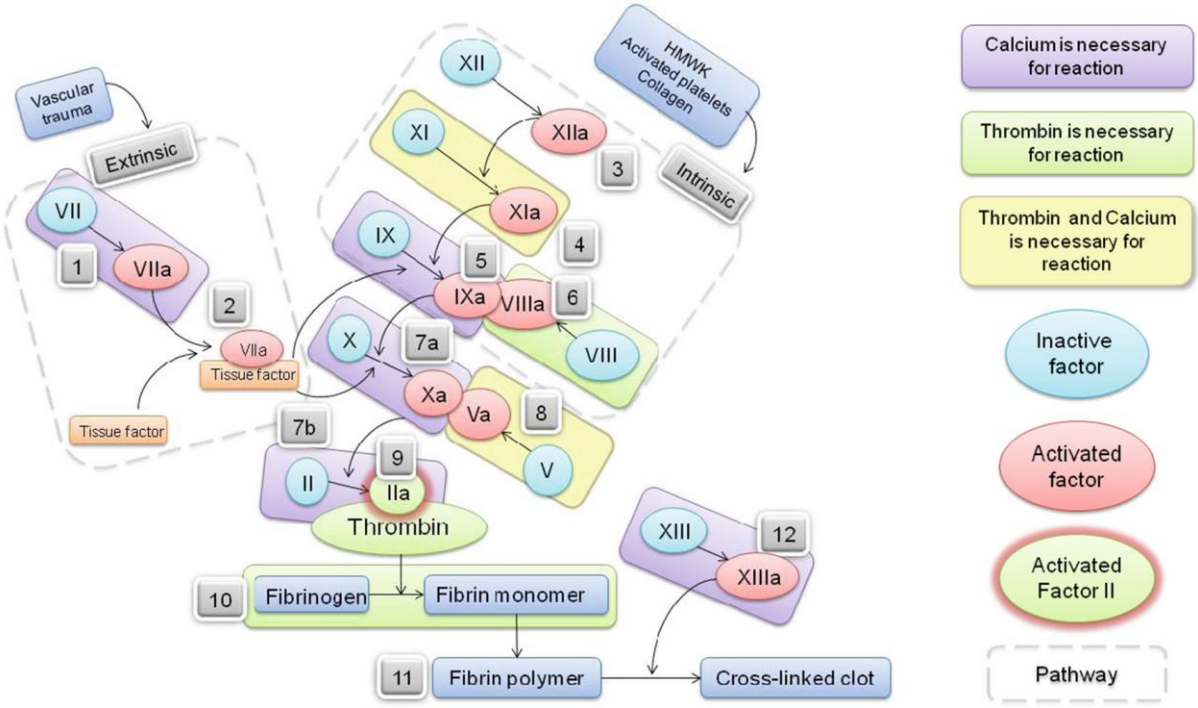


Figure 2.4: Outline of the cascade model of coagulation.[40] Factor VII is activated to its active form, VIIa, in response to vascular injury or trauma. Tissue factor that is visible on the vascular subendothelium is bound by factor VIIa. Factor XII is simultaneously activated to its active form, XIIa, by collagen, activated platelets, and high molecular weight kininogen. Thrombin, calcium, and factor XIIa convert factor XI into XIa, which in turn activates factor IX into IXa. Factor VIII is converted into its active form, VIIIa, by thrombin. Calcium facilitates both the intrinsic and extrinsic pathways that activate factor X to Xa. Xa is activated by the factor VIIa-TF complex in the extrinsic pathway and by the factor IXa-VIIIa complex in the intrinsic pathway. Thrombin also converts factor V to its activated form, Va. In conjunction with calcium, the Xa-Va complex catalyses the conversion of prothrombin (factor II) to thrombin (factor IIa). Thrombin cleaves fibrinogen (factor I) into fibrin monomers (factor Ia). Finally, factor XIII is activated to XIIIa by calcium, and factor XIIIa promotes the crosslinking of fibrin polymers, forming a stable fibrin network.[40]

This coagulation model, centred on cells expressing TF and platelets, highlights the interactions between these components and various coagulation factors. These cells function as catalysts, significantly accelerating the activation of coagulation factors by a factor of one thousand. In turn, thrombin establishes a feedback mechanism that further activates additional platelets, which aggregate through the action of fibrinogen and von Willebrand factor.[47]

2.4.2 Blood components

2.4.2.1 Platelets and Platelet-Poor Plasma

Platelets are myeloid blood cells that lack a nucleus and perform a variety of physiological roles. Under typical physiological conditions, they travel through blood vessels without engaging with non-activated vascular endothelium.[48] Platelets congregate at locations of endothelial damage to aid in haemostasis by attracting

additional platelets, thereby creating a cohesive platelet plug that acts as a physical barrier.[49] This process promotes the production of thrombin and provides a surface for coagulation, ultimately contributing to the prevention of blood loss.[49, 50]

Blood fractions with different platelet concentrations are known as PPP.[51] The platelet count of PPP is 0.5×10^8 platelets/mL.[51] Platelet-poor plasma is obtained by washing and centrifuging human blood at different speeds.[51] Platelet-poor plasma, which is composed of thrombin, fibronectin, and fibrinogen, has a lower platelet content than normal blood.[51] Platelet-poor plasma participates in haemostasis and coagulation and acts as a vector for cell attachment.[51] Based on previous studies, PPP can maintain cell growth and survival, encourage cell processes linked to wound healing, and speed up fibroblast migration and proliferation.[51] Platelet-poor plasma is also used to promote tissue regeneration.[51] Platelet-poor plasma exhibits characteristics similar to those of commercially manufactured fibrin glue, possessing both adhesive and haemostatic capabilities[52]. It is important to note that while PPP contains reduced concentrations of fibrinogen compared to these industrial adhesives, it still demonstrates effective adhesive properties.[52] The production of PPP is essential for obtaining reliable test results, which are vital for accurately diagnosing and managing conditions such as haemophilia, deep vein thrombosis, and other disorders related to coagulation.[53]

2.4.2.2 Fibrinogen

Fibrinogen plays a crucial role in the preservation of haemostasis and is classified as an acute phase protein involved in the blood coagulation process. This process culminates in the development of a fibrin clot, which is initiated by the transformation of fibrinogen into fibrin, followed by the cross-linking of fibrin strands.[54, 55] In the human body, fibrin clots spontaneously form due to vascular damage.[54]

Fibrinogen plays a vital role in platelet aggregation, acting as the main substrate for plasma coagulation and contributing to the formation of a mesh network that strengthens blood clots. However, fibrinogen is one of the most susceptible coagulation factors in individuals experiencing severe injuries. During the initial phases of trauma, the consumption of fibrinogen rises significantly, while its synthesis does not increase correspondingly, leading to critically diminished levels in circulation.[56]

The higher risk of elevated intracranial pressure in patients with severe TBI, is associated with low fibrinogen levels. Based on a study done by Lv et al.[56], when trauma patients' fibrinogen levels dropped below 2.29 g/L, each unit increase in fibrinogen concentration decreased the trauma patient mortality rate by 0.8%.[56] In a study performed by Chitsaz et al.[57], a significant difference in fibrinogen levels was noted among different stroke types, including patients with ICH and ischemic stroke. Previous studies have shown that higher fibrinogen levels are associated with an increased risk of stroke.[57] This increase in fibrinogen is acknowledged as a contributing factor to ischemic stroke. As a result, elevated serum fibrinogen levels encourage the formation of atheromatous plaques in the arteries, which indirectly aids in the onset of ischemic stroke.[57]

Porous networks called fibrin clots play crucial roles in wound healing, haemostasis, and fibrinolysis.[58] Fibrin clots can deform without rupture, thus they possess extreme extensibility and compressibility.[58] Fibre diameter, branching, network density, and the extent of factor XIIIa-mediated fibrin cross-linking all influence clot characteristics.[58]

2.4.2.3 Thrombin

In the primary haemostatic process, thrombin plays a crucial role in activating platelets. In the secondary haemostatic process, it mediates the transformation of fibrinogen into fibrin.[59] This occurs when thrombin proteolyzes fibrinogen, releasing fibrinopeptide A.[54] Additionally, fibrinopeptide B, a second peptide, is broken down from fibrin by thrombin.[54] Thrombin plays a crucial role in forming a thrombus, which is essential for stopping bleeding and facilitating the development of a hematoma following the infiltration of blood into the brain parenchyma.[59]

Thrombin generation warrants assessment in cases of identified coagulopathy, as it signifies the ultimate common pathway in coagulation. Additionally, the activity of thrombin, whether dependent on platelets or independent of them, may serve as a more significant indicator of coagulation status than the traditional prothrombin time and activated partial thromboplastin time measurements.[60]

The transformation of fibrinogen into fibrin by thrombin is crucial in the context of ICH as it plays a vital role in halting the initial bleeding and constraining the expansion of hematomas once blood infiltrates the parenchyma.[59] However, thrombin plays a dual

role in ICH; it promotes haemostasis and exhibits neurotoxic properties. This neurotoxicity is characterised by the induction of DNA fragmentation and the potential disruption of the BBB.[59] The accumulation of the hematoma can exert pressure on surrounding healthy brain structures, leading to elevated intracranial which may result in brain herniation and, in severe cases, death.[59] Platelet activation and coagulation processes are influenced in real-time by the local movement of platelets, coagulation factors, and inhibitors, particularly in the context of ongoing blood flow.[61]

2.4.3 Hemorheology

The flow and deformation behaviour of blood and its constituent elements such as red blood cells, white blood cells, and platelets—are the subject of hemorheology.[62] Blood is classified as fluid connective tissue made up of different kinds of cells, including platelets, red blood cells, and white blood cells, as well as a liquid intercellular substance called plasma.[62] Blood can be viewed from a rheological perspective as a two-phase liquid or as a solid-liquid suspension, with the solid phase consisting of the cellular components.[62] Blood is a non-Newtonian suspension, so a single viscosity value cannot adequately capture its fluidity.[62] Hemorheology is therefore defined by its shear-thinning viscosity, a broad viscoelastic behaviour in response to temporary flow deformations, and the presence of a non-zero yield stress.[63] In critical bleeding, plasma fibrinogen concentration frequently becomes the first coagulation factor to drop to a pathologically low level. It also plays a significant role in determining the thickness of the viscoelastic test tracing.[47] The thickness of the viscoelastic tracing can serve as a reference for adjusting fibrinogen levels through the administration of fibrinogen concentrate or cryoprecipitate in cases of severe haemorrhage. However, it is important to note that laboratory assessment of plasma fibrinogen concentration provides a more precise and specific measurement, as the thickness of the viscoelastic tracing is also affected by platelet levels.[47]

In pathophysiological conditions linked to acute phase reactions, plasma viscosity levels are elevated and are a nonspecific indicator of disease processes. This rise is directly related to plasma's protein content. In disease processes, acute-phase reactants like fibrinogen play a major role in the nonspecific increase in plasma viscosity.[62] Fibrin have unique properties as it is a viscoelastic polymer. Thus, it has both reversible elastic characteristics and irreversible plastic or viscous properties.[58] In arterial shear, the viscoelastic polymer characteristics prevents damage to fibrin

under harsh conditions, due to characteristics such as increased stiffness at high strains or straining stiffening.[58]

damage

The viscoelastic nature of a thrombus that partially obstructs a blood vessel will influence the response of the flowing blood, determining whether the thrombus will undergo reversible or irreversible deformation, rupture, or lead to embolization.[64]

2.5 Thrombosis

Pathological thrombosis in veins or arteries arises when the coagulation process is activated without any accompanying vascular damage, or when coagulation is not properly regulated at locations where injury has occurred.[49] Pathological thrombosis can be initiated through two distinct mechanisms: the tissue factor pathway or the contact pathway, which involves the activation of factor XII upon exposure to foreign materials.[65] In both scenarios, thrombin generation during the initiation phase activates factor XI, triggering an amplification cascade. This cascade significantly increases thrombin levels, facilitating the further development and expansion of the pathological thrombus within the vascular system.[65]

The three main categories of factors that are believed to contribute to thrombosis—hypercoagulability, hemodynamic alterations, and endothelial damage—are referred to as Virchow's triad.[66] Virchow's triad theory emphasises the dangers of thrombosis resulting from impaired blood circulation.[67] Figure 2.5 illustrates the components of Virchow’s triad. Presently, this triad is understood in relation to platelets and coagulation factors (with lesser contributions from erythrocytes and leukocytes), the endothelial layer, and the phenomena of blood turbulence and venous stasis.[43] In individuals experiencing S-ICH, venous stasis occurs due to immobility and hemiplegia.[68] Additionally, endothelial damage may arise from invasive surgical procedures, while hypercoagulability can be attributed to the use of dehydrating, haemostatic, and antifibrinolytic agents.[68] In trauma and critical care patients, venous thromboembolism (VTE) events, such as deep vein thrombosis and pulmonary embolism, are a serious concern.[69] The elevated incidence of VTE among individuals with trauma is believed to result from a variety of mechanistic factors, such as blood stasis, vascular injury, the presence of microparticles, and the activation of innate immune responses.[69] Compared to patients who have experienced general trauma,

those who have experienced TBI are even more vulnerable to VTE.[69] In patients with ICH, malignancy and coagulopathy serve as possible risk factors for VTE.[70]

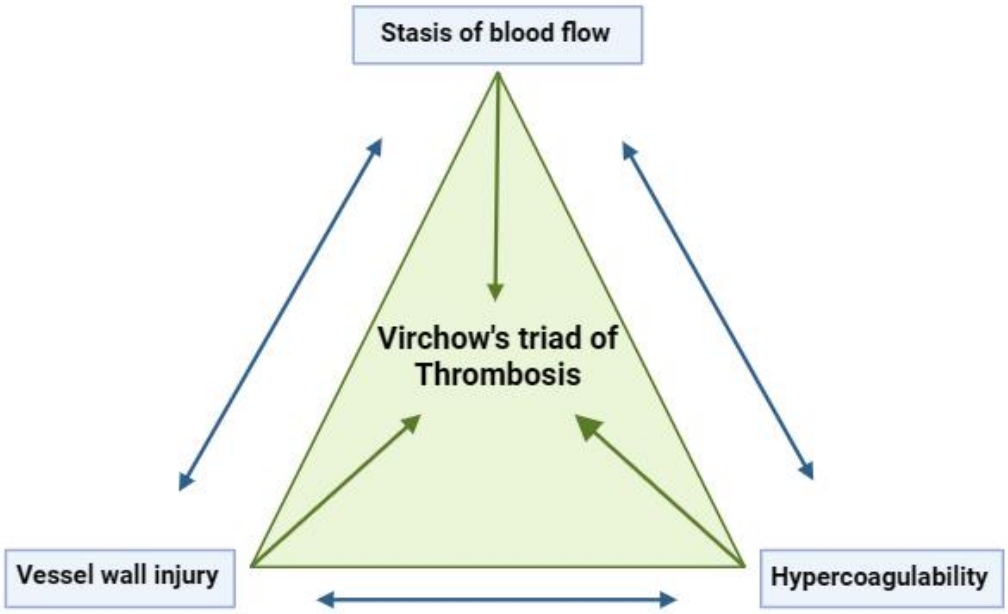


Figure 2.5: Illustration of Virchow's triad. Describes three parameters, stasis of blood flow, vessel wall injury and hypercoagulation as the main contributing factors to thrombus formation. (adjusted from 64, diagram created with BioRender.com)

