# Investigating clotting changes in patients from Steve Biko Academic Hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood

**by**

**Shené Ferreira**

**18008306**

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**Department of Physiology**

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| Supervisor | Co-supervisor | Co-supervisor | HOD |
|---|---|---|---|
| Prof Janette Bester | Prof Llewellyn Padayachy | Dr Sajee Alummoottil | Prof Annie Joubert |

# Abstract

Patients with intracerebral haemorrhage (ICH) have an increased risk of experiencing clotting changes when compared to healthy individuals, with recent research indicating that a bleeding event may enhance the prothrombotic effects of ICH. Although the effects of inflammation on the properties of whole blood (WB) in these patients have been studied extensively, there is a scarcity of research on the effects of ICH on the haemorheological-, and morphological properties of WB in patients with ICH. Therefore, this study utilised microscopy and viscoelastic techniques to examine clotting in these patients, in order to obtain a better understanding of the changes in clot formation in ICH patients. This may give more insight into thrombotic risk assessment and management. Whole blood from traumatic ICH (TICH) and non-traumatic ICH (NTICH) patients were compared to healthy controls. For a haematological overview of the ICH patients, routine clinical test results were utilised. Light microscopy (LM) was used to quantify the amount of deformed red blood cells (RBCs) present in each patient group. Scanning electron microscopy (SEM) was used to study the ultrastructural changes in blood cells and formed clots. Thromboelastography (TEG®) was used to study the changes in clot kinetics during clot formation. Results from the full blood count and C-reactive protein (CRP), demonstrated a tendency toward inflammation in both patient groups. No significant difference was seen in RBC deformation in both groups compared to the controls, indicating there was no significant RBC deformation in the patient groups. Ultrastructural studies on RBCs using SEM in both patient groups showed fine membrane changes and increased aggregation when compared to healthy controls. Platelets (PLTs) also appeared to be spread and fibrin fiber formation was disorganised. Viscoelastic results showed that clots formed faster in ICH patients, with increased strength and rigidity, thus revealing a hypercoagulable nature during clotting in these patient groups. The results of this study have revealed the marked differences in coagulation and associated blood components in TICH and NTICH patients compared to healthy controls. They provide a greater understanding of clot dynamics that could contribute to an increased risk of thrombotic events, traceable through viscoelastic techniques. This justifies further investigation into the utilisation of these techniques in a clinical, point-of-care setting, in order to enhance the prevention and management of thrombotic events in these patients.

**Keywords:** Intracerebral haemorrhage, Bleeding event, Coagulation system, Haemorheology, Scanning electron microscopy, Thromboelastography®

# Declaration

I, Shené Ferreira, hereby declare that this research dissertation "Investigating clotting changes in patients from Steve Biko Academic Hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood" is my own work and has not been presented for any degree at another University.

Signed: ……… ……….

Date: …………20-11-2023…………..

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

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# List of abbreviations

| | |
|---|---|
| **A** | α-angle |
| **AA** | Arachidonic ccid |
| **AD** | Alzheimer’s disease |
| **ADP** | Adenosine diphosphate |
| **AHT** | Arterial hypertension |
| **AR** | Axial ratios |
| **AT** | Antithrombin |
| **ATP** | Adenosine triphosphate |
| **AVM** | Arteriovenous malformation |
| **BBB** | Blood brain barrier |
| **BDMP/s** | Brain derived microparticle(s) |
| **BP** | Blood pressure |
| **$Ca^{2+}$** | Calcium |
| **CAA** | Cerebral amyloid angiopathy |
| **$CaCl_2$** | Calcium choride |
| **CBF** | Cerebral blood flow |
| **Cd** | Cadmium |
| **CGRP** | Calcitonin gene-related peptide |
| **CM** | Cavernous malformation |
| **CNS** | Central nervous system |
| **COX-2** | Cyclooxygenase-2 |
| **Cr** | Chromium |
| **CRP** | C-reactive protein |
| **CSF** | Cerebrospinal fluid |
| **CT** | Computed tomography |
| **CVT** | Cerebral venous thrombosis |
| **DAMP** | Danger-associated molecular patterns |
| **DIC** | Disseminated intravascular coagulation |
| **FBC** | Full blood count |
| **G** | Shear elastic modulus |
| **GCS** | Glasgow Coma Scale |
| **GPVI** | Glycoprotein VI |
| **Hb** | Haemoglobin |
| **HCT** | Haematocrit |
| **HIV** | Human immunodeficiency virus |
| **HMDS** | Hexamethyldisilazane |
| **5-HT** | 5-hydroxytryptamine |
| **ICH** | Intracerebral haemorrhage |
| **ICP** | Intracranial pressure |
| **ICU** | Intensive care unit |
| **IL** | Interleukin |
| **IL-1β** | Interleukin-1β |
| **INR** | International Normalised Ratio |
| **ISF** | Interstitial fluid |
| **IVH** | Intraventricular haemorrhage |
| **K** | Kinetics |

| | |
|---|---|
| **LM** | Light microscopy |
| **LPS** | Lipopolysaccharide |
| **MA** | Maximum amplitude |
| **Max** | Maximum |
| **MCH** | Mean corpuscular haemoglobin |
| **MCHC** | Mean corpuscular haemoglobin concentration |
| **MCV** | Mean corpuscular volume |
| **Min** | Minimum |
| **MMPs** | Matrix metalloproteinases |
| **MP** | Microparticles |
| **MRA** | Magnetic Resonance Angiogram |
| **MRI** | Magnetic Resonance Imaging |
| **MRTGG** | Maximum Thrombus Generation |
| **NF-κB** | Nuclear factor-κB |
| **NHLS** | National Health Laboratory Services |
| **NHRD** | National Health Research Database |
| **NO** | Nitric oxide |
| **NT** | Non-traumatic |
| **NTICH** | Non-traumatic intracerebral haemorrhage |
| **NSAID** | Nonsteroidal anti-inflammatory drugs |
| **$O_2$** | Oxygen |
| **$OsO_4$** | Osmium tetroxide |
| **PAI-1** | Plasminogen activator inhibitor 1 |
| **PBS** | Phosphate buffered saline |
| **PLA 2** | Phospholipase A2 |
| **PLT/s** | Platelet/s |
| **PPP** | Platelet-poor-plasma |
| **$pO_2$** | Partial pressure of oxyegn |
| **PS** | Phosphatidylserine |
| **R** | Reaction time |
| **RBC/s** | Red blood cell/s |
| **RCVS** | Reversible Cerebral Vasoconstriction Syndrome |
| **ROS** | Reactive oxygen species |
| **SA** | South Africa |
| **SAH** | Subarachnoid haemorrhage |
| **SBAH** | Steve Biko Academic Hospital |
| **SD** | Standard deviation |
| **SEM** | Scanning electron microscopy |
| **SF** | Serum ferritin |
| **SVD** | Small Vessel Disease |
| **TBI** | Traumatic brain injury |
| **T2D** | Type 2 diabetes |
| **TEG** | Thromboelastography |
| **TF** | Tissue factor |
| **TFPI** | Tissue factor pathway inhibitor |
| **TICH** | Traumatic intracerebral haemorrhage |
| **TMRTGG** | Time to Maximum Thrombus Generation |
| **TNF-α** | Tumour necrosis factor alpha |

| | |
|---|---|
| **tPA** | Tissue plasminogen activator |
| **TTG** | Total Thrombus Generation |
| **UP** | University of Pretoria |
| **vWF** | von Willebrand factor |
| **WB** | Whole blood |
| **WBC/s** | Whole blood cell/s |

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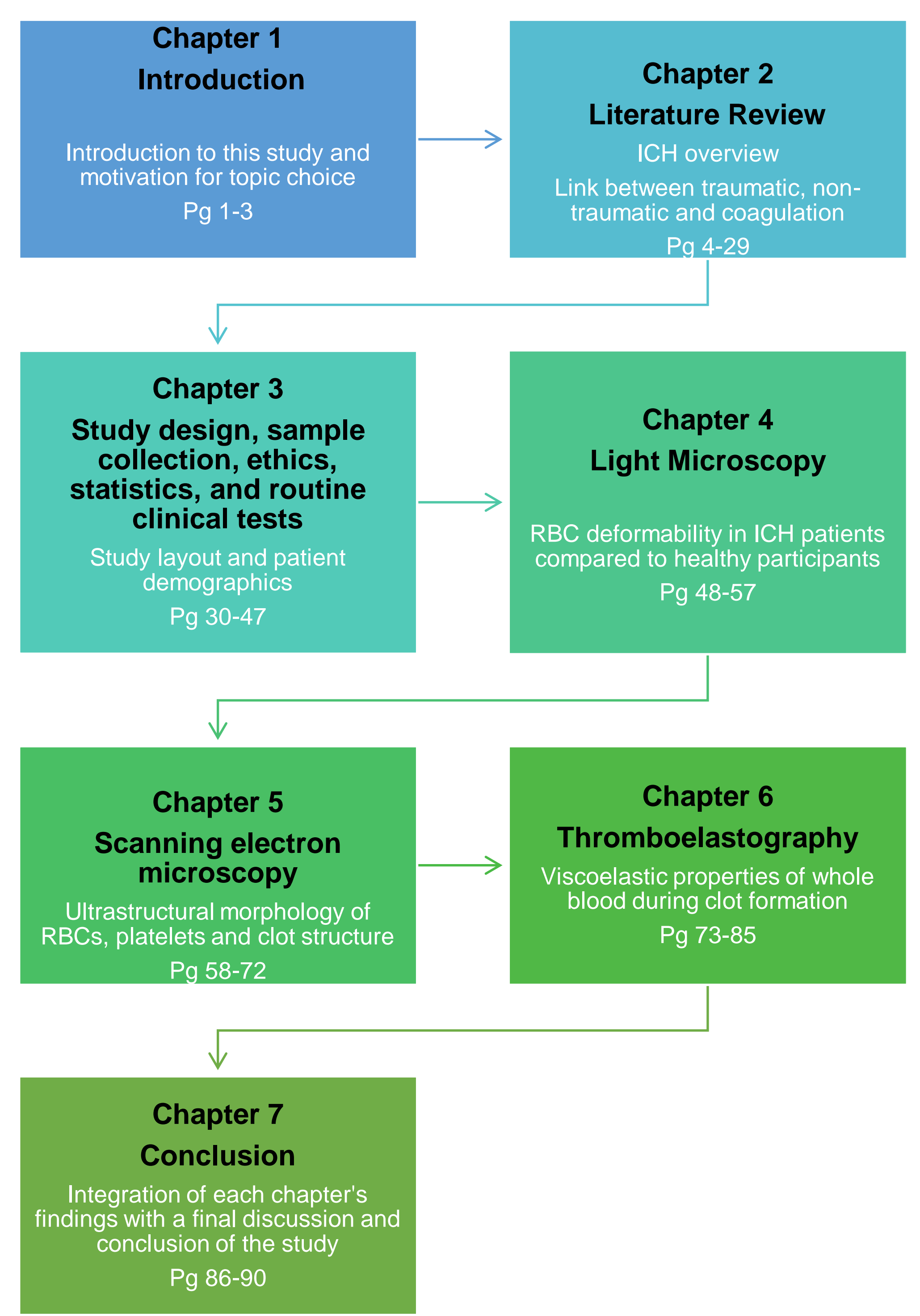
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# Chapter 1: Introduction

Intracerebral haemorrhage (ICH) is caused by a loss of vascular integrity, which leads to the rupture of a cerebral vessel, resulting in bleeding within the brain parenchyma[1-2]. Traumatic events and spontaneous vessel rupture both have the potential to harm cerebral blood vessels. Traumatic brain injury (TBI) has been defined as brain damage induced by an external impact, such as penetration by an item or blast wave. Traumatic brain injury is most commonly caused by sports-related injuries, blast exposures, transport accidents, and falls[3]. The two main pathoetiologies of spontaneous ICH are cerebral amyloid angiopathy (CAA) and chronic hypertension. Blood loss from tumors and vasculopathy, as well as the haemorrhagic conversion of an ischemic stroke or venous thrombosis, are examples of secondary pathoetiologies. Coagulopathy, platelet (PLT) dysfunction, and usage of illegal drugs can cause ICH or worsen its symptoms[4].

Every year, almost three million people worldwide experience ICH. Although the incidence of ICH is substantially lower than that of ischemic stroke, it causes a 50% larger global burden of disability. Intracerebral haemorrhage has a 30-day case fatality rate of more than 40%, and the majority of survivors have lasting impairments[5]. Due to the high likelihood of poor outcomes, physicians caring for patients with ICH frequently use clinical severity scores early on to estimate the risk of mortality or future disability in order to determine the aggressiveness of medical care[5].

The most prevalent symptom of ICH is a sudden onset focused neurological impairment, which is detected by the site of the haemorrhage and subsequent oedema. This is frequently coupled with a decline in the conscious status of the patient, as measured by the Glasgow Coma Scale (GCS). Other typical symptoms and indicators include headache, nausea/vomiting, seizures, and a high diastolic blood pressure (BP) (>110 mmHg). Clot extension into the ventricles can result in obstructive hydrocephalus, which is characterised by signs and symptoms of increased intracranial pressure (ICP), such as postural headaches, papilledema, nausea, vomiting, diplopia, disorientation, and a reduced consciousness level[2].

Intracerebral haemorrhage can be diagnosed by a doctor using blood tests and a GCS score, as well as brain imaging techniques. Non-contrast brain computed tomography

(CT) is commonly used as the reference standard for ICH diagnosis since it is quick, highly sensitive, and specific for all kinds of ICH[6].

In the presence of ICH, healing mechanisms are engaged as with any tissue injury, leading to the development of accompanying inflammation as a typical side effect of tissue damage repair. Haemostasis is a response mechanism that stops blood loss at an injury site by creating a clot that first slows down the blood flow before stopping it altogether[7]. The inflammatory response, which is characterised by an accumulation and activation of inflammatory cells, starts as soon as blood components are present in the parenchyma. Following this, a variety of cytokines, chemokines, free radicals, and other potentially harmful compounds are released by activated inflammatory cells. Cell death occurs during the ICH process, resulting in a new phase of the inflammatory response. To further stimulate the immune system, dead/damaged cells release a wide range of pro-inflammatory factors. Danger-associated molecular patterns (DAMPs) generated by dead cells can cause leukocyte infiltration into the brain, aggravating the inflammatory damage[7].

The body is known to become hypercoagulable during an increase in inflammation, increasing the likelihood of clot formation. These clots, or thrombi, are directly responsible for thrombotic events such as deep vein thrombosis, venous thromboembolism, pulmonary embolism, strokes, and heart attacks[8]. Thus, ICH patients have an increased risk of developing thrombosis as complication. This being said, a comprehensive investigation into each component of the haemostatic system will provide a better understanding of the clotting abnormalities in these patients and may aid in the development of better patient management [8].

The aim of this study was to assess clot formation in ICH patients by studying the viscoelastic characteristics, as well as the morphological properties of whole blood (WB) in these patients. To assess each patient's inflammatory status and overall haematological profile, a full blood count (FBC) and C-reactive protein (CRP) test results were obtained from hospital records. The results were compared to normal reference ranges to evaluate their profiles. Red blood cell (RBC) axial ratios (AR) were calculated to determine the degree of cell deformation and were compared to healthy controls. Scanning electron microscopy (SEM) was utilised to examine the fine ultrastructural characteristics of RBCs, PLTs, and fibrin fibers in ICH patients

compared to healthy controls. Thromboelastography® (TEG®) was utilised to investigate clot kinetics in patients and controls. Very high magnifications were used to show RBC membrane ultrastructure that was not visible with light microscopy (LM). Results of the FBC measurements revealed that six of the nine parameters were not within the normal reference range. Axial ratios from the LM indicated no significant difference for both ICH 0patient groups compared to healthy controls respectively. Scanning electron microscopy results for the traumatic ICH (TICH) group revealed changes compared to the control group. Slight RBC shape change, RBC aggregation, PLT presence and spreading with long pseudopods, interaction between PLT and RBCs, and less structured branching with finer webbed fibers were observed. The non-traumatic ICH (NTICH) group displayed modest RBC shape change, RBC aggregation, shedding of microparticles (MPs), RBC and PLT aggregation, long pseudopod formation, and a disturbed network uniformity with fiber tangles and fusions. The TEG technique was used to confirm the hypercoagulative status in ICH patients and to define dynamic changes in order to identify a clot profile in these patients. Both ICH patient groups showed significant difference in their kinetics (K), maximum amplitude (MA), shear elastic modulus (G), maximum thrombus generation (MRTG) and total thrombus generation (TTG) parameters. Together, these methods led to a better comprehension of how clot dynamics and component changes occur in a hypercoagulative state, particularly in ICH patients.

Based on the findings, we were able to tease out and characterise some of the actions of the role players in the development of hypercoagulability in ICH patients. Also, to identify further avenues of investigation that could improve our understanding. The findings of this study were aimed to strengthen the existing understanding of ICH-associated hypercoagulability, with the goal of developing management and treatment plans that not only save the lives of ICH patients but also to maintain their quality of life. This study added to the current body of information by expanding our understanding of the haemorheological and morphological consequences of ICH on WB. Future research should incorporate further biochemical quantitative analysis to uncover and explain more of the activation mechanisms.

# Chapter 2: Literature review

## 2.1 Chapter objectives

In this chapter, current literature was reviewed to showcase the background on the current topic.

### 2.1.1 Introduction

The burden of neurological conditions in sub-Saharan Africa has been increasing over the years, causing disproportion compared to developed countries[9]. Africa accounts for 25% of the global burden and has the world's largest human genetic diversity, with important inferences for understanding neurological disorders. Although early prognosis in neuroscience is critical, Africa has lagged behind in this subject, owing primarily to insufficient funding, an overwhelmed healthcare system, and a lack of infrastructure[10].

South Africa (SA) is a low to middle-income country with limited access to health care and a continually high rate of trauma, mostly due to interpersonal violence[11-12]. This implies that a remarkably proportion of TBI's in SA is secondary to penetrating trauma, although great detail has not been documented[13].

Intracerebral haemorrhage is commonly found in, male sex, younger age groups, advanced age groups and low-and middle-income countries[14]. Intracerebral haemorrhage has a high fatality rate of 40% at one month and 54% at one year, where only 12% - 39% of survivors achieve functional independence[15]. The incidence rate, from a 36 population-based epidemiology study of ICH per 100,000 person-years was 20 in Hispanics, 52 in Asians, 23 in Blacks, and 24 in Whites[16]. For all ages, men showed a higher annual incidence rate per 100,000 persons than women; 5.9 vs. 5.1 for ages 35 - 54 years, 37.2 vs. 26.4 aged 55-74 years, and 176.3 vs. 140.1 aged 75 - 94 years[15].

## 2.2 Intracerebral haemorrhage

### 2.2.1 Intracerebral haemorrhage: A general overview

An ICH refers to the rupturing of a blood vessel within the brain parenchyma[17]. Although blood may leak into the subarachnoid space or ventricular system, it always

begins in the brain tissue[18]. Once bleeding occurs, it can lead to the cellular architexture disruption inside the brain, with the mass of the haematoma increasing intracranial pressure which can compress the brain regions. This compression may hinder blood flow and eventually lead to brain herniation (movement of brain tissue)[19]. Intracerebral haemorrhage can either occur due to complications from a pre-existing lesion, known as a secondary ICH, or in the absence of an underlying lesion, which is a primary ICH and the most common type[17].

To diagnose a suspected ICH patient, the treating doctors will need to establish symptoms of the patient as well as, the medical history that includes previous medical problems, medication usage and family history. The neurological symptoms experienced by ICH patients usually aggrevate over minutes or hours. The most common symptoms include[16]:

- Headaches which is attributed to increased ICP or the presence of blood in the cerebrospinal fluid (CSF)
- Vomiting in 50% of hemispheric ICH patients, however commonly in cerebellar haemorrhages due to ICP rising
- Decreased level of consciousness caused by compression of the thalamus or brainstem
- Coma in cases that involve the brainstem reticular activating system.
- Seizures
- Neurological deterioration
- Sensorimotor deficits is common in patients with supratentorial ICH, involving the brainstem and thalamus.
- Cortical dysfunction such as neglect, hemianopia (loss of one half of a vertical visual field) and aphasia (difficulty with language or speech) in lobar ICH

Intracerebral haemorrhage can be classified as either a TICH and NTICH[20]. Traumatic intracerebral haemorrhage occurs as a result of a physical and traumatic event, such as a violent movement to the head, causing the tensile to strain and the brain to surpass vascular resistance, resulting in haemorrhage or damaged blood vessels[21]. TICH can be divided into either primary brain injury caused by direct force on the skull or secondary brain injury following on from initial trauma[22]. This life-threatening

condition is more common in adults compared to children and accounts for 27% of strokes world-wide.

Non-traumatic intracerebral haemorrhage is defined as a spontaneous event with a focal collection of blood within the brain parenchyma or ventricular system. This condition results from several underlying vasculopathies and overelapping risk factors that play a role in the rupturing of vessels[23]. Non-traumatic intracerebral haemorrhage, like TICH, can be divided into two categories, the primary NTICH that that occurs without concomitant vascular malformation or coagulation pathologies and the secondary that occurs as a vascular malformation result[21]. A study done in Cameroon to determine the clinical profile and prognosis of NTICH patients concluded that the occurrence of NTICH was 37% with male predominance (64%). Deep coma contributed to 30%, while basal ganglion had the most frequent location of haemorrhage (82%). Intraventricular blood effusion, mass effect, cerebral oedema and herniation occurred in 31.4%, 25.7%, 8.8%, and 5.0% respectively. Hypertension (57.5%) was the most prevalent cause for NTICH and had a mean length hospitalization of 9.0 $\pm$ 7.7 days. A climbing fatality rate was noted after 24 hours, during admission, month one and month three being 9.6%, 39.9%, 46.0%, 59.8% respectively. Patients with NTICH mostly died within three months while 50% of survivors after six months show poor outcome[24].

### 2.2.2 Types of haemorrhages

Although ICH is the second most common type of stroke (15 - 30%), it is also seen as the most deadly form when comparing it to other haemorrhages[25]. Table 1 illustrates the various forms of haemorrhages that can occur[26]. Bleeding may either occur within the skull (outside the brain tissue itself), referring to an epidural, subdural and subarachnoid haemorrhage (SAH), or within the brain tissue, known as an intracerebral and intraventricular haemorrahage (IVH)[26]. For the purpose of this study the main focus will remain on intracerebral bleeds. Once the location of the bleed is identified, the extent of the damage can be determined.

**Table 1: The different type of haemorrhages[26]**

| Type | Location |
|---|---|
| Epidural haemorrhage | Bleeding between the skull and the dura mater. |

| Subdural haemorrhage | Bleeding between the dura mater and arachnoid membrane. |
|---|---|
| Subarachnoid haemorrhage | Bleeding between the arachnoid membrane and the pia mater. |
| Intracerebral haemorrhage | Bleeding inside the lobes, pons, and cerebellum. (including the brainstem) |
| Intraventricular haemorrhage | Bleeding inside the brain's ventricles, specific to where cerebrospinal fluid is produced |

Intracerebral haemorrhage can further be defined by their location being either deep (ganglionic), lobar or infratentorial[23]. Deep ICH is located within the internal capsule, basal ganglia, brainstem and cerebellum; lobar ICH is located in the the cortical-subcortical areas[27] and infratentorial involving the cerebellum and/or brainstem[23]. Statistical analyses were done in population-based registries, from the United Kingdom and North Manhatten, revealed that deep ICH cases accounted for 60 - 65% whereas lobar only accounted for 31 - 40% of ICH cases[23]. Deep ICH is often caused by deep perforating vasculopathy occurring in small perforating arterioles. These arterioles originate in the middle cerebral artery and basilar artery, explaining the location[23]. Lobar ICH results from the rupturing of small to medium sized arterial perforators either in the cortex or subcortical areas[28]. Figure 1 indicates the difference between the deep and lobar locations where ICH is found.

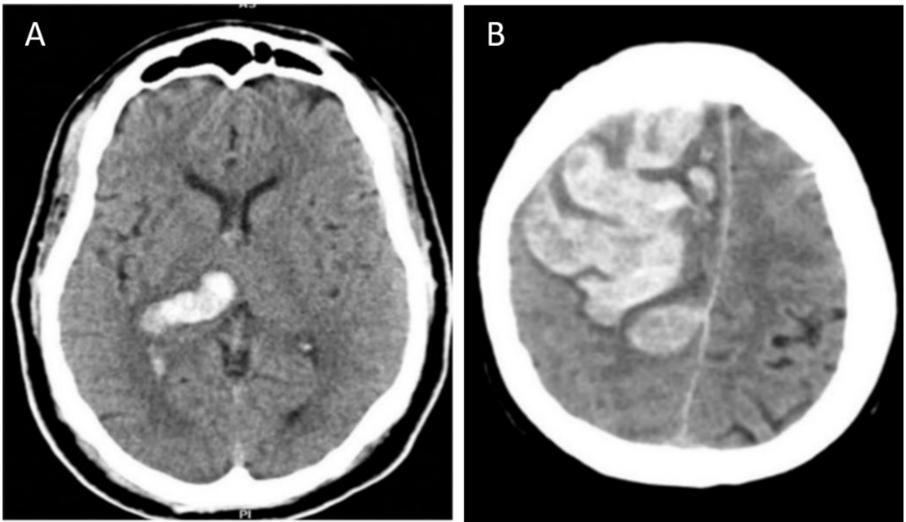


**Figure 1: Ganglionic or deep (A) versus lobar (B) intracerebral haemorrhage.** A: Supratentorial, intracerebral (intraparenchymal) haemorrhage which originated within the right thalamus and extends into the right lateral ventricle. Etiology hypertension. B: Supratentorial right hemisphere large right lobar haematoma with mass effect, cerebral edema, and midline shift. Etiology cerebral amyloid angiopathy[27].

Since TICH and NTICH are influenced by different risk factors, it is import to understand these risk factors since they provide aetiologic insights and can become targets for preventative strategies[17]. As mentioned, TICH is caused by traumatic events with risk factors including, severe car accidents, violence to the head and falls. The most important risk factors for NTICH are summarised in Table 2.

**Table 2: Common causes of non-traumatic intracerebral haemorrhage[23]**

| Aetiology | Main features |
| --- | --- |
| Deep perforating vasculopathy | - Hypertension is an important risk factor<br>- Mostly associated with deep ICH |
| Brain AVM | - History of seizures and focal neurological deficits<br>- DSA is used for the diagnosis |
| CVT | - Prothrombotic disorders, cancer, haematological diseases, vasculitis and inflammatory systemic disorders, pregnancy, infections, and other local causes |
| Dural arteriovenous fistula | - Pulsatile tinnitus<br>- Often is an acquired lesion |
| Cerebral CM | - Previous epilepsy<br>- Diagnosis made by MRI |
| CAA | - Lobar ICH without other detected cause<br>- Classified according to the Boston criteria |
| Haemorrhagic transformation | - Patients presenting with cerebral infarction<br>- Risk factors: early doses of anti-thrombotic agents |
| Brain tumours | - History of tumours and radiological evience of other cerebral metastases |
| Primary or systematic vasculitis | - Presence of laboratorial or radiological features suggestive of vasculitis<br>- Increased CSF cell count<br>- To confirm diagnosis, neuropathological evidence of cerebral vasculitis is required |
| RCVS | - Thunderclap headache<br>- Differential diagnois with subarachoid haemorrhage |
| Infective endocarditis | - Fever and changes in WBC count<br>- Evidence of systemic embolism |

Arteriovenous malformation (AVM), cerebral amyloid angiopathy (CAA), cavernous malformation (CM), cerebrospinal fluid (CSF), cerebral venous thrombosis (CVT), digital subtraction angiography (DSA), intracerebral haemorrhage (ICH), magnetic resonance imaging (MRI), reversible cerebral vasoconstriction syndrome (RCVS), white blood cell (WBC)

Pathophysiology of ICH is related to the rupturing of cerebral arterioles in brain parenchyma, with deep perforating vasculopathies (also known as small vessel disease (SVD)) playing a large role. These deep perforating vasculopathies can be due to arteriosclerosis and CAA causing fragile arterioles with poor contractile capability, increasing the risk of rupture[29]. Cerebral amyloid angiopathy can result from amyloid protein deposition in cortical arterioles[27]. Arterial hypertension (AHT) is another risk factor present in ± 60% of NTICH, triggering arteriole wall changes that can lead to vascular obstructions[18-19]. Primary NTICH usually results from a weakened vascular wall due to degenerative processes secondary to AHT or CAA, whereas secondary NTICH results from vessels containing wall abnormalities[18].