2.6 Bleeding disorders

Significant risks of ICH can be caused by bleeding disorders, with high mortality and morbidity rates.[71, 72] Haemophilia is a hereditary condition marked by a disruption in the blood coagulation process, stemming from a lack of specific clotting factors. The two main forms of this disorder are haemophilia A, which arises from a deficiency in factor VIII (FVIII), and haemophilia B, which is attributed to a deficiency in factor IX.[73] Approximately 5% of individuals with severe haemophilia A may suffer from ICH, whereas the occurrence is less frequent in patients with haemophilia B.[71] Intracerebral haemorrhage represents a significant complication in haemophilia, particularly in patients diagnosed with the severe variant of the condition.[71] In cases of moderate and mild severity of haemophilia, haemorrhaging typically arises following instances of trauma, surgical interventions, or other invasive medical procedures.[71]

Von Willebrand disease (VWD), named in honour of the Finnish physician Erik Adolf von Willebrand, represents the most prevalent inherited bleeding disorder, impacting roughly 1–2% of the global population.[73] This condition is primarily transmitted in an autosomal dominant manner and is characterised by a deficiency of von Willebrand

factor. A large multimeric glycoprotein encoded by chromosome 12 plays a crucial role in platelet adhesion and aggregation mechanisms.[73] The severity of the bleeding usually falls between mild and moderate, though type 3 VWD is particularly susceptible to potentially fatal bleeding.[71] Intracerebral haemorrhage affects 10% of patients with type 3 VWD. Type 2 VWD has a rate of 2%, while type 1 VWD has a rate of 1%.[71]

The primary contributor to morbidity and mortality in individuals with bleeding disorders is ICH.[71, 72] However, implementing a suitable therapeutic approach can markedly reduce the incidence of ICH and enhance the overall quality of life for affected patients.[71, 72]

2.7 Treatment for intracerebral haemorrhage

To specifically customise haemostatic treatment, the primary goals are identifying patients who received antithrombotic medications and using haemostatic therapies to stop the ongoing bleeding.[25, 74] While treatment for anticoagulation or antiplatelet-associated ICH aims to reverse the antithrombotic effects, treatment for S-ICH should increase blood clot formation to promote haemostasis.[74] Intracerebral haemorrhage continues to require innovative therapeutic strategies and enhanced utilization of existing methods across all dimensions of the condition, including primary and secondary prevention, acute inpatient management, and post-stroke rehabilitation and recovery.[75] The American Society of Anaesthesiologists/American Heart Association guidelines for treating patients with ICH are summarized in Table 2.5. Class I recommendations for ICU (Intensive care unit) management are listed.[18]

Table 2.5: Class I recommendations from AHA/ASA guidelines for optimal clinical management of patients with ICU relevant for neurocritical care.[18]

Class I recommendations	
General monitoring	A doctor with acute care experience in neurology should oversee clinical treatment and monitoring in an ICU or stroke unit.
Arterial blood pressure management	Acute lowering of SAP to 140 mm Hg is safe and can improve functional outcome for ICH patients with SAP between 150 and 220 mm Hg who are not contraindicated for acute BP treatment.
	(i) Platelet replacement or coagulation, or both when needed

Haemostasis and coagulopathy, antiplatelet agents, and DVT prophylaxis	(ii) If the INR is elevated due to VKA, discontinue VKA therapy, replace vitamin K-dependent factors, and administer vitamin K to correct INR.
	(iii) Pneumatic leg compressions on occasion, starting at hospitalization, to avoid venous thromboembolism
Glucose management	Glucose should be monitored. Avoiding both hyperglycaemia and hypoglycaemia is advised.
Seizures and antiseizure drugs	Antiseizure medications should be used to treat clinical seizures. Antiseizure medications should be administered to patients with an alteration in mental status who are found to have electrographic seizures
Management of medical complications	To reduce the risk of pneumonia, all patients should undergo a formal dysphagia screening before beginning oral intake.
Prevention of recurrent ICH	Blood pressure should be controlled in all ICH patients. Procedures to control BP should begin immediately after ICH
Blood pressure (BP); deep vein thrombosis (DVT); intracerebral haemorrhage (ICH); intensive care unit (ICU); international normalised ratio (INR); systolic arterial pressure (SAP); vitamin K antagonist (VKA)	

2.8 Study rationale

Intracerebral haemorrhage is a neurological disorder that is a major health problem due to the high fatality and morbidity rate. Intracerebral haemorrhage remains a multifaceted medical challenge that requires continuous research, innovative strategies, and a collaborative multidisciplinary approach. A comprehensive understanding of its prevalence, underlying mechanisms, diagnostic methods, and treatment options is essential for enhancing patient outcomes and mitigating its significant impact on individuals and healthcare systems.

2.9 Aim and objectives

This study aimed to investigate the viscoelastic and ultrastructural properties in PPP of ICH patients from Steve Biko Academic Hospital (SBAH) to identify any coagulation changes. The analysis compared two patient groups with spontaneous and traumatic intracerebral haemorrhage, against a control group.

The following objectives were used to achieve the aim of this study:

1. To obtain the inflammation status of ICH patients by using the CRP and PCT taken from patient records and comparing them to healthy reference ranges.
2. To study the ultrastructural morphology of fibrin fibres during clot formation of ICH patients compared to control individuals using SEM.
3. To determine the fibrin fibre thickness of ICH patients compared to control individuals using the ImageJ software.
4. To determine fibrin fibre branching ICH patients compared to control individuals using Fractal Analysis System.
5. To analyse the viscoelastic properties of clot formation in ICH patients compared to control individuals using TEG®.

Chapter 3: Study design, sample collection, patient demographics and routine clinical analyses

3.1 Study design and setting

This research utilized a prospective study conducted in a laboratory setting. The investigation analysed ex vivo blood samples from two populations: a control group comprising 37 healthy individuals and an experimental group of 51 intracerebral haemorrhage patients, which was further divided into two subgroups, 31 S-ICH and 20 T-ICH patients. The statistician consulted indicated that each group needed a minimum of 20 participants.

Patient recruitment took place at the Neurosurgery Department at SBAH, and letters of approval were obtained (Addendum 2 and 6) for the recruitment. Prof LC Padayachy and Dr C Grobbelaar, under the supervision of Prof Padayachy, were the recruiting doctors who determined which patients needed blood samples to be taken.

Once the recruiting doctors identified the patients, the principal investigator approached the patients before sample collection to request their participation in the study. Participants were informed about the study and the informed consent documents were explained to the participants (Addendums 3 and 4). This information session was held in private. The participants only signed the consent forms and consented to participate once they understood the scope of the study and what was required from them. Participants were informed that they were under no obligation to take part in the study and that they had the right to refuse to participate at any time during the study without explanation or prejudice. Participants were informed that this would not affect the quality of their medical treatment. There were no cost implications to the participants. Information about control and patient groups was handled anonymously, results were kept confidential and were provided only upon request to patients, and recruiting physicians were notified if significant abnormalities were found. Blood samples were obtained during regular blood tests, only one additional tube was drawn without causing any inconvenience to the participant. The recruiting doctor collected the blood sample in a 4 mL citrate tube using a 3.2% (0.105 M) buffered sodium citrate solution acquired from Lasec.

Due to the vulnerability of the patients needed for this study, the research ethics committee approved a consent waiver in situations where the patient was in the ICU, unconscious and unable to provide consent after regaining consciousness, or deceased. However, for patients who regained consciousness, a delayed consent process was implemented following medical clearance from their treating physician. These patients were promptly informed of their inclusion in the study and advised of their right to withdraw without any impact on their standard of care. (Addendum 4).

To ensure a sample from a homogeneous population, background information was provided in the form of a questionnaire to capture demographic data.

This study was conducted as a component of a larger study and in cooperation with Miss Shené Ferreira, who investigated the viscoelastic and ultrastructural properties of the WB components.

3.2 Ethical approval

To work with patients at SBAH, the study received both ethical approval (ethics number 298/2022) and National Health Research Database (NHRD) approval (approval number GP_202210_003). The Declaration of Helsinki and the ethical standards established by the Faculty of Health Sciences Research Ethics Committee were followed in this case (approval letters found in Addendum 1 and 2).

3.3 Division of the study population

As seen in Figure 3.1, there were three groups in the study: one control group and two patient groups.

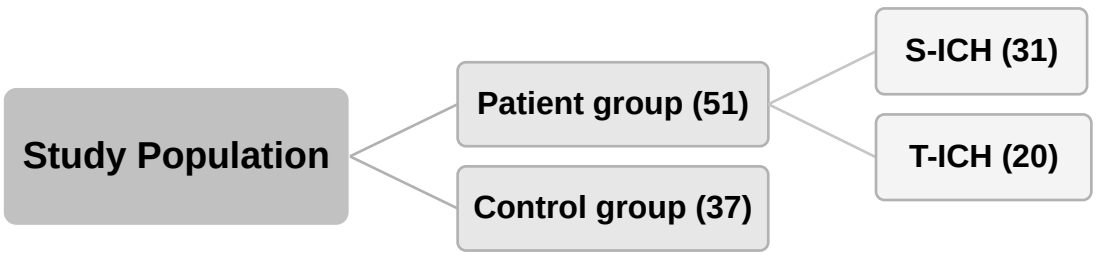


Figure 3.1: Diagram of the study population group division. Spontaneous intracerebral haemorrhage (S-ICH), Traumatic intracerebral haemorrhage (T-ICH)

3.4 Sampling Criteria and Procedures

3.4.1 Control group

Recruitment:

Willing family, friends, and colleagues at SBAH and Prinshof campus at the University of Pretoria (UP) were recruited to serve as a control group if they met the study's inclusion criteria. A control group was selected from a comparable population to guarantee group homogeneity. All participants were individuals with no suspicion of ICH.

***Inclusion criteria**:*

- Ability to provide informed consent
- Biological sex: Male and female
- Age range: 20–60 years[76]

***Exclusion criteria**:*

- Smoking any tobacco or consumption of related products
- Use of chronic medications
- Presence of any condition associated with chronic inflammation negatively impacting the coagulation system
- Use of medications affecting coagulation (e.g. Aspirin/Nonsteroidal anti-inflammatory drug (NSAIDs), etc.)
- Any acute or long-term illness that is known (such as ICH, stroke, cancer, auto-immune disorders etc.)
- History of an immunocompromised status.
- Usage of herbal or vitamin supplements, corticosteroids, anti-inflammatory, anti-coagulative, or anti-platelet medications within two weeks before sample collection date
- Human immunodeficiency virus (HIV) positive (only if the status was known)
- Excessive alcohol consumption (defined as five or more drinks on the same occasion at least once in the past 30 days)[77]

3.4.2 Intracerebral haemorrhage patient group

Recruitment:

Blood samples from patients diagnosed with ICH by a qualified medical doctor were collected for this study. The samples were then separated into S-ICH and T-ICH patient groups. As described in the selection criteria, patients should have had no other sign of infection or inflammation unrelated to ICH.

Inclusion criteria:

- Able to provide informed consent if conscious or waiver of consent applied
- Biological sex: Male and female
- Age range: 20–60 years[76]
- Intracerebral haemorrhage diagnosis by a medical doctor

Exclusion criteria:

- Smoking any tobacco or consumption of related products
- Chronic medication use that negatively impacts the coagulation system.
- Any condition that may manifest as chronic inflammation
- History of an immunocompromised status.
- Patients that received any intravenous fibrinolytic, intravenous, or subcutaneous heparin (ordinary or unfractionated)
- Ischaemic strokes that present with cerebral bleeding
- Brain tumours that present with intracerebral bleeds
- Patients that are on chemotherapy medication, anticoagulant medication or antiplatelet medication, warfarin, or any novel oral anticoagulants (e.g. Rivaroxaban, Apixaban, Dabigatran or Edoxaban)
- HIV positive (obtained from patient files; status must be known)
- Excessive alcohol consumption (defined as five or more drinks on the same occasion at least once in the past 30 days)[77]

A medical professional took blood from the central venous line. Only while doing standard testing on ICU patients was this carried out. The technique that was used for drawing blood from a central line:

1. Informed the nursing staff that blood was drawn from the patient's central line (only during routine blood testing).

2. Confirmed whether there were any problems with the central line such as bleeding, discharge, or redness around the central line.

3. The procedure was explained to the patient (if awake).

4. The sister caring for the patient was informed and prepared a central venous pressure (CVP) pack/set.

5. The doctor was dressed in the appropriate Personal Protective Equipment (PPE), such as an apron, theatre cap and mask.

6. The doctor scrubbed as one would for theatre while the sister prepared for the procedure, e.g., local anaesthetic, heparinised saline, and nylon stitch.

7. The doctor usually used the subclavian vein (but rotated sites).

8. The doctor cleaned the area with alcohol and a drape, observing a sterile technique throughout the whole process.

9. The doctor then inserted the CVP. Secured it with a nylon suture. Dressed the area with transparent dressings and noted the date of insertion.

10. The doctor removed it/rotated the site every 7 days.

11. All solutions infusing were turned off for one full minute (if applicable).

12. The needleless connector was cleansed with three alcohol swabs and wiped for one full minute.

13. The blood draw syringe was attached to a needleless connector, and 5 mL of blood was aspirated and discarded. The catheter was clamped when the syringe was removed.

14. A new syringe was attached, the clamp was removed, and the total amount of blood required was aspirated. The catheter was clamped when the syringe was removed.

15. The appropriate tubes were filled with blood.

16. The catheter was flushed with 10 mL of 0.9% sodium chloride. The catheter was clamped after the blood drawn.

17. The procedure was documented in the patient’s medical record.

The sterile process avoided infections from central venous lines, and this line was more stable.

The patient recruiting procedure, from patient identification to sample processing, is summarized in Figure 3.2.

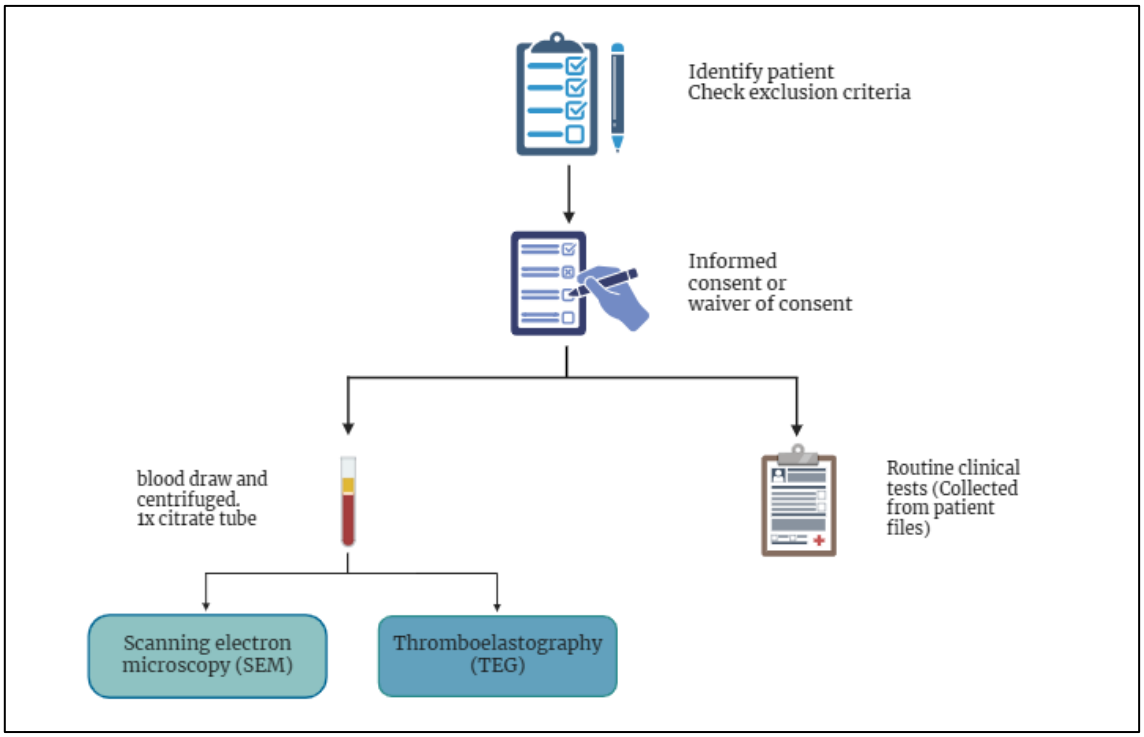


Figure 3.2: The patient recruitment process, from patient identification to sample analysis. (Diagram created with biorender)

3.5 Participant demographics

To stabilise the patient, stop the bleeding, and avoid more issues, treating ICH frequently calls for an urgent, multimodal strategy. Medications are crucial for the treatment plan in ICH patients, and we did not prevent any patients from being treated while recruiting them for the study. The drugs that a number of individuals were taking when their blood was drawn are shown in Table 3.1. It is important to consider a range of factors related to the patient's specific condition to determine the medications to be administered and the route of administration. These factors include the severity and location of bleeding, underlying medical conditions, and other relevant considerations. Since no negative effects of these medications on coagulation have been demonstrated in the literature and they cannot be considered confounding factors, patients taking them were included in the study. Table 3.1 lists references shown by research to have no negative effects on coagulation.

Table 3.1: A summary of the demographics and medication use of the study participants.

Control Group (n=37)				
Age range (years)	**Number of patients in age group (n=37)**	**Biological sex**	**Type of medication (n= number of patients taking medication)**	**Medication brand**
20 – 29	11	F(3) M(8)	High blood pressure (n=1)	Indapamide[78]
30 – 39	10	F(6) M(4)		
40 – 49	10	F(6) M(4)		
50 – 59	5	F(2) M(3)		
60 – 69	1	F(1) M(0)		
Spontaneous ICH (n=31)				
Age range (years)	**Number of patients in age group (n=31)**	**Biological sex**	**Type of medication (n= number of patients taking medication)**	**Medication brand**
20 – 29	1	F(1) M(0)	Pain medication (n=27)	Panado, Tramadol[21], Perfalgan, Morphine[79]
30 – 39	5	F(1) M(4)	Anti-epileptic (n=27)	Epilim, Phenytoin[80]
40 – 49	9	F(3) M(6)	Anti-hypertensive medication (n=23)	Amloc, Ridaq, Enalapril[81], Hydralazine[82], Amlodipine[83], Carloc[84], Labetalol infusion, Atenolol, Propranolol
50 – 59	6	F(3) M(3)	Proton pump inhibitor (n=19)	Pantoloc[85], Pantosec, Lansoprazole
60 – 69	6	F(4) M(2)	Antiemetics (n=5)	Maxolon[86]
70 – 79	3	F(1) M(2)	Diuretic (n=5)	Mannitol[87], Cardura XL, Lasix
80 – 89	1	F(1) M(0)	Antibacterial agent	Meropenem[88]

			(n=2)	
			Corticosteroid (n=2)	Dexamethasone[89], Solu cortef
			Anti-psychotic (n=1)	Risperdal[90]
			Anti-cholesterol (n=1)	Simvastin[91]
			Calcium channel blocker (n=1)	Nimodipine[92]
			Calcium deficiency (n=1)	Calcium gluconate[93]
			Constipation (n=1)	Lactulose[94]
			Insulin (n=1)	Actrapid[95]
			Nasal congestion (n=1)	Afrin[96]
			Sedative drug (n=1)	Dormicum[97]
Traumatic ICH (n=20)				
Age range (years)	**Number of patients in age group (n=20)**	**Biological sex**	**Type of medication (n= number of patients taking medication)**	**Medication brand**
20 – 29	9	F(1) M(8)	Pain medication (n=18)	Panado, Tramadol[21], Morphine[79], Perfalgan, Paracetamol
30 – 39	5	F(0) M(5)	Anti-epileptic drug (n=16)	Phenytoin[80], Epilim
40 – 49	4	F(1) M(3)	Proton pump inhibitor (n=14)	Pantoloc[85]
			Antibacterial/antibiotic agents (n=9)	Augmentum[98], Imipenem[99], Rocephin, Kefzol[100]
50 – 59	0	F(0) M(0)	Sedative drug (n=8)	Dormicum[97], Precedex

60 – 69	1	F(0) M(1)	Antiemetics (n=6)	Maxolon[86], Metaclopramide[101]
70 – 79	1	F(0) M(1)	Diuretic (n=5)	Mannitol[102]
			Anti-psychotic (n=1)	Risperidone[103]
			High blood pressure (n=1)	Pharmapress[104]
			Vitamin B1 deficiency (n=1)	Thiamine[105]
Intracerebral haemorrhage (ICH), female (F), male (M)				

3.6 Statistical analyses

A statistician from UP carefully examined the study and experimental procedure details. Addendum 5 contains a letter providing statistical clearance.

Statistical analysis for the C-reactive protein (CRP) and Procalcitonin (PCT) parameters was performed on GraphPad Prism Version 10.1.0 for Windows. The Shapiro-Wilk test was used to test for normality. Nonparametric data was tested using the Mann-Whitney test showing the median (min, max); for parametric data, the t-test was used with a mean (SD). Descriptive statistics were conducted considering the possibility of some parameters following a normal distribution while others did not. To determine if each parameter was within, above, or below the normal reference range, the mean or median was compared to the reference ranges.

3.7 Routine clinical tests

The inflammation status of the ICH patients was obtained by using the CRP and PCT taken from patient records and comparing it to healthy reference ranges.

Inflammatory markers:

Every patient undergoes various routine tests as part of any medical evaluation that occurs before a diagnosis is made. To guide future research, testing, and diagnosis, these standardised tests are utilised to better understand the body's reaction to the illness or condition. Of these tests, the biomarkers of inflammation, such as CRP and PCT, have been analysed via immunoassays and have been acknowledged as reliable markers of infection or sepsis.[106] Elevated levels of these biomarkers of inflammation can be seen in patients with ICH.[13, 107] Procalcitonin (PCT) is widely used as a diagnostic marker for severe infection and sepsis and has been associated with

mortality in a range of clinical contexts, such as sepsis, cancer, and trauma.[108] Researchers have investigated the predictive significance of serum PCT in individuals with neurological conditions such as epilepticus status, ischaemic stroke, subarachnoid haemorrhage, and intracerebral haemorrhage.[108] C-reactive protein is a sensitive indicator of inflammation across a broad spectrum of diseases.[109] Prior research has indicated that increased levels of CRP are associated with a higher likelihood and greater intensity of ischemic stroke in patients.[109] The inflammatory markers were tested as part of the patients' routine clinical testing at SBAH, through the National Health Laboratory Services (NHLS). Routine blood testing at the NHLS is carried out according to the standard procedure, typically within four hours of blood collection. Blood levels of C-reactive protein (CRP) and procalcitonin (PCT) were assessed via immunoassay techniques to evaluate cellular abnormalities in ICH patients. These inflammatory markers were measured and evaluated against established reference ranges, detailed in Table 3.2 along with their corresponding units of measurement.

C-reactive protein: CRP is an inflammatory protein synthesised by the liver in response to inflammation, infection, or bodily injury.[109, 110] It can play a role in amplifying inflammation in the cerebral blood vessels and the development of brain damage by activating the complement cascade, initiating the migration of white blood cells, and inducing the expression of adhesion molecules through a positive feedback loop.[110] Studies have shown that the advancement of ICH has resulted in an inflammatory response due to the bleeding of cerebral blood vessels. However, there is limited evidence regarding the correlation between CRP and ICH.[109] Patient records were used to obtain the CRP values for each patient.

Procalcitonin: The peptide precursor of the calcitonin hormone, known as PCT, is synthesised by the parafollicular cells of the thyroid and the neuroendocrine cells of the lung and intestine. Bacterial infections induce this synthesis.[106] The predictive value of infection is notably higher when considering PCT levels. The development and severity of infection are closely tied to a poor prognosis in patients with severe head injuries.[111] Each patient's PCT levels were taken from their medical records.

Table 3.2: Inflammatory markers reference ranges for adults. Adapted from Nehring et al.[112] and du Plessis et al.[113]

Inflammatory markers	Reference range	Units	Interpretation

C-reactive protein (CRP)	<10	mg/L	↑: Inflammatory conditions including burns, trauma, infection surgery, autoimmune disease and neoplastic disease
Procalcitonin (PCT)	0.0 – 0.05	ug/L	Normal range.
	0.06 – 0.49	ug/L	May indicate: - absence or minimal systemic inflammatory response - regional inflammation - chronic inflammatory processes - autoimmune disorders - viral infections - mild to moderate localised bacterial infections
	0.5 – 1.99	ug/L	Potential bacterial infection. Nonbacterial aetiologies (such as invasive fungal infections, burns, severe trauma, cardiogenic shock, major surgery, small cell lung cancer, viral infections, *Plasmodium falciparum* malaria, etc.) have to be excluded.
	2 – 10	ug/L	Systemic fungal, bacterial or parasitic infection is likely. Also compatible with severe burns or polytrauma.
	>10	ug/L	Typically indicate severe conditions such as sepsis, severe sepsis, septic shock (predominantly bacterial in origin), or multi-organ failure.

3.7.1 Results

The following results on inflammatory markers were obtained from patient files at SBAH. The CRP and PCT values in the S-ICH and T-ICH groups were not within the normal reference range. In the S-ICH group, 74% had an increased CRP, and 29% had an increased PCT. In the T-ICH group, 95% had an increased CRP, and 40% had an increased PCT. Table 3.3 provides a summary of these results.

Table 3.3: Descriptive statistics of the inflammatory markers for the patient groups according to the reference ranges.

CRP			
Group	**Median (min; max)**	**Normal range**	**Outcome**
S-ICH	66 (4;392)	<10 mg/L	Increased CRP
T-ICH	142 (11;314)		Increased CRP
PCT			
Group	**Median (min; max)**	**Normal range**	**Outcome**
S-ICH	0.24 (0.03;13.12)	0.0 – 0.05 ug/L	Increased PCT
T-ICH	0.27 (0.03;7.35)		Increased PCT

Parameter values above the reference ranges are indicated in red, Spontaneous intracerebral haemorrhage (S-ICH), traumatic intracerebral haemorrhage (T-ICH), C-reactive protein (CRP), Procalcitonin (PCT).

3.7.2 Discussion

Based on the population demographics the control group comprised 37 participants with a mean age of 36.78 ± 11.58 years. In this control group, 19 were males and 18 were females. The S-ICH group comprised 31 participants with a mean age of 52.26 ± 13.71 years. There was a significant difference observed in the age between the S-ICH patient group compared to the control group with a P-value<0.0001. In this S-ICH group, 17 were males, and 14 were females. The T-ICH group comprised 20 participants with a mean age of 36.15 ± 13.37 years. In this T-ICH group, 18 were males, and 2 were females. There was no significant difference observed in the age between the T-ICH patient group compared to the control group with a P-value=0.7429.

According to the findings of the inflammatory biomarkers investigation, the CRP and PCT readings were not within the normal ranges. Both the PCT and CRP medians were higher than the normal ranges in the S-ICH group. As a result of the bleeding event in ICH, the blood components form a growing hematoma and activate inflammatory cells.[109] An acute inflammatory response triggering systemic inflammation can occur several minutes after ICH because of the physiological and pathological response to the hematoma.[109, 114] Based on a study done by Wang et al.[109], patients suffering from ICH exhibit a comparable inflammatory response pathway during the secondary injury to that of ischemic stroke patients following the initial injury. The microglia are activated during the secondary phase, leading to the infiltration and release of blood-derived inflammatory cytokines like tumour necrosis

factor-alpha (TNF)-α and interleukin-1β.[109] According to a study by Kumar et al.[110], greater CRP levels are substantially linked to a 30-day mortality following S-ICH.

In the T-ICH group, the medians for the CRP and PCT levels were above the normal range. According to Mas-Celis et al.[115], 60% of trauma patients die prior to hospital arrival, and an additional 60% of deaths occur within the first few hours after admission. Moreover, injury-related causes account for 10% of infectious disease-related fatalities, representing 54% of mortality after 48 hours, and 76% after 7 days of admission.[115] Following bacterial infection, CRP is secreted within 4 to 6 hours and reaches its peak levels within 36 hours.[111] In contrast, serum PCT levels exhibit persistence over prolonged periods, enabling the observation of variations in the clinical symptoms of the disease. As a result, PCT can serve as a predictive indicator for the inflammatory status in trauma patients.[111, 115]

An important factor to consider when taking note of these biomarkers is the changes that may occur in the results over the course of the bleeding events and different types of treatment. This should be an important consideration in future related studies.