### 2.2.3 Diagnosis of intracerebral haemorrhage

During diagnosis routine blood tests such as for FBC, urea, liver function, electrolytes, and glucose[18], as well as coagulation analyses to determine the activated partial thromboplastin time and International Normalised Ratio (INR) are performed. Haemorrhage growth is usually associated with high glucose and creatinine levels and can lead to a poor functional prognosis, while haemorrhages associated with anticoagulant treatment increase the risk of mortality and require treatment to reverse the disorder[18]. After the patient is assessed, diagnostic tests may aid to determine the bleeding source.

The GCS is used as a descriptive measurement of impaired consciousness in acute and trauma patients. The scale assesses patients according to components of responsiveness: verbal, motor and eye-opening responses[30]. Each component gives a clear picture of the state of the patient and can be combined to give a summarised description, nevertheless give a useful summary of the overall severity[31]. The levels of response in the components are 'scored' from one, (indicating no response), to four (eye-opening response), five (verbal response) and six (motor response) being normal. The GSC score thus has a total between three and 15, with 15 being the highest score[31]. The GCS score is simplistic and seen as the most useful initial evaluation[32].

There are various standerdised methods that can be used to provide a clear and accurate diagnosis of an ICH patient. Firstly, a non-contrast CT scan is highly

sensitive, non-invasive, and used to look at the anatomical structure in the brain to pinpoint the bleeding[21]. A CT scan allows one to identify the location of the haemorrhage[32] and can accurately estimate the volume and expansion of the bleeding, as shown in Figure 2. Since the growth of haemorrhages is associated with neurological deterioration, CT angiography using contrast may help to identify patients at higher risk for haemorrhage expansion based on spot sign (isolated contrast in the haemorrhage)[18].

Second, magnetic resonance imaging (MRI) scans employ a magnetic field in conjunction with radio-frequency waves to provide a detailed image of the soft tissue within the brain[33]. This scan is used to sensitively detect blood during the initial phase of the haemorrhage, but also has the capability of detecting old haemorrhages[34].

Thirdly, a magnetic resonance angiogram (MRA) is a technique used to inject contrast into the bloodstream, enabling the examination of blood vessels and brain structures[21]. Along with these techniques, secondary prevention methods can also be implemented to prevent ICH.

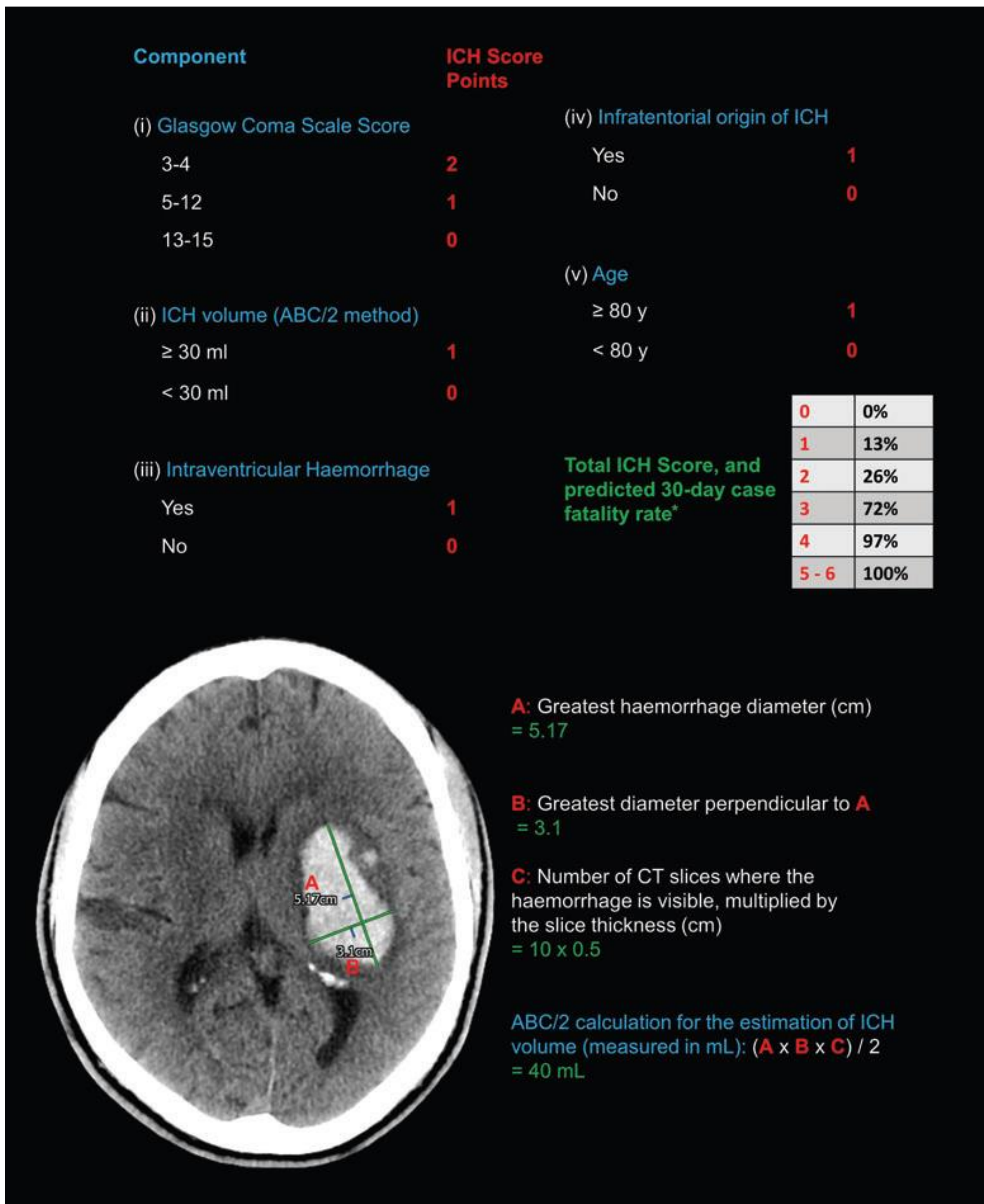


**Figure 2: Illustation of intracerebral haemorrhage score calculation[32]**

### 2.2.4 Secondary injury due to intracerebral haemorrhages

Secondary injury in the context of an ICH refers to damage to brain tissue that occurs in addition to the initial injury caused by the haemorrhage. Due to multiple mechanisms, this bleeding can result in a variety of secondary injuries: mass effect,

haematoma expansion, inflammation, blood clot formation, excitotoxicity, oxidative stress and oedema[35]. Secondary brain injuries are neurological emergencies, particularly if the clotting mechanism is extremely active, which can result in excessive clot formation within the brain. These clots can occlude small blood vessels, reducing blood supply to the brain and causing ischemia[35]. This can aggravate brain injury and raise the possibility of consequences[35]. Following an ICH, the release of blood products and haemoglobin (Hb) breakdown products might cause an inflammatory reaction in the brain. This inflammation can permeate the blood-brain barrier (BBB), allowing immune cells and proteins to enter and cause severe damage to brain tissue[35]. Clotting tendencies may have a role in the inflammatory response. In some circumstances, clot development within the brain can raise ICP. Ischemia and decreasing neurological function can result from elevated ICP, which compresses brain tissue and impedes cerebral blood flow (CBF)[36]. Table 3 summarises some recommendations for secondary prevention.

**Table 3: Recommendations for secondary prevention of ICH recurrence[18]**

| Prevention | Recommendation |
|---|---|
| BP control | Maintaining the blood pressure of patients with ICH below 120/80 mm Hg. |
| Anticoagulants | Careful management, as well as the possibility of discontinuing or switching to safer alternatives, should be examined. Do not administer anticoagulants after a lobar ICH in cases with non-valvular atrial fibrillation. Patients may receive antiplatelet drugs as an alternative. |
| Surgical treatment | Evaluate surgical treatment according to the risk of bleeding. Deep haemorrhages should be monitored and in case of rebleeding surgery should be considered. |
| Treatments for AVMs | Treatments include endovascular, surgical or radiosurgical depending on the surgical risk, size and location of the lesion |

Arteriovenous malformation (AVM), blood pressure (BP), intracerebral haemorrhage (ICH)

The most important goal of acute management is to stabilise the patient, ensuring they survive the initial ICH and to prevent any secondary brain injury[32]. Figure 3 depicts the "time is brain" principle and shows that managing the consequences of ICH is a critical focus of acute care. The location of ICH has a significant impact on decision making. Given the increased risk of brainstem compression and herniation symptoms in the limited region of the posterior fossa, neurosurgical surgery is often suggested for infratentorial bleeding[32]. Raised ICP might be caused by the mass effect of the bleed, peri-haematomal oedema, or hydrocephalus. In this situation, treating hydrocephalus

alone with external ventricular drainage is not advised and could even be hazardous, particularly if the basal cisterns are compressed[32].

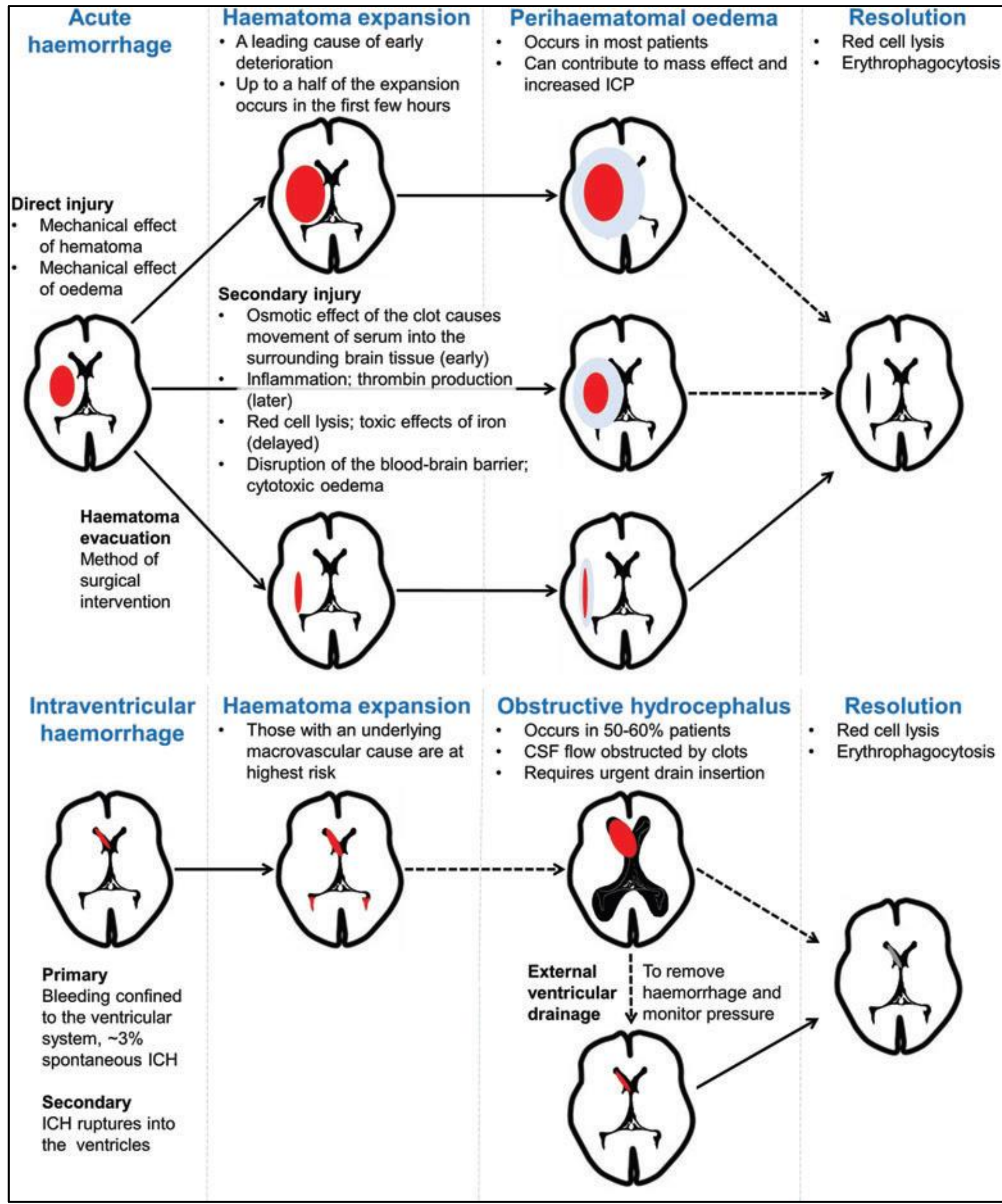


**Figure 3: Outline of the time course and mechanisms of secondary brain injury in intracerebral haemorrhage (including intraventricular haemorrhage)[32]**

In conclusion, secondary injury is closely related to the first vascular rupture and bleeding event due to the effects of the initial damage can trigger a number of negative processes that can result in further tissue damage and difficulties.

## 2.3 Bleeding event

The cerebral vasculature is composed of extremely specialized systems that guarantee continuous brain perfusion required to satisfy the extremely high demand for oxygen ($O_2$) and glucose by neurons and glial cells. The entire pial surface is covered in a dense, redundant network of arteries, from which penetrating arteries enter the cortex and connect with the neurovascular units. Cerebral arteries play a critical role in the drainage of interstitial fluid (ISF) and solutes like amyloid-beta in addition to supplying blood to the brain parenchyma[37].

The ongoing maintenance of a healthy brain depends heavily on the cerebral vessels. Although damage to brain tissue is the main concern in TBI, nearly all severe cases involve some degree of blood vessel damage. Any damage to or functioning of the vasculature places neural tissue at risk[38].

In addition to exposing brain tissue to the comparatively uncontrolled composition of WB, which can disturb the homeostasis of the central nervous system (CNS), vessel disruption frequently prevents efficient perfusion and causes severe rises in ICP. Even in the absence of haemorrhage, pathophysiological changes in CBF are frequent[38].

Different blood vessel cells react differently to the presence of extravascular blood. The outermost layer (the adventitia) comprises perivascular nerve axons wrapped in a collagen sheath; the media contains smooth muscle; and the internal layer (the intima) contains endothelial cells and the basement membrane[39]. Oxyhaemoglobin, which is released by lysed RBCs, is harmful to all three layers. Nerve fiber loss has been described in the adventitia following ICH. This sort of denervation is likely to result in a loss of neurogenic control of cerebral arteries as well as impaired autoregulation[39]. Myonecrosis is defined in the media as the loss of contractile protein in smooth muscle, as well as a rise in the amount of interstitial collagen. These processes work together to cause arterial constriction that is not caused by vasospasm[39]. After a haemorrhage, matrix metalloproteinases (MMPs) are released in the intima layer. The MMPs destroy the basement membrane and tight junction proteins, causing the BBB to rupture. The permeability of the BBB is also affected by local chemicals secreted by various cell types. Perivascular nerve fibers emit calcitonin gene-related peptide (CGRP), 5-hydroxytryptamine (5-HT), and substance P, which contribute to mast cell histamine production and inflammation[39].

## 2.4 Blood components and coagulation

Given the increased risk of thromboembolic events in ICH patients, it is critical to first understand the physiology of coagulation before addressing the thrombogenic effects of ICH. Coagulation is the cessation of bleeding caused by a series of physiological responses[40]. The participation of blood components and how their interactions affect coagulability determines whether coagulation is effective or dysfunctional. Before explaining the process of coagulation seen in figure 4, a brief discussion of the components of WB and their role in coagulation is given.

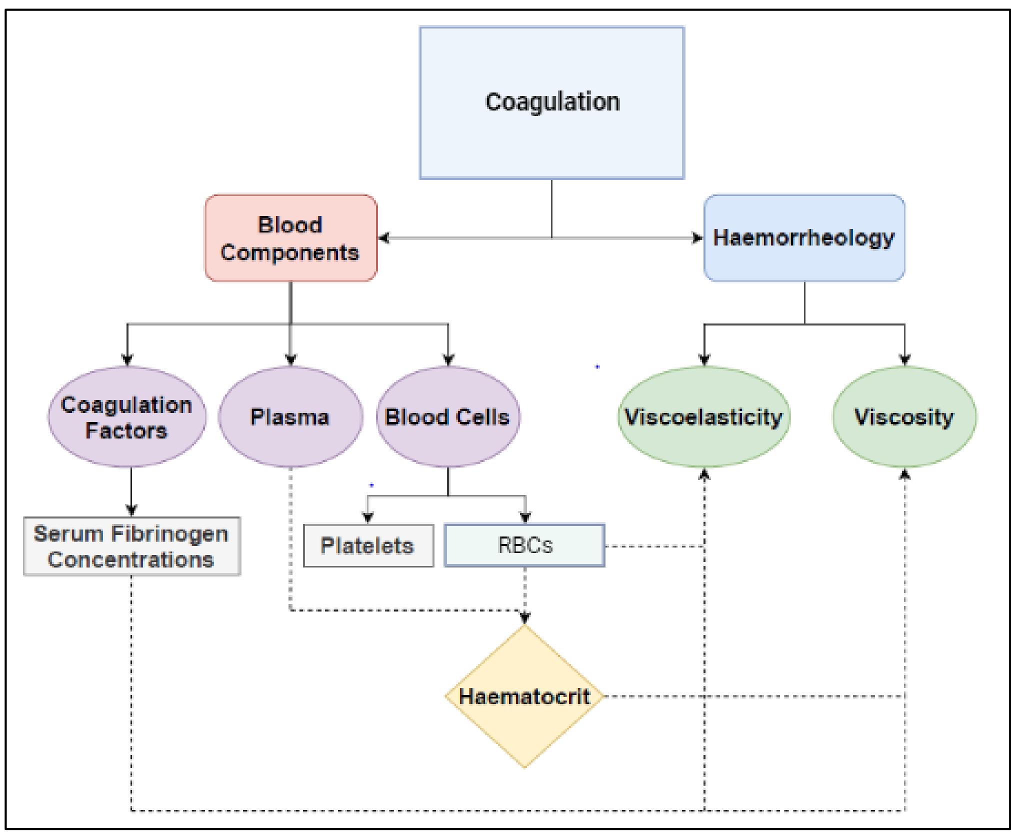


**Figure 4: An illustration of coagulability and the factors that affect it[41].** (Diagram created with biorender).

### 2.4.1 Blood components

#### 2.4.1.1 Platelets

Platelets, also referred to as thrombocytes, are formed in the bone marrow and remain there to undergo fragmentation. They are not genuine cells, but rather smaller, non-circulating megakaryocytes[42]. Platelets are key components in coagulation and

function to (1) produce the initial PLT plug, (2) create a catalytic surface for fibrin production and (3) to assist in clot retraction[43].

Platelet formation and function are inextricably linked, with considerable cytoskeleton remodeling taking place when a dormant, spherical PLT is activated. Platelets become activated when they adhere to collagen, exposed from the tissue injury site, and tissue factor (TF) presenting components in the endothelium matrix, resulting in $Ca^{2+}$ influx into the PLT[44]. The surface area of the PLT is increased by cytoskeletal reorganisation, creating a larger surface for PLT aggregation to create the primary PLT plug and a larger catalytic surface for the assembly of the different coagulation components that fuel fibrin formation. The main factors governing PLT function and their prothrombotic contribution are their reactivity and activation[44]. The enzymatic component of coagulation occurs in three overlapping stages, according the cellular model of coagulation[45]. The PLTs in this model stand out as they serve as the catalyst for the activation and complexation of the coagulation factors[46]. The PLT adhesion, activation, secretion, and procoagulant activity must be sufficient before these three stages since they potentiate one another to create an environment that promotes the synthesis of fibrin. Platelet secretory products promote secondary PLT activation, and PLT aggregation[47], as well as procoagulant activity while collagen and TF enhance PLT adhesion and activation[48-49]. The von Willebrand factor (vWF), known as a plasma protein, assists in platelet adhesion, platelet aggregation and stabilisation of factor VII[50]. In the coagulation cascade, the exposure of procoagulant phospholipids on the PLT membrane surface catalyzes the building of enzyme complexes[51]. During secondary coagulation, a stable PLT clot forms as a result of the thrombin-mediated conversion of fibrinogen to fibrin. Although blood and vascular cell surfaces also produce thrombin, the PLT membrane is the favored location for secondary coagulation because it has receptors with high-affinity clotting factor binding sites and lipid assembly rafts[49]. Platelet activation causes anionic phosphatidylserine (PS) to transfer from the inner PLT membrane leaflet to the outside PLT membrane leaflet, resulting in PLT procoagulant activity. The PS exposure on aggregated PLT creates a procoagulant catalytic surface for the formation of procoagulant complexes, resulting in the generation of thrombin[52].

Platelets play a physiological function in a variety of processes, including inflammation, immunological responses, and haemorrhage, in addition to coagulation and

thrombosis[53]. There exists an established link between haemorrhage, inflammation and PLT activation. In an article published by Frontera et al., it was found that SAH patients had hypoperfusion which initated a cascade of events including diffuse upregulation of endothelial adhesion molecules, PLT activation, and inflammation, that end in microthrombosis, ischemia, vasogenic edema and early brain injury[54]. Platelets contribute to the inflammatory cascade. Activated PLTs express P-selectin and produce chemokines/cytokines that increase leukocyte adherence and transmigration at PLT deposition sites. Additionally, neutrophils emit substances that encourage PLT activation. Activated PLTs and inflammatory cells can contribute to further endothelial damage in areas far from the origin of vessel rupture by continuing the cycle of microthrombosis and inflammation[54].

#### 2.4.1.2 Red blood cells

Over the last few decades, mounting evidence has suggested that RBCs play biologically significant roles in coagulation and thrombosis[55-56]. While endothelial cells, PLTs, and coagulation factors are principally responsible for clotting and thrombin generation, RBCs assist through their biochemical, biomechanical, and rheological features[57]. The involvement of RBCs in coagulation can thus be characterized in terms of their qualitative and quantitative features, as well as their interactions with other cellular and molecular components of the coagulation system.

Red blood cell structure and function are intricately linked, thus understanding their structure is essential to comprehend their function. These cells are typically small, anuclear, discoid, and biconcave-shaped cells produced in the bone marrow, stimulated by erythropoietin[58]. Their typical lifespan is around 100 to 120 days following formation and maturation[59]. These cells have high surface-area-to-volume ratios and are densely packed with haemoglobin, contributing significantly to blood viscosity, one of the primary drivers of blood rheology[56]. Blood viscosity, which restricts blood flow, may be influenced by many factors such as the haematocrit (HCT) (amount of circulating RBCs)[56]. The physical interaction of PLTs with blood vessel surfaces is directly affected by HCT-related blood viscosity, with PLT adhesion increasing as HCT increases under flow conditions[56]. Red blood cells increase blood viscosity by producing linear stacks of cells, rouleaux, or other three-dimensional aggregates at

static or low shear rates[55-56]. Additionally, blood displays a shear-thinning behavior in which shear rate rises while blood viscosity falls and *vice versa*[60-61].

Red blood cells are deformable, owing to their biconcave shape. This property enables them to effectively flow through blood vessels that are smaller than their size[56,62]. Membrane rigidity is affected by membrane deformability (governed by the cytoskeleton and cellular metabolic energy) and cytoplasmic viscosity (determined by haemoglobin concentration)[56]. A decreased deformability in RBCs impairs their ability to cross the microcirculation, resulting in decreased PLT transport towards the vascular endothelium. These cells interact with PLTs, through chemical signaling and sticky RBC-PLT contacts, to help control their reactivity[56]. Red blood cells release adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in reaction to mechanical deformation or low partial pressure of oxygen ($pO_2$) concentrations, increasing PLT activation and degranulation. Extracellular Hb, produced by haemolysis, stimulate PLTs by decreasing nitric oxide (NO) bioavailability. Haemoglobin is a potent NO scavenger that prevents NO from reducing PLT function[56]. Since the structure and biochemical makeup of RBCs have been determined, their role in coagulation can be examined.

During successful haemostasis, adequate prothrombotic surfaces are required for the assembly of the prothrombinase complex and the production of thrombin during the coagulation cascade[56]. Cells that expose PS, a negatively charged substance present in the inner leaflet of cellular membranes, produce prothrombotic surfaces[56,62]. In order to provide enough prothrombotic surfaces, RBCs first enhance PLT migration and flow towards the blood vessel periphery, positioning them in the perfect position for fast attachment to exposed collagen or TF. Second, PS-flips on RBCs generate prothrombotic surfaces during haemostasis, ensuring appropriate PLT activation and coagulation cascade activity[56]. It has been established that RBC membranes are particularly vulnerable to inflammation, especially cytokine overexpression and the oxidative damage that follows. Red blood cells are important health indicators because of their plasticity and shorter lifespan[63]. Due to their lack of a nucleus, mitochondria, and the capacity for restorative activities like protein synthesis, RBCs are particularly susceptible to oxidative stress. Although normal-sized RBCs have a capacity for regeneration 250 times greater than their oxidative potential, in some pathological settings, the antioxidant system in them is insufficient for the level of oxidative stress

they suffer[64]. This level of oxidative stress will disrupt the action of membrane-bound enzymes, ion transport, and lipid bilayer membrane characteristics, ultimately leading to RBC eryptosis[64]. Eryptosis is known as a type of cell death in RBCs characterized by cell shrinkage, membrane blebbing, and membrane PS translocation to the outer leaflet of the RBCs lipid bilayer. An extensive *in vivo* model of inflammation in disease is provided by RBC sensitivity and eryptosis[63,65]. Pretorius *et al.* suggested that alterations in the RBC membrane caused by inflammation might be used as a measure of the overall inflammatory condition of the body[66-67]. Inflammatory mediators have been demonstrated to raise the concentrations of several procoagulants while reducing the amounts of endogenous anticoagulants in the blood[66].

#### 2.4.1.3 Fibrinogen

Hepatocytes produce the soluble glycoprotein fibrin, which circulates in plasma at a concentration of 1.5 to 3.5 g/L[68]. A fibrin clot is created by the cleavage of fibrinogen into fibrin, which allows fibrin polymerization to occur, as a result of the interplay of 12 coagulation factors[69]. The thrombin-catalyzed cleavage of fibrinogen, which reveals binding sites in the central nodule of the protein, transforms fibrinogen into insoluble fibrin. The central binding sites are complementary to constitutively accessible binding sites at the end of the protein, enabling fibrin polymerization to take place through half-staggered interactions that cause two-stranded protofibrils to aggregate laterally and longitudinally to form fibers[69]. Once the fibers have developed and branched, plasma transglutaminase Factor VIIIa covalently crosslinks the fibrin polymer to produce a mature, mechanically stable fibrin clot[69].

While serum fibrinogen concentration alone plays a significant role in blood viscosity, serum fibrinogen concentration in combination with efficient fibrin polymerization is crucial for guaranteeing appropriate fibrin clot formation. An increase in serum fibrinogen concentration is associated with an increase in blood viscosity[70]. The rise in serum fibrinogen concentration is linked to the acute phase of inflammation in tissue damage, as the haemostatic response is crucial for blood loss prevention during tissue damage[71]. Now that the roles of blood components in coagulation have been established, it is important to discuss haemorheology and how it affects coagulation,

since these factors are involved in three of the four factors that determine blood viscosity.

### 2.4.2 Haemorheology

Haemorheology is the study of the plasmatic and cellular components of blood as well as its biophysical and flow characteristics[72]. An important factor in successful tissue perfusion is maintaining a balance between the rheological characteristics of blood, since disruptions to this equilibrium play critical roles in a number of disease processes. Blood is categorised as a non-Newtonian fluid and is made up of two phases: the plasma, which is an aqueous solution of proteins, salts, and organic molecules, and the blood cells, which are created elements suspended in the plasma[73]. The two main rheological characteristics of blood are viscoelasticity and blood viscosity[72].

Blood viscosity is a biophysical property of blood that measures the resistance to flow. The amount of friction between the layers of blood as they pass through the vessels and the amount of friction between the blood and the vessel walls are both influenced by blood viscosity. Viscosity is influenced by HCT, plasma viscosity, and the mechanical characteristics of RBCs (deformability and aggregation), which influence the shear forces brought on by flow[74-75]. The most significant factor affecting blood viscosity is HCT, which has the capacity to raise blood viscosity by 4% with a single unit increase[76]. The quantity of plasma proteins, particularly fibrinogen and immunoglobulins, which promote RBC aggregation in the microcirculation, as well as its water content and macromolecular components, regulate plasma viscosity[76].

The ability of RBCs to alter their shape in response to deforming forces is referred to as RBC deformability[75]. A stress-strain relationship rather than a single parameter is needed to define deformability. The direction in which the strain is applied, as well as the amplitude and pace of the stress, determine the rate, extent, and manner in which the RBC deform. Blood viscoelasticity is a biophysical characteristic of blood that is mostly a result of the elastic energy accumulated during RBC deformation as blood circulates through the body[77]. In short, the concentration and behavior of RBCs as they deform, aggregate, and dissociate in response to local flow conditions is the key determinant of blood rheology.