3.7.3 Conclusion

Inflammatory blood tests, using CRP and PCT as markers of inflammation, were conducted to assess the inflammatory profile of the S-ICH and T-ICH patient groups. These biomarkers provided insight into the overall inflammatory status of the patients. The CRP and PCT levels substantiated the elevated state of inflammation in the S-ICH and T-ICH patient groups. The results of inflammatory markers indicated abnormalities in the systemic inflammation profiles of the patient groups, highlighting the impact of ICH on the haemostatic system, as assessed through methods such as SEM and TEG® in the next two chapters.

Chapter 4: Scanning Electron Microscopy and fibre quantification

4.1 Chapter objectives

The research will be guided by the following objectives in this study:

1. To study the ultrastructural morphology of fibrin fibres during clot formation of ICH patients compared to the control group using SEM.
2. To determine the fibrin fibre thickness of ICH patients compared to the control group using the ImageJ software.
3. To determine fibrin fibre branching ICH patients compared to the control group using Fractal Analysis System.

4.2 Introduction

Scanning electron microscopy is an ultrastructural, sensitive technique that uses a focused beam of electrons to scan the surface of a sample.[116, 117] This technique analyses specific changes to fibrin packaging that are involved in clotting.[116] The process of fibre branching creates a three-dimensional network.[118] By using SEM , a detailed image of the two-dimensional surface topology is obtained, making it a valuable tool for analysing the overall structure of blood clots and providing critical information on the fibrin fibre networks within them.[117] This includes the fibrin fibre distribution, diameter, and branching; these images can also observe the network pore size or porosity.[119] Based on studies done by Wang et al.[120], the benefit of using PPP is that individual exposure to the clot's fibrin fibre is visible for SEM evaluation.

Protofibril connections are linked to the diameter of the fibrin fibre. When the fibrin fibre diameter increases, the connections of the protofibrils change, as opposed to protofibrils that are joined in a uniform manner of a homogeneous cross-section.[118] The density of the protofibril decreases with an increase in the fibrin fibre diameter; thus, protofibrils in thin fibres are compacted, whereas those in thick fibres are more loosely arranged. Protofibrils aggregate laterally to form fibres when they are long enough.[64] The fibrin fibres' physical structure and mechanical behaviour are important properties for the formation of blood clots and lysis.[118] It was demonstrated by Liang et al.[121], that fibrin’s low-density isotropic network structure allows it to be considered a foam with fluid in its pores.[121]

A study done by Pretorius et al.[54], has shown that SEM can be used to visualise fibrin fibre clots. They observed that thin, minor fibres entwined with the thick major fibres to form a mesh. It was also previously demonstrated that this network alters to form dense matted fibrin deposits during inflammatory conditions like diabetes and thromboembolic ischemic stroke.[40, 54] The results of this study, along with additional research, corroborate clinical observations indicating that changes in plasma fibrinogen levels may act as a nonspecific marker for the presence of inflammatory processes within the body.[54] Consequently, alterations in fibrinogen levels associated with inflammation can be effectively visualised using SEM by introducing thrombin to PPP.[54]

According to Daraei et al.[119], the clot structure and stability may be used to evaluate the occurrence and treatment of bleeding disorders. ImageJ (public domain Java-based image processing program created at the National Institutes of Health: http://rsbweb.nih.gov/ij/) can be used to accurately analyse the fibrin fibre diameters and the porosity of fibrin clots using SEM images.[119]

Scanning electron microscopy is an instrumental technology in clinical research and basic science.[117] Although fixation, dehydration, drying, and sputter coating during the clot preparation process for SEM may cause minor clot structure changes, fibre diameter and porosity measurements are repeatable, and comparisons between patient and control clots are highly informative.[119] Therefore, in this study, SEM was used to identify the ultrastructural changes that occur during clot formation in ICH patients compared to controls. This made it easier to pinpoint particular modifications linked to the various elements involved in clot formation.

4.3 Materials and Methods

4.3.1 Materials, reagents and equipment

The following materials were utilised for SEM, ImageJ and Fractal dimensions:

- 2 – 20 µL Eppendorf pipette
- 10 mm glass coverslips (Lasec)
- 200 µL pipette tips (Lasec)
- 24 well plates (Lasec)
- Curved tip forceps
- Human thrombin donated by the South African National Blood Service

- Phosphate buffered saline (Sigma-Aldrich)
- Hexamethyldisilazane (Sigma-Aldrich)
- Osmium tetroxide (Sigma-Aldrich)
- Ethanol (Sigma-Aldrich)
- Aluminium plates
- Graphite rods
- Carbon tape
- Quorum Q150T coating unit (Quorum Technologies, Lewes, United Kingdom)
- Zeiss Gemini Ultra Plus FEG SEM (Carl Zeiss Microscopy, Munich, Germany)
- ImageJ (public domain Java-based image processing program created at the National Institutes of Health: http://rsbweb.nih.gov/ij/)
- Fractal dimension (branching) analysis software (Copyright© 1998-2006 NILGS, NARO; Coded by Hiroyuki SASAKI)

4.3.2 Method

Sample preparation

As outlined in Chapter 3, blood was drawn from the patient and control groups in 4.5 mL citrate tubes. The samples in this study were analysed within a 4-hour period from the time of collection. The single-step centrifugation method Rikket et al.[122] described was optimised to obtain PPP. The blood was centrifuged at 622 x *g* for 15 minutes to obtain the PPP. The plasma was removed 1 cm above the buffy coat. Two samples were prepared for each participant. Two different glass coverslips were smeared with 10 µL of PPP for the fibrin clot network analysis. The fibrin networks were then activated by adding 5 µL of thrombin to the coverslips. The coverslips were put into separate wells of a 24-well plate after being allowed to dry at room temperature for roughly three minutes. For 15 minutes, the smears were submerged gently in 1X phosphate-buffered saline (PBS) solution (pH 7.4) containing 0.01 M phosphate buffer and 0.154 M sodium chloride. After this washing process, the smears were fixed with 4% formaldehyde for at least 30 minutes. Followed by washing three times in PBS for three minutes each. The samples were then fixed for secondary fixation by covering in 1% osmium tetroxide (OsO4) in a fume hood for 15 minutes. The samples were then rewashed three times with PBS for three minutes each. The samples were placed in increasing ethanol concentrations (i.e., 30%, 50%, 70%, 90%, and three times with

100% ethanol) for three minutes in order to gradually dehydrate them. After this step, the samples were immersed in Hexamethyldisilazane (HMDS) for 30 minutes in a fume hood. After the samples were lifted and reinserted into the 24-well plate, the HMDS was removed and replaced with a single drop of HMDS. The samples were then left in the fume hood to dry overnight.

The samples were then mounted onto aluminium plates, using double-sided carbon tape. The samples were prepared for placement inside the Quorem Q150T Coating Unit after the graphite rods were loaded. Next, the required pre-set sputter coating protocol was chosen, and the coating unit was sealed. The graphite rods were heated to produce a fine carbon dust that covered the samples after the proper vacuum was created inside the chamber. The Zeiss Crossbeam 540 FEG-SEM or Zeiss Ultra Plus FEG-SEM was used to study the ultrastructural morphology of the samples using the InLens detector at 2 kV.

The objects of interest in this study were predominantly the fibrin networks. For each sample, the following properties of PPP were noted:

1.) Presence of clots

2.) Branching of the fibrin networks

3.) Density of the fibrin networks

4.) Presence of dense and matted fibrin network deposits

For clots, the fibrin network was studied. A series of micrographs were taken for sample analysis of SEM preparations that represented features of interest in the study. A minimum of five representative images were taken to show the general overview of the sample at magnifications 10.00 KX Magnification and, 20.00 KX Magnification respectively. Features of interest were identified and observed differences were then noted and analysed between the healthy controls and the ICH patient groups.

Determination of fibrin fibre thickness

Image J, a public domain Java-based image processing program created at the National Institutes of Health: http://rsbweb.nih.gov/ij/, was used to measure the thickness of the fibrin fibres. For every sample, 50 fibres were measured, and five micrographs were randomly taken. Once the program was opened, the micrograph

was imported, and the line function was selected on the toolbar. A line was drawn across the width of the fibre, and the letter M was pressed on the keyboard to record the measurement (length shown in the red block in Figure 4.1). Once all 50 measurements were taken, the data was imported into an Excel sheet. In Excel, the measurement was divided by the measurement of the scale bar (1 µm or 2 µm depending on the image scale) and multiplied by 1000 to obtain the measurement in nm. The results were noted and discussed between the healthy controls and the ICH patient groups.

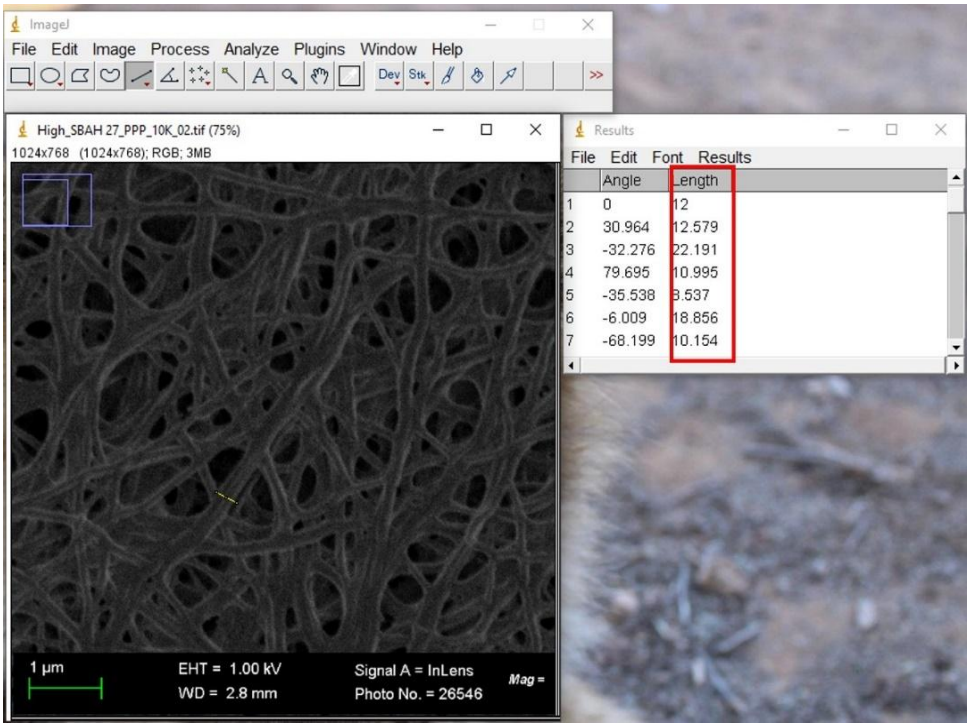


Figure 4.1:Using ImageJ to measure fibrin fibre thickness.

Determination of fibrin fibre branching

The Fractal Analysis System (Copyright© 1998-2006 NILGS, NARO; Coded by Hiroyuki SASAKI) was used to measure the branching of the fibrin fibre networks. The same micrographs used to measure the fibrin fibre thickness were used to determine the fibrin fibre branching. In ImageJ, the micrograph was cropped to measure 1024 in width and 512 in height and saved as a bitmap (BMP) file. The BMP file of the micrograph was then imported into the Fractal Analysis System and set to grey scale. This was done so that the fractal dimensions could be analysed. The data was recorded in an Excel spreadsheet.

4.4 Statistical analysis

Statistical analysis for the SEM parameters was performed on GraphPad Prism Version 10.1.0 for Windows. The Shapiro-Wilk test was used to test for normality. Nonparametric data was tested using the Mann-Whitney test showing the median (min, max); for parametric data, the t-test was used with a mean (SD). A P-value < 0.05 was considered significant.

4.5 Results

The results in this chapter are based on the SEM micrographs used to examine the biophysical properties of the fibrin clot networks. A general overview of the area of interest was based on the low magnification of the images. In contrast, a high magnification provided more detailed information regarding the fibrin network and interaction of the fibres.

4.5.1 Fibrin fibres

Figure 4.2 A-F illustrates a selection of micrographs of the fibrin fibres of the control group. The fibrin fibres were uniformly distributed across the samples, establishing a distinctly observable fibrin network, with defined areas of branching evident within the fibrin fibres.

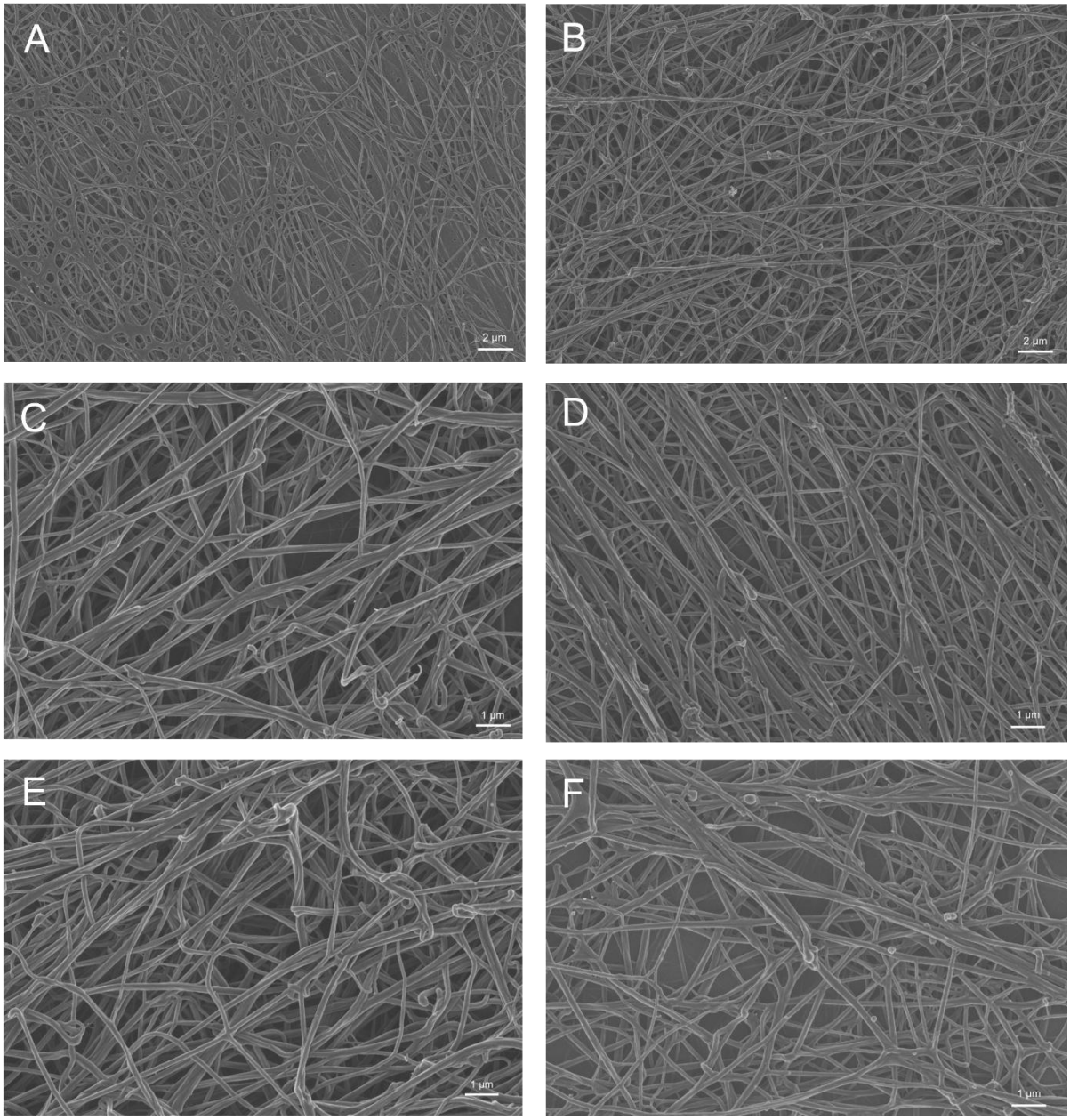


Figure 4.2:SEM results of fibrin fibres in the control group. A to B: 10KX magnification of fibrin fibres **(low magnification).** C to F: 20KX magnification of fibrin fibres **(high magnification).**

Figure 4.3. A-F illustrates a selection of micrographs of the fibrin fibres of the S-ICH patient group. The fibrin fibres in this group appeared to be thinner in size. The network of fibrin fibres is dispersed uniformly. The fibrin fibres created small net-like structures in A, C and D. Fusion of the fibres was observed in images C, D and F.

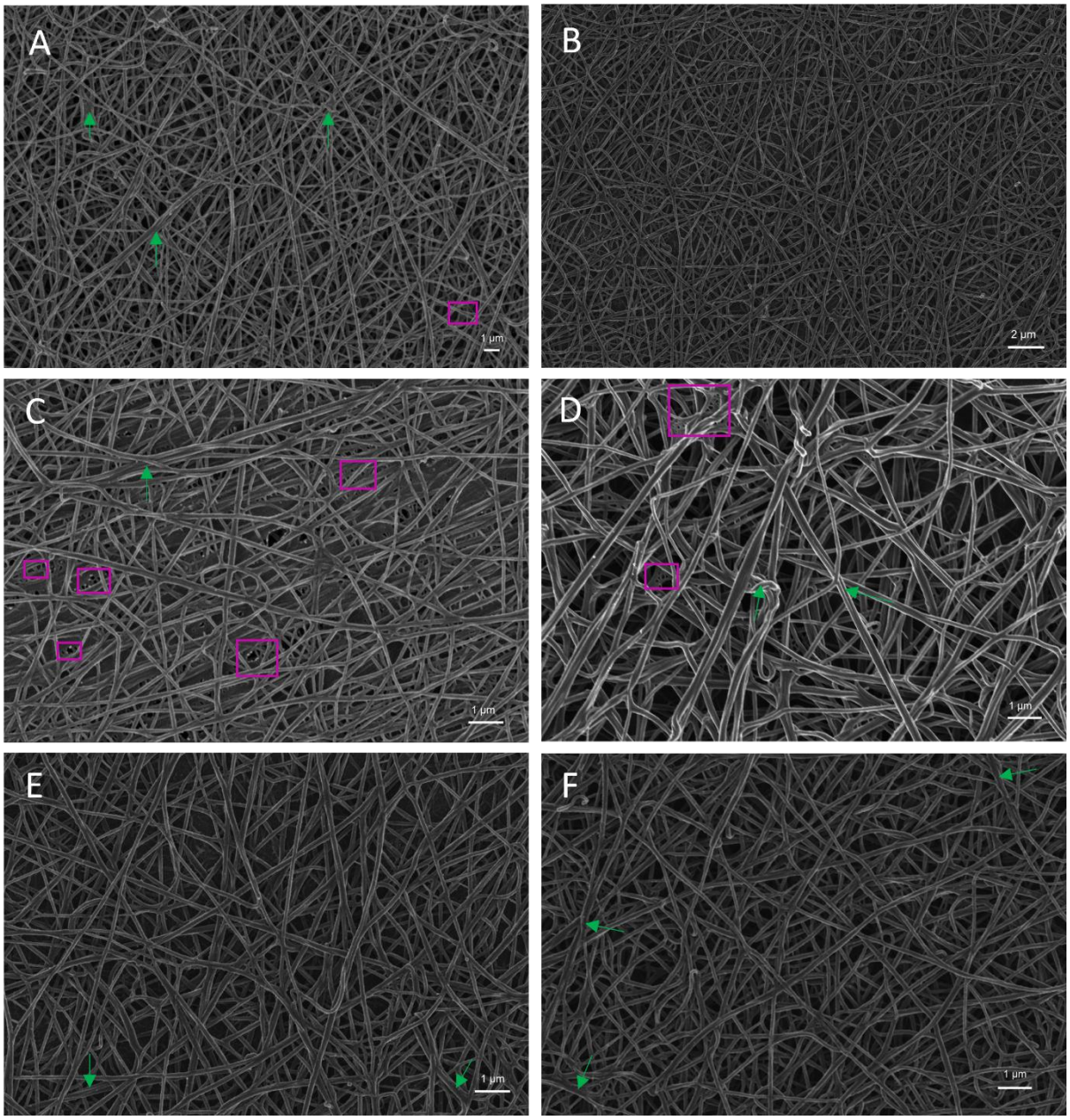


Figure 4.3:**SEM results of fibrin fibres in the S-ICH group.** A to B: 10KX magnification of fibrin fibres (**low magnification**). C to F: 20KX magnification of fibrin fibres (**high magnification**). The purple squares indicate a net-like structure created by the fibres. The green arrows indicate fusion of fibres.

Figure 4.4. A-F illustrates a selection of micrographs of the fibrin fibres of the T-ICH patient group. An inconsistent fibrin fibre network structure was observed; some fibres appeared to be thin, while other fibres were slightly thicker in size. Net-like structures and fibrin fibre fusion were seen in Figures 4.4. A – B. Fibrin fibre interlocking, and fusion were seen in all six micrographs.

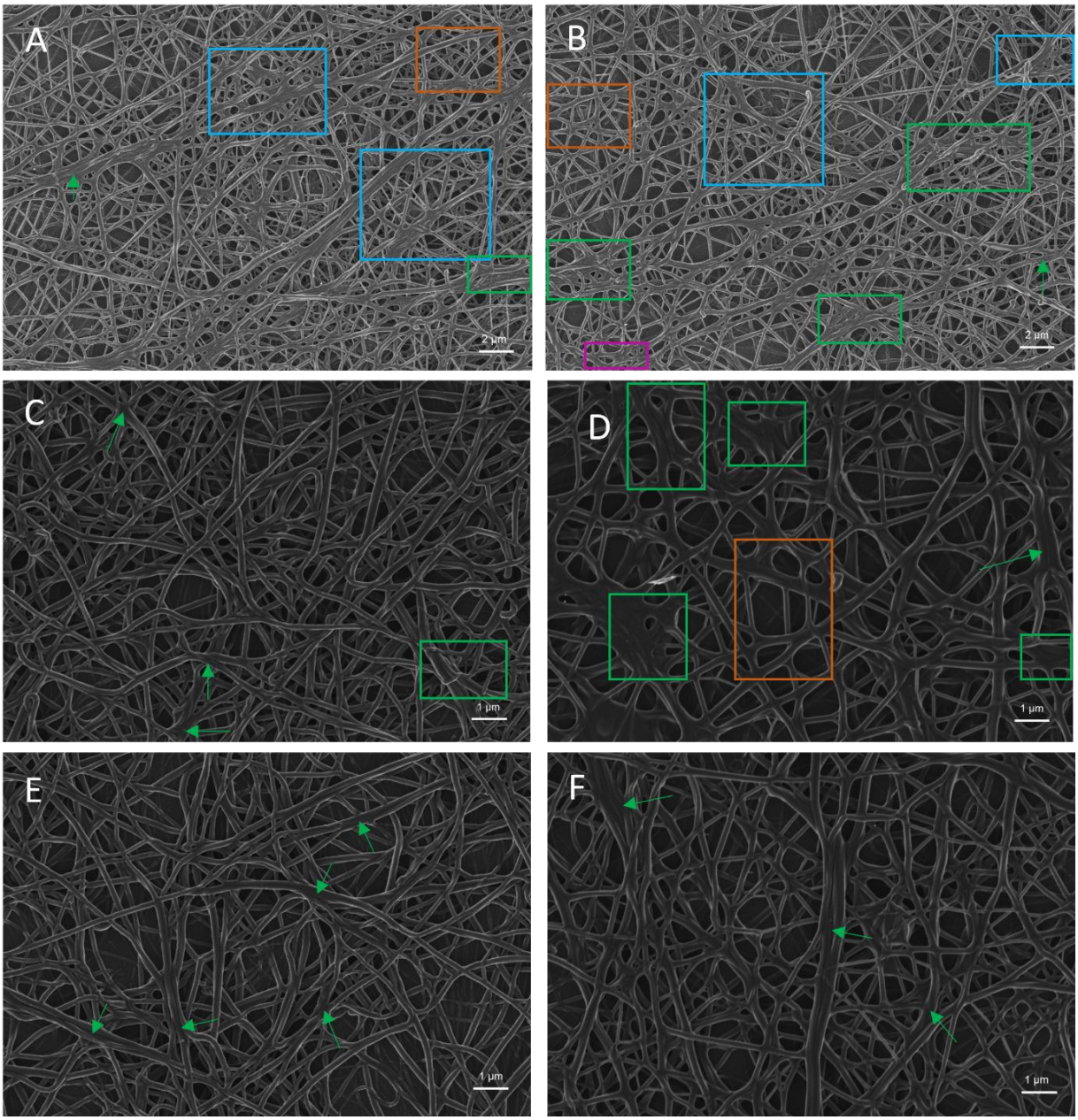


Figure 4.4: SEM results of fibrin fibres in the T-ICH group. A to B: 10KX magnification of fibrin fibres **(low magnification).** C to F: 20KX magnification of fibrin fibres **(high magnification).** The orange squares indicate a combination of thick and thin fibres. The purple squares indicate a net-like structure created by the fibres. The green arrows and squares indicate fusion of fibres. The blue squares indicate a combination of fusion and net-like structures of the fibres.

4.5.2 Fibrin fibre thickness

Quantification of the fibrin characteristics was done in addition to the morphological results that were obtained. The statistical data on fibrin fibre thickness passed the normality test. Comparing these findings to the control group, no significant differences were seen in any of the patient groups. However, based on the qualitative data, the fibres' sizes seemed to vary. The data in Table 4.1 presents a summary of these results. Note that patient numbers will not be sequential, as all patients in the patient

groups were chosen at random and subdivided into various groups after receiving a diagnosis.

Table 4.1: A summary of the descriptive statistics of the fibrin fibre thickness for the healthy controls (Cont), the spontaneous intracerebral haemorrhage (S-ICH) patients, and the traumatic intracerebral haemorrhage (T-ICH) patients.

Fibrin fibre thickness			
Group	**Mean (SD) Add measurement unit**	**P-value**	**Significant**
Cont *vs*. S-ICH	174.5 (20.19) vs 167.5 (27.93)	0.2948	ns
Cont *vs*. T-ICH	174.5 (20.19) vs 183.4 (31.11)	0.2500	ns
Significance indicated as: * = p<0.05; ** = p<0.01; *** = p<0.001; ns – not significant. Standard deviation (SD).			

4.5.3 Fibrin fibre branching

Fractal Analysis System was used to determine the fibrin fibre branching in each of the three groups. The nonparametric Mann-Whitney U test provided statistical data for the fibrin fibre branching. These results revealed no significant difference in the S-ICH patient group when compared to the control group; there was a statistical difference in the T-ICH patient group compared to the control group. The data in Table 4.2 presents a summary of these results.

Table 4.2: A summary of the descriptive statistics of the fibrin fibre branching for the healthy controls (Cont), the spontaneous intracerebral haemorrhage (S-ICH) patients, and the traumatic intracerebral haemorrhage (T-ICH) patients.

Fibrin fibre branching			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	2.57 (2.32; 2.64) vs 2.54 (2.32; 2.71)	0.1775	ns
Cont vs T-ICH	2.57 (2.32; 2.64) vs 2.44 (2.27; 2.66)	0.0010	***
Significance indicated as: * = p<0.05; ** = p<0.01; *** = p<0.001; ns – not significant. Standard deviation (SD).			

4.6 Discussion

When the fibrin networks of the patient groups were morphologically compared to the control group, it was found that the S-ICH group produced fibrin network structures

that appeared to be denser fibrin networks, while the T-ICH group produced fibrin network structures that appeared to be slightly looser packed networks. These findings are similar to the results observed in a study performed by Łopatka et al.[123], where individuals under the age of 50 who experienced ICH of unknown origin exhibited a pro-haemorrhagic fibrin clot phenotype. This phenotype was characterised by a prolonged clot formation time, reduced density of the fibrin fibre network, and an accelerated lysis rate when compared to appropriately matched control subjects.[123] The fibrin fibre samples from the control group had normal fibrin networks with fibres that had a consistent size and length. The S-ICH group displayed fibrin network structures that appeared to be thin, fused and tangled into clusters of fibres compared to those found in the control group. The T-ICH group displayed fibrin network structures that were thick, fused and tangled into clusters of fibres, along with net-like structures, compared to those found in the control group. Similarly, in a study done by van Rooy et al.[124], the networks exhibited a greater density and a more disorganised arrangement characterised by sticky, thick fibres that tended to cluster. This phenomenon is likely attributable to an increased lateral aggregation of the protofibrils. Such findings align with previous research indicating that denser fibrin networks correlate with decreased clot permeability, which in turn affects the process of clot lysis.[124] In a study done by Undas et al.[55], it was demonstrated that altered clot properties are associated with stroke. They observed that fibrin clots formed faster and had a compact structure with thicker fibres compared to healthy controls.[55] To provide more details on the potential effects of clot formation, these results were quantified in addition to the morphological observations.

According to the data presented in Table 4.1, there was no significant difference in fibrin fibre thickness between the S-ICH patient group compared to the control group with a P-value=0.2948, even though the fibres appeared thinner structurally (Figure 4.3). There was no significant difference in fibrin fibre thickness between the T-ICH patient group compared to the control group, with a P-value=0.2500, even though the fibres appeared thicker morphologically (Figure 4.4). This could suggest that the thickness of the fibrin fibre is unaffected by ICH. As described in research done by Mihalko and Brown.[125], low thrombin concentrations result in coarse, unbranched networks of thick fibres, whereas high thrombin concentrations produce dense, highly

branched network structures with thin fibres.[125] This is one way that thrombin concentrations are known to affect clot structure.