### 2.4.3 Coagulation

Coagulation is a critical, complicated interplay of cellular, haemorheological, enzymatic, and proteinaceous mediators that leads to PLT activation and aggregation, the coagulation cascade, and fibrinolysis. The cornerstones of coagulation include RBCs, PLTs, enzymes, and coagulation factors, specifically prothrombin and fibrinogen[78].

The homeostatic reaction to vascular injury, coagulation, takes place as a primary and secondary reaction[79]. A number of mechanisms work together to reduce blood loss and promote tissue healing. The primary haemostatic response works to create a fragile PLT aggregate, which is then strengthened by the secondary haemostatic response which converts soluble fibrinogen into insoluble fibrin[78]. Additionally, as part of the primary haemostatic response, ionized calcium ($Ca^{2+}$) and a negatively charged phospholipid bilayer are produced. These two crucial substances are needed for the proper polymerization of fibrin and the transformation of fibrinogen into fibrin[80]. Primary coagulation is achieved through (1) vascular spasm and (2) PLT adhesion, activation, aggregation, and secretion[81-82]. The injured blood vessels contract after vascular injury to stop the flow of blood. Vascular injury exposes the collagen matrix of the vascular subendothelium to the blood inside the vessel. Von Willebrand factor allows the PLTs to cling to the injured endothelium[82]. Von Willebrand factor is a blood glycoprotein that, by complexing with PLTs and collagen, promotes PLT adhesion. Platelet glycoprotein Ib-IX-V receptor complexes bind to vWf, while the vWf glycoprotein VI (GPVI) receptor and integrin α2β1 bind to collagen[83].

Platelet activation is brought by PLT-collagen contact, which permits degranulation[48]. Platelet granules also include the less effective activators ADP and arachidonic acid (AA). Degranulation provides for localised, magnified PLT activation, allowing PLTs to aggregate at the injury site[48]. Platelet activation also initiates secondary coagulation[48].

Secondary coagulation is achieved through coagulation factor activation and, finally, fibrinolysis[48,84]. Platelets serve an important role in secondary coagulation by acting as a catalytic surface for the formation of the tenase and prothrombinase complexes, resulting in a thrombin burst, and by secreting $Ca^{2+}$, which is essential for the activation

of numerous coagulation factors. The goal of the thrombin burst is to stimulate additional PLT activation, which then cleaves soluble fibrinogen to produce insoluble fibrin networks. The fibrin networks function to temporarily block the injured vessel, preventing blood loss, minimizing pathogenic invasion, and promoting wound healing by attracting and encasing immune cells[48]. The enzymatic breakdown of the fibrin networks is known as fibrinolysis. It is activated and strengthened in a manner similar to the coagulation cascade, allowing for the maintenance of vascular patency after healing. A haemostatic response that can effectively stop blood loss is created as a result of the sequential interactions between the various haemostatic response elements and the injured endothelium. These components will be discussed as they relate to the haemostatic response.

#### 2.4.3.1 Cellular model

The cell-based model, which improved on its predecessor, the classical coagulation cascade model, by more accurately depicting *in vivo* coagulation, did so by taking into account the role that cells play in clot production and regulation. Three separate yet overlapping stages—initiation, amplification, and propagation—were used to illustrate haemostatic physiology, as seen in figure 5. The cell-based model, which was created at the turn of the millennium, continues to serve as the basis for contemporary knowledge and clinical practice in blood clotting and thrombosis.

Initiation occurs when circulating blood is exposed to an endothelial matrix composed of collagen- and TF-presenting subendothelial fibroblasts or smooth muscle cells[45]. Circulating proconvertin (factor VII) rapidly attaches to the TF of the TF-presenting cells, becoming activated (factor VIIa). The TF-VIIa unit then functions to activate plasma thromboplastin component (factor IX) and circulatory factor X. In order to avoid being inactivated by antithrombin (AT) III and tissue factor pathway inhibitor (TFPI), factor Xa stays on the surface of the TF-presenting cell. It works in conjunction with proaccelerin (factor V) to convert a small amount of prothrombin to thrombin, resulting in a thrombin burst. The initial thrombin burst activates coagulation factors and PLTs, starting the second phase of coagulation, known as amplification[45,85].

During amplification, the thrombin burst increases PLT adhesion and activation and activates factor V, antihemophilic factor (factor VIII), and factor XI. Degranulation of

activated PLTs results in the release of partially activated factor V, AA, serotonin, and ATP. Amplification ensures that activated factors V, VIII, and XI are bound to activated PLT surfaces in order for the propagation stage to start[45]. The building of the tenase and prothrombinase complexes is critical to the last stage. The assembly takes place on the active PLT cell surface[45,85].

When the propagation stage begins, factor IXa dissociates from the TF-presenting cell and binds to its receptor, which is expressed on active PLTs. The tenase complex is formed when the factor IXa-receptor unit complexes with the VIIIa bound to the activated PLT[45]. The tenase complex activates factor X, which then combines with factor Va to produce the prothrombinase complex. The prothrombinase complex generates a significant thrombin burst, which allows for the enzymatic conversion of fibrinogen to fibrin and the activation of fibrin stabilizing factor (factor XIII)[45,85].

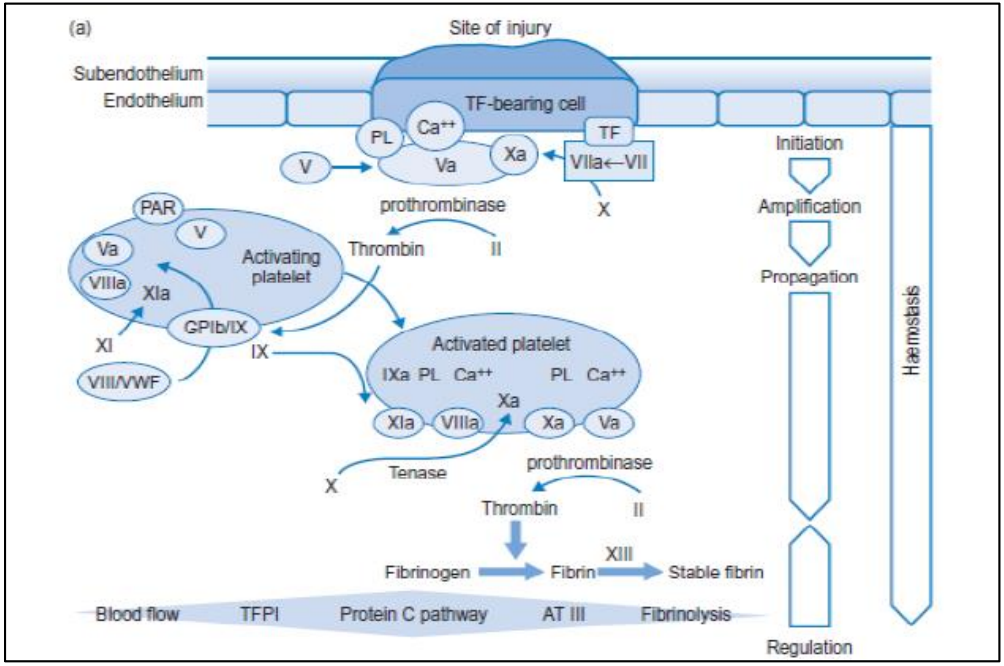


**Figure 5: A diagram of the cellular model of coagulation.** The interaction of the proteinaceous and PLT components that results in a stable fibrin clot is significant[82].

Fibrinolysis is the final step in the coagulation process that happens after wound healing. An enzymatic cascade destroys the fibrin network of the clot, allowing the entire fibrin clot to dissolve. The breakdown of the fibrin clot is critical for preserving vascular integrity[86]. Plasmin is the enzyme that breaks down the fibrin clot.

Plasminogen, its precursor, forms a ternary complex with fibrin and tissue plasminogen activator (tPA) to activate plasminogen and produce its active enzyme, plasmin[87]. Plasminogen can break fibrin, a wide range of clotting factors, and plasma proteins that are present in the clot. It also has a broad substrate specificity. Plasmin activity starts concurrently with the initiation of the coagulation cascade[78]. Vascular endothelial cells control the plasmin activity, which moves more slowly than the coagulation cascade to permit the formation of the PLT plug and fibrin mesh.

Coagulation is a complex process with many important steps that rely on the healthy generation and operation of different mediators[88]. If the normal physiological production or activity of these mediators is disrupted, the integrity of the haemostatic response is altered. The next section will cover thrombosis, a pathologic process that happens when the mediators involved in coagulation become dysfunctional.

## 2.5 Thrombosis

Thrombosis is the formation of a blood clot (thrombus) within a blood vessel, which results in the partial or total blockage of the vessel, reducing blood flow[89]. Thrombosis is complex and takes place when the endogenous haemostatic and anticoagulant systems are out of balance, or when the processes that control clot formation and lysis are out of proportion. As shown in Figure 6, three common criteria summarise the risk of thrombosis and are referred to as "Virchow's triad" - (1) endothelial damage, (2) hypercoagulability, and (3) vessel stasis[90-91]. Virchow's Triad interacts with thrombosis pathophysiology in a variety of ways.

Endothelial injury causes inflammation, which promotes the generation of proinflammatory, and prothrombotic cytokines, TF exposure and production, enhanced PLT activation, and increased adhesion molecule production and expression[92]. Hypertension, inflammation, oxidative stress, and high shear stress all pose a threat to endothelial integrity and are factors often associated with ICH[93].

Hypercoagulability is a wide haematological concept referring to an increased risk of thrombosis caused by elevated concentrations or function of prothrombotic substances in the bloodstream[90]. Hypercoagulability can be caused by a number of haemostatic system changes, including an increase in inflammatory factors, oxidative stress, changes in blood viscosity, changes in blood composition, increased PLT

reactivity, increased proinflammatory cytokine and procoagulant production, and a decrease or deficiency in endogenous anticoagulants.

Vessel stasis occurs as a result of vessel occlusions disrupting the smooth laminar flow of blood, raising the shear rate required to circumvent the occlusion and, as a result, increasing turbulence downstream of the occlusion. It is sometimes caused by limited movement, which is commonly seen in old age as disability or a sedentary lifestyle, as well as reduced flow and haemorheological dysfunction due to vessel fibrosis or remodelling. Any one or a combination of these factors could coexist and cause thrombosis, especially if they are exacerbated by modifiable factors including poor diet, smoking, and stress[93].

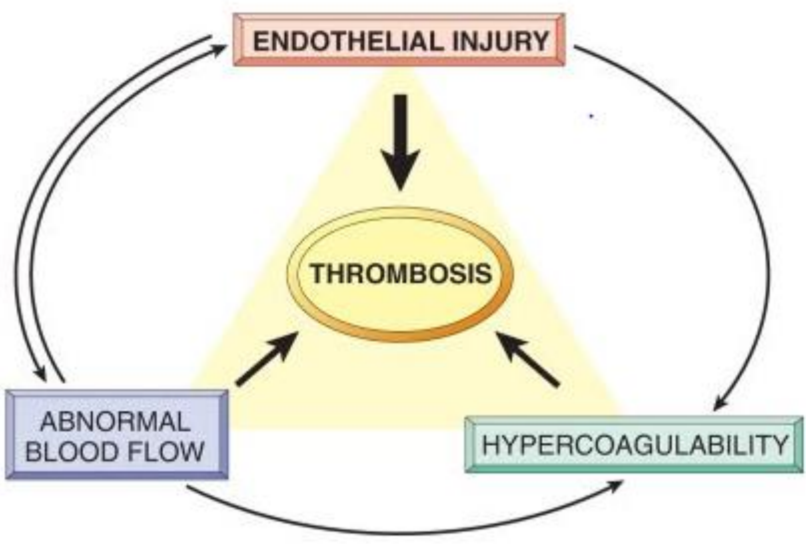


**Figure 6: Virchow's triad. Thrombosis could result from a combination of endothelial dysfunction, hypercoagulability, and or vessel stasis[90].** (Diagram created with biorender).

## 2.6 Could intracerebral haemorrhage be associated with thrombotic tendencies?

An inflammatory response in the body attempts to remove and limit an initial stimulation against injury, through phagocytosis and activation of the inflammasome (which causes apoptosis), in order to promote tissue regeneration and scarring[94]. An overactive inflammatory response, albeit meant to be useful and protective, can actually cause or contribute to disease pathology and tissue damage. Once deployed,

activated cells target distant areas that are reacting to the inflammatory stimulation as well as the original site of inflammation[94].

Neuroinflammation, a secondary injury, contributes significantly to the neurologic impairment seen in ICH patients. The presence of intraparenchymal blood triggers this, which initiates cytotoxic, excitotoxic, oxidative, and inflammatory pathways. When blood components are recognised within the parenchyma, an acute inflammatory response occurs, defined by the mobilization and activation of inflammatory cells[95].

Microglia and astrocytes are thought to be the first inflammatory cells to respond to extravasated blood. Danger-associated molecular pattern factors generated by harmed cells can cause microglia to overreact. High quantities of inflammatory cytokines and cytotoxic substances are consequently produced by overactive microglia, which contribute to additional neurodegeneration[3].

Microglia activation results in the infiltration of numerous circulating immune cells, most notably macrophages and T lymphocytes. This causes the release of inflammatory cytokines (e.g., interleukin-1β [IL-1β] and tumor necrosis factor-α [TNF-α]), chemokines, free radicals, and potentially hazardous compounds, all of which are coordinated by the transcription factor nuclear factor-κB (NF-κB). The NF-κB targets include cytokines, chemokines, adhesion molecules, cell surface receptors, and inflammatory enzymes (for example, inducible nitric oxide synthase [iNOS], cyclooxygenase-2 [COX-2], and phospholipase A2 [PLA2]). These chemicals, along with the byproducts of cell death, stimulate resident and migrating lymphocytes, increasing lymphocyte infiltration and perpetuating the inflammatory response cycle. There is growing proof that this inflammatory reaction causes oedema by increasing BBB permeability around the haematoma, which worsens the mass effect, speeds up the process of cell death through secondary ischaemia, and causes additional inflammatory insults to the nearby brain tissue[8,94-95].

Following ICH and neuroinflammation, coagulopathy and hypercoagulability are frequent[39]. The risk of death from traumatic brain injury is multiplied by ten in the presence of coagulopathy, and the chance of substantial morbidity is multiplied by thirty. When brain arteries are damaged, a protein called TF, the main physiological initiator of the coagulation cascade which is found in endothelial cells and leukocytes, is released into the bloodstream. Cerebral vessels are a major contributor to the

development of CNS and systemic coagulopathy and have a large reservoir of TF[39]. The typical mechanisms that prevent excessive coagulation may be overwhelmed by the enormous amount of TF produced[96]. Antithrombin, clotting factors, and PLTs are all depleted in cases of severe bleeding. Plasminogen activator-inhibitor (PAI-1) expression increases concurrently, blocking the fibrinolytic mechanism[39]. The early coagulopathy found in TBI has also been linked to tissue hypoperfusion and protein C pathway activation. These elements play a part in disseminated intravascular coagulation (DIC), which affects up to 25% of individuals with severe TBI. Within hours following damage, DIC-induced coagulopathy may manifest. On the other hand, the production of micro- and macro-vascular fibrin thrombi might result from TF-dependent activation of coagulation. In studies of ICH, excessive micro-thrombi development is suggested to be the cause of delayed ischaemia since it may restrict flow in small vessels. It has been demonstrated that thrombin is a strong inducer of PLT and endothelial cell production of MPs[97]. Microparticles are tiny vesicles with a bilayer plasma membrane and a little amount of cytoplasm that express surface antigens from the originating cell. Numerous forms of apoptogenic, procoagulant, or proinflammatory stimulation result in the production of MP. These stimuli cause membrane budding, which results in the release of MPs, and the migration of procoagulant phospholipids, such as phosphotidylserine, to the outer leaflet of the plasma membrane. To facilitate the building of clotting enzyme complexes on MP surfaces, phosphotidylserine produces an extra procoagulant surface. Additionally, MPs act as a reservoir for circulating TF and improve the TF/factor VIIa complex's catalytic performance[39].

Through the thrombin receptor, protease-activated receptor 1 (PAR1), which is present on endothelial cells, thrombin not only facilitates the development of fibrin clots but also induces vasoconstriction. When thrombin cleaves activated PAR1, it causes vasoconstriction and increases its own expression, making the vessel more sensitive to thrombin. Vasoconstriction, which prevents bleeding but may exacerbate ischaemia after TBI, is potentiated by these processes. The activation of PAR1 also directly initiates inflammation, increases BBB permeability, oedema, and cell death, as is the case with many of the pathways examined[39].

## 2.7 Treatment

The standard treatment for ICH consists of a combination of medicinal and surgical treatments aimed at stabilising the patient, limiting additional bleeding, and preventing or treating possible complications. It should be noted that the specific treatment approach may differ depending on the condition of the patient, the amount and location of the haemorrhage, and other individual considerations.

The primary goal of pre-hospital care is to send the patient to the nearest emergency department by providing airway, breathing, and circulatory support[98]. A comprehensive history from any witnesses or family/caregivers at the scene of the incident is always useful because it may reveal pertinent information on trauma, medical, and substance history. Early aggressive medical care in an acute hospital setting has been proven to have a direct impact on morbidity and death after an ICH[99].

Oral anticoagulants can both increase the risk of ICH recurrence and prevent thromboembolic events[100], therefore a weighted decision must be made against the risk of future ICHs. Anticoagulants should also be suspended during the acute phase, except if the patient is at high risk for thromboembolic events.

## 2.8 Study rationale

In summary, the comprehensive review of current literature has provided clear evidence about ICH and the association with coagulopathies. Intracerebral haemorrhage is a catastrophic brain injury with high morbidity and fatality rates, making it a major public health problem. Intracerebral haemorrhage is still a complex and difficult medical challenge that necessitates ongoing research, innovation, and a multidisciplinary approach. Understanding its prevalence, pathogenesis, diagnosis, and treatment choices is critical for improving patient outcomes and lowering the considerable burden it takes on individuals and healthcare systems. Gaining more insights in this field, especially coagulopathies, holds promise for more effective diagnostic and management options to provide better outcomes for patients affected by ICH.

## 2.9 Aim and objectives

The aim of this study was to investigate coagulation changes in patients from Steve Biko Academic Hospital (SBAH) with ICH by studying the viscoelastic and ultrastructural properties of WB. Traumatic ICH and NTICH patient groups were compared to the control group respectively.

The following objectives were used to achieve the aim:

[1] To obtain a FBC from patient records for each ICH patient for a haematological profile and compared to healthy reference ranges

[2] To determine the degree of RBC deformation in ICH patients compared to healthy individuals by calculating AR using LM and ImageJ.

[3] To study the ultrastructural and morphological changes of RBCs, PLTs, and clot structure in ICH patients compared to healthy individuals, using SEM.

[4] To measure the viscoelastic properties of WB in ICH patients compared to healthy individuals using TEG®.

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

## 3.1 Study design and setting

This study was a patient centered, laboratory-based analytical study. Sample analysis was done on blood *ex vivo*, obtained from a control group with a total number of 40 healthy control individuals, and two patient groups of 20 TICH and 31 NTICH patients. A minimum of 20 participants per group was required according to the statistian consulted.

Patient recruitment took place at SBAH, at the Department of Neurosurgery. The recruiting doctors, Prof Padayachy and Dr Craig Grobbelaar under the supervision of Prof Padayachy, identified the patients from whom blood samples were collected. After the doctors identified the patient, the principle investigator approached the patient to request participation. If the patient was willing to participate an information session was held in private with no time constraint on making a decision. Samples were collected by the recruiting doctor in a 4 mL citrate tube with 3,2% (0.105 M) buffered sodium citrate solution (Lasec).

Background information was included in the consent form to record the demographics of the population which ensured sampling from a homogenous population. This information was also used to describe the population in the study.

This research project formed part of a larger study and was conducted in conjunction with Ms Andrea Lenting who investigated the ultrastructural and viscoelastic properties of platelet-poor-plasma (PPP) focusing on fibrinogen on coagulation changes.

## 3.2 Ethical approval

This study was approved by the MSc committee and research ethics committee of the Faculty of Health Sciences, University of Pretoria (UP), as well as the National Health Research Database (NHRD) (approval nr GP_202207_094) for permission to work with patients in SBAH. Ethical clearance, 261/2022, was granted for this project (Addendum 1).

The following ethical concerns were considered for this study. No participant was obligated to participate. No participant was treated differently if they decided not to participate. All contact sessions with the participant were conducted privately. All the results were kept confidential and made available to the patient on request. If any serious abnormalities were detected the recruiting doctor was informed. There was no costs involved for the participant. The participant could withdraw from participating at any time during the course of the study without explanation and prejudice. Simple language was used to explain to the participant in order for the them to fully understand the purpose of the study and what their participation entailed. The blood sample collection was done during routine blood tests, therefore just one extra tube was drawn at no extra inconvenience to the participant.

## 3.3 Division of the study population

The study consisted of three groups and was divided into one control group and two patient groups, as shown in Figure 7.

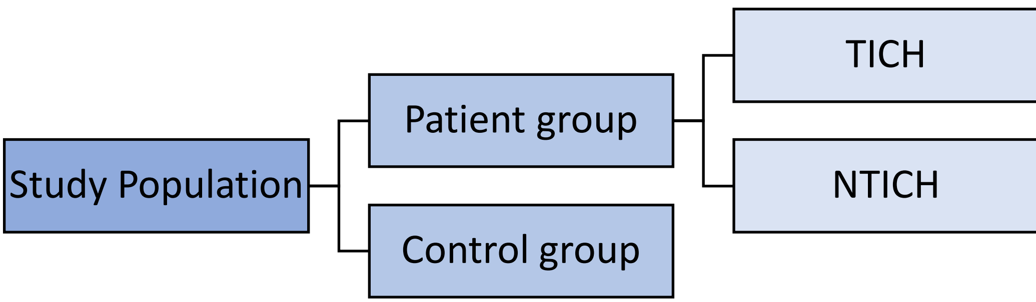


**Figure 7: Study population group division**

## 3.4 Sampling criteria and procedures

### 3.4.1 Control group

Healthy participants were recruited from family, friends, and colleagues at SBAH to donate blood and serve as a control group. The participants were deemed healthy if they did not have any known acute or chronic condition or took any chronic medication. All participants underwent an information session and were required to sign a consent

form prior to sample collection (Addendum 2). The recruitment of a control group from a similar population ensured homogeneity between the groups to limit confounding factors such as different socioeconomic statuses, biological sex, and age. The following criteria have been selected to best imitate a control group with respect to all conditions

***Inclusion criteria:***

- Able to provide informed consent
- Biological sex: Male or female
- Ages between 20 - 60 years[101]

***Exclusion criteria:***

- Smoking of any tobacco or uses any related product
- Usage of any of chronic medication
- Any condition which could present with chronic inflammation
- Any known acute or chronic condition (such as ICH, stroke, cancer, auto-immune disorders etc.)
- Usage of medication that influences coagulation (e.g. Aspirin/ Nonsteroidal anti-inflammatory drugs (NSAID), etc)
- History of an immune-compromised status
- Usage of chemotherapy medication, anti-platelet, or anti-coagulative specific medication within two weeks prior to sample collection date
- Human immunodeficiency virus (HIV) positive (if status is known)
- Excessive consumption of alcohol (drinking five or more drinks on the same occasion on at least one day in the past 30 days)[102]

### 3.4.2 Patient group

The recruiting doctor facilitated patient consultation in collaboration with the principal investigator to explain the purpose of this study, obtained the necessary informed consent as well as the blood samples. Samples from patients from any subtype of ICH was collected. The samples were then separated according to TICH or NTICH incidence. Approval was obtained by the NHRD to access the medical records of these patients to obtain the necessary clinical information. All patient information was

handled confidentially and anonymised. As described in the criteria below, patients should have had no other sign of infection or inflammation not related to ICH.

***Inclusion criteria:***

- Biological sex: Male and female
- Ages between 20 and 60 years[101]
- Diagnosed with ICH

***Exclusion criteria:***

- Smoking of any tobacco or uses any related product
- Usage of any chronic medication that had a negative effect on the coagulation system (induced coagulation)
- Any condition which could present with chronic inflammation
- Has a history of an immune-compromised status
- Usage of chemotherapy medication, anti-platelet, or anti-coagulative specific medication within two weeks prior to sample collection date
- HIV positive (if status is known)
- Excessive consumption of alcohol (drinking five or more drinks on the same occasion on at least one day in the past 30 days)[102]
- Ischaemic strokes that present with cerebral bleeding
- Brain tumours that present with intracerebral bleeds

All participants were required to sign an informed consent form prior to sample collection. In cases were the patient was in intensive care unit (ICU), the research ethics committee approved a waiver consent in the case where the patient was unconscious and incapable of providing consent after regaining consciousness, or deceased. The delayed consent was done if the patient regained consciousness and was able to provide consent. The patient was deemed able to give consent upon the medical clearance from the assessment done by the treating clinician. The patient was informed of the patient's inclusion in the research study as soon as reasonably possible and was advised about his/her right to withdraw from the study without a change in their quality of care (Addendum 3). Blood was drawn from the central venous line by a medical doctor following the standard procedure. This was only done

during routine tests for patients in ICU. This line is more stable, and the sterile procedure prevents central venous line infections.

Figure 8 illustrates a summary of the chain of events from blood sample collection, through sample analyses to obtain the study data.

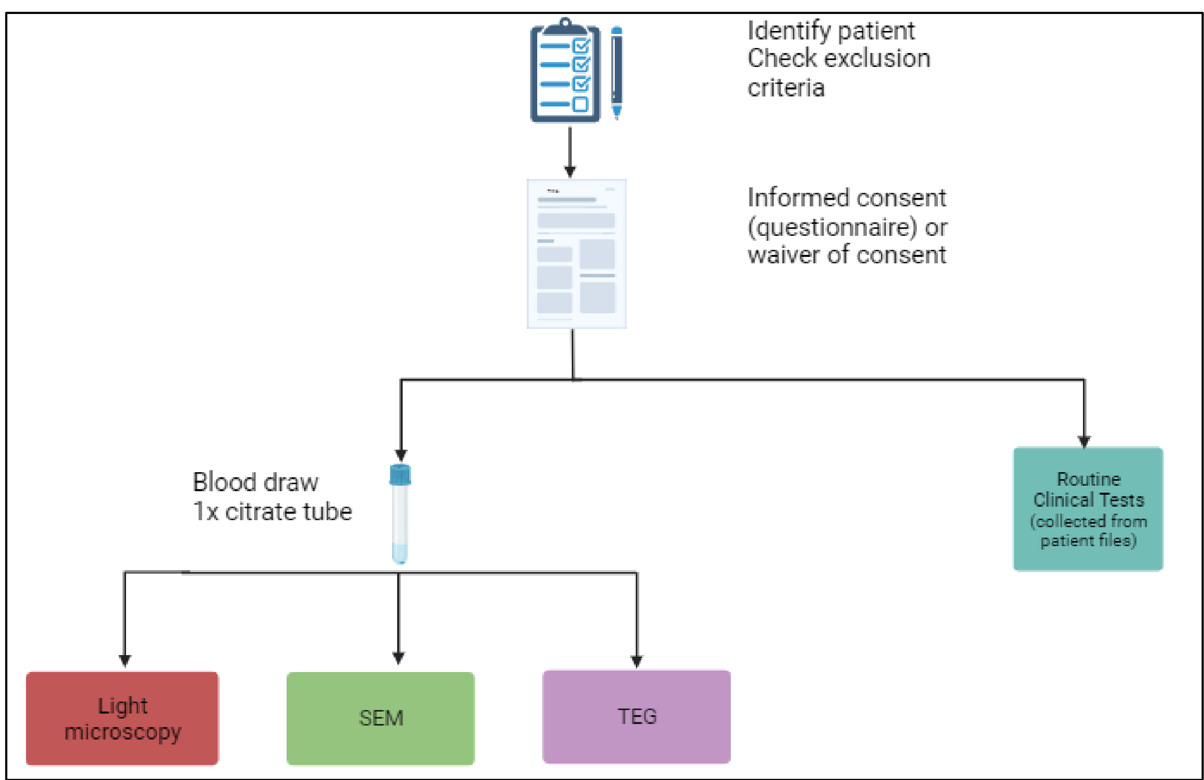


**Figure 8: Events that followed during patient recruitment from patient identification to sample analysis.** (Diagram created with biorender)

## 3.5 Demographics

The treatment of ICH often entails an immediate and multimodal approach aimed at stabilising the patient, decreasing bleeding, and preventing future problems. Medications are essential in this therapeutic plan and we did not prevent any treatment of patients to take place in order to recruit patients. Table 4 contains a list of medications that some of the patients were taking at the time of blood collection. It is important to highlight that the medications used and how they were administered were determined by the specific condition of each patient, which included the amount and location of the haemorrhage, underlying health conditions, and other considerations. Patients using these medications were included in the study as it has been shown by

literature not to have a negative effect on coagulation and could not be seen as a confounding factor. References were provided in table 4 that were confirmed by research not to have a negative effect on coagulation.

**Table 4: Control and patient demographics and medication use**

| Control Group (n= 40 ) | | | | |
|---|---|---|---|---|
| Age range | | Biological sex | Type of medication | Medication brand |
| 18-28 | 16 | M(10) F(6) | High blood pressure (n=1) | Indapamide[103] |
| 29-38 | 12 | M(7) F(5) | | |
| 39-48 | 7 | M(4) F(3) | | |
| 49-58 | 5 | M(3) F(2) | | |
| Traumatic ICH group (n= 20) | | | | |
| Age range | | Biological sex | Type of medication | Medication brand |
| 18-28 | 9 | M(8) F(1) | High blood pressure (n=3) | Pharmapress[104] |
| 29-38 | 4 | M(6) F(0) | Proton pump inhibitor (n=14) | Pantoloc[105] |
| 39-48 | 3 | M(2) F(1) | Diuretic (n=4) | Mannitol[106] |
| 49-58 | 2 | M(1) F(0) | Anti-epileptic drug (n=16) | Phenytoin[107], Epilim |
| 59-68 | 1 | M(1) F(0) | Pain medication (n=18) | Panadol, Tramadol[108], Morphine[109], Perfalgan, Paracetamol[110] |
| 69-78 | 1 | M(1) F(0) | Sedative drug (n=8) | Dormicum[111], Precedex[112] |
| | | | Antiemetics (n=6) | Metaclopramide[113] |
| | | | Antibacterial/antibiotic agents (n=7) | Augmentum[114], Imipenem[115], Rocephin, Kefzol[116] |
| | | | Vitamin B1 deficiency (n=1) | Thiamine[117] |
| | | | Anti-psychotic (n=1) | Risperdal[118] |
| Non-traumatic ICH group (n=31 ) | | | | |
| Age range | | Biological sex | Type of medication | Medication brand |
| 29-38 | 5 | M(3) F(2) | Anti-hypertensive medication (n=22) | Amloc[119], Ridaq, Enalapril, Hydralazine[120], |

| | | | | Amlodipine, Carloc[121], Lebetalol infusion, Atenolol, Propranolol |
|---|---|---|---|---|
| 39-48 | 10 | M(7) F(3) | Antiemetics (n=5) | Metaclopramide[113] |
| 49-58 | 5 | M(3) F(2) | Anti-epileptic (n=27) | Epilim, Phenytoin[122] |
| 59-68 | 7 | M(2) F(5) | Nasal congestion (n=1) | Afrin[123] |
| 69-78 | 3 | M(2) F(1) | Calcium deficiency (n=1) | Calcium gluconate[124] |
| 79-88 | 1 | M(0) F(1) | Anti-cholesterol (n=1) | Simvastin[125] |
| | | | Anti-psychotic (n=1) | Risperdal[126] |
| | | | Antibacterial agent (n=2) | Meropenem[127] |
| | | | Corticosteroid (n=2) | Dexamethasone[128], Solu cortef |
| | | | Diuretic (n=4) | Mannitol[129], Cardura XL, Lasix[130] |
| | | | Calcium channel blocker (n=1) | Nimodipine[131] |
| | | | Insulin (n=1) | Actrapid[132] |
| | | | Pain medication (n=27) | Panado,Tramadol[108], Perfalgan[110] |
| | | | Proton pump inhibitor (n=18) | Pantoloc[105], Pantosec, Lansoprazole[133] |
| | | | Constipation (n=1) | Lactulose[134] |
| | | | Sedative drug (n=1) | Dormicum[111] |

Intracerebral haemorrhage (ICH), male (M), female (F)

## 3.6 Statistical analysis

This study and all experimental procedural details were discussed with a statistician from UP. A letter granting statistical clearance has been provided (Addendum 4).

The statistical analysis of the FBC and CRP was done using GraphPad Prism 8 for windows. The data were first tested for normality with the Shapiro-Wilk normality test. Given that some parameters might be normally distributed and others not, descriptive

statistics were done. The mean and standard deviation (SD) were used to describe parametric data, while the minimum (min), median and maximum (max) were used for non-parametric data. The mean or median for each parameter was assessed according to the reference ranges and then used to determine if the parameter were below, within or above the normal reference range.

## 3.7 Routine clinical tests

The FBC is a common investigative tool that assists in the diagnosis of various conditions, as well as give insight into the overall health of an individual[135]. It is a frequently used automated clinical technique that enables the consulting clinician to obtain a comprehensive clinical picture of the patient by providing details like the presence of inflammation, infection, anaemia, or impaired haemostatic functioning, all of which are pertinent in further diagnoses and investigation[136]. The haematology analysis was conducted as part of the patients' routine clinical testing at SBAH, through the National Health Laboratory Services (NHLS). The NHLS follows the standard procedure for routine blood testing within four hours after blood drawl. This was used to measure nine haematological parameters using a haematology analyser to investigate differences in cell numbers and abnormalities of the cellular components in ICH patients and assessed according to normal reference ranges. The parameters, unit of measurement and reference ranges are outlined in Table 5.

**Red blood cell count:** The RBC count measures the number of RBCs in circulation. The value obtained is dependent on physiological factors. The RBC count is dependent on mechanisms that stimulate or inhibit RBC production. Several factors, like an increase in tissue oxygen requirement and red cell mass, can stimulate RBC production and increase the RBC count, while chronic bleeding, malnutrition and bone marrow dysfuntion would inhibit RBC production and thus reduce the RBC count[137]. A low RBC count is linked to poorer outcomes following ICH. These findings are thought to be the result of decreased brain oxygenation[138].

**White blood cell count:** The WBC count (leukocyte count) measures the number of WBCs in circulation. White blood cells are part of the immune system and help the body fight against infection and other diseases[139]. A WBC count is often used to help

diagnose a disorder related to having a low/or high WBC count. Disorders related to a low WBC count, known as leukopenia, include diseases of the immune system, lymphoma and diseases of the liver or spleen. Disorders related to a high WBC count, known as leukocytosis, can include autoimmune and inflammatory diseases, bacterial or viral infections, cancers and allergic reactions[140]. White blood cells interact with PLTs, endothelium, and coagulation factors and are widely acknowledged as key contributors to coagulation in normal and pathological situations. Acute leukocytosis changes the haemostatic balance in favor of coagulation and may thus play an essential role in clotting following an ICH[141]. Leukocytosis is connected with ICH, and the inflammatory activity reflected by leukocytosis may have a role in ICH severity. Multiple investigations have found that higher WBC counts are associated with more severe ICH, as defined by decreased consciousness, increased baseline haematoma volume, and the presence of intraventricular haemorrhage[141].

**Platelet count:** A PLT count measures the average number of PLTs in the blood. Adequate amount of circulating PLTs is needed to maintain vascular integrity and to ensure thrombus formation at sites of injury[142-143]. It is critical to examine the PLT count as prior research has shown that PLT hypofunction and low PLT count might lead to poor coagulation[144-145], while increased PLT counts promote fibrin synthesis and thrombus formation[146]. Platelet count has a strong relationship with clot strength and can thus be give an indication of the coagulation potential of ICH patients. Platelet concentration is measured using an automated PLT analyser based on electrical impedance principle[147].

**Mean corpuscular volume (MCV):** The MCV measures the average volume of RBCs in the blood and forms part of the RBC indices[148]. The MCV is used as part of the morphological classification of anaemia. Physiologically, the availability of folic acid, iron and vitamin $B_{12}$ is important for a MCV count. An iron deficiency will result in microcytes (RBCs with decreased volumes), while a vitamin $B_{12}$ or folic acid deficiency, or both, will result in macrocytes (RBCs with increased volumes)[149]. A normal MCV count combined with a low RBC count may indicate that the low RBC count is the result of blood loss[140].

$$MCV = HCT/RBC \times 10$$

**Mean corpuscular haemoglobin (MCH):** The MCH measures the average mass of haemoglobin that is contained in a single RBC from a sample of blood. This is calculated by dividing the measured haemoglobin concentration by the RBC count. The MCH depends on the factors that determine the RBC and Hb concentration[150]. This parameter can be used to detect RBC morphological variations (such as an increase in MCH can indicate macrocytosis)[151].

$$MCH = Hb/RBC \times 10$$

**Mean corpuscular haemoglobin concentration (MCHC):** The MCHC is a measurement of the concentration of Hb relative to the unit volume of packed RBCs. The MCHC is similar to the MCH, however takes the volume of the RBCs into account when being calculated. Alternatively, the MCHC can be explained as the percentage of an RBC that consists of Hb. To calculate the MCHC, the Hb concentration is divided by the RBC or HCT and then multiplied by 100. The MCHC depends on the factors that determine the RBC and Hb concentration[152].

$$MCHC = (Hb/HCT) \times 100$$

**Haemoglobin concentration:** The Hb concentration provides the concentration of Hb available for oxygen transport within the RBCs[150]. The concentration is measured following lysis of the RBCs. The haemoglobin content of RBCs depends on RBC, since it is produced in the cells, making the Hb concentration an indirect measure of the RBC. Factors stimulating or inhibiting RBC production would have the same effect on Hb concentration. Previous research has linked low Hb levels to poor outcomes in individuals with ICH, and these patients are more likely to have larger haematomas[153-155].

**Haematocrit:** The HCT measures the ratio of RBCs to the total blood volume. An automated haematology analysis calculates the HCT by multiplying the MCV with the RBC. The HCT is important for determining blood viscosity and thus the shear rate of blood[156]. The HCT depends on the factors that determine the RBC, MCV, as well as the hydration level.

$$HCT = RBC \times MCV$$

**C-reactive protein:** C-reative protein, produced in the liver, is a sensitive inflammation marker. Hours after the commencement of ICH, a cascade of inflammatory processes occurs around the haematoma, and the harmful neuroinflammation contributes to haematoma expansion and worsens the result[157]. Another study discovered that a high CRP level is associated with the recurrence of ICH during the acute phase of hospitalisation, implying that an initial inflammation response may have a poor effect on the haematoma and disrupt the stability of cerebral vascular haemodynamics[158]. The CRP values for each patient was obtained from patient records.

**Table 5: Full Blood Count and C-reative protein reference ranges for adults**

| Parameters | Reference ranges | Unit of measurement |
|---|---|---|
| Red blood cell | $4.19 – 5.85 \times 10^{12}$ | Cells/L |
| White blood cell count | $3.92 – 10.40 \times 10^{9}$ | Cels/L |
| Platelet count | $171 – 388 \times 10^{9}$ | Cells/L |
| Mean corpuscular volume | 83.1 – 101.6 | fL |
| Mean corpuscular haemoglobin | 27.8 – 34.8 | pg |
| Mean corpuscular haemoglobin concentration | 33.0 – 35.0 | g/dL |
| Haemoglobin | 13.4 -17.5 | g/dL |
| Haematocrit | 0.390 - 0.510 | L/L |
| C-reactive protein | <10 | mg/L |

### 3.7.1 Results

The two groups are denoted as $T_{pat}$ for the TICH group and $NT_{pat}$ for the NTICH group. Note that all patients were first randomly collected and only after diagnosis catergorised into TICH and NTICH, thus the patient numbers in the various groups won't be sequential. Missing data in the tables denote data that was not found or tests that were not performed since all FBC values were recorded from patient files. Mean or median values of each parameters were compared to the reference ranges. The comments on the results only included above or below the normal range. Raw data will be shown in Table 6 and summary statistics for each parameter will be shown in Table 7.

**Table 6: The full blood count data obtained from patient files for the traumatic and non-traumatic intracerebral haemorrhage patients**

| $T_{pat}$ ICH group | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Patient code** | **RBC count ($10^{12}$/ L)** | **WBC count ($10^{9}$/ L)** | **PLT count ($10^{9}$ / L)** | **MCV (fL)** | **MCH (pg)** | **MCHC (g/dL)** | **Hb (g/dL)** | **HCT (L/L)** | **CRP (mg/L)** |
| Patient 3 | 4.46 | 7.33 | 293 | 85.7 | 28.9 | 33.8 | 12.9 | 0.382 | |
| Patient 9 | 3.79 | 10.07 | 358 | 88.7 | 28.0 | 31.5 | 10.06 | 0.336 | 268 |
| Patient 12 | 4.17 | 11.80 | 189 | 97.8 | 33.3 | 34.1 | 13.9 | 0.408 | 1 |
| Patient 14 | 3.06 | 5.10 | 198 | 91.2 | 30.4 | 33.3 | 9.3 | 0.279 | 207 |
| Patient 16 | 3.60 | 14.55 | 200 | 84.2 | 26.7 | 31.7 | 9.6 | 0.303 | 163 |
| Patient 17 | 3.15 | 7.54 | 84 | 82.2 | 27.0 | 32.8 | 8.5 | 0.259 | 260 |
| Patient 19 | 2.77 | 8.36 | 103 | 93.9 | 31.8 | 33.8 | 8.8 | 0.260 | 87 |
| Patient 22 | 3.96 | 7.73 | 177 | 87.1 | 28.8 | 33.0 | 11.4 | 0.345 | 39 |
| Patient 24 | 4.76 | 13.39 | 212 | 69.7 | 20.6 | 29.5 | 9.8 | 0.332 | |
| Patient 31 | 3.55 | 20.11 | 221 | 100.0 | 31.3 | 31.3 | 11.1 | 0.355 | 127 |
| Patient 35 | 5.29 | 8.37 | 281 | 85.3 | 28.4 | 33.3 | 15.0 | 0.451 | 142 |
| Patient 37 | 3.73 | 4.52 | 92 | 85.0 | 27.9 | 38.8 | 10.4 | 0.317 | 103 |
| Patient 43 | 4.58 | 16.01 | 322 | 84.3 | 26.4 | 31.3 | 12.1 | 0.386 | 11 |
| Patient 44 | 3.29 | 10.21 | 129 | 90.9 | 30.1 | 33.1 | 9.9 | 0.299 | 134 |
| Patient 48 | 4.77 | 10.58 | 228 | 91.0 | 29.8 | 32.7 | 14.2 | 0.434 | 97 |
| Patient 49 | 5.09 | 17.25 | 312 | 85.7 | 27.9 | 32.6 | 14.2 | 0.436 | 27 |
| Patient 51 | 3.74 | 8.71 | 287 | 92.5 | 31.0 | 33.5 | 11.6 | 0.346 | 220 |
| Patient 54 | 4.47 | 7.95 | 145 | 91.1 | 30.4 | 33.4 | 13.6 | 0.407 | 31 |
| Patient 55 | 3.61 | 7.26 | 151 | 95.0 | 31.9 | 33.5 | 11.5 | 0.343 | 151 |
| Patient 57 | 4.67 | 16.34 | 248 | 100.6 | 34.7 | 34.5 | 16.2 | 0.470 | |
| Patient 61 | 3.17 | 9.81 | 133 | 94.0 | 31.2 | 33.2 | 9.9 | 0.298 | 243 |
| **$NT_{pat}$** | | | | | | | | | |
| **Code** | **RBC count ($10^{12}$/ L)** | **WBC count ($10^{9}$/ L)** | **PLT count ($10^{9}$/ L)** | **MCV (fL)** | **MCH (pg)** | **MCHC (g/dL)** | **Haemoglobin (g/dL)** | **Haematocrit (L/L)** | **CRP (mg/L)** |
| Patient 1 | | | 423 | 96.2 | 30.7 | 31.9 | | 0.501 | 48 |
| Patient 2 | 5.24 | 11.35 | 264 | 93.1 | 31.7 | 34.0 | 16.6 | 0.488 | 36 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patient 4 | 3.90 | 8.65 | 157 | 90.3 | 27.7 | 30.7 | 10.8 | 0.352 | |
| Patient 5 | 5.93 | 7.81 | 349 | 61.7 | 18.7 | 30.3 | 11.1 | 0.366 | |
| Patient 6 | 3.72 | 11.60 | 288 | 96.2 | 32.5 | 33.8 | 12.1 | 0.358 | 9 |
| Patient 7 | 4.47 | 10.67 | 172 | 83.7 | 27.7 | 33.2 | 12.4 | 0.374 | 285 |
| Patient 8 | 3.02 | 16.32 | 327 | 94.7 | 28.8 | 30.4 | 8.7 | 0.286 | 66 |
| Patient 10 | 4.88 | 13.55 | 267 | 91.6 | 29.3 | 32.0 | 14.3 | 0.447 | |
| Patient 11 | 4.47 | 9.65 | 201 | 80.0 | 26.3 | 32.9 | 11.8 | 0.359 | |
| Patient 18 | 4.37 | 10.37 | 198 | 86.5 | 26.5 | 30.7 | 11.6 | 0.378 | 81 |
| Patient 21 | 5.56 | 15.44 | 383 | 100.7 | 31.8 | 31.6 | 17.7 | 0.560 | 4 |
| Patient 23 | 5.02 | 9.77 | 161 | 86.3 | 26.9 | 31.2 | 13.5 | 0.433 | 329 |
| Patient 25 | 3.75 | 14.07 | 213 | 90.9 | 27.7 | 30.5 | 10.4 | 0.341 | 54 |
| Patient 27 | 3.88 | 5.49 | 219 | 96.9 | 33.2 | 34.3 | 12.9 | 0.376 | |
| Patient 28 | 4.11 | 13.09 | 208 | 93.2 | 29.9 | 32.1 | 12.3 | 0.383 | 81 |
| Patient 29 | 4.22 | 5.70 | 243 | 93.6 | 30.6 | 32.7 | 12.9 | 0.395 | 5 |
| Patient 30 | 5.54 | 5.08 | 334 | 91.2 | 31.2 | 34.3 | 17.3 | 0.505 | 9 |
| Patient 32 | 4.01 | 18.92 | 257 | 91.8 | 28.9 | 31.5 | 11.6 | 0.368 | 245 |
| Patient 34 | 3.29 | 11.55 | 169 | 94.5 | 30.4 | 32.2 | 10.0 | 0.311 | 62 |
| Patient 36 | 5.29 | 6.88 | 214 | 89.0 | 24.4 | 31.8 | 15.0 | 0.471 | 5 |
| Patient 39 | 4.30 | 26.93 | 137 | 97.9 | 31.2 | 31.8 | 13.4 | 0.421 | 329 |
| Patient 40 | 4.62 | 5.39 | 239 | 89.2 | 30.1 | 33.7 | 13.9 | 0.412 | 99 |
| Patient 41 | 3.72 | 10.83 | 101 | 92.2 | 29.8 | 32.4 | 11.1 | 0.343 | 224 |
| Patient 42 | 4.92 | 5.25 | 192 | 74.0 | 22.6 | 30.5 | 11.1 | 0.364 | |
| Patient 45 | 5.33 | 9.34 | 351 | 82.9 | 27.0 | 32.6 | 14.0 | 0.442 | 6 |
| Patient 47 | 4.67 | 16.09 | 223 | 95.1 | 30.8 | 32.4 | 14.4 | 0.444 | 213 |
| Patient 50 | 5.27 | 8.81 | 289 | 91.8 | 28.7 | 31.2 | 15.1 | 0.484 | 6 |
| Patient 52 | 4.45 | 17.34 | 225 | 87.4 | 29.2 | 33.4 | 13.0 | 0.389 | |
| Patient 53 | 5.51 | 10.58 | 382 | 90.6 | 30.9 | 34.1 | 17.0 | 0.499 | |
| Patient 58 | 5.22 | 14.94 | 139 | 77.2 | 24.1 | 31.3 | 12.6 | 0.403 | 22 |
| Patient 59 | 4.44 | 6.78 | 303 | 89.6 | 30.9 | 34.4 | 13.7 | 0.398 | 16 |

Parameter values above the reference ranges are indicated in red, while parameters below the reference ranges are indicated in blue. Abbreviations defined: C-reactive protein (CRP), haemoglobin (Hb), haematocrit (HCT), intracerebral haemorrhage (ICH), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), non-traumatic group ($NT_{pat}$), platelet (PLT), red blood cell (RBC), traumatic group ($T_{pat}$), white blood cell (WBC).

In the dataset, four of the nine parameters did not pass the Shapiro-wilk normality test; hence, the median (min;max) for these five parameters was used to determine whether they were above, below or within the normal range. The remaining five parameters did pass the Shapiro-wilk normality test; hence, the mean and SD was used. Of the nine parameters, six were not within the normal reference range. Table 7 outlines the results.

**Table 7: Descriptive statistics of the full blood count for the traumatic- and non-traumatic intracerebral haemorrhage group were assessed according to the reference range**

| RBC count | | | |
|---|---|---|---|
| Group | Mean (SD) | Normal range | Outcome |
| $T_{pat}$ | 4.0 (±0.7) | 4.19 – 5.85 x $10^{12}$ cells/ L | Slightly decreased RBCs |
| $NT_{pat}$ | 4.6 (±0.7) | | Slightly decreased RBCs |
| **WBC count** | | | |
| Group | Mean (SD) | Normal range | Outcome |
| $T_{pat}$ | 10.8 (±4.2) | 3.92 – 10.40 x $10^{9}$ cells/ L | Slightly above normal range |
| $NT_{pat}$ | 11.3 (±4.9) | | Slightly above normal range |
| **PLT count** | | | |
| Group | Mean (SD) | Normal range | Outcome |
| $T_{pat}$ | 214.0 (±76.7) | 171 – 388 x $10^{9}$ cells/ L | Not affected |
| $NT_{pat}$ | 246.1 (±80.8) | | Not affected |
| **MCV** | | | |
| Group | Median (min, max) | Normal range | Outcome |
| $T_{pat}$ | 91.0 (69.7;100.6) | 83.1 – 101.6 fL | Not affected |
| $NT_{pat}$ | 91.2 (61.7;100.7) | | Not affected |

| MCH | | | |
|---|---|---|---|
| Group | Median (min, max) | Normal range | Outcome |
| $T_{pat}$ | 30 (20.6;34.7) | 27.8 – 34.8 pg | Not affected |
| $NT_{pat}$ | 29.3 (18.7;33.2) | | Not affected |
| **MCHC** | | | |
| Group | Median (min, max) | Normal range | Outcome |
| $T_{pat}$ | 33.3 (29.5;38.8) | 33.0 – 35.0 g/dL | Not affected |
| $NT_{pat}$ | 32.1 (30.3;34.4) | | Slightly decreased MCHC |
| **Hb** | | | |
| Group | Mean (SD) | Normal range | Outcome |
| $T_{pat}$ | 11.8 (±2.1) | 13.4 – 17.5 g/dL | Decreased Hb |
| $NT_{pat}$ | 13.1 (±2.2) | | Slightly decreased Hb |
| **HCT** | | | |
| Group | Mean (SD) | Normal range | Outcome |
| $T_{pat}$ | 0.36 (±0.06) | 0.390 – 0.510 L/L | Slightly decreased HCT |
| $NT_{pat}$ | 0.41 (±0.06) | | Not affected |
| **CRP** | | | |
| Group | Median (min, max) | Normal range | Outcome |
| $T_{pat}$ | 127.0 (1.0;268.0) | <10 mg/L | Increased CRP |

| | | | |
|---|---|---|---|
| $NT_{pat}$ | 54.0 (4.0;329.0) | | Increased CRP |

Parameter values above the reference ranges are indicated in red, while parameters below the reference ranges are indicated in blue. C-reactive protein (CRP), haemoglobin (Hb), haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), non-traumatic group ($NT_{pat}$), platelet (PLT), standard deviation (SD), red blood cell (RBC), traumatic group ($T_{pat}$), white blood cell (WBC).

### 3.7.2 Discussion

A review of the results from the FBC analysis revealed a change in six of the nine parameters assessed according to the normal ranges. In the $T_{pat}$ ICH group, the means for RBC, Hb and HCT were all slightly below the normal reference range, with WBC mean above and CRP median levels well above the normal range. As a result of the bleeding event, the TICH patients may have a haemorrhagic anaemic profile, which could explain the decreases in RBC count, Hb, and HCT[159]. Depending on the underlying aetiology, anaemia has a very diverse pathophysiology. For example, in acute haemorrhagic anaemia the dilution of the remaining RBCs during the restoration of blood volume with intracellular and extracellular fluid causes anaemia. Red blood cells are made in the bone marrow and released into circulation. Every day, 1% of RBCs are taken out of circulation and an imbalance between RBC production and elimination or destruction can lead to anaemia[160]. Badireddy *et al*. found that increased RBC destruction due to blood loss (acute haemorrhage, trauma, menorrhagia, and chronic gastrointestinal blood losses) and haemolytic anaemia (infection, blood transfusion-related, microangiopathic, and defects in RBC membrane production) are main mechanisms involved in this condition. A second mechanism that can be deficient or defective is erythropoiesis[159]. Although these patients do not display indications of acute anaemia, the observed changes in FBC values show the chances of developing this if the cause of the haemorrhage is not resolved.

Haemoglobin is the most accurate diagnostic measure of haemorrhagic anaemia since it is necessary for oxygen transport, and most cases of anaemia will result in a drop of haemoglobin[161]. Bruns *et al.* conducted a study on Hb levels and concluded that haemorrhage in trauma patients are associated with an early decrease in the Hb levels and are actively bleeding[162]. Additionally, anaemia has been linked to modifications in brain metabolism or oxygenation, which indicate that low Hb levels in patients with

severe TBI are one of the most significant indicators of the emergence of ischaemic regions[163]. The quantity and size of RBCs may also have an impact on the HCT. If the number of cells decreases, as in a haemorrhage, the HCT may decrease. Whether or not there are fewer cells, the HCT can also fall when the size of individual cells diminishes, as in microcytic anaemias[161].

The CRP level is a defining characteristic of the inflammatory status of patients and widely used as a systemic marker of inflammation[164]. The elevated CRP levels in the $T_{pat}$ ICH group indicate a increased state of inflammation and correlated with a higher 30-day mortality rate, as well as an additional 8% ICH score accuracy improvement[165]. According to Rajapathy *et al.*, the GCS and overall survival at six months are worse when CRP levels are greater than 5 mg/dL at admission and 72 hours after admission[165]. Increased CRP levels are commly caused by severe infections, while other possible causes include severe tissue damage and a poorly controlled autoimmune disease. The increased WBC count (leukocytosis) in the TICH group may suggest that the body is fighting an infection, an allergic reaction, or inflammation[166].

Assessing the FBC and CRP values of the $NT_{pat}$ ICH group according to the reference range there were changes in the RBC, WBC, MCHC, Hb and CRP parameters. Only the WBC count mean and CRP median were above normal reference range, while the RBC and Hb mean, and MCHC median were below normal range. The $NT_{pat}$ ICH group patients could also possibly have an anaemic profile, due to the decrease in Hb, and increased inflammation due to elevated CRP levels. As a measure of the average concentration of Hb in RBCs, the slight decrease in MCHC (hypochromia) suggests that there is somewhat less Hb per unit of RBCs, and as a result, the ability of the tissue to supply oxygen may be impaired. Suprasanna *et al.* discovered that the progression of intracerebral haematoma was related with statistically significant declines in Hb, RBC count, HCT, PLT count, and PLT crit (volume occupied by PLTs in the blood as a percentage)[167].

Finally, the slightly higher WBC count corresponds to the higher CRP level observed in the NTICH group. This increase could be caused by tissue damage from the blood vessel wound site. Hu *et al.* conducted a study which concluded that higher WBC counts were associated with an increased risk of fatal stroke[166]. In case-control studies, greater WBC counts upon admission have been associated with negative

outcomes, including a higher risk of stroke mortality[168], ischaemic stroke[169], and haemorrhagic stroke[170].

### 3.7.3 Conclusion

A haematology analysis was included for the purpose of determining the general haematological profile of both the $T_{pat}$ ICH and $NT_{pat}$ ICH patient groups and gaining insight on their overall haematological status. The results for both groups indicated a potential lowered tissue oxygenation capacity due to a slight decrease in Hb levels and MCHC. Based on the CRP values, it was confirmed that both patient groups have an increased state of inflammation. The slight increased WBC count in the both groups were also consistent with tissue damage, producing more WBC needed for wound healing and protection against possible infection. The haemorheological implications of this data indicate a general decrease in the blood viscosity of ICH patients. Human blood, as detailed in Chapter 2, is a non-Newtonian fluid with shear-thinning behaviour; at low shear stress (as a result of a lower cell count), blood viscosity is high, whereas at high shear stress or shear rate, blood viscosity is low[171]. Given that these patient groups had decreased blood viscosity, it is possible that the shear rates, and hence shear stress, were high. These alterations in blood composition may cause endothelial dysfunction as well as positive PLT priming, thereby enhancing hypercoagulability. In terms of Virchow's triad, these findings suggested a possible increase in the risk of thrombosis. While these parameters are useful in assisting with diagnoses and treatment approaches, they do not provide specific information about possible coagulopathy of the patients. These tests did indicate some abnormalities in the haematological profile of the patients, therefore techniques such as LM, SEM and TEG® can be used to futher investigate the impact on the haemostatic system.