There was no significant difference in fibrin fibre branching between the S-ICH patient group and the control group, with a P-value=0.1775 (Table 4.2). The branching of the fibrin fibres between the two patient groups appeared to be similar (Figure 4.2 and Figure 4.3). However, the fibrin fibre branching was found to be statistically significant in the T-ICH group, compared to the control group, with a P-value=0.0010 (Table 4.2). There appeared to be less branching of the fibrin fibres in the T-ICH group (Figure 4.4). A clot with thin fibrin fibres and tightly packed fibrin fibres with small pores is more impermeable, less elastic, and more resistant to fibrinolysis.[118] The networks of clots made of thicker fibres are typically less stiff and looser, making them more permeable and vulnerable to fibrinolysis.[119]

According to previous research, changes in the polymerisation dynamics of fibrin impact the porosity of the network, the fibre thickness, and the fibrin structure's branching arrangement, subsequently influencing the mechanical properties of the clot.[118] Based on clot lysis research on fibrin fibre size by Ignjatovic et al.[126], it can be assumed that clot lysis in patients with S-ICH may occur at a slower rate compared to healthy controls due to thinner fibres that are tightly packed. In contrast, clot lysis in patients with T-ICH may occur faster than healthy controls due to thicker fibres that are loosely packed. An increase in clot lysis was observed during the acute phase following TBI; however, the duration of its persistence remains uncertain.[127] Therefore, changes observed in the fibrin networks formed in the blood of ICH patients will potentially have an effect on clot lysis. The increase in lysis time will increase the risk of embolus formation.[125] This, however, will need to be investigated further using clot lysis studies.

The fibrous networks of SEM images are often analysed manually; this process may be tedious and could introduce user bias and limit the sample size of this type of analysis.[119] Faster image analysis and the mitigation of some of these issues would be made possible by a dependable, automated technique for analysing fibrous networks in SEM images. To investigate the structure of blood and plasma clots in haemostasis and thrombosis, standardisation protocols involving automated analysis methods would be beneficial.[119]

4.7 Conclusion

Qualitative and quantitative methods were used in this chapter to study the morphological changes in fibrin fibres due to ICH. Closer interlocking strands and disrupted network uniformity were observed in the patient groups. The results in this chapter suggest that profile abnormalities exist in the coagulation process in ICH patients. Technologies such as SEM, ImageJ, and Fractal analysis systems should be used more frequently in general practice to assist with the early detection of devastating conditions such as ICH or to monitor the health status of patients while on treatment. As a crucial element of Virchow's triad, hypercoagulation will also be covered in Chapter 5, along with a review of the changes in clot formation that have been noticed.

Chapter 5: Thromboelastography®

5.1 Chapter objectives

The research was guided by the following objective:

To analyse the viscoelastic properties of clot formation in ICH patients compared to control individuals using TEG®

5.2 Introduction

Hellmut Hartert developed the haemostatic function for blood samples utilising TEG® in 1948.[128] Thromboelastography® is known as a point of care (POC) viscoelastic test and research technique since it detects real-time changes in viscosity and elasticity of WB or PPP during the blood clotting process.[116, 129, 130] Based on studies done by Kawano-Castillo et al.[131], it was noted that the TEG® may be used to identify significant changes during clotting in ICH patients as hypercoagulable trends may be induced in response to ICH.[131]

The TEG® technique, which is widely used to evaluate comprehensive clotting functions, operates on a simple principle.[129] A 0.36 mL blood sample is placed in a cup with a pin suspended and analysed using the TEG® auto analyser. The cup spins at a 4° 45' angle around the pin, connected by the development of the clot as it forms.[128] The transducer detects the change in tension caused by the pin.[128]

Clot formation and fibrinolysis are dynamically assessed by TEG® as it continuously measures and graphically displays the changes in viscoelasticity at all stages of the developing and resolving clot.[129] The TEG® system not only gives the corresponding clot duration data, but it also creates a complete picture of the viscoelastic changes that occur during the clot formation process.[44] Figure 5.1 illustrates the representative trace that shows various TEG® parameters of a healthy individual and a hypercoagulative individual using PPP.

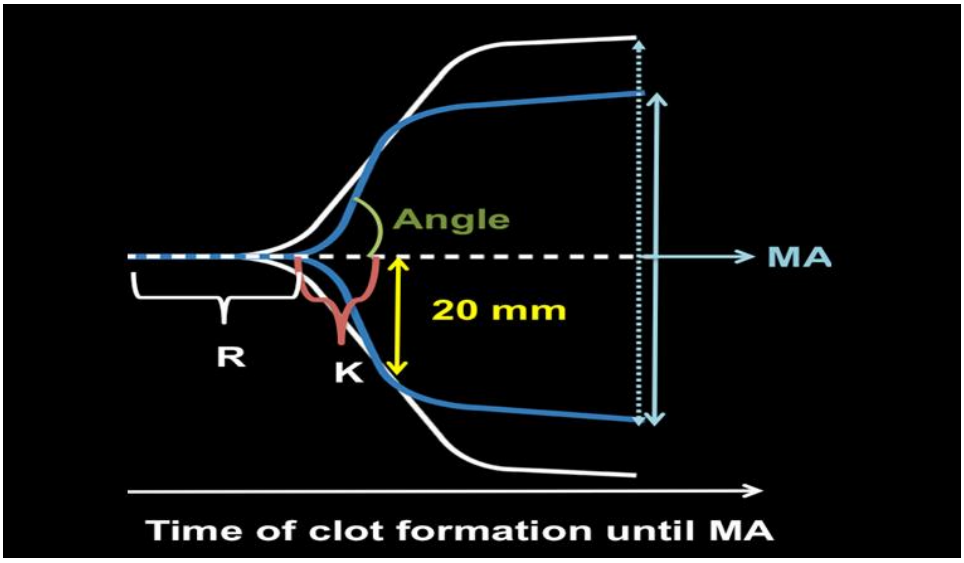


Figure 5.1: TEG® trace of a healthy individual (blue) and a hypercoagulable individual (white) using PPP.[116] R; reaction time (minutes), K; kinetics (minutes), MA; maximum amplitude (mm).

The degree of pathology associated with the coagulation status can be assessed using clotting parameters from the TEG® results.[116] These parameters assist in providing information on the kinetics and strength of the clot that was formed.[129] Table 5.1 illustrates the TEG® clot parameters of PPP and describes each parameter, based on studies done by Pretorius et al.[116] When variations in the measured TEG® parameters are observed below or above the normal ranges, the clotting may be identified as hyper- or hypocoagulable. Table 5.1 provides an example of interpreting the TEG® results for PPP samples.

Table 5.1: Thromboelastography® clot parameters for platelet-poor plasma. Table adapted from Pretorius et al.[116, 132]

Parameters	Description	Hypercoagulable	Hypocoagulable
R: reaction time (min)	The latency period from the commencement of the test to the initial formation of fibrin (amplitude of 2 mm), i.e. initiation time.	↓	↑
K: kinetics (min)	Time taken to achieve a specific level of clot strength (amplitude of 20 mm), i.e. amplification.	↓	↑
A (alpha): Angle (slope between the traces represented by R and K) (degrees)	The angle quantifies the speed of fibrin accumulation and cross-linking, thereby evaluating the rate at which clot formation occurs, i.e. thrombin burst.	↑	↓

MA: Maximal Amplitude (mm)	The maximum clot size indicates the ultimate strength of the fibrin clot, representing the overall stability of the clot. The larger the MA, the more hypercoagulable the clot.	↑	↓
G: Clot strength/ Shear elastic modulus strength (d/sc)	G-value is a log-derivation of the MA and is meant to also represent the clot strength. An increased G-value is linked to a hypercoagulable condition, which subsequently heightens the risk of developing venous thromboembolic disease.	↑	↓
MRTG: Maximum rate of thrombus generation (dcs)	The maximum velocity of clot growth observed or maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in dynes per cm^{-2}	↑	↓
TMRTG: Time to maximum rate of thrombus generation (min)	The time interval observed before the maximum speed of the clot growth.	↓	↑
TTG: Total thrombus generation (dcs)	The clot strength: the amount of total resistance (to the movement of the cup and pin) generated during clot formation. The total area beneath the velocity curve throughout the process of clot formation signifies the quantity of clot strength produced during the growth of the clot.	↑	↓

Thromboelastography® analysis is currently used in diagnostic and treatment algorithms as it effectively determines the coagulation profile of Trauma Induced Coagulopathy (TIC).[130] Based on studies done by Walsh et al.[128], it was noted that there was an increase in the use of TEG® for diagnosing TIC and patients that required massive transfusions.[128] Meier et al.,[16] conducted one of the first studies employing TEG® to compare coagulation status in patients with ICH, whether or not they had renal dysfunction.[16] The study found that patients with ICH and lower creatinine clearance exhibited stronger clots (increased MA and G) and quicker clot formation (decreased K and increased angle), suggesting hypercoagulability.[16] This hypercoagulable state in WB was also described in a study done by Kawano-Castillo et al.[131] Their findings revealed that patients with spontaneous ICH had a faster initial clot formation and were hypercoagulable compared to controls.[131] Concluding that clotting may be faster and stronger in immediate response to ICH.[131]

Ultimately, PPP exhibits both viscous and elastic properties, making it a viscoelastic fluid. This viscoelasticity is due to the presence of various plasma proteins, such as

fibrinogen, globulins, and albumin, which can form a loose network structure.[133] Platelet-poor plasma can display both liquid (lysis) and solid (clotting) features under varying conditions due to its combination of elastic and viscous qualities. Therefore, the process of TEG® allows for the evaluation of the changes in viscoelastic properties of PPP. These changes can be assessed while providing essential insights into coagulation and possible bleeding risks in ICH patients.

5.3 Materials and Methods

5.3.1 Materials, reagents and equipment

The following materials were utilised for TEG®:

- 0.2 M calcium chloride ($CaCl_2$) (Barker Medical, 7003)
- TEG® 5000 (Haemoscope Crop., Niles, IL, USA)
- 2 – 20 µL Eppendorf pipette
- 100 – 1000 µL Eppendorf pipette
- TEG® cups and pins (Vertice)
- 2 – 20 µL pipette tips (Lasec)
- 100 – 1000 µL pipette tips (Lasec)

5.3.2 Method

Platelet-poor plasma was obtained and used as described in Chapter 4. Following the manufacturer's instructions, the TEG® assays were carried out using the TEG® 5000 computer-controlled apparatus. A calibration test was performed before each sample was analysed to make sure the torsion wire was in good working order and that the pin connecting it to the oscillating cup holder was level. After that, the cup and pin were loaded into the TEG® in accordance with the instructions.

To initiate the coagulation process for each blood sample, 340 µL of PPP from each tube was placed in a TEG® cup, together with 20 µL of 0.2 M $CaCl_2$. Since this investigation mainly concerned clot formation, the procedure was permitted to proceed until MA was achieved. (Figure 5.1 and Table 5.1).

The TEG® parameters, as listed in Table 5.1, were recorded in an Excel spreadsheet for every sample.

5.4 Statistical analysis

Statistical analysis for the TEG® parameters was performed on GraphPad Prism Version 10.1.0 for Windows. The Shapiro-Wilk test was used to test for normality. Nonparametric data was tested using the Mann-Whitney U test showing the median (min, max); for parametric data, the t-test was used with a mean (SD). A P-value < 0.05 was considered significant.

5.5 Results

The following results were obtained from the TEG® analysis. Four of the eight TEG® parameters in the S-ICH group were found to be significantly different from the control group. In the S-ICH group, the medians for the MA, G, MRTG and TTG parameters increased compared to the control medians. Significant differences were seen between the T-ICH group and the control group for three of the eight TEG® measures. In the T-ICH group, the medians for the MA, MRTG and TTG parameters increased compared to the control medians. Table 5.2 provides a summary of these results.

Table 5.2: A summary of the descriptive statistics for all the parameters of the healthy controls (Cont), the Spontaneous intracerebral haemorrhage (S-ICH) patients, and the traumatic intracerebral haemorrhage (T-ICH) patients.

Reaction Time (R)			
Group	**Median (min; max) Unit?**	**P-Value**	**Significant**
Cont *vs*. S-ICH	15.70 (4.30; 45;80) *vs*. 12.60 (5.30; 44.90)	0.1525	ns
Cont *vs*. T-ICH	15.70 (4.30; 45.80) vs 11.25 (2.20; 47.20)	0.1321	ns
Kinetics (K)			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	2.30 (0.80; 7.20) vs 1.90 (0.90; 11.90)	0.6567	ns
Cont vs T-ICH	2.30 (0.80; 7.20) vs 2.65 (1; 29.40)	0.9317	ns
α-Angle (A)			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	57.25 (28.20; 87.80) vs 53.30 (11.30; 72.70)	0.1848	ns
Cont vs T-ICH	57.25 (28.20; 87.80) vs 52.40 (7.60; 74.50)	0.7107	ns

Maximum Amplitude (MA)			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	45.20 (19.20; 93) vs 63.90 (37.20; 78.80)	0.0015	** Increased
Cont vs T-ICH	45.20 (19.20; 93) vs 61.55 (32.90; 81.50)	0.0252	* Increased
Shear Elastic Modulus (G)			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	4.50 (1.20; 66.50) vs 8.80 (3; 18.60)	0.0234	* Increased
Cont vs T-ICH	4.50 (1.20; 66.50) vs 8.00 (2.50; 22)	0.1304	ns
Maximum Rate of Thrombus Generation (MRTG)			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	5.52 (2.1; 13.31) vs 8.95 (1.4; 15.48)	0.0080	** Increased
Cont vs T-ICH	5.52 (2.10; 13.31) vs 7.48 (0.40; 23.97)	0.0303	* Increased
Time to Maximum Rate of Thrombus Generation (TMRTG)			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	17.17 (6.17; 46.33) vs 15.33 (6.75; 43.83)	0.4350	ns
Cont vs T-ICH	17.17 (6.17; 46.33) vs 15.29 (3.0; 65.83)	0.5889	ns
Total Thrombus Generation (TTG)			
Group	**Median (min; max)**	**P-Value**	**Significant**
Cont vs S-ICH	399.4 (119.3; 5841) vs 887.1 (297.9; 1862)	0.0020	** Increased
Cont vs T-ICH	399.4 (119.3; 5841) vs 801.6 (242.5; 2221)	0.0328	* Increased
Significance indicated as: * = p<0.05; ** = p<0.01. ns – not significant. Minimum (min), maximum (max).			