# Chapter 4 : Light microscopy

## 4.1 Chapter objectives

In this chapter the following objective will direct the research:

To determine the degree of RBC deformation in ICH patients compared to healthy individuals by calculating AR using LM and ImageJ.

## 4.2 Introduction

Light microscopy, also known as optical microscopy, is a technique used to view samples through the magnification of a lens with visible light. This technique was first invented in the 17$^{th}$ century and overtime became a standard tool in the study of cell morphology[172]. Using this microscope to examine a stained blood smear is useful in providing a complete haematological picture from a morphological perspective.

Healthy RBCs are shaped discoid and biconcave with no cellular organelles. When deviations are present, in shape, color or size, it can indicate possible pathophysiology[173]. Examples of this includes indentifying poikilocytosis, known as morphological abnormal RBCs in peripheral blood smears, that indicates the possibility of abnormal blood rheology[174]. Table 8 provides examples of RBC morphologies that can be identified using LM and can commonly be found in peripheral blood smears[175]. Crucial to RBC functioning is their ability to deform since RBCs are larger than the vascular diameter of the microcirculation[176]. It is known that blood viscoelasticity exists due to the elastic energy created by the defomation of RBCs, thus a decrease in RBC deformability can result in a significant increase in blood viscosity and resistance in microvascular flow[177].

**Table 8: Summarising the variation in red blood cell morphology commonly seen in blood smears. Adapted from Red Blood Cell Morphology[175]**

| Common Poikilocytes in Peripheral Blood Smears | |
|---|---|
| **RBC morphology** | **Morphological definition** |
| Acanthocyte (spur cell) | RBC with irregularly distributed, variation in size and pointy projections |
| Anisocytosis | Population of RBCs showing variation in size |
| Bite cell/ Blister cell | RBC has semi-circular indentation in cytoplasmic border. 'Roof' to indentation =blister cell or no roof = bite cell. |
| Burr cell | RBC with equal sized, round projections from the surface |
| Elliptocyte | RBC with a long oval shape |
| Irregularly Contracted Cell | Small and dark RBC with no central pallor. Its outer margin is not spherical. |
| Macrocytes | Abnormally large RBC with small area of central pallor |
| Microcytes | Abnormally small RBC with large area of central pallor |
| Ovalocyte | RBC that are oval or egg shaped |
| RBC agglutination | RBC adhering to each other, resembling a bunch of grapes |
| Rouleaux formation | RBC adhering to each other, resembling stacked coins |
| Schistocyte | RBCs that appear to be fragmented, showing angular morphology and no area of central pallor. |
| Sickle cell | RBC with a lunar or boat shape |
| Spherocyte | RBC that is smaller and darker than normal, and no area of central pallor. |
| Stomatocyte | RBC that has a linear area of central pallor |
| Target cell | RBC with a red area inside its central pallor |
| Tear drop cells | RBCs that is tapered to a point at one of its ends |

Red blood cell (RBC)

Red blood cells operate as free radical scavengers, protecting human tissues from oxidative damage. Prolonged exposure to oxidative components produces more free radicals than can be neutralised, resulting in oxidative damage to RBCs, particularly the phospholipid bilayer membranes. The asymmetrical distribution is disrupted in pathological situations and under oxidative stress, resulting in membrane scrambling and the "PS-flip" phenomena[178-179]. Thus, deformed RBCs may be the result of oxidative stress.

Various previous studies made use of LM to investigate RBC morphology. Sulette *et al.*[180] primarily wanted to determine if females', with episodic migraine-with-aura, blood were changed due to inflammation and whether their coagulability state changed during the phases of migraine. They also critically evaluated RBC morphology for indicators of oxidative stress, using LM. Results from LM indicated increased AR of RBCs of the female migraineurs, showing deviation from the normal discoid shape. The LM changes in RBC morphology of migraine patients typically indicated biophysical changes, although agglutination and microrouleaux formation indicated aberration at the biochemical level. They concluded that observed variations in the

RBC size, shape (quantified with LM) and distribution were most likely the result of oxidative stress[180].

Another study, by Bester *et al.*[181], made use of LM to study general morphology of RBCs and their AR to determine shape variations. The authors analysed trends between high and normal serum ferritin (SF) in individuals with Alzheimer's disease (AD) and hypothesised that unliganded iron contributes to coagulation complications seen in AD, as well as changes seen in RBC morphology. Results showed that a large fraction of RBCs of AD individuals with high SF have an extended, non-discoid RBC shape and pointed extensions. The study concluded that the increased number of deformed cells in high SF AD individuals may be due to membrane and cytoskeletal architectural changes that possibly led to cells losing their ability to maintain or return to their discoid shape. They also showed that RBC ultrastructure is changed significantly in the presence of iron overload[181].

Light microscopy is generally used to study cell morphology and to give an accurate overview of the distribution of cells in the sample[182]. In addition, LM can also be used to quantitatively determine the degree of deformation of the cells by calculating AR of RBCs. Therefore, in this study LM analysis was used to quantify the RBCs deformation in each patient group. A few LM micrographs were also added to provide a general overview since RBCs contribute to viscoelastic properties of blood rheology[183].

## 4.3 Materials and methods

### 4.3.1 Materials, reagents and equipment

The materials that were used for LM and axial ratio measurements:

- 4.5 mL Sodium citrate tubes (Lasec)
- Zeiss AXIO Imager.M2 Light Microscope (Carl Zeiss Microscope, Munich, Germany)
- Glass microscope slides (Lasec)
- 2 - 20 µL Eppendorf pipette
- 200 µL pipette tips (Lasec)
- Methanol (Sigma-Aldrich)

- Methylene blue stain (Sigma-Aldrich)
- Eosin stain (Sigma-Aldrich)
- Entellan (Sigma-Aldrich)
- Cover slips (Lasec)
- Image J

### 4.3.2 Method

Sample preparation

A volume of 10 µL WB was pipetted onto a glass microscopy slide. Another microscope slide was placed onto the blood drop at a 30˚ angle, allowing the blood drop to spread along the slide. The angled microscope slide was then pulled across the length of the miscroscope slide, creating a thin blood smear. Once the smear had dried, the microscope slide was placed in methanol for 5 minutes to fix the blood cells on the slide; and air dried at room temperature. After the sample was dry, the sample was stained with methylene blue and eosin respectively. The sample was first stained with methylene blue for 5 minutes and the sample was rinsed with running tap water until the water was clear of the stain; again left to air dry. After the sample was completely dry, the sample was placed in eosin for 30 seconds and rinsed again under running tap water till the water was clear of the stain and left to air dry. Once the sample was completely dry, entellan was used to adhere a coverslip onto the slide. The LM was used to view and photograph the RBCs.

Determination of axial ratios

Axial ratios of RBCs were measured using five representative micrographs taken on a 100x magnification per sample. The measurements were done in Image J (Image J is a public domain, Java-based image processing program developed at the National Institute of Health: http://rsbweb.nih.gov/ij/). Ten RBCs were arbitrarily identified across each micrograph and their AR were measured. These measurements were imported to Microsoft Excel.

To measure an axial ratio of a RBC, the diameter of the RBC was measured twice, with the measurement lines crossing each other perpendicularly. The diameters were then divided by using the longest of the diameters as the numerator. Perfectly circular

RBCs will provide an axial ratio measurement of one, while deviations in RBCs shape will result in axial ratio measurements greater than one and translate into a deformed cell.

Figure 9 illustrates how the AR were calculated. The length of line A was used as the numerator where line B was used as the denominator.

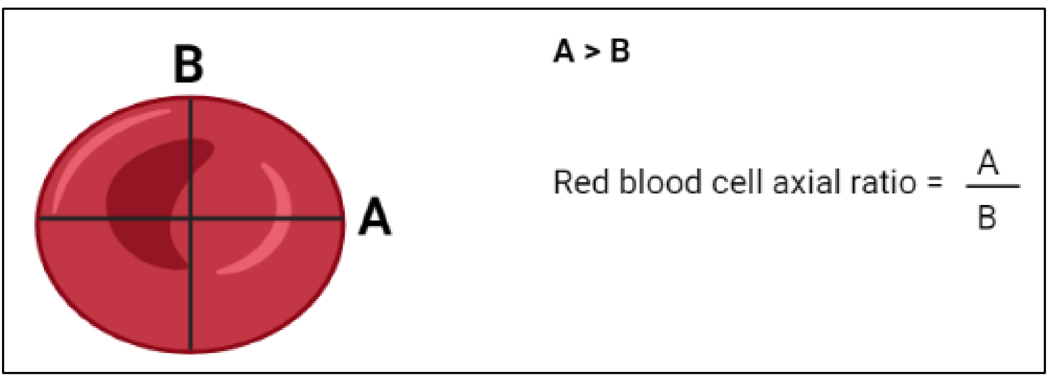


**Figure 9: The measurement and calculation of red bloo cell axial ratios**

## 4.4 Statistical analysis

The statistical analysis for AR was done using GraphPad Prism 8 for windows. The data was first tested for normality using the Shapiro-Wilk normality test. Descriptive statistics were done and the mean and SD were used to describe parametric data, while the median and min and max were used for non-parametric data. Both patient groups was compared to the the control group using a Mann-Whitney test. The dataset did not pass the Shapiro-wilk normality test; thus, the Mann-Whitney t-test was performed to compare the median (min;max) of both the $T_{pat}$ ICH and $NT_{pat}$ ICH groups to the control group. A p-value $<0.05$ was considered significant.

## 4.5 Results

The three groups were denoted as Control , $T_{pat}$ for the traumatic ICH group, and $NT_{pat}$ for the non-traumatic ICH group.

### 4.5.1 Light microscopy morphology

The main objective was not to study the RBC morphology using LM, however, a few micrographs were included to show an overview of the blood cells as analysed using LM. The micrographs were selected (approximately two to three micrographs per individual) as illustrative representations of each group. In Figure 10, 11 and 12 the samples were magnified 100 times, indicating the overall morphology of the three groups.

#### 4.5.1.1 Control Group

A comparable distribution of RBC morphologies could be seen overall in the micrographs of the control group, shown in figure 10. The discoid RBC shape is predominant in the smear, with cell size remaining constant throughout all of the samples.  The RBCs were uniformily distributed across the micrographs, with limited cell interactions.

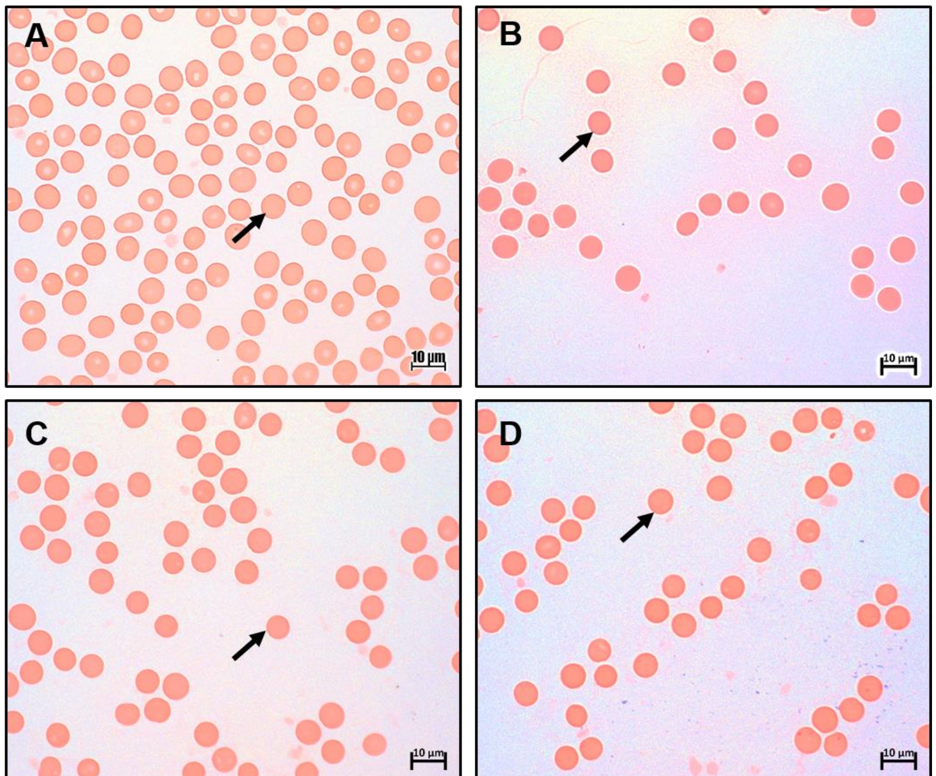


**Figure 10: Light microscopy micrographs from the control group.** Micrographs A-D: overall discoid cells, indicated by the black arrows.

#### 4.5.1.2 Traumatic intracerebral haemorrhage group

The micrographs of this group showed little irregular distribution of RBC morphologies, shown in figure 11. The typical discoid shape was common in the smears, although some abnormal shaped cells were found in the samples. These samples displayed slight morphological diversity across the slide.

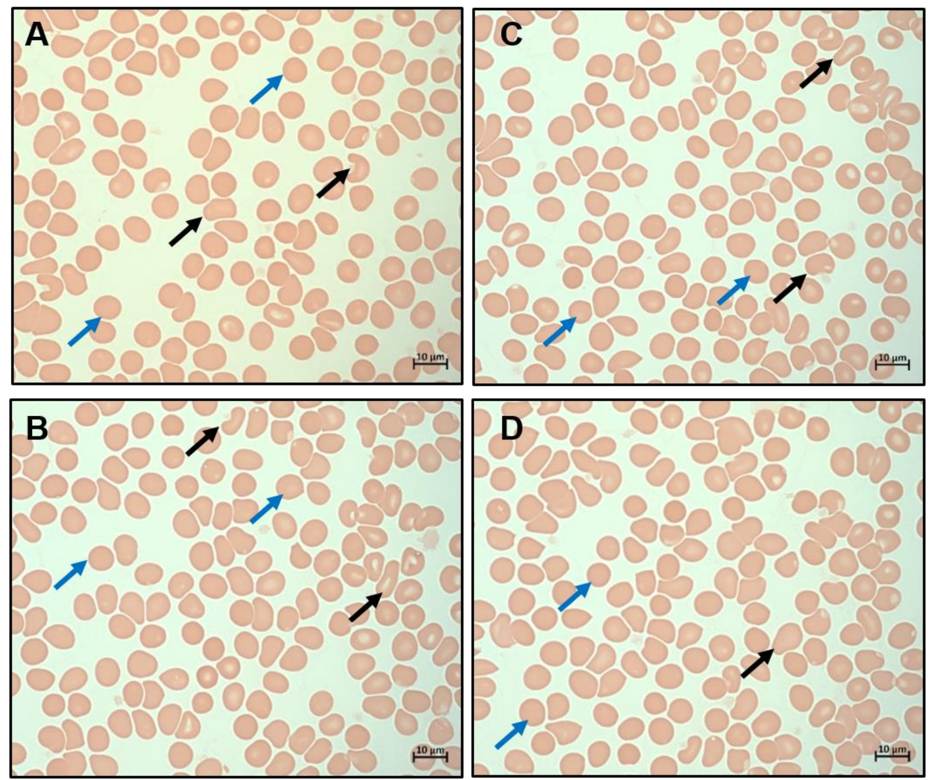


**Figure 11: Light microscopy micrographs from the traumatic intracerebral haemorrhage group.** Micrographs A-D: blue arrows indicate discoid RBCs with normal AR values, while the black arrows indicate abnormal RBCs with increased AR values.

#### 4.5.1.3 Non-traumatic intracerebral haemorrhage group

The micrographs from this group revealed slightly uneven distribution of RBC morphologies, shown in figure 12. The usual discoid shape was observed in the smears, with some abnormal shaped cells found.

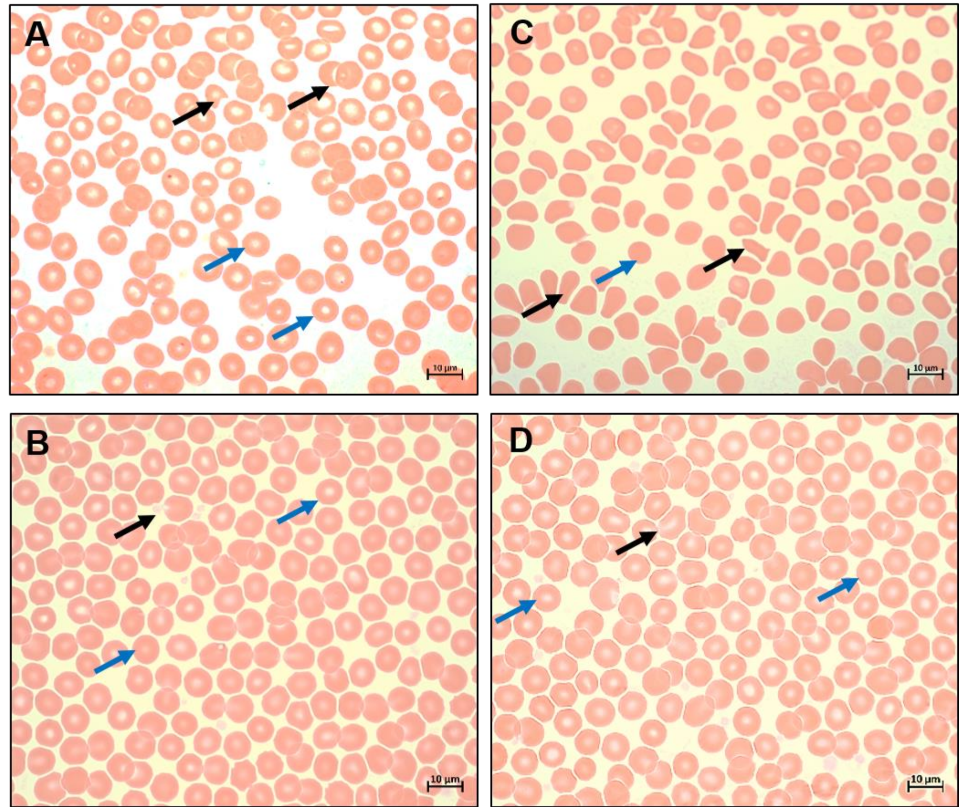


**Figure 12: Light microscopy micrographs from the non-traumatic intracerebral haemorrhage group.** Micrographs A-D: The RBCs appeared to be normally shaped and sized, as indicated by the blue arrows, with the presence of some abnormal RBCs indicated by the black arrows.

### 4.5.2 Red blood cell axial ratios

The data is presented as a summary table (Table 9) of the descriptive statistics of each of the three groups. The statistical analysis revealed no significant differences when both patient groups were compared to the control group. The AR of 50 RBCs per individual were measured. All three groups had a median axial ratio of 1.1. In the the control group, 6.70% of the 2000 RBCs measured had an axial ratio precisely to 1.00. Of the 1000 RBCs measured in the $T_{pat}$ ICH group, 6.50% and 6.26% of the 1550 RBCs measured in the $NT_{pat}$ ICH group had AR equal to 1.00.

**Table 9: Descriptive statistics of the red blood cell axial ratios of the control, traumatic- and non-traumatic intracerebral haemorrhage groups**

| **Red Blood Cell Axial Ratios** | | | | |
|---|---|---|---|---|
| Group | Median (min;max) | Normal value | P-value | Outcome |
| Control vs $T_{pat}$ | 1.1 (1.1;1.2) vs 1.1 (1.0;1.2) | 1.00 | 0.0656 | Limited presence of deformed RBCs |
| Control vs $NT_{pat}$ | 1.1 (1.1;1.2) vs 1.1 (1.1;1.3) | | 0.0521 | Limited presence of deformed RBCs |

minimum (min), maximum (max), traumatic group, ($T_{pat}$), non-traumatic group ($NT_{pat}$).

## 4.6 Discussion

Light microscopy is a basic technique for low magnification sample investigation, since it provides an overall picture and a starting point for the rest of the study. Through LM, it was clear that the general morphology of RBCs in both patient groups displayed minor noticeable shape changes. These changes showed little differences when compared to RBC morphology seen in the control group. The RBCs studied from the control group were discoid and consistent in size. These minor deviations from normal morphology could be attributed to mechanical alterations resulting from sample preparation such as increased adhesion potential.

The RBCs studied from the $T_{pat}$ ICH group showed morphology that deviated slightly from that of the control group. Irregular shaped RBCs were scattered throughout the samples in this patient group. Abnormal cells such as bite cells are frequently formed as a result of fragmentation or oxidative damage[184].

The RBC morphology studied from the $NT_{pat}$ ICH group also indicated minor deviations when compared to the control group. Marked shape changes and RBC aggregation was observed. Red blood cell aggregation occurs when the negative charge of the RBCs decreases, most usually due to an increase in the presence of globulins such as fibrinogen and immunoglobulins, which is frequently caused by inflammation[185].

Axial ratios are an excellent quantitative tool for determining the degree of cell deformation. When compared to the control group, the p-values for both the $T_{pat}$ and $NT_{pat}$ ICH groups showed no significant difference. However, both p-values were

borderline of significance, indicating that a small number of cells may have had deformation and a larger sample size could have provided a more definitive outcome.

## 4.7 Conclusion

In conclusion, there is limited evidence to substantiate the variations in RBC morphology seen in both the $T_{pat}$ and $NT_{pat}$ ICH group. Although no significant differences were found between controls and patient groups, visual evaluation of micrographs identified some degree of shape change. It should be highlighted that the degree of cell deformation seen in the control group was consistent with what was seen in the patient groups. The findings in this chapter emphasise the importance to further investigate the changes seen with LM. The following ultrastructural studies will allow the examination of the cells on a higher magnification and provide the ability to identify alterations not visible with LM.

# Chapter 5: Scanning electron microscopy

## 5.1 Chapter Objectives

In this chapter the following objective will direct the research:

Investigating the ultrastructural morphology of RBCs and PLTs in ICH patients and the RBC-PLT interactions, as well as clot structure and fibrin network using SEM.

## 5.2 Introduction

Scanning electron microscopy is a high resolution microscope that produces micrographs by scanning the surface of a sample with a focused beam of electrons. The sample is specially prepared and coated with an electron dense material, such as carbon[186]. Once the electrons make contact with the sample, they excite and are emitted as different signals which is detected by an array of detectors within the SEM chambers. The signals are interpreted using computer algorithms into highly detailed photographic micrographs.

The application of SEM may be useful to investigate the ultrastructure of RBCs and their surface morphology, as well as cellular interactions in the blood of ICH patients[187]. Changes to RBC shape and size can indicate pathology for example increased or decreased elasticity[188-189], and can provide a summary of the overall health of RBCs. Membrane typography and the presense of shedding or MP can also be studied using the SEM. All these factors impact coagulation and the blood flow of patients[190]. Lastly, SEM can be used to study fibrin networks which are responsible for clot stability until damage tissue is repaired.

Previous research done by Bester *et al.*[191], made use of this technique to study WB and plasma in AD patients. The researchers argued that bacteria cell wall components, lipopolysaccharide (LPS) might be the cause of the on-going and low-grade inflammation, distinctive of AD. The SEM results indicated discoid RBCs and overactivated PLTs, typically seen during systemic inflammation. In addition extensive fibrin networks for the AD individuals were indicated. Eryptotic RBCs were also present, characterised by shrinkage, blebbing and phospholipid scrambling of the cell

membrane. The SEM results could thus be used to support the theory of increased coagulability in AD patients and highlight the problem areas.

Venter et al.[192] used SEM to examine the effects of heavy metals cadmium (Cd) and chromium (Cr) alone and in combination on the morphology of RBCs. Scanning electron microscopy was used to study RBCs, PLTs and fibrin fiber structure. The authors concluded that Cd and Cr alone and in combination caused morphological changes to RBCs, PLTs and fibrin fibre[192]. Platelet activity seen in the metal groups, mostly due to oxidative stress and inflammation, lead to more loose or bended fibres and caused pathological thrombi. The fiber thickness present in the metal groups further contributed to the alterations of clot formation, and may cause reduction in the lysis of clots[192].

Scanning electron microscopy analysis of this study also examined the ultrastructural changes during clot formation in ICH patients when compared to controls. This aided to identify specific changes that related to the different components in clot formation.

## 5.3 Materials and methods

### 5.3.1 Materials, reagents and equipment

The materials that were used for SEM are the following:

- 10 mm Glass coverslips (Lasec)
- 200 µL pipette tips (Lasec)
- 2 - 20 µL Eppendorf pipette
- Human thrombin donated by the South African National Blood Service
- 24 Well plates (Lasec)
- Curved tip foreseps
- 0.01 M Phosphate buffered saline (Sigma-Aldrich)
- Formaldehyde (Sigma-Aldrich)
- Osmium tetroxide (Sigma-Aldrich)
- Hexamethyldisilazane (Sigma-Aldrich)
- Aluminium plates
- Carbon tape
- Graphite rods

- Quorum Q150T Coating Unit (Quorum Technologies, Lewes, United Kingdom)
- Zeiss Gemini Ultra Plus FEG SEM ( Carl Zeiss Microscopy, Munich, Germany)

### 5.3.2 Method

All samples for SEM were prepared using a standard method[193-194]:

A volume of 10 µL WB was placed directly on a glass coverslip to make smears of RBCs; another 10 µL of blood was placed on a coverslip and mixed with 5 µL thrombin to create a WB clot. After the smears were made, the coverslips were placed into individual wells of a 24 well plate and left to air dry for 1 minute at room temperature. The samples were then washed by gently immersing for 15 minutes in phosphate buffered saline (PBS) solution (pH 7.4). Subsiquently, the samples were fixed with 4% formaldehyde for 30 minutes, followed by a wash step where the samples were washed three times in PBS for 3 minutes each. After the three washes, a secondary fixation was done by covering the samples in 1% osmium tetroxide ($OsO_4$) for 15 minutes. The samples were again washed three times with PBS for 3 minutes each and then dehydrated with 30%, 50%, 70%, 90% and three times 100% ethanol; all for 3 minutes each. After dehydration the samples were immersed in 1,1,1,3,3,3-Hexamethyldisilazane (HMDS) for 30 minutes, the HMDS was removed and a drop of HMDS was added to the sample and were left to dry over night at room temperature. After drying, the samples were mounted on metal plates and coated with carbon to provide conduction of the samples under the microscope. The SEM viewing was done at the Microscopy and Microanalysis unit at UP, Pretoria, South Africa. A Zeiss ULTRA plus FEG_SEM with InLens capabilities was used to study the morphology of blood cells and the clots and micrographs were taken at 2 kV.

Specific characteristics were studied for each sample. This included:

1.) The overall shape of the RBCs and PLTs

2.) The typography of the RBC membrane

3.) Presence or shedding of MPs

4.) Presence of dense, matted fibrin network deposits

5.) Cell interactions

6.) Platelet spreading and pseudopodia formation

Sample analysis consisted of a series of representative micrographs being taken of each sample. For all samples a minimum of 10 micrographs were taken. From these, features of interest were noted according to the properties of interest described. Micrographs obtained from healthy controls were then compared to those obtained form the ICH patient groups.

## 5.4 Results

The SEM data are displayed as micrographs and are entirely observational. The samples were viewed at 5000, 10000 and 20000 times machine magnification, during which ultrastructural trends within the groups were noted. All samples were micrographed; the micrographs that best reflect each group are presented. The three groups were denoted as Control, $T_{pat}$ for the traumatic ICH group, and $NT_{pat}$ for the non-traumatic ICH group.

### 5.4.1 Red blood cell ultrastructure and platelet presence

The criteria used to evaluate each sample and the findings from each group are summarised in table 10 and 11. Some degree of PLT activation is expected as during sample preparation the glass coverslips are known to induce contact activation.

**Table 10: Descriptive summary of the criteria used to evaluate the samples, as well as the results obtained from the red blood cell scanning electron microscopy**

| RBC morphology | | | |
|---|---|---|---|
| Criterion | Control | $T_{pat}$ ICH group | $NT_{pat}$ ICH group |
| Overall morphology | Circular and biconcave disc shaped | Mostly circular with slight RBC change | Circular with RBC shape change |
| Observed membrane texture | Slightly globular surface | Slightly globular surface | Slightly globular surface |
| Cell interaction | RBCs evenly distributed, limited interaction | RBC aggregation present | RBC aggregation present |
| Shedding of MPs | Not present | Limited presence | Present |

Intracerebral haemorrhage (ICH), microparticles (MPs), non-traumatic group ($NT_{pat}$), red blood cells (RBCs), traumatic group ($T_{pat}$).

**Table 11: Descriptive summary of the criteria used to evaluate the samples, as well as the results obtained from the platelet scanning electron microscopy**

| PLT morphology and interaction | | | |
|---|---|---|---|
| Criterion | Control | $T_{pat}$ ICH group | $NT_{pat}$ ICH group |
| Overall morphology | Round in shape with some degree of spreading | Platelet presence with aggregation, more pronounced spreading | Platelet presence with aggregation, more pronounced spreading |
| Pseudopodia | Relatively short pseudopodia | Clear pseudopod formation, relatively long | Clear pseudopod formation, relatively long |
| PLT-PLT interaction | None | Extensive | Extensive |
| RBC-PLT interaction | Limited interaction | Extensive | Extensive |

Intracerebral haemorrhage (ICH), non-traumatic group ($NT_{pat}$), platelet (PLT), red blood cell (RBC), traumatic group ($T_{pat}$).

### 5.4.1.1 Control group

Figure 13 exhibit the representative micrographs from the control group. In these micrographs the majority of the cells have a normal morphology. The RBCs appeared to have a slight globular membrane and no RBC-RBC interactions. Minimal PLT presence and pseudopod formation was seen throughout the control group. These PLTs had a spherical form, minimal spreading, and no PLT-PLT interactions.

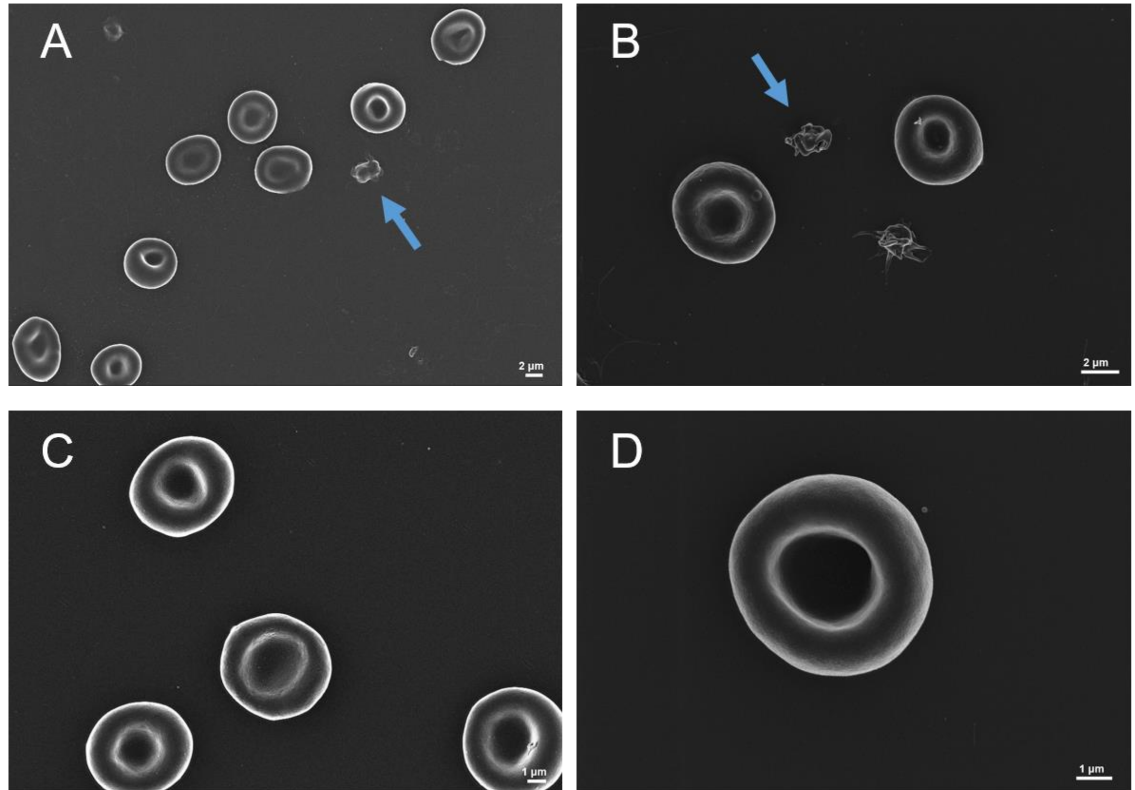


**Figure 13: Scanning electron microscopy micrographs from the control group.** Micrographs A-D representing a healthy control RBC population. Micrograph A&B: normal biconcave RBCs with limited PLT presence (blue arrows). C: Evenly distributed RBCs. D: high magnification of a RBC showing slightly globular membrane.

### 5.4.1.2 Traumatic intracerebral haemorrhage group

Figure 14 illustrate the representative micrographs of RBCs and PLTs in the $T_{pat}$ ICH group**.** Overall, circular RBCs with minor shape changes were identified, as illustrated by the green arrows. The yellow arrows indicate a changed membrane with a globular surface appearing porous. More PLTs were seen with extended pseudopodia, showing PLT-PLT interactions, as demonstrated by the blue block. Some RBC-PLT interaction and RBC aggregation was observed in this group, as indicated by the red and white arrows respectively.

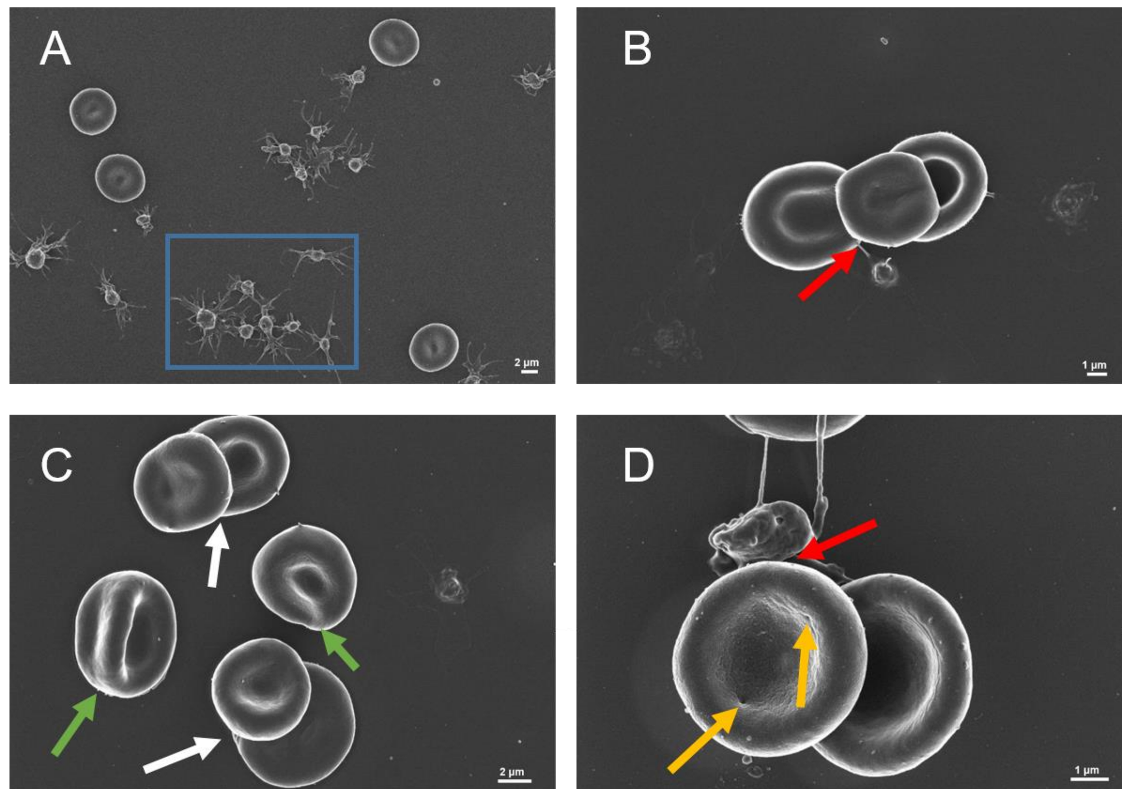


**Figure 14: Scanning electron microscopy micrographs from the traumatic intracerebral haemorrhage group.** Micrographs A-D representing RBCs and PLTs. Micrograph A: increased PLT presence, extended pseudopodia and PLT-PLT interactions (blue block). B&D: increased RBC-PLT interactions (red arrows). C: minor RBC shape changes (green arrows) and RBC aggregation (white arrows). D: high magnification showing the globular and porous membrane appearance (yellow arrows).

### 5.4.1.3 Non-traumatic intracerebral haemorrhage group

Figure 15 illustrates the representative micrographs of RBCs and PLTs in the $NT_{pat}$ ICH group. The RBCs were mostly round and biconcave, and slight shape modification were visible. The membranes of some cells had a globular appearance, as shown by the yellow arrow. The possible presence of MPs found, which is indicated by pink arrows and was shown to be less prominent. Platelet-platelet interaction and extensive PLT aggregation were also observed in these samples, as indicated by the blue blocks. The red arrow indicates a PLT spreading with extensive pseudopod fusing with RBCs, resulting in RBC-PLT interaction. These samples also showed RBC aggregation as indicated by the white arrows.

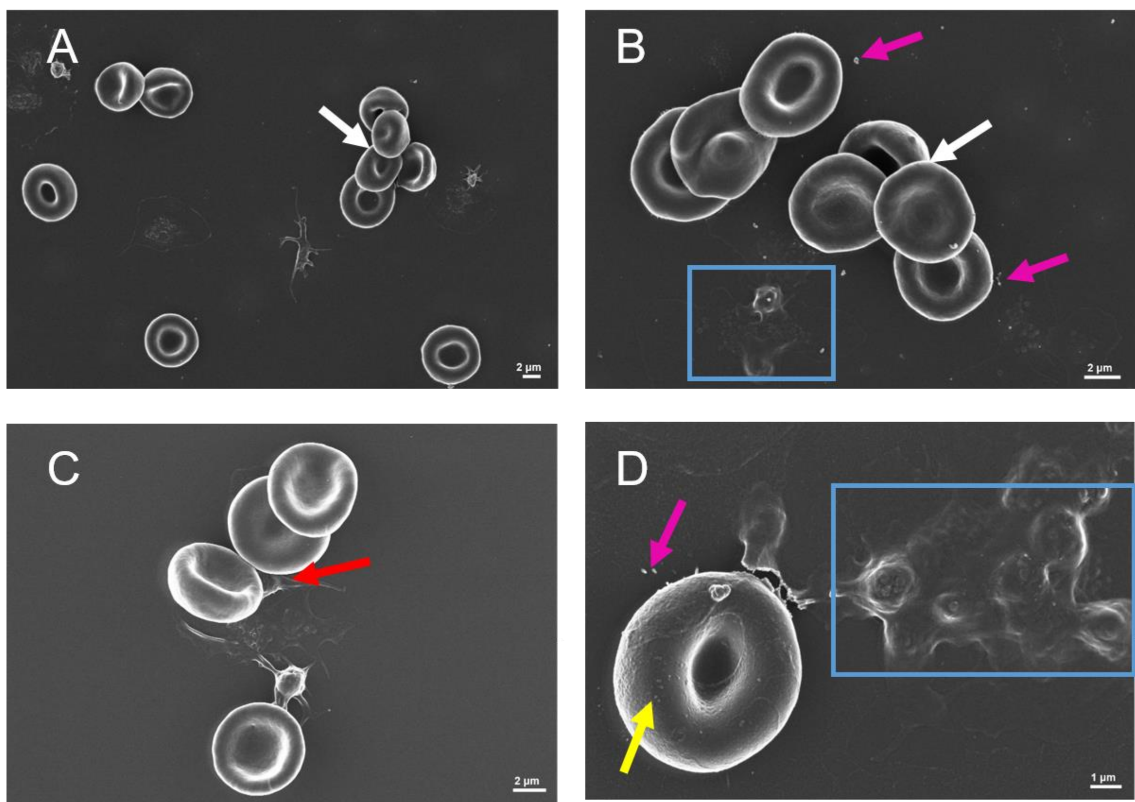


**Figure 15: Scanning electron microscopy micrographs from the non-traumatic intracerebral haemorrhage group.** Micrographs A-D representing RBCs and PLTs. Micrograph A&B: RBC aggregation (white arrows). B&D: possible presence of MPs (pink arrows) and PLT-PLT interactions (blue blocks). C: spreaded PLT pseudopods fusing to RBCs (red arrow). D: textured and globular membrane surface (yellow arrow).

### 5.4.3 Fibrin architecture

The fibrin architecture of the three groups were compared based on observations between interactions of the RBCs and the fibrin net, and the overall structure of the fibers. The criteria used to evaluate each sample and the findings from each group are summarised in Table 12.

**Table 12: Descriptive summary of the clot morphology according to the set criteria**

| Fibrin architecture and network | | | |
|---|---|---|---|
| Criterion | Control | $T_{pat}$ ICH group | $NT_{pat}$ ICH group |
| Overall morphology | Organised and structured branching | Disorganised lattices, less structured branching | Disturbed network uniformity |
| Overall composition | Range of fibre thickness seen | Fibrin network more finely webbed | Fiber tangles and fusion |

| RBC interaction | RBCs maintained their shape when interacting with the fibrin net | Slight RBC shape change | Slight RBC shape change |
|---|---|---|---|

Intracerebral haemorrhage (ICH), non-traumatic group ($NT_{pat}$), red blood cell (RBC), traumatic group ($T_{pat}$).

#### 5.4.3.1 Control group

The branching of the fibers observed in the control group produced a lattice-like structure with geometric like corners, indicated by the blue arrow. The RBCs trapped within the lattices did not deform or collapse under the fibers entrapping them, indicating robust and complete membrane integrity. Figure 16 illustrate the representative micrographs of the fibrin clots from the control group.

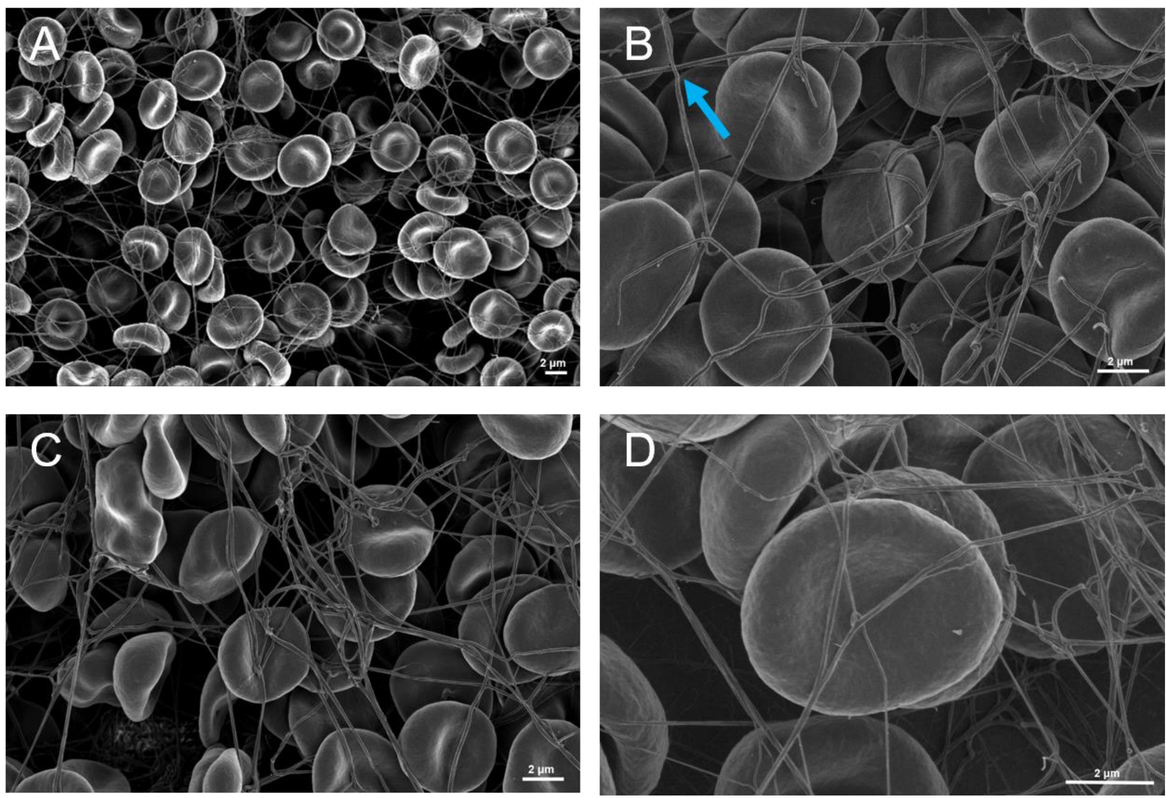


**Figure 16: Scanning electron microscopy micrographs from the control group.** Micrographs A-D representing fibrin clots. Micrograph A-D: RBCs entrapped between the fibers and exhibit limited deformation. Most fibers show angular corners and a lattice-like structure (blue arrow).

#### 5.4.3.2 Traumatic intracerebral haemorrhage group

Figure 17 illustrates the representative micrographs of the fibrin clots from the $T_{pat}$ ICH group. The fibrin fibers are disorganised and more finely webbed as shown by the yellow arrows. These finer webs create tightly tangled areas in between, indicated by the blue block. Limited RBC deformation were present.

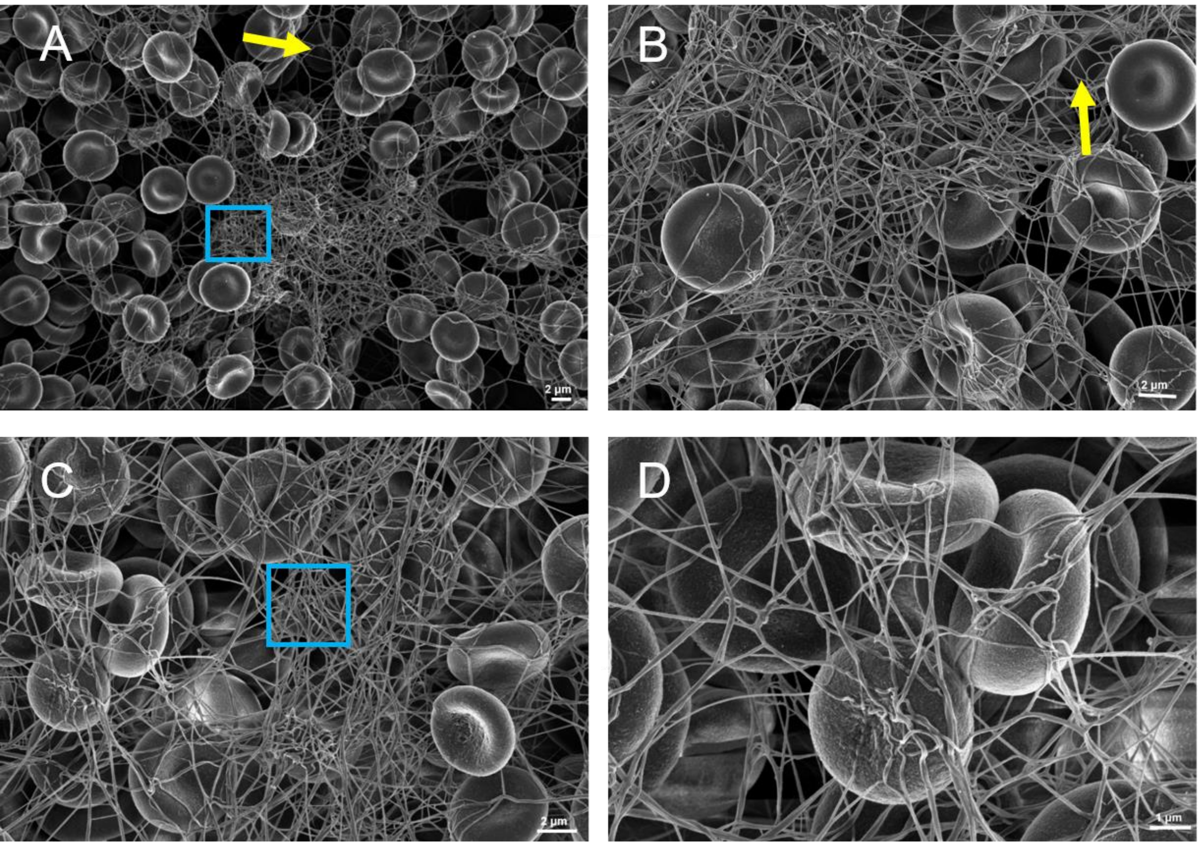


**Figure 17: Scanning electron microscopy micrographs from the traumatic intracerebral haemorrhage group.** Micrographs A-D representing fibrin clots. Micrograph A-D: finely webbed fibrin fibers (yellow arrows) and tightly tangled areas (blue blocks).

#### 5.4.3.3 Non-traumatic intracerebral haemorrhage group

Figure 18 illustrates the representative micrographs of the fibrin clots from the $NT_{pat}$ ICH group. The fibrin structures observed in this group indicated an altered network uniformity with fiber tangles and fusions indicated by the blue blocks. The fibers had less structured branches which grew sparce, leaving open holes in the fibrin network as shown by the yellow arrows. The RBCs showed some degree of deformation as seen by green arrows indicating the minor deformation.

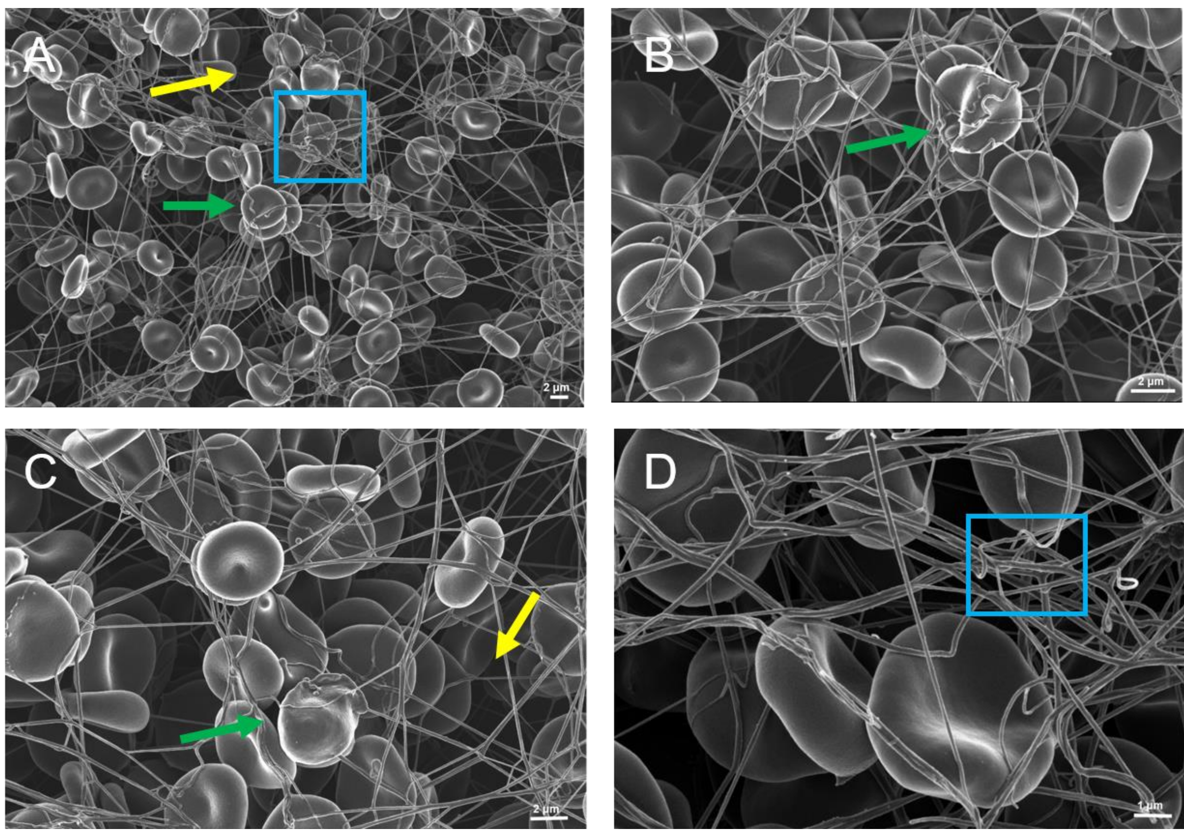


**Figure 18: Scanning electron microscopy from the non-traumatic intracerebral haemorrhage group.** Micrographs A-D representing fibrin clots. Micrograph A&D: fibrin fiber tangles (blue blocks). A&C: large open areas in the net (yellow arrows). A,B&C: slight RBC deformation (green arrows).

## 5.5 Discussion

The importance of investigating RBC morphology is highlighted by the fact that RBC shape is essential for optimal oxygen carrying capacity. The observations made from representative micrographs of the samples in the control group served as the baseline to which the observations in the $T_{pat}$ ICH and $NT_{pat}$ ICH groups were compared. The control group indicated normal RBC morphology, RBC membrane typography, RBC interaction, PLT morphology, PLT pseudopodia formation, PLT interaction, and fibrin clot formation consistent with earlier investigations[195].

Both the $T_{pat}$ and $NT_{pat}$ ICH groups showed slight RBC morphological changes when compared to the control group. One of the major causes of extensive variation in size, shape, and distribution are oxidative stress and hyperosmotic shock-induced by overexpression of inflammatory mediators, both of which accompany persistent systemic inflammation and lead to eryptosis[179,196]. Red blood cells have been found to

undergo both biochemical and biophysical changes during inflammation. Biochemical changes are characterized by disruptions in the molecular organization of the plasma membrane and RBC function, whereas biophysical alterations are characterized by changes in the overall structural arrangement and RBC morphology, which result in changes in RBC mechanics. In most cases, inflammatory chemicals interact with RBC membranes, causing structural alterations[197].

When there is increased inflammation, RBCs aggregate more frequently, forming rows or stacks where they fit into one another due to their discoid structure; known as rouleaux formation. The presence of fibrinogen is an important major plasma protein involved in the formation of the rouleaux. Enhanced fibrinogen levels result in enhanced rouleaux development[198]. The binding of fibrinogen to the RBC membrane influences not just aggregation but also RBC viscosity. This increased viscosity will contribute to poor blood flow, making movement of RBCs in the microvascular, such as capillaries challenging[198].

Membrane typography of $T_{pat}$ ICH RBCs appeared slightly porous and globular, however no distinct difference were noted between this patient group compared to control RBC membrane topography. Red blood cell membrane typography from the $NT_{pat}$ ICH group contained possible presence of MPs. Increased inflammatory conditions produce both membrane asymmetry loss and MP release in RBCs via PS externalisation[62]. Previous research has found that a large number of MPs are released into the bloodstream following an acute brain damage[199]. Microparticles were discovered to have an important role in peripheral inflammatory development, thrombosis, endothelial dysfunction, and angiogenesis. Microparticles and brain derived microparticles (BDMPs) generated from microglia/macrophages can promote cerebral inflammation[199].

Although increased WBCs were not visible in the SEM micrographs, the FBC count indicated an increased number of WBCs. The presence of increased WBC is another intriguing characteristic of clot shape that has previously been described[200]. White blood cells release proteases and oxygen metabolites that influence neighboring cells, including RBCs, which may experience membrane changes. The RBC may experience physical and/or molecular damage when exposed to an oxidative and proteolytic environment, such as an increase in membrane bound Hb and a change in

band three profile. Because the protrein band-3 connects the lipid bilayer to the cytoskeleton, any changes to its profile may destabilise membrane structure and, as a result, the ability of the RBC cytoskeleton to induce membrane deformation. Finally, this may have an effect on clot contraction and thrombi compactness. Since the membrane integrity of RBCs showed some indications of disruption in the $NT_{pat}$ ICH group, the increased WBC count seen in their FBC could be responsible.

Platelet adhesion and aggregation were previously seen in response to TBI due to increased vWF[3]. The endothelium rapidly secretes vWF into the plasma and basement membrane following vascular damage. The interaction of vWF with the PLT receptor (GPIb-IX) results in PLT spreading, pseudopodia formation and integrin-dependent signaling[3], all observed in both patient groups.

Platelets from the $NT_{pat}$ ICH group were aggregated and spread, which differed from control PLTs. Increased PLT presence may affect blood flow by facilitating adherence of more PLTs and RBCs to the blood vessel wall, as well as PLT aggregation, resulting in a decreased shear rate and an increased viscosity[201]. The formation of PLT aggregates was previously documented by Page *et al*. where they studied the effect of TNF-α and C3 on coagulation[202]. Tumor necrosis factor-α was introduced to either WB or plasma in the Page study participants in order to establish a low-grade chronic inflammatory condition. As a result, significant PLT aggregation and clumping occurred in their samples. This could imply that an enhanced inflammatory status, as found in both of the ICH patient groups, has a significant impact on PLTs. Although these PLTs appeared to be more activated, this can only be validated using specialised PLTs activation tests, which were not performed during this investigation.