The superimposed TEG® tracing showing a representative graph from each of the three groups is illustrated in Figure 5.2. The tracings from the S-ICH and T-ICH groups are shown by the green and purple lines, respectively, while the white line represents the tracing from the control group. The sole objective is to demonstrate the variations in the viscoelastic profile shape between the groups.

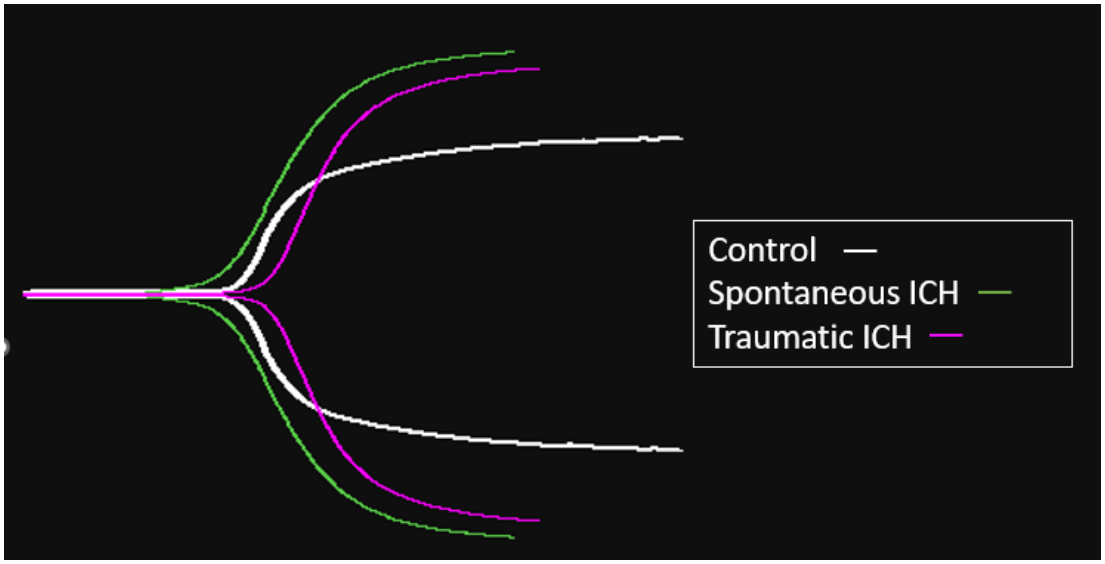


Figure 5.2: Superimposed thromboelastography® tracings of all three groups.

5.6 Discussion

The TEG® results presented in this chapter demonstrate a significant difference in the viscoelastic parameters of clot formation between the control and patient groups. There are two sections to the discussion of these findings. The first section examines the overall results for the S-ICH and T-ICH groups compared to the control group, which generates a clot formation "profile" for these groups. In the second section, hypercoagulability will be identified in the S-ICH and T-ICH groups.

The MA, G, MRTG and TTG parameters were significantly increased in the S-ICH group. The MA demonstrating the ultimate strength of the fibrin clot, was found to be significantly different as this parameter was increased in the S-ICH group. This indicates increased fibrin fibre interaction / thickness, resulting in a more rigid and dense clot.[116, 128] Clot strength can be assessed using the TTG parameter.[134] These changes in TTG and MRTG indicate an altered or abnormal process of polymerisation occurring in the soluble plasma protein fibrinogen, resulting in the formation of insoluble fibrin fibres.[135]

A clot with a higher density under pressure was indicated by a significant increase in the G parameter. The morphological observations of the fibrin fibre networks observed in the SEM investigations of the S-ICH group are consistent with the TEG® results. Based on the contribution of platelets and fibrin networks, previous research has shown that G is the best indicator of clot elasticity, showing whether the clot is rigid or elastic.[136]

In the T-ICH group, there was a significant increase in the MA, MRTG, and TTG parameters. The MA, indicating the stability of the clot and ultimate strength, was found to be significantly different as this parameter was increased in the T-ICH group compared to the control group. There was an increase in the MRTG parameter, indicating a trend of increased clot growth in T-ICH patients. Significant changes in MRTG and TTG suggest certain modifications that occur when fibrin is formed from fibrinogen.[137] The coagulation or clotting parameters, besides their primary role in indicating the coagulation process, also provide insights into the intricate interactions among various blood components such as cells and plasma proteins.[135]

Noticeably, there were similarities between the parameter changes observed in the T-ICH group and the S-ICH group. It can be concluded that the effect on the haemostatic system is consistent regardless of the original cause of ICH. Based on these results, the clot profile for the S-ICH and T-ICH groups can be described as a clot that has increased strength and rigidity compared to the control group. However, a noticeable difference between the S-ICH and T-ICH groups was a significant increase in the G parameter in the S-ICH group. This observation indicates an increase in the overall effectiveness of the clot in the S-ICH group compared to the control group.[136]

As discussed previously in this chapter, a key contributing factor to thrombus generation is hypercoagulability. Based on the data gathered, an increase or decrease in TEG® parameters, as shown in Table 5.1, can be used to detect hypercoagulability trends. Based on TEG® analysis, these changes can indicate hypercoagulable patient groups compared to the control group.[116] In previous studies, a hypercoagulable state was revealed in the early stage of ICH based on viscoelastic testing.[138] Viscoelastic tests such as TEG® are recommended to fully describe coagulopathy after TBI. The increase in PCT and CRP levels seen in this study for both patient groups is consistent with earlier research showing a hypercoagulable profile linked to inflammation.[139]

5.7 Conclusion

When comparing S-ICH and T-ICH groups to healthy controls, the viscoelastic parameters of clot formation acquired with TEG® revealed significant differences in multiple parameters. The viscoelastic parameters of these patient groups demonstrate a “clot profile” characterised by increased rigidity and strength in contrast to healthy controls. The SEM analyses supported the clot rigidity, potentially making clot lysis more challenging. A hypercoagulable state was observed for both patient groups, with four of the eight measured TEG® parameters increasing significantly in the S-ICH group, and three of the eight measured TEG® parameters increasing significantly in the T-ICH group. The increase in the inflammatory markers in Chapter 3 supports the observation of a hypercoagulable state. Consequently, their risk of thrombosis may increase.

The use of TEG® as a viscoelastic technique for identifying hypercoagulation and possible thrombosis risk is thus justified. Further studies should be done to determine reference ranges for changes in viscoelastic tests using PPP. To guide patient management and identify patients at risk of thrombosis based on the original cause of their diagnosis, it is imperative to have larger sample sizes in each group and to conduct the study over a longer period of time.

Chapter 6: Conclusion

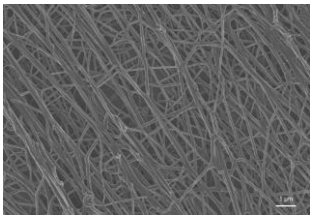
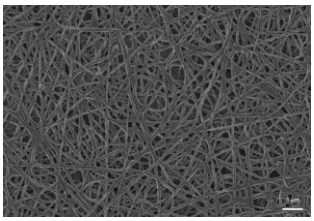
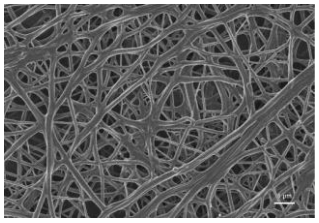
There is a significant worldwide effort to improve the care of patients with ICH. This condition is a major contributor to global morbidity and mortality due to its high 1-month case-fatality rate of about 40% and its poor long-term prognosis.[2, 11] Despite ICH representing a smaller proportion of strokes globally (10–30%), it imposes a heavier burden of disability-adjusted life years compared to ischemic stroke, particularly due to its high prevalence in low- and middle-income nations.[11] What seems to be lacking is that acute ICH currently has no established medical treatments. Although, surgery is commonly recommended for patients with ICH, there is ongoing debate on the appropriate timing and roles of different surgical techniques.[11]

This study aimed to examine the viscoelastic and ultrastructural characteristics of PPP by examining the clotting changes in patients with ICH. The inflammatory markers increased in both patient groups, suggesting that inflammation may occur in ICH patients. The findings of the SEM analysis showed that there were minor structural and network-related alterations in the fibrin fibres. However, there was no statistical difference in fibrin thickness and only a statistical difference in the T-ICH group regarding fibrin branching. The TEG® results revealed that the clots produced in the patient groups were denser and more rigid compared to the control group.

A summary of the main conclusions drawn from the qualitative and quantitative analyses revealed that ICH led to moderate modifications in specific coagulation components. However, the collective impact of these individual alterations produced a substantial transformation in the viscoelastic and ultrastructural characteristics observed during clot formation in both the S-ICH and T-ICH patient groups. Table 6.1 summarises the significant findings from each analysis and comparisons between the control and S-ICH and T-ICH groups, respectively.

Table 6.1: A summary table of the significant results from the comparison between the control and patient groups.

	Control	S-ICH	T-ICH
Inflammatory markers			
CRP <10 mg/L	x	The median CRP levels were higher than the normal range (median = 66)	The median CRP levels were higher than the normal range (median = 142)

PCT ug/L	x	The median PCT levels were higher than the normal range (median = 0.24)	The median PCT levels were higher than the normal range (median = 0.27)
SEM			
Fibrin fibres	 Organised and structured fibrin network	 Thin fibrin fibres with a tightly packed fibrin network	 Thick and thin fibrin fibres, with a loosely packed fibrin network
TEG®			
MA	Each patient group was compared to the control parameter separately.	Increased fibrin(ogen) interaction resulting in a more rigid and denser clot	Increased fibrin(ogen) interaction resulting in a more rigid and denser clot
G	Each patient group was compared to the control parameter separately.	Increased clot rigidity	Increased clot rigidity
MRTG	Each patient group was compared to the control parameter separately.	Increased clot growth	Increased clot growth
TTG	Each patient group was compared to the control parameter separately.	Increased clot strength	Increased clot strength

Spontaneous intracerebral haemorrhage (S-ICH), traumatic intracerebral haemorrhage (T-ICH), x indicates values not recorded, C-reactive protein (CRP), procalcitonin (PCT), scanning electron microscopy (SEM), thromboelastography® (TEG®), maximum amplitude (MA), shear elastic modulus (G), maximum rate of thrombus generation (MRTG), total thrombus generation (TTG)

One of the most frequent and avoidable complications of S-ICH is VTE.[68] However, there are inconsistencies between the treatment strategies of ICH and VTE. Anticoagulation and preventing recurrent thrombosis are the main goals of treating VTE, whereas haemostasis and preventing hematoma expansion are the main goals of treating ICH.[68] A deeper comprehension of the mechanisms underlying ICH-associated brain injury, the relationship between this injury and subsequent cognitive decline, as well as a more thorough understanding of the neuro repair processes following a stroke, will be essential for the advancement of new therapeutic interventions.[17]

Reiterating the literature, blood coagulation is accelerated and intensified in hypercoagulation. Since the inflammatory process plays a role in both acute and chronic brain damage following ICH, hypercoagulation is associated with ICH.[140]

The findings of this investigation will contribute to our understanding of clotting changes in PPP from ICH patients. Firstly, the results indicated that there was spontaneous activation of coagulation, perhaps due to the inflammatory profile of the S-ICH and T-ICH patient groups. The increase of the inflammatory parameters, CRP and PCT, can probably be linked to the activation of coagulation resulting in the increased strength of the clots for ICH patients. These findings are supported by the literature, which indicates that proinflammatory cytokines exhibit a significant increase in levels during metastasis.[141] The findings from TEG® and SEM are supported by existing literature, which indicates that proinflammatory cytokines, such as IL-6—significantly increasing only in cases of metastasis—can activate coagulation components by enhancing the expression of tissue factor (TF).

The results indicate that during clot formation, fibrin networks in both ICH patient groups underwent ultrastructural changes. The fibrin network observed in the ICH patient groups indicates a mesh structure characterised by distinct open areas among the individual branching elongated fibres; this relates to the TTG parameter.[116] The fibrin fibre thickness measured relates to the MA parameter as it depicts the elongated fibres that exhibit branching.[116]

Hypercoagulability was identified in S-ICH and T-ICH patient groups, based on the results of this study. Eight viscoelastic parameters were measured, which resulted in more rigid and denser clots in both patient groups. This hypercoagulable state can be linked to an increase in thrombosis due to the activation of the complement system.[142]

The results of this study indicated that a risk stratification approach concentrating on critical components of the haemostatic system could be beneficial for the management of ICH patients. Such a strategy may facilitate the development of tailored treatment plans for individual patients with ICH.

Although significant results were obtained, there are some limitations in this study These include the study's time constraint, a restricted patient history because of the patient's state of awareness, a small sample size because of the rigorous exclusion criteria, and the desire to participate. To guarantee comprehensive data detail of patients with ICH, future research can overcome these constraints by using a larger sample size over a longer time period. The difference in sex and age range between the control and ICH patient groups could be seen as a limitation due to the

convenience sampling in this study. This can be addressed in future studies by utilising patients of comparable ages and genders to demonstrate how diversity and perspectives improve the scientific quality and social significance of research. Assessing the structural and functional characteristics of clots in patient plasma samples that were formed *ex vivo* with or without procoagulant and/or anticoagulant agents can shed light on the efficacy of different treatment approaches for individual patients.[125] Future studies could be used to measure which cytokines are increased in ICH patients. This elevation in cytokines subsequently activates coagulation factors through enhanced expression of TF.[141]

Thromboelastography® and SEM have been demonstrated as effective methods for tracking the dynamics of clot formation and morphological alterations. These changes are essential for comprehending thrombotic risk, and it makes sense to validate these methods further in a clinical context. Incorporating viscoelastic analyses, such as atomic force microscopy and rheological assessments, alongside SEM and TEG® evaluations of clots, may provide further insights into the coagulability of the clot.[116] Enzyme-linked immunosorbent assays could be used to measure the baseline fibrinogen levels or TF involved in inflammation and coagulation. Turbidimetry tests could be used to measure clot formation and lysis of ICH patients. The possible mechanisms by which ICH causes a further prothrombotic shift could also be investigated in future research by looking at the influence of the circulating coagulation factors, as well as the other blood determinants such as WB viscosity, and the morphological changes of the blood components.

Addendum 1: Ethics approval and renewal certificates, ethics reference no. 298/2022

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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**

29 September 2022

Approval Certificate
New Application

Dear Miss A Lenting

Ethics Reference No.: 298/2022
Title: Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

The **New Application** as supported by documents received between 2022-05-23 and 2022-09-28 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-09-28 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 2023-09-29.
- Please remember to use your protocol number (298/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

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

We wish you the best with your research.