Both patient groups displayed varied fibrin clot formation that lacked the typical lattice-like appearance. The fibrin fibers of the $T_{pat}$ ICH group seemed disorganised and appeared denser. This group showed moderate RBC deformation, which was most likely caused by fibrin fibers entrapping the RBCs tightly distorting their membranes. Pretorius *et al.*[203] discovered that moderate iron loading increases thrombus formation following arterial injury, increases vascular oxidative stress, and impairs vasoreactivity. As both RBCs and coagulation factors are exposed, the probable production of hydroxyl radicals or the excess iron itself may affect the ultrastructure of RBCs and fibrin fibers[203]. Fibrin fibers from the $NT_{pat}$ ICH group were less organised,

with fiber tangles and fusions that left big voids in the network. The RBCs appeared deformed, due to the fibers exerting pressure on the RBC surfaces, suggesting a decreased membrane integrity.

These findings have several implications, including alterations in blood viscosity and the risk of thrombus development. Changes in RBC shape, as well as RBC deformation in fibrin fiber networks, suggest a possible impact on membrane integrity. This deformation may be a contributing factor to viscosity changes, as deformed RBCs may increase the turbidity of blood flow, lowering the shear rate of flow in the blood vessel and resulting in increased viscosity[176]. One of the mechanisms described in Virchow's Triad that increases the chance of thrombus development is altered blood flow[204].

Changes in the density of these fibrin networks generated in the blood of $T_{pat}$ ICH patients may have an effect on clot lysis, which is a natural process of clot breakdown. Increased fibrin synthesis may lengthen the time to lyse, increasing microclot formation and persistent clots. Thicker fibers usually increase thrombotic events[205]. This, however, will require additional research using clot lysis investigations.

## 5.6 Conclusion

To highlight the findings mentioned in this chapter, the clot components of both TICH and NTICH patients differ significantly from those of healthy controls. Notable findings include modest alterations in RBC morphology, RBC aggregation, increased PLT presence, and enhanced fibrin fiber production with closer interlocking strands and disrupted network uniformity. The consequences of these results may include changes in rheology caused by variations in blood flow, facilitated by enhanced RBC deformation and aggregation, ultimately increasing blood viscosity. Alterations to the vascular wall, encouraged by increased PLT aggregation, and hypercoagulation all enhance the likelihood of spontaneous thrombus formation. It is not unexpected, that increased blood viscosity and fibrin concentration are associated with, and significant predictors of, cardiovascular disease [198].

Red blood cells are essential to our existence since they deliver adequate amounts of $O_2$ to organs . These cells operate due to their well-known discoid structure and $O_2$

carrying capability. If the RBCs' structure is disrupted, the $O_2$ carrying capacity may be reduced. As a result, these structural alterations may have a significant impact on normal tissue perfusion and give rise to complications such as hypoxia or ischaemia events.

Although there are some discernible variations between the two patient groups and the control group, it is crucial to note that SEM data is qualitative and hence subjective. The incorporation of quantitative methodologies provides a more acurate measure of the functional properties of these blood components. Results in this section indicated that the coagulation system appears to be increasingly activated in both ICH patient groups. As hypercoagulation is another key component of Virchow's triad, this, along with a discussion on observed changes to clot formation will be revisited in Chapter 6.

# Chapter 6: Thromboelastography®

## 6.1 Chapter Objectives

In this chapter the following objective directed the research:

To measure the viscoelastic properties of WB during clot formation in ICH patients compared to healthy control individuals using TEG®.

## 6.2 Introduction

Thromboelastography® is a viscoelastic test, developed by Hartert in 1948[206], used as a point of care instrument, to rapidly assess the rate and intensity of clot formation to clot lysis, as well as the viscous and elastic interactions of blood components[207-208]. Many researchers believe that this method is preferable to conventional coagulation tests when analysing the hypercoagulable state as well as the bleeding risk in patients with haemophilia, postpartum haemorrhage, and patients having cardiac or brain trauma surgery[209-211].

Thromboelastography® functions to quantitatively measure the ability of WB to form a clot [212]. The aim of TEG® is to identify and measure the dynamic changes in blood viscoelastic characteristics that occur during clot formation under low shear stress.

The thromboelastograph contains two chambers (37˚C) in which a cup containing a clotting activator and a WB sample is placed, as well as a detection pin suspended in the center of the cup[213]. The cup oscillates around the detection pin, where the formation of a blood clot induces movement of the pin; which is detected and recorded as a function of time. During coagulation, the formed clot adheres to the cup and detection pin, inducing marked movement of the pin due to resistance (increased blood viscosity)[213-214]. The noticeable rise in the viscoelastic and mechanical characteristics of the blood reflects the formation of the three-dimensional fibrin mesh and PLTs components of the blood. The amplitude of the pin motion increases as the blood viscoelasticity increases. As fibrinolysis starts, the fibrin mesh and PLT clot will detach and cause the clot to lose contact with the detection pin. A thromboelastogram is an image of the recorded amplitude of movement of the pin as a fuction of time and is used to illustrate induced movement of the pin (Figure 19)[215].

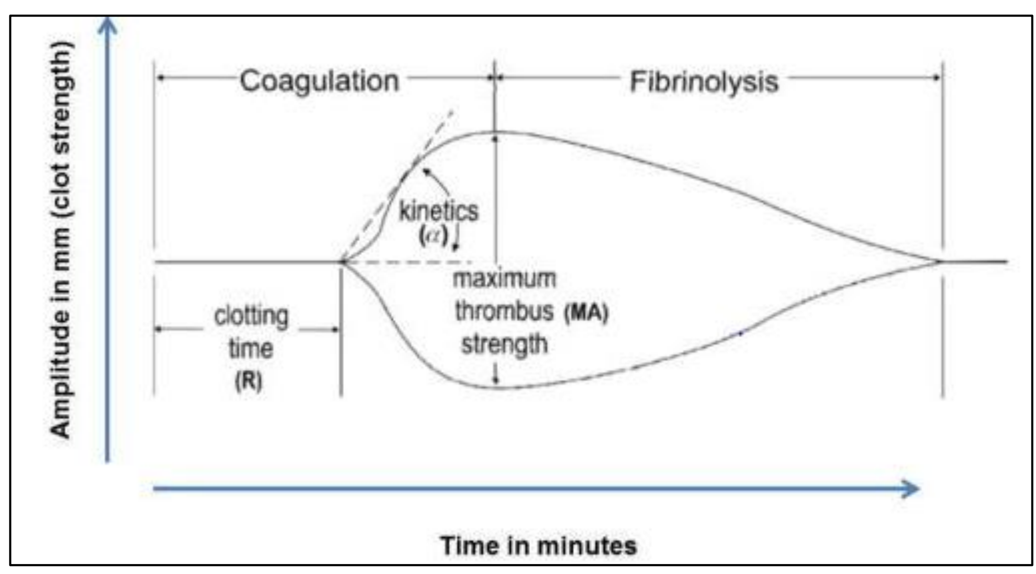


**Figure 19: A thromboelastogram graph showcasing normal clot formation and lysis [215]**

Analysing various parameters in the TEG® together provide useful information about the haemorheological status of an individual; whether coagulation is normal, or hypo- or hypercoagulable. Furthermore, individual TEG® parameters also provide deeper insight of the contribution of the different coagulation components such as, PLTs, RBCs and fibrin fibres to clot size and strength. The TEG® measures eight parameters and is summarised in table 13[216,217].

**Table 13: A summary of the thromboelastography parameters, reference values, and biological significance. Adapted from Shaydakov et al. published on StatPearls[216]**

| Parameters | Description | Reference values | Biological significance |
|---|---|---|---|
| **Reaction time (R)** | Time passed from initiation of the test to the first detectable clot formation; amplitude of 2 mm. | 9-27 min | **Signifies the activation phase of coagulation**: the time required to activate the intrinsic pathway and fibrin deposition. Reflects an individual's ability to generate thrombin. Coagulation factor dependent. Indicative of the functioning of enzymatic clotting factors during initial clot formation |
| **Kinetics (K)** | Time passed from initiation of clotting to the formation of a clot with strength that correlates with a size of 20 mm | 2-9 min | **Signifies the amplification phase of coagulation**: the speed at which fibrin deposits and cross-links (speed of clot formation). Fibrinogen dependent. |
| **α-Angle (A)** | The angle between the reaction time and an imaginary line constructed from the time of clotting initiation to the point of maximum clot formation speed. | 22-58 degrees | **Signifies the propagation phase of coagulation**: maximum speed at which thrombin is generated and fibrin is deposited. Fibrinogen dependent. |

| **Maximum Amplitude (MA)** | Maximum amplitude of the curve or the thromboelastogram. | 44-64 mm | **Signifies the termination phase of coagulation**:maximal mechanical strength of the clot. Platelet dependent. |
|---|---|---|---|
| **Shear Elastic Modulus (G)** | Rigidity of the clot calculated from the amplitude of the clot. | 3.6-8.5 d/sc | **Signifies the elasticity of the clot**: provides a measure of density and rigidness, which in turn provides and indication of the clot's penetrability to fibrinolytic substances. |
| **Maximum Thrombus Generation (MRTG)** | Maximum velocity of clot formation. | 0-10 dcs | **Signifies the maximum rate at which the clot formed**: the peak rate at which the haemostatic system functions |
| **Time to Maximum Thrombus Generation (TMRTG)** | Time taken to reach the maximum velocity of clot growth from initiation of coagulation. | 5-23 min | Signifies the quality of the interaction of the components of the haemostatic system |
| **Total Thrombus Generation (TTG)** | Total resistance of the clot during clot formation, from initiation of coagulation until TEG® is complete | 251-1014 dcs | Signifies the total strength of the formed clot |

Thromboelastography (TEG®)

The overall clot stiffness is measured by the MA, which is also used to assess both primary and secondary coagulation[218]. It is also useful in guiding PLT transfusion[219], with an MA < 50 to 55 mm indicating ineffective PLT involvement in coagulation which signals PLT transfusion. A lower MA, on the other hand, indicates both reduced PLT counts and dysfunction[218]. The reaction (R) time measures the duration from the start of measurement to the start of clot formation. A longer R-time is linked to a reduced concentration of clotting factors, which denotes the requirement for factor replacement. The K time indicates the time taken from the start of clot formation to the time the clot firmness reaches an amplitude of 20 mm. An α-angle (A) of < 45 ° indicates dysfunctional fibrinogen and calls for fibrinogen replacement. Maximum rate of thrombus generation, time to maximum thrombus generation (TMRTG), and TTG are parameters determined from variations in the elastic modulus G, a parameter that indicates changes in amplitude and consequently the build-up of strength over time[220].

Table 14 outlines a summary of the ways the viscoelastic profile changes depending on the change of a TEG® parameter.

**Table 14: Interpreting the changes in clotting parameters in hypercoagulable and hypocoagulable state[221]**

| Parameter | Hypercoagulable | | Hypocoagulable | |
|---|---|---|---|---|
| **R** | ↓ | Clot initiation occurs faster | ↑ | Clot initiation occurs slower |
| **K** | ↓ | Clot reaches a set (20 mm) strength faster | ↑ | Clot reaches a set (20 mm) strength slower |
| **A** | ↑ | An increased thrombin burst resulting in more cross-linking of fibrin fibre | ↓ | A decreased thrombin burst resulting in more cross-linking of fibrin fibre |
| **MA** | ↑ | Increased PLT and/or fibrin(ogen) interaction resulting in a denser clot that is more rigid | ↓ | Decreased PLT and/or fibrin(ogen) interaction resulting in a denser clot that is more rigid |
| **G** | ↑ | Increased clot density (thus decreased deformity under pressure) | ↓ | Decreased clot density (thus increased deformity under pressure) |
| **MRTGG** | ↑ | Increased maximum velocity of thrombus formation | ↓ | Decreased maximum velocity of thrombus formation |
| **TMRTGG** | ↓ | Shorter time from initiation to maximum velocity | ↑ | Longer time from initiation to maximum velocity |
| **TTG** | ↑ | Increased total clot strength | ↓ | Decreased total clot strength |

α-angle (A), kinetics (K), maximum amplitude (MA), platelet (PLT), reaction time (R), shear elastic modulus (G), maximum thrombus generation (MRTG), time to maximum thrombus generation (TMRTG), total thrombus generation (TTG).

A study done by Pretorius *et al.*[222] using type 2 diabetes patients (T2D) on thromboelastic parameters, made use of TEG® which suggested that analysis can be used as a point-of-care tool to establish a comprehensive clotting profile for T2D patients[222]. Panigada *et al.* used TEG® to study the effects of COVID-19 on the coagulation system of these COVID-19 patients[223]. The findings revealed that patients who tested positive for COVID-19 had a significant increase in coagulability compared to healthy controls[223]. Supporting the individualised patient-centered approach, de Villiers *et al.*[224] suggested a combination of viscoelastic methodologies, including TEG® and SEM, to improve diagnosis and management of thrombo-embolic ischaemic stroke. The results indicated that a TEG® could be useful in detecting TF pathology[224].

To review the idea of viscoelasticity presented in chapter 2, WB is referred to as a viscoelastic fluid, as it possesses both viscous and elastic qualities, referring to its capacity to flow and its ability to return to its original shape after deformation. These qualities are easily detected by considering the changes in blood that occur during clot formation, from a liquid to a solid (clotting) and back to a liquid (lysis). The changes in

viscoelastic properties that occur during this process can be evaluated and provide useful information on coagulation and potential coagulopathy. Therefore, the standard viscoelastic parameters obtained during TEG® allow for both holistic and systems analysis.

## 6.3 Materials and methods

### 6.3.1 Materials, reagents and equipment

The materials that were used for TEG® are the following:

- TEG® 5000 (Haemoscope Corp., Miles, IL, USA)
- TEG® cups and pins (Vertice)
- 0.2 M Calcium chloride (Vertice)
- 200 µL pipette tips (Lasec)
- 1000 µL pipette tips (Lasec)
- 2 - 20 µL Eppendorf pipette
- 100 - 1000 µL Eppendorf pipette

### 6.3.2 Method

The TEG® assays were performed using a TEG 5000 computer-controlled device according to the manufacturer's guidelines. Citrated blood drawn can be safely analysed within 8 hours, however. the samples from this study were analysed within 4 hours of collection.

Before sample analysis, a calibration test was performed to ensure that the torsion wire was functioning optimally, and the pin attached to the torsion wire was level in relation to the oscillating cup holder. A QC test was done on a monthly basis. After the calibration step, a test cup was loaded and filled with 340 µL of WB, together with 20 µL of 0.2 M calcium chloride ($CaCl_2$) to activate the coagulation process[195,225]. The specialised TEG® computer programme monitored and terminated the coagulation and clot kinetics after MA was reached, since only clot formation was relevant to this study. The results were reported numerically and as a graphical tracing.

## 6.4 Statistical analysis

The statistical analysis for TEG® was done using GraphPad Prism 8 for Windows. The data were first tested for normality with the Shapiro-Wilk normality test. The descriptive statistics were done showing the mean and SD for parametric data, while the min, median and max were used for non-parametric data. The median for all eight parameters in both patient groups was compared to the median of all eight parameters in the control group using a Mann-Whitney analysis. A p-value <0.05 was considered significant.

## 6.5 Results

The three groups were denoted as Control , $T_{pat}$ for the traumatic ICH group, and $NT_{pat}$ for the non-traumatic ICH group.

The results are summarised in table 15, 16 and 17. Note that all patients were first randomly collected and only after diagnosis catergorised into the different groups, thus the patient numbering will not be sequential.

**Table 15: Raw thromboelastography data for the control group**

| Control group | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Participant code** | **R** | **K** | **A** | **MA** | **G** | **MRTG** | **TMRTG** | **TTG** |
| Control 1 | 13.8 | 3.3 | 55.9 | 67.5 | 10.4 | 4.2 | 21.2 | 1061.3 |
| Control 2 | 13.3 | 4.2 | 50.7 | 66.5 | 9.9 | 4.0 | 19.1 | 1011.0 |
| Control 3 | 12.4 | 5.2 | 45.2 | 58.0 | 6.9 | 3.0 | 19.6 | 694.4 |
| Control 4 | 13.8 | 6.2 | 39.3 | 63.5 | 8.7 | 3.0 | 23.6 | 869.5 |
| Control 5 | 9.6 | 5.2 | 49.6 | 49.5 | 4.9 | 2.4 | 15.6 | 497.8 |
| Control 6 | 9.8 | 4.8 | 52.2 | 65.0 | 9.3 | 2.9 | 19.3 | 944.8 |
| Control 7 | 10.4 | 6.8 | 45.0 | 55.0 | 6.1 | 1.8 | 19.5 | 622.2 |
| Control 8 | 11.2 | 4.2 | 51.4 | 68.5 | 10.9 | 4.6 | 17.7 | 1109.9 |
| Control 9 | 7.4 | 2.8 | 60.1 | 64.0 | 8.9 | 4.8 | 11.8 | 898.9 |
| Control 10 | 9.0 | 2.8 | 61.9 | 65.5 | 9.5 | 4.2 | 14.0 | 958.5 |
| Control 11 | 8.3 | 3.1 | 60.2 | 54.5 | 6.0 | 3.6 | 13.3 | 598.7 |
| Control 12 | 9.9 | 4.9 | 50.8 | 55.5 | 6.2 | 2.4 | 15.4 | 626.5 |
| Control 13 | 8.5 | 3.2 | 57.9 | 67.5 | 10.4 | 4.4 | 14.6 | 1048.7 |
| Control 14 | 16.5 | 9.6 | 39.8 | 56.5 | 6.5 | 2.2 | 28.5 | 659.7 |
| Control 15 | 7.2 | N/A | 36.8 | 14.5 | 0.8 | 0.6 | 0.3 | 84.1 |
| Control 16 | 12.7 | 4.4 | 49.6 | 61.5 | 8.0 | 3.1 | 17.6 | 807.5 |
| Control 17 | 8.9 | 3.1 | 59.9 | 57.0 | 6.6 | 5.4 | 12.1 | 664.5 |
| Control 18 | 13.2 | 2.3 | 57.9 | 61.6 | 8.0 | 6.2 | 16.1 | 802.9 |
| Control 19 | 9.8 | 2.6 | 54.0 | 64.6 | 9.1 | 6.8 | 13.8 | 934.3 |
| Control 21 | 11.8 | 3.7 | 43.8 | 59.4 | 7.3 | 4.1 | 16.2 | 734.6 |

| Control 22 | 16.2 | 5.7 | 35.6 | 58.7 | 7.1 | 3.1 | 23.4 | 717.1 |
|---|---|---|---|---|---|---|---|---|
| Control 23 | 12.0 | 2.4 | 58.2 | 62.7 | 8.4 | 5.7 | 15.2 | 844.3 |
| Control 24 | 5.3 | 1.6 | 64.7 | 63.4 | 8.6 | 7.7 | 7.3 | 867.8 |
| Control 25 | 9.9 | 3.2 | 47.3 | 52.2 | 5.5 | 3.9 | 12.8 | 552.9 |
| Control 26 | 13.2 | 4.1 | 41.7 | 61.0 | 7.8 | 3.3 | 19.4 | 786.6 |
| Control 27 | 11.8 | 3.7 | 25.7 | 50.1 | 5.0 | 3.5 | 14.8 | 503.0 |
| Control 28 | 7.2 | 3.3 | 33.3 | 56.8 | 6.6 | 3.6 | 9.1 | 660.2 |
| Control 29 | 12.2 | 1.3 | 75.6 | 72.0 | 12.9 | 13.5 | 15.1 | 1313.1 |
| Control 30 | 7.9 | 6.1 | 40.5 | 63.5 | 8.7 | 4.4 | 17.9 | 884.7 |
| Control 31 | 21.6 | 8.8 | 39.3 | 52.5 | 5.5 | 2.2 | 30.6 | 557.5 |
| Control 32 | 12.5 | 3.2 | 57.0 | 60.0 | 7.5 | 4.6 | 16.6 | 759.5 |
| Control 33 | 14.4 | 5.1 | 43.4 | 63.0 | 8.5 | 2.9 | 18.5 | 859.9 |
| Control 34 | 12.2 | 3.6 | 55.6 | 62.0 | 8.2 | 4.7 | 16.3 | 825.3 |
| Control 35 | 14.1 | 2.8 | 61.5 | 62.0 | 8.2 | 5.5 | 17.5 | 821.4 |
| Control 36 | 16.3 | 4.8 | 47.2 | 57.5 | 6.8 | 3.8 | 23.0 | 683.4 |
| Control 37 | 11.8 | 3.6 | 54.5 | 59.5 | 7.4 | 4.3 | 16.6 | 732.9 |
| Control 38 | 10.9 | 10 | 425 | 42.5 | 3.7 | 2.2 | 18.3 | 381.5 |
| Control 39 | 6.1 | 2.8 | 63.4 | 51.5 | 5.3 | 3.8 | 9.8 | 533.4 |
| Control 40 | 63.0 | 2.6 | 68.1 | 56.0 | 6.4 | 6.0 | 10.7 | 635.1 |
| Control 41 | 21.5 | 7.5 | 40.0 | 45.5 | 4.2 | 1.9 | 29.7 | 422.9 |

Parameter values above the reference ranges are denoted in red, while parameters below the reference ranges are denoted in blue. N/A denotes an error on the TEG® analyser. α-angle (A), kinetics (K), maximum amplitude (MA), reaction time (R), shear elastic modulus (G), maximum thrombus generation (MRTG), time to maximum thrombus generation (TMRTG), total thrombus generation (TTG), thromboelastography (TEG®).

**Table 16: Raw thromboelastography data for the traumatic intracerebral haemorrhage group**

| $T_{pat}$ ICH group | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Patient code** | **R** | **K** | **A** | **MA** | **G** | **MRTG** | **TMRTG** | **TTG** |
| Patient 3 | 11.2 | 2.5 | 55.1 | 67.1 | 10.2 | 5.9 | 17.8 | 1020.4 |
| Patient 9 | 11.2 | 2.3 | 57.6 | 67.0 | 10.1 | 8.0 | 15.1 | 1019.9 |
| Patient 12 | 8.8 | 2.1 | 61.3 | 68.6 | 10.9 | 7.4 | 12.0 | 1097.6 |
| Patient 14 | 9.7 | 2.6 | 59.3 | 73.6 | 13.9 | 11.2 | 14.4 | 1403.1 |
| Patient 16 | 9.8 | 2.3 | 56.7 | 61.6 | 8.0 | 5.9 | 12.5 | 806.3 |
| Patient 19 | 5.9 | 1.5 | 67.6 | 63.9 | 8.9 | 7.8 | 7.2 | 891.6 |
| Patient 22 | 14.2 | 2.8 | 54.1 | 61.4 | 8.0 | 5.2 | 17.8 | 800.0 |
| Patient 24 | 17.3 | 2.8 | 57.8 | 75.0 | 15.0 | 9.7 | 23.2 | 1512.9 |
| Patient 31 | 14.8 | 3.1 | 54.5 | 76.3 | 16.1 | 12.0 | 21.6 | 1628.7 |
| Patient 35 | 16.9 | 4.1 | 43.6 | 66.6 | 10.0 | 4.2 | 23.0 | 1003.1 |
| Patient 37 | 8.4 | 2.8 | 52.2 | 50.5 | 5.1 | 3.7 | 9.7 | 513.9 |
| Patient 43 | 7.8 | 1.6 | 50.1 | 78.3 | 18.0 | 15.0 | 11.0 | 1814.2 |
| Patient 44 | 13.2 | 3.6 | 47.6 | 68.8 | 11.0 | 6.8 | 19.4 | 1109.8 |
| Patient 48 | 3.2 | 1.1 | 73.8 | 73.0 | 13.5 | 12.0 | 4.8 | 1359.5 |
| Patient 49 | 1.4 | 1.4 | 62.2 | 55.5 | 6.2 | 7.6 | 2.3 | 625.8 |
| Patient 51 | 6.8 | 1.6 | 67.6 | 73.0 | 13.5 | 9.1 | 9.4 | 1359.5 |
| Patient 54 | 4.0 | 1.5 | 67.8 | 65.7 | 9.6 | 7.0 | 5.5 | 960.4 |
| Patient 55 | 23.1 | 8.4 | 26.9 | 68.5 | 10.9 | 4.7 | 42.3 | 1092.5 |
| Patient 57 | 22.3 | 11.6 | 16.2 | 56.3 | 6.4 | 1.6 | 38.3 | 647.3 |
| Patient 61 | 9.4 | 2.4 | 62.4 | 81.7 | 22.3 | 17.55 | 15.83 | 2249.6 |

Parameter values above the reference ranges are denoted in red, while parameters below the reference ranges are denoted in blue. N/A denotes an error on the TEG® analyser. α-angle (A), kinetics (K), maximum amplitude (MA), reaction time (R), shear elastic modulus (G), maximum thrombus generation (MRTG), time to maximum thrombus generation (TMRTG), total thrombus generation (TTG).

**Table 17: Raw thromboelastography data for the non-traumatic intracerebral haemorrhage group**

| $NT_{pat}$ ICH group | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Patient code** | **R** | **K** | **Alpha** | **MA** | **G** | **MRTG** | **TMRTG** | **TTG** |
| Patient 1 | 28.8 | 12.8 | 17.0 | 57.6 | 6.8 | 1.3 | 50.7 | 684.0 |
| Patient 2 | 8.0 | 2.8 | 38.2 | 62.9 | 8.5 | 4.9 | 13.9 | 848.0 |
| Patient 4 | 5.7 | 2.2 | 43.6 | 62.7 | 8.4 | 6.2 | 8.3 | 842.5 |
| Patient 5 | 9.2 | 1.8 | 66.8 | 72.6 | 13.2 | 11.3 | 12.4 | 1328.0 |
| Patient 6 | 5.6 | 1.4 | 67.9 | 67.1 | 10.2 | 9.8 | 7.4 | 1027.0 |
| Patient 7 | 9.7 | 1.9 | 64.0 | 69.4 | 11.3 | 8.7 | 13.1 | 1141.5 |
| Patient 8 | 11.0 | 2.3 | 61.2 | 82.8 | 24.0 | 18.3 | 19.2 | 2414.6 |
| Patient 10 | 10.8 | 3.0 | 53.2 | 68.1 | 10.7 | 5.7 | 15.5 | 1072.0 |
| Patient 11 | 10.0 | 2.3 | 57.8 | 71.8 | 12.7 | 8.1 | 13.9 | 1281.8 |
| Patient 18 | 13.5 | 3.1 | 55.4 | 70.8 | 12.1 | 9.2 | 19.3 | 1222.3 |
| Patient 21 | 13.0 | 2.7 | 54.9 | 68.9 | 11.1 | 6.5 | 17.8 | 1114.5 |
| Patient 23 | 10.5 | 2.2 | 61.1 | 70.4 | 11.9 | 8.1 | 14.6 | 1197.0 |
| Patient 25 | 9.3 | 2.2 | 58.2 | 66.5 | 9.9 | 6.6 | 12.3 | 997.7 |
| Patient 27 | 12.9 | 3.3 | 56.2 | 80.2 | 20.3 | 14.4 | 20.9 | 2043.0 |
| Patient 28 | 14.1 | 2.2 | 65.3 | 79.8 | 19.8 | 16.9 | 19.1 | 1989.0 |
| Patient 29 | 11.8 | 3.2 | 52.8 | 63.2 | 8.6 | 6.3 | 16.3 | 863.1 |
| Patient 30 | 14.8 | 4.9 | 41.2 | 60.2 | 7.6 | 4.1 | 22.1 | 764.8 |
| Patient 32 | 11.4 | 1.8 | 67.8 | 78.0 | 17.7 | 17.4 | 15.2 | 1791.2 |
| Patient 34 | 9.8 | 2.8 | 50.4 | 58.7 | 7.1 | 4.4 | 13.1 | 713.6 |
| Patient 36 | 8.6 | 3.7 | 47.6 | 60.2 | 7.6 | 3.9 | 14.6 | 757.9 |
| Patient 39 | 11.0 | 2.2 | 61.0 | 70.9 | 12.2 | 8.6 | 14.7 | 1226.1 |
| Patient 40 | 5.4 | 1.1 | 73.2 | 72.6 | 13.2 | 14.8 | 7.1 | 1332.3 |
| Patient 41 | 9.3 | 1.8 | 65.4 | 66.2 | 9.8 | 7.4 | 11.3 | 985.5 |
| Patient 42 | 7.9 | 1.8 | 65.4 | 71.5 | 12.6 | 9.4 | 11.2 | 1258.0 |
| Patient 45 | 4.2 | 1.3 | 69.7 | 71.8 | 12.7 | 9.3 | 6.5 | 1274.6 |
| Patient 47 | 17.9 | 4.7 | 36.0 | 63.2 | 8.6 | 4.1 | 26.9 | 862.6 |
| Patient 50 | 7.7 | 1.9 | 63.3 | 68.8 | 11.0 | 7.8 | 10.7 | 1108.6 |
| Patient 52 | 14.8 | 1.8 | 68.0 | 87.0 | 33.3 | 33.5 | 19.6 | 3366.9 |
| Patient 53 | 14.7 | 5.2 | 38.3 | 68.1 | 10.7 | 5.2 | 22.1 | 1071.5 |
| Patient 58 | 6.0 | 2.2 | 41.8 | 62.9 | 8.5 | 5.6 | 8.8 | 850.2 |
| Patient 59 | 13.8 | 5.9 | 33.1 | 66.0 | 9.7 | 3.5 | 27.3 | 977.4 |

Parameter values above the reference ranges are denoted in red, while parameters below the reference ranges are denoted in blue. N/A denotes an error on the TEG® analyser. α-angle (A), kinetics (K), maximum amplitude (MA), reaction time (R), shear elastic modulus (G), maximum thrombus generation (MRTG), time to maximum thrombus generation (TMRTG), total thrombus generation (TTG).

The representative TEG® tracing from each of the three groups were superimposed, are displayed in figure 20. The white line illustrates the tracings from the control group,

while the green and purple line illustrate the tracings from the $T_{pat}$ and $NT_{pat}$ ICH patient group, respectively. This is purely used to illustrate the change in the viscoelastic profile shape between the groups.

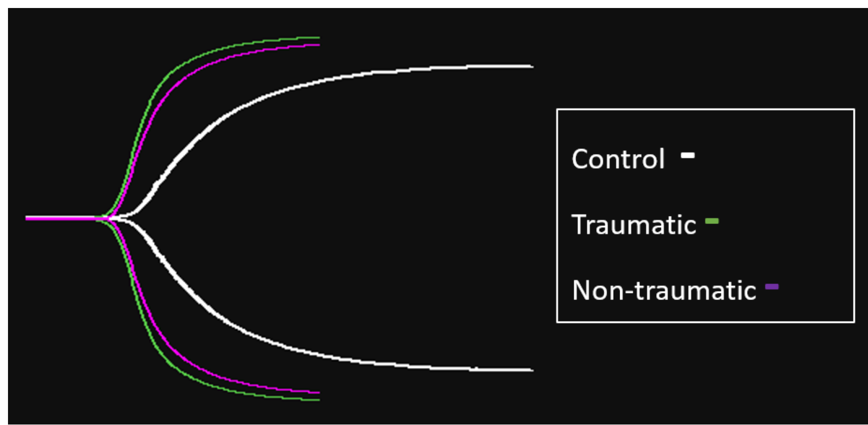


**Figure 20: Thromboelastography superimposed tracings of all three groups**

Five of the eight TEG® parameters in both the $T_{pat}$ ICH and $NT_{pat}$ ICH group were found to be significantly different from controls. In both patient groups, K had a decreased median, whereas MA, G, MRTG and TTG had increased medians compared to control medians. Table 18 provides a summary of the results.

**Table 18: Descriptive statistics for all the parameters for the control, traumatic and non-traumatic group**

| Reaction time (R) | | | |
|---|---|---|---|
| Group | Median (min;max) | Normal Range | P-value |
| Control vs $T_{pat}$ | 11.8 (5.3;63) vs 9.8 (1.4;23.1) | 9-27 min | 0.3357 |
| Control vs $NT_{pat}$ | 11.8 (5.3;63) vs 10.5 (4.2;28.8) | | 0.2763 |
| **Kinetics (K)** | | | |
| Group | Median (min;max) | Normal Range | P-value |
| Control vs $T_{pat}$ | 3.7 (1.3;10) vs 2.5 (1.1;11.6) | 2-9 min | **0.001 |
| Control vs $NT_{pat}$ | 3.7 (1.3;10) vs 2.2 (1.1;12.8) | | ***<0.0001 |

| α-Angle (A) | | | |
|---|---|---|---|
| Group | Median (min;max) | Normal Range | P-value |
| Control vs $T_{pat}$ | 51.1 (25.7;425) vs 57.2 (16.2;73.8) | 22- 58 degrees | 0.1184 |
| Control vs $NT_{pat}$ | 51 (25.7;425) vs 57.8 (17;73.2) | | 0.1173 |
| **Maximum amplitude (MA)** | | | |
| Group | Median (min;max) | Normal Range | P-value |
| Control vs $T_{pat}$ | 59.8 (14.5;72) vs 67.8 (50.5;81.7) | 44-64 mm | ***<0.0001 |
| Control vs $NT_{pat}$ | 59.8 (14.5;72) vs 68.8 (57.6;87) | | ***<0.0001 |
| **Shear Elastic Modulus (G)** | | | |
| Group | Median (min;max) | Normal Range | P-value |
| Control vs $T_{pat}$ | 7.4 (0.0;12.9) vs 10.6 (5.1;22.3) | 3.6–8.5 d/sc | ***<0.0001 |
| Control vs $NT_{pat}$ | 7.4 (0.0;12.9) vs 11 (6.8;33.3) | | ***<0.0001 |
| **Maximum rate of thrombus generation (MRTGG)** | | | |
| Group | Median (min;max) | Normal Range | P-value |
| Control vs $T_{pat}$ | 3.9 (0.6;13.5) vs 7.5 (1.6;17.6) | 0-10 dcs | ***<0.0001 |
| Control vs $NT_{pat}$ | 3.9 (0.6;13.5) vs 7.8 (1.3;33.5) | | ***<0.0001 |
| **Time to maximum rate of thrombus generation (TMRTGG)** | | | |
| Group | Median (min;max) | Normal Range | P-value |
| Control vs $T_{pat}$ | 16.5 (0.3;30.6) vs 14.8 (2.3;42.3) | 5-23 min | 0.3016 |
| Control vs $NT_{pat}$ | 16.5 (0.3;30.6) vs 14.6 (6.5;50.7) | | 0.2329 |
| **Total thrombus generation (TTG)** | | | |

| Group | Median (min;max) | Normal Range | P-value |
|---|---|---|---|
| Control vs $T_{pat}$ | 747.1 (84.1;1313) vs 1056 (513.9;2250) | 251-1014 dcs | ***<0.0001 |
| Control vs $NT_{pat}$ | 747.1 (84.1;1313) vs 1109 (684;3367) | | ***<0.0001 |

Significance indicated as: *- <0.01, ** - <0.001, ***- <0.0001. minimum (min), maximum (max), traumatic group ($T_{pat}$), non-traumatic group ($NT_{pat}$).

## 6.6 Discussion

The TEG® results obtained indicated significant differences between the viscoelastic parameters of clot formation in the control group compared to the $T_{pat}$ ICH group and $NT_{pat}$ ICH group respectively. The discussion of these findings are divided into two sections: first, creating a clot formation "profile" by comparing the results of the $T_{pat}$ ICH group and $NT_{pat}$ ICH group to the control group; and second, identifying hypercoagulability in patient groups.

In the $T_{pat}$ ICH group the results showed that the time to clot initiation does not differ, however once initiated, the clot forms at a faster rate, has increased crosslinking of fibers and reaches a set (20 mm) strength faster than in healthy controls. This trend suggests that the clot initiation is not impacted, however the rate of clot formation is faster, possibly facilitated by the rate of fibrin cross-linkage[216].

The MA indicates maximal mechanical strength of the clot, which is PLT dependent. Maximum amplitude reflects a more rigid, denser and stronger clot. The SEM results are consistent with the result of this parameter. The stronger clot may be due to the increased interactions between the blood cells and the denser fibrin network observed. This was confirmed by the significant TTG results of the $T_{pat}$ ICH group. The G parameter represented a clot with an increased density under pressure. These outcomes are consistent with the morphological findings of the fibrin networks seen with the SEM analyses. Changes in fibrin structure, deposition, and interaction affect clot stiffness, which results in an increase in clot rigidity or stability. A more rigid clot poses the risk of a thromboembolic event due to endogenous anticoagulants and fibrinolytics with a reduced ability to penetrate and lyse the clot, posing a risk for vessels occlusion and ischaemia[226].

Fibrinogen levels are well known to rise with inflammation[198]. Increased fibrinogen levels, as well as other parameters (increased oxidative stress and iron levels), produce aberrant fibrin fiber development during a thrombotic event. Individual fiber widths are substantially smaller than those of typical fibrin fibers, leading RBCs to alter shape and become stuck in the abnormal mesh[227].

Tutwiler *et al.*[228] confirmed that increased fibrinogen levels associated with ischaemic stroke can decrease clot contraction *in vitro*. Greater fibrinogen concentration results in greater fibrin mass and more fibers that are not connected with active PLT-PLT aggregates, which can inhibit clot contraction. In stroke patients, for example, fibrin and RBCs can undergo oxidative and proteolytic alterations, resulting in the production of densely matted deposits of fibrin and RBCs, which have been linked to persistent thrombi. The altered fibrin structure associated with ischaemic stroke results in decreased clot permeability and a slower rate of lysis[228]. The MRTG parameter indicates that there is a trend of increased clot growth with possible hyperreactivity or dysregulation of one or more component of the haemostatic system.

Notebly, the parameter changes in the $NT_{pat}$ ICH group were similar to those seen in the $T_{pat}$ ICH group. This could imply that regardless of the initial cause of vascular injury, the effect on the haemostatic system is consistent. Of the five parameters that were significant, MA, G, MRTG and TTG had increased medians, whereas K had a decreased median. Thus, the results from the TEG® analysis of the viscoelastic profiles of both patient groups compared to the control group indicated a hypercoagulable profile[229]. The clot profiles can be decribed as faster forming clots, which ultimately develop clots of increased strength and rigidity.

In the early stages of trauma, the hypercoagulable condition has been reported[230]. This was assumed to be caused mostly by tissue injury and accompanying inflammation, which has been linked to increased thrombin generation uncontrolled by TF. An acute phase reaction with an increase in PLT activity and fibrinogen levels was seen, and this acute phase reaction, according to Liu C *et al.*, resulted in hypercoagulative TEG® patterns[230]. The increase in CRP levels observed in this study for both patient groups is thus consistent with previous findings linking inflammation as a trigger to the hypercoagulable profile.

## 6.7 Conclusion

The results obtained from the TEG® analyses showed that both the $T_{pat}$ ICH - and $NT_{pat}$ ICH group present with hypercoagulability tendencies based on the K, MA, G, MRTG and TTG parameters. The clot formation profiles in both patient groups were shown to be faster, increasing their potential risk of thrombosis. Clot rigidity was similarly increased in both groups, and was supported by the SEM analyses, potentially making clot degradation more challenging. Platelets activation, as a result of the increased inflammation, led to interaction with RBCs and fibrin to generate stronger, more rigid clots. This was further reinforced by the increased PLT spreading and extensive pseudopodia observed in SEM micrographs. Therefore, from the evidence it can be said that the clots produced in ICH patients are prothrombotic and PLTs as well as fibrin are key role players in this tendency. This should be taken in consideration during patient management. The implications of these findings suggest that microclots can form, affecting perfusion in fine capillary networks, and that further research is required to elucidate this.

# Chapter 7: Conclusion

Integrating the significant results from the quantitative and qualitative analyses, it was observed that ICH resulted in moderate alterations in individual aspects of coagulation, however, the cumulative effect of the individual changes resulted in a considerable change in the viscoelastic profiles during clot formation of both TICH and NTICH patient groups.

On a physiological level the haemostatic system of the patient groups showed increased viscoelasticity with PLTs and RBCs aggregation  and slightly lower Hb concentration. The impact of these changes may result in microvasculature occlusion due to RBC-PLT interaction, which results in abnormal fibrin network development and vessel blockage. Hypoxia, tissue damage, and inflammation may result from such occlusions, and if not resolved promptly or combined with comorbidities, coagulation may aggrevate. However, as observed in the TEG® results, the clots that formed in both ICH groups were rigid, that might require more effort from the natural fibrinolytic enzymes to dissolve the clot. The significant results obtained from all of the analyses and comparisons between the control group and TICH and NTICH respectively are summarised in table 19.

**Table 19: Summary of the significant results from the different analyses employed**

| | Control | TICH | NTICH |
|---|---|---|---|
| **FBC** | | | |
| **RBC** (4.19 – 5.85 $x10^{12}$ cells/L) | x | The mean RBC count were below the normal range (mean = 4.0) | The mean RBC count were below the normal range (mean = 4.6) |
| **WBC** (3.92 – 10.40 $x10^{9}$ cells/L) | x | The mean WBC count were above the normal range (mean = 10.8) | The mean WBC count were above the normal range (mean = 11.3) |
| **MCHC** **(**33.0 – 35.0 g/dL) | x | The median MCHC were within the normal range (median = 33.3) | The median MCHC were below the normal range (median = 32.1) |

| **Hb** (13.4 – 17.5 g/dL) | x | The mean Hb concentration were below the normal range (mean = 11.8) | The mean Hb concentration were below the normal (mean = 13.1) |
| --- | --- | --- | --- |
| **HCT** (0.390 – 0.510 L/L) | x | The mean HCT were below the normal range (mean = 0.36) | The mean HCT were within the normal range (mean = 0.41) |
| **CRP** <10 mg/L | x | The median CRP count were above the normal range (median = 127.0) | The median CRP count were above the normal range (median = 54.0) |
| **LM** | | | |
| **RBC axial rarios** | Control compared individually to each patient group. | No significant deformation of RBCs (p= 0.0656) | No significant deformation of RBCs (p= 0.0521) |
| **SEM** | | | |
| **RBC and PLT ultrastructure** | Regular shaped RBCs with limited PLTs visible. | Some RBC morphology alterations, more PLTs visible, RBC aggregation and RBC-PLT interaction | Some RBC morphology alterations, more PLTs visible, microparticle present, RBC aggregation and RBC-PLT interaction |
| **Fibrin clot sructure** | Organised and structured branching. RBCs maintained their shape within the fibrin network | Disorganised lattices, less structured branching. Fibers more finely webbed | Uneven network uniformity, fiber tangles and fusions |

| | | | |
|---|---|---|---|
| **TEG®** | | | |
| **K** | Control compared individually to each patient group | Fiber cross-linking occurred at a faster rate | Fiber cross-linking occurred at a faster rate |
| **MA** | Control compared individually to each patient group | Increased PLT and/or fibrin interaction resulting in a denser clot that is more rigid | Increased PLT and/or fibrin interaction resulting in a denser clot that is more rigid |
| **G** | Control compared individually to each patient group | Increased clot rigidity | Increased clot rigidity |
| **MRTG** | Control compared individually to each patient group | Increased maximum velocity of thrombus formation | Increased maximum velocity of thrombus formation |
| **TTG** | Control compared individually to each patient group | Increased total clot strength | Increased total clot strength |

x- values not recorded. α-angle (A), C-reactive protein (CRP), full blood count (FBC), haemoglobin (Hb), haematocrit (HCT), kinetics (K), maximum amplitude (MA), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), maximum thrombus generation (MRTG), non-traumatic group ($NT_{pat}$), platelet (PLT), reaction time (R), red blood cell (RBC), shear elastic modulus (G), standard deviation (SD), scanning electron microscopy (SEM), red blood cell (RBC), traumatic group ($T_{pat}$), thromboelastography (TEG®) time to maximum thrombus generation (TMRTG), total thrombus generation (TTG), white blood cell (WBC).

Intracerebral haemorrhage-associated thrombosis is still one of the leading causes of treatment complications in ICH patients, with its severity varying based on a variety of circumstances[231-232]. The purpose of this study was to investigate clotting changes in patients with ICH by studying the viscoelastic and ultrastructural properties of WB.

To reiterate literature, hypercoagulation is a condition in which the haemostatic system is prone to coagulation, resulting in rapid and enhanced blood coagulation. In ICH patients, hypercoagulation has been demonstrated to be elevated, which may be promoted by ICH-associated inflammation[233].

Even though a patient does not present with a thrombotic event during this condition, there may be subclinical changes that have an impact on the normal functioning of the haemostatic system. First, there is a need for additional research employing more in-depth and exact analytical methodologies to understand the specific triggers, and

second, a foundation for developing a preventative treatment approach. Based on these findings, specific strategic guidelines should be developed to identify the trigger in each patient and perhaps be incorporated in patient monitoring. This could also lead to more personalised care for patients.

The findings of this investigation will add to the existing body of information about the impact of ICH on coagulation alterations in two ways. To begin with, the findings demonstrate that TICH and NTICH are associated with a change in blood composition as well as moderate RBC morphology, presumably due to deep vein thrombosis, venous thromboembolisms, and pulmonary embolisms[90]. Platelets seemed to be more prominent in the patient groups, with finely webbed and uneven fibrin networks, and RBC alteration when in contact with fibrin fibers. Notebly, while there were no differences in the quantity of PLTs between the patient and control groups, as noted in chapter three, the PLTs in the patient groups tend to be more adhesive, and hence their presence appeared more visible on the slides. This suggests that overactivation of the coagulation system and inflammation at the injury site could attract additional PLTs to adhere or activate PLTs downstream.

Second, the findings revealed that both patient groups had ultrastructural abnormalities in RBCs and fibrin morphology, as well as hypercoagulability. Eight viscoelastic parameters were investigated, which resulted in faster forming, rigid clots in both patient groups, with microclots as a predicted consequence. Targeted therapeutics, such as PLT or fibrin specific treatment, can be investigated to reduce this risk by preserving blood artery integrity and flow, reducing clot strength and rate of formation, and delaying the rate of thrombin generation. The findings suggested that a risk stratification strategy that specifically focuses on key role players in the haemostatic system might be useful to be included in the management of these patients. This can be used to guide patient-specific treatment

This study had several limitations, such as a difference in age range between control and the patient groups due to the fact that we made use of convenient sampling, a small sample size due to strict exclusion criteria, time limit and willingness of participation; and a limited patient history due to the state of patient consciousness. Further research can overcome these limitations by including a larger sample size in order to provide more indept detail to the data. That being said, TEG® and SEM have

been shown to be effective tools for monitoring clot formation dynamics and morphological changes. These alterations are critical in understanding thrombotic risk, and more clinical validation of these approaches is recommended.

Future research approaches that will provide greater insight into the data include confocal laser scanning, flow cytometry, and atomic force microscopy. Confocal laser scanning can be used to identify PLT activation and microparticles, whilst atomic force microscopy can measure membrane topology and elasticity. Flow cytometry can also be used to quantify PLT activity. Enzyme-linked immunosorbent assays, to measure the concentrations of activating factors such as fibrinogen levels or TF that are involved in coagulation and inflammation. Future studies could also examine the potential mechanisms through which ICH results in a further prothrombotic shift by examining whether PLT reactivity arises after ICH, whether other blood viscosity determinants like plasma viscosity are impacted by ICH, whether ICH affects the concentration of circulating coagulation factors, and whether the morphological changes brought on by ICH can be measured.

# Addendum 1: Approval and renewal certificate, ethics reference nr: 261/2022

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**

6 June 2022

**Approval Certificate**
**New Application**

Dear Miss S Ferreira

**Ethics Reference No.: 261/2022**
**Title: Investigating clotting changes in patients from Steve Biko Academic Hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood**

The **New Application** as supported by documents received between 2022-04-26 and 2022-06-01 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-06-01 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 2023-06-06.
- Please remember to use your protocol number (261/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

**Ethics approval is subject to the following:**

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

We wish you the best with your research.

**Yours sincerely**

**On behalf of the FHS REC, Professor Werdie (CW) Van Staden**
MBChB, MMed(Psych), MD, FCPsych(SA), FTCL, UPLM
***Chairperson:** Faculty of Health Sciences Research Ethics Committee*

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

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

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**

16 February 2023

**Approval Certificate**
**Annual Renewal**

Dear Miss S Ferreira,

**Ethics Reference No.: 261/2022 – Line 2**
**Title: Investigating clotting changes in patients from Steve Biko Academic Hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood**

The **Annual Renewal** as supported by documents received between 2023-01-31 and 2023-02-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-02-15 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 2024-02-16.
- Please remember to use your protocol number (261/2022) 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

# Addendum 2: Information leaflet and informed consent for healthy control group and data collection sheet

## Information leaflet and informed consent form (Healthy Participants)

**Study Title***:* ***Investigating clotting changes in patients from Steve Biko Academic Hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood***

**Sponsor**: Thuthuka Trust

**Principal Investigator**: Miss Shené Ferreira
Department of Physiology, University of Pretoria
0817236709

**Ethical clearance number**: To be obtained.

**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.

**2) The nature and purpose of this study**

The researcher is investigating coagulation changes in whole blood of intracerebral haemorrhage (ICH) patients that might identify with bleeding risk, using ultrastructural and viscoelastic techniques, compared to individuals without ICH risk. This will allow us to understand if ICH patients have hypercoagulation and changed blood clotting properties. To

do this research, we will use specialized microscopes (scanning electron microscope and light microscope) to look at the structure of the red blood cells; as well as equipment that tests blood clotting properties (called a thromboelastograph) to determine the degree to which clotting is changed in the blood. We will compare the results to those of participants who have ICH. Your blood sample will be used as part of a control group of healthy controls, meaning that you do NOT have ICH, but will be compared to participants who do have ICH.

**3) Explanation of procedure**

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

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

A cotinine test will also be performed to confirm the use of tobacco or tobacco related products. From the whole blood drawn, 2 mL of plasma will be stored at -80 degrees celcius and only after sample recruitment is done the cotinine ELISA test will be conducted and the smokers will be excluded from the sample population

The samples will be used, within four hours of collection, to do scanning electron microscopy, light microscopy and thromboelastography.

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

**4) Future genetic 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 phlebotomist or trained nurse.

**6) Possible benefits of this study.**

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

**7) Compensation**

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

**8)** I understand that if I do not want to participate in this study, I will still receive standard treatment for my illness.

**9) Your rights as a research participant.**

Your participation in this trial is entirely voluntary and you may refuse to participate or stop at any time without stating any reason.

**10) Ethics approval**.

This Protocol (261/2022) was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, and telephone numbers 012 356 3084 / 012 356 3085. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

**11) Information**

If you have any questions concerning this study, please contact Miss Shené Ferreira tel: 081 723 6709

**12) 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 patients remain unidentifiable. The hard copies of all your records will be kept in a locked facility at the Department of Physiology at The University of Pretoria**.**

**13) Consent to participate in this study**

- **I have received, read, or have had read to me in a language I understand and understood the above-written information about the study, before signing consent.**
- **I have had adequate time to ask questions and I have no objections to participating in this study.**
- **I am aware that the information obtained in the study, including personal details, will be anonymously processed and presented in the reporting of results.**
- **I understand that I will not be penalized in any way should I wish to discontinue the study and that withdrawal will not affect my further treatments.**
- **I am participating willingly.**

…………………………………………………………………………. ……………..

Participant's name and signature Date

…………………………………………………………………………. ……………..

Investigators name and signature. Date

…………………………………………………………………………. ……………..

Witness name/ Dr name and signature. Date

**Participant code……………………**

**Verbal patient informed consent**

(Applicable when patients cannot read or write)

I, the undersigned, ………………………………………..…have read and have explained fully to the patient, named …………………………………. and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his illness. The patient indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her treatment.

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

……………………………………………………………………………… ……………..

Participant's name and signature Date

……………………………………………………………………………… ……………..

Investigators name and signature. Date

……………………………………………………………………………… ……………..

Witness name/ Dr name and signature. Date

**Data Capture Sheet – Healthy control group**

**Investigating clotting changes in patients from Steve Biko Academic Hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood**

**Shené Ferreira 081 723 6709**

**Healthy control Group**

<table>
<tr><td colspan="2">Date Captured:<br>DD / MM / YYYY</td><td colspan="2">Allocated Study ID (e.g. C1):<br>*(Will be allocated by Investigator)*</td></tr>
<tr><td colspan="4">**Personal Information**</td></tr>
<tr><td colspan="4">Age:</td></tr>
<tr><td colspan="4">**Medical Information & History**</td></tr>
<tr><td colspan="2">Do you smoke tobacco or any related product?<br>(If yes, for whole long?)</td><td colspan="2">☐ Yes *Length of time:*<br>☐ No</td></tr>
<tr><td colspan="2">Would you say that you have consumed 5/more drinks on the same occasion in the past 30 days?</td><td colspan="2">☐ Yes<br>☐ No</td></tr>
<tr><td colspan="2">Do you make use of contraceptives?</td><td colspan="2">☐ Yes<br>☐ No</td></tr>
<tr><td colspan="4">Do you have or have you ever had any of the following conditions?</td></tr>
<tr><td colspan="2">☐ Diabetes<br>☐ High Blood Pressure<br>☐ Heart Problems<br>☐ Heart attacks<br>☐ Inflammatory conditions</td><td colspan="2">☐ Stroke<br>☐ Arthritis<br>☐ Other *Specify:* ___________<br>☐ Allergies *Specify:* ___________</td></tr>
<tr><td>*HIV Status:*</td><td colspan="3">☐Positive ☐Negative ☐Unknown</td></tr>
<tr><td colspan="4">Medication history:</td></tr>
<tr><td colspan="4">☐Yes ☐No Are you taking any chronic medication? *Specify:* ______________________<br><br>*Have you taken any of the following within the last 2 weeks?*<br><br>☐Yes ☐No Vitamin-supplements</td></tr>
</table>

☐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*** | ***MRTTG*** | ***TMRTTG*** | ***TTG*** |
| | | | | | | | |
| **FBC parameters** | | | | | | | |
| **RBC count** | **WBC count** | **PLT count** | **MCV** | **MCH** | **MCHC** | **Haemoglobin** | **Haematocrit** |
| | | | | | | | |
| **Scanning Electron Microscopy** | | | | | | | |
| **Features of Interest** | | | | | | **Control Image** | |
| | | | | | | | |
| | | | | | | | |
| **Light Microscopy** | | | | | | | |
| **Average Axial Ratio** | | | | | | | |

# Addendum 3: Informed leaflet and informed consent for ICH patient group and data collection sheet

**Information leaflet and informed consent form (Intracerebral haemorrhage patients- delayed consent)**

**Study Title**: Investigating clotting changes in patients from an academic hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood

**Sponsor**: Thuthuka Trust

**Principal Investigator**: Shené Ferreira
Department of Physiology, University of Pretoria
081 723 6709

**Ethical clearance number**: 261/2022.

**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 coagulation changes in whole blood of intracerebral haemorrhage (ICH) patients that might identify with bleeding risk, using ultrastructural and viscoelastic techniques, compared to individuals without ICH risk.. This will allow us to understand if intracerebral haemorrhage patients in South Africa have changed blood clotting or bleeding tendencies For this research, we will use specialized microscopes (scanning electron microscope and light microscope) to look at the structure of the red blood cells, PLTs and fibrin fibres; as well as equipment that tests blood clotting properties (called a thromboelastography) to determine the degree of change in clotting in the blood. We will compare the results to those of participants who do not have ICH.

**3) Explanation of procedure**

This study involves answering some questions with regards to your health and any illnesses, examination of you, and only after consent taking some blood samples.

In the case of the patient being in ICU. Blood will be drawn from the central venous line by a medical doctor. 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 doctor from the neurosurgery department into a citrate tube, each containing 4mL of blood or the equivalent of one teaspoon.

The principle investigator will do a waiver consent for unconscious patients and delayed consent 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, within four hours of collection, to do scanning electron microscopy, light microscopy, and thromboelastography.

A cotinine test will also be performed to confirm the use of tobacco or tobacco related products. From the whole blood drawn, 2 mL of plasma will be stored at -80 degrees Celcius and only after sample recruitment is done the cotinine ELISA test will be conducted and the smokers 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) Future genetic 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, Shene Ferreira. 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 phlebotomist or trained nurse.

**6) Possible benefits of this study.**

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

**7) Compensation**

You will not be paid to take part in the study. There are no costs to you to participate in this study. I understand that if I do not want to participate in this study, I will still receive standard treatment for my illness.

**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 (261/2022) 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 has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

**10) Information**

If you have any questions concerning this study, please contact Miss Shené Ferreira tel: 081 723 6709

**11) Confidentiality**

All information obtained during the course of this study will be regarded as confidential. Each participant that is taking part will be provided with an alphanumeric coded number e.g. A001. 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 patients 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 for me 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 penalized in any way should I wish to discontinue with the study and that my withdrawal will not affect further treatment.
- 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 enrollment 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 patient informed consent**

(Applicable when patients cannot read or write)

I, the undersigned, …………………………………………...…have read and have explained fully to the patient, named …………………………………..., the information consent document, which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his illness. The patient indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his treatment.

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 patient has agreed to participate in this study.

……………………………………………………………………………… ……………..

Participant name and signature Date

……………………………………………………………………………… ……………..

Investigator name and signature. Date

……………………………………………………………………………… ……………..

Doctor/Witness name and signature. Date

**Data Capture Sheet – Patient Group**

**Investigating clotting changes in patients from Steve Biko Academic Hospital with intracerebral haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood**

**Shené Ferreira 081 723 6709**

**Patient group**

| Date Captured