Yours sincerely

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

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

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YUNIBESITHI YA PRETORIA

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.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Faculty of Health Sciences **Research Ethics Committee**

18 May 2023

Approval Certificate
Annual Renewal

Dear Miss A Lenting,

Ethics Reference No.: 298/2022 – Line 1
Title: Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

The **Annual Renewal** as supported by documents received between 2023-04-18 and 2023-05-17 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-05-17 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 2024-05-18.
- Please remember to use your protocol number (298/2022) 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
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

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 USA Federal wide Assurance.
- FWA 00002567, Approved on 19 March 2022 and Expires: 18 March 2027
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through 01/31/2026 and Expires: 01/30/2026.

Faculty of Health Sciences **Research Ethics Committee**

16 May 2024

Approval Certificate
Annual Renewal

Dear Miss A Lenting,

Ethics Reference No.: 298/2022 – Line 2
Title: Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

The **Annual Renewal** as supported by documents received between 2024-04-10 and 2024-05-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-05-15 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 2025-05-16.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (298/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.

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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Gezina 0031, South Africa
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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 on 19 March 2022 and Expires: 18 March 2027
- IORG #: IORG0001762 OMB No. 0990-0279 Approved to expire [illegible] 2026 and Expires 04/30/2026.

Faculty of Health Sciences **Research Ethics Committee**

19 November 2024

Acknowledgement Certificate
Research Completed or Terminated

Dear Miss A Lenting,

Ethics Reference No.: 298/2022 – Line 3
Title: Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

The **Research Completed Report** as supported by documents received between 2024-10-28 and 2024-11-18 for your research, was acknowledged by the Faculty of Health Sciences Research Ethics Committee on 2024-11-18 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).

Research Ethics Committee
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University of Pretoria, Private Bag X323
Gezina 0031, South Africa
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E-mail: [illegible]
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Dikarolo tsa Maphelo

Addendum 2: NHRD approval letter, NHRD reference no. GP_202210_003

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

STEVE BIKO ACADEMIC HOSPITAL

Enquiries: Dr JS Mangwane
Tel No: +2712 3452018
Fax No: +2712 354 2151
E-mail: joseph.mangwane@gauteng.gov.za

For attention: janette Bester

NHRD Ref Number: GP_202210_003

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

TITLE: Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

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

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

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

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

STATUS OF APPLICATION:
Approved

Date: 2022-10-27

Dr. J S. Mangwane
Manager: Medical Service

Addendum 3: Information leaflet, informed consent and data collection sheet for healthy control group

Information leaflet and informed consent form (Control participant)

Study Title: Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma.

Sponsor: Thuthuka Trust

Principal Investigator: Miss. Andrea Lenting
Department of Physiology, University of Pretoria
073 499 8711

Ethical clearance number: 298/2022

Date and time of first informed consent discussion: …….

Date and time

Dear prospective participant

Dear Mr. / Mrs.....

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. 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 the link between the ultrastructural and viscoelastic properties of patients with intracerebral haemorrhage (brain bleed) to identify any blood clotting changes that might be linked with the complications of intracerebral haemorrhage. This will allow us to understand if intracerebral haemorrhage patients in South Africa have changed blood clotting or bleeding tendencies. To do this research, we will use a specialised microscope (scanning electron microscope) to look at the structure of a blood clot; as well as equipment that tests blood clotting properties (thromboelastography®) to determine the degree to which clotting is changed in the blood. We will compare the results to those of participants who have intracerebral haemorrhage. **Your blood sample will be used as part of a control group, meaning**

that you do NOT have intracerebral haemorrhage, but will be compared to participants who do have intracerebral haemorrhage.

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, examination of yourself before taking blood, and only after giving consent taking some blood samples.

One tube of blood will be drawn by a qualified nurse or medical doctor from the neurosurgery department into a citrate tube, containing 4 mL of blood or the equivalent of one teaspoon.

The samples will be used to do scanning electron microscopy, thromboelastography® as well as measuring a blood clotting protein and a marker for nicotine (fibrinogen and cotinine) levels.

The cotinine test will be done to confirm the use of tobacco or tobacco-related products. From the blood drawn, 2 mL of plasma will be stored at -80 °C and only after sample recruitment is completed the cotinine ELISA test will be conducted. If the levels are higher than 10 ng/mL, which indicates the use of tobacco or tobacco-related products the sample will be excluded from the sample population.

The blood drawl process will only be done once the participants have been identified by a medical doctor from the neurosurgery department, the blood drawl process will only be done once and will not interrupt any other routine treatment, and no follow-up tests will be required.

4) **Possible future testing**

The samples that you give to this study could one day lead to discoveries using methods and tests not included in this protocol, 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 intracerebral haemorrhage patients compared to healthy individuals in order to identify any abnormalities. These tests may only be identified after the results from this study have been obtained. To that end, we would like to keep the samples for as long as they are deemed useful for research purposes. This research could potentially be used for purposes not specified above for up to 5 years of collection. Ethical approval will be obtained before any further testing on the residual samples will be done. You may specify a shorter period of time for the study principal investigator to keep the samples.

You have the right to withdraw your consent at any time and may request that the samples you give to the study be destroyed. If you choose to do so, contact the study principal investigator, Andrea Lenting. 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 from this study, the study may help us to improve the treatment and understanding of intracerebral haemorrhage in the future. Many of these tests are done routinely on participants 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) Your rights as a research participant.

Your participation in the research 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.

9) Ethical approval

This protocol (298/2022) 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 have been granted by that committee (ethical clearance number). The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving humans/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

10) Information

If you have any questions concerning this study, please contact Miss Andrea Lenting tel: 073 499 8711

11) Confidentiality

All information obtained during the course of this study will be treated as confidential. Each participant that is taking part will be provided with an alphanumeric coded number e.g. A001 that will ensure the confidentiality of information 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**.**

12) 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 penalised 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

…… ……..

Doctor/Witness name and signature. Date

Participant code……

Verbal participant informed consent

(Applicable when participant cannot read or write)

I, the undersigned, ……..have read and have explained fully to the participant named …… , 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/her 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.

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

Data Capture Sheet – Healthy control group

Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

Andrea Lenting 073 499 8711

Healthy control Group

Date Captured: DD / MM / YYYY	Allocated Study ID (e.g., C1): *(Will be allocated by Investigator)*
Personal Information	
Age:	
Medical Information & History	
Do you smoke tobacco or any related product? (If yes, for whole long?)	☐ Yes *Length of time:* ☐ No
Would you say that you have consumed 5/more drinks on the same occasion in the past 30 days?	☐ Yes ☐ No
Do you use contraceptives?	☐ 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 (drugs or substances that reduces inflammation (redness, swelling and pain) in the body.

☐Yes ☐No Anti-coagulants

Other:_____

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-coagulants

Other:_____

Experimental test results

Thromboelastography®

R	K	Angle	MA	G	MRTG	TMRTG	TTG

Scanning Electron Microscopy

Features of Interest	Reference Image

ImageJ

Average of fibrin fibre thickness of 50 fibres per sample	

Addendum 4: Information leaflet, informed consent and data collection sheet for ICH patient group

Information leaflet and informed consent form (ICH patients – delayed consent)

Study Title: Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

Sponsor: Thuthuka Trust

Principal Investigator: Miss. Andrea Lenting
Department of Physiology, University of Pretoria
073 499 8711

Ethical clearance number: 298/2022

Date and time of first informed consent discussion: …….

Date and time

Dear prospective participant

Dear Mr. / Mrs .....

We would like to invite you 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 in this study. Before you agree for you to take part in this study you should fully understand what is involved. You will be counselled before you can provide consent on behalf of the participant. If you have any questions, which are not fully explained in this document, do not hesitate to ask the investigator. You should not agree for you 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. The principal investigator will go through the informed consent with the participant.

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 the link between the ultrastructural and viscoelastic properties of patients with intracerebral haemorrhage (brain bleed) to identify any blood clotting changes that might be linked with the complications of intracerebral haemorrhage. This will allow us to understand if intracerebral haemorrhage patients in South Africa have changed blood clotting or bleeding tendencies. To do this research, we will use a specialised microscope (scanning electron microscope) to look at the structure of a blood clot; as well as equipment that tests blood clotting properties

(thromboelastography®) to determine the degree to which clotting is changed in the blood. We will compare the results to those of participants who do not have intracerebral haemorrhage.

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, examination of you before taking blood, and only after giving consent, taking some blood samples.

In the case of the patient being in ICU. drawn from the central venous line by a medical doctor. This will be done during routine tests for patients in ICU. Therefore, only when these tests are requested will we take additional citrate tubes for our analyses. One tube of blood will be drawn by a qualified nurse or medical doctor from the neurosurgery department into a citrate tube, containing 4 mL of blood or the equivalent of one teaspoon.

The principal investigator will do a delayed consent for unconscious patients and when the patient regains consciousness and is able to provide consent. The patient will be deemed able to give consent upon the medical clearance from the treating clinician's assessment. The patient will be informed of the patient's inclusion in the research study as soon as reasonably possible and will be advised about his/her right to withdraw from the study without a change in their quality of care. All the data of the patient will then be removed from the study. The samples will be used to do scanning electron microscopy, thromboelastography® as well as measuring a blood clotting protein and a marker for nicotine (fibrinogen and cotinine) levels.

The cotinine test will be done to confirm the use of tobacco or tobacco-related products. From the blood drawn, 2 mL of plasma will be stored at -80 ºC and only after sample recruitment is completed the cotinine ELISA test will be conducted. If the levels are higher than 10 ng/mL, which indicates the use of tobacco or tobacco-related products the sample will be excluded from the sample population.

The blood drawl process will only be done once the participants have been identified by a medical doctor from the neurosurgery department, the blood drawl process will only be done once and will not interrupt any other routine treatment, and no follow-up tests will be required.

4) Possible future testing

The samples that you give to this study could one day lead to discoveries using methods and tests not included in this protocol, 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 intracerebral haemorrhage patients compared to healthy individuals in order to identify any abnormalities. These tests may only be identified after the results from this study have been obtained. To that end, we would like to keep the samples for as long as they are deemed useful for research purposes. This research could potentially be used for purposes not specified above for up to 5 years of collection. Ethical approval will be obtained before any further testing on the residual

samples will be done. You may specify a shorter period of time for the study principal investigator to keep the samples.

You have the right to withdraw your consent at any time and may request that the samples you give to the study be destroyed. If you choose to do so, contact the study principal investigator, Andrea Lenting. 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 potential risk and discomfort associated with drawing blood from a vein, which can result in bruising and bleeding, and less commonly infection and bleeding at 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 from this study, the study may help us to improve the treatment and understanding of intracerebral haemorrhage in the future. Many of these tests are done routinely on participants 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) Your rights as a research participant.

Your participation in this study is entirely voluntary. You can refuse to participate or stop at any time during the study without giving any reason. Should you wish not to participate your care will not be compromised and your management / treatment will not differ in any way to those participating. All services usually provided to patients in the critical care unit will be provided to you no matter if you decide to participate or not.

9) Ethics approval.

This protocol (298/2022) 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 have been granted by that committee (ethical clearance number – 298/2022). The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving humans/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

10) Information

If you have any questions concerning this study, please contact Miss Andrea Lenting tel: 073 499 8711

11) Confidentiality

All information obtained during the course of this study will be treated as confidential. Each participant that is taking part will be provided with an alphanumeric coded number e.g. A001 that will ensure the confidentiality of information 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**.**

12) Consent to participate in this study

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

…… ……..

Participant's name and signature Date

…… ……

Doctor/Witness name and signature. Date

Participant code……

STATEMENT BY RESEARCHER OBTAINING INFORMED CONSENT:

I declare that the information document has been read by or accurately read out to the potential participant. I confirm that I have to the best of my ability made sure that the participant understands all the procedures outlined therein to be undertaken on enrolment of the participant in the study.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by them have been answered correctly and to the best of my ability. I confirm that the participant has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this informed consent form has been provided to the participant.

_____ _____

Investigator's Name (Please print) Date

_____ _____

Investigator's Signature Date

Verbal participant informed consent

(Applicable when participant cannot read or write)

I, the undersigned, ……., have read and have explained fully to the participant, named …… , the informed consent document, 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.

The participant has indicated that he/she understands and that the participant will be free to withdraw from the study at any time for any reason and without jeopardizing his/her treatment / management.

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

…….	……..
Participant's name and signature	Date
…….	……..
Investigator's name and signature	Date
…….	……
Doctor/Witness name and signature.	Date

Data Capture Sheet – ICH patient group

Investigating clotting changes in intracerebral haemorrhage patients from Steve Biko Academic Hospital by studying the viscoelastic and ultrastructural properties of platelet-poor plasma

Andrea Lenting 073 499 8711

ICH patient group

Date Captured: DD / MM / YYYY	Allocated Study ID (e.g., C1): *(Will be allocated by Investigator)*
Personal Information	
Hospital number	Age:
Medical Information & History	
Do you smoke tobacco or any related product? (If yes, for whole long?)	☐ Yes, Length of time: ☐ No
Would you say that you have consumed 5/more drinks on the same occasion in the past 30 days?	☐ Yes ☐ No
Do you use contraceptives?	☐ 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 (drugs or substances that reduces inflammation (redness, swelling and pain) in the body.

☐Yes ☐No Anti-coagulants

Other:_____

Experimental test results

Thromboelastography®

R	K	Angle	MA	G	MRTG	TMRTG	TTG

Scanning Electron Microscopy

Features of Interest	Reference Image

ImageJ

Average of fibrin fibre thickness of 50 fibres per sample	

Addendum 5: Letter of statistical support

Faculty of Health Sciences
Department of Immunology

Letter of Statistical Clearance

Tuesday, April 05, 2022

This letter is to confirm that the MSc student with the Name: **A Lenting**, Student No: **15375928** studying at the University of Pretoria discussed the project with the title; **An *ex vivo* study on the viscoelastic and ultrastructural properties of platelet poor plasma in intracranial haemorrhage patients** 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 PAA Meyer
Head of Department: Immunology
ReaCom appointed Biostatistician
University Pretoria

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Addendum 6: Letter of Approval from Head of Neurosurgery.

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FACULTY OF HEALTH SCIENCES
DEPARTMENT OF NEUROSURGERY
STEVE BIKO ACADEMIC HOSPITAL
26/06/2022

To whom it may concern

I hereby grant permission for the study titled '**An *ex vivo* study on the viscoelastic and ultrastructural properties of platelet poor plasma in intracranial haemorrhage patients.**' to be carried out in my department.

PROFESSOR LC PADAYACHY
HEAD: DEPARTMENT OF NEUROSURGERY
UNIVERSITY OF PRETORIA
STEVE BIKO ACADEMIC HOSPITAL

Addendum 7: Turnit in report

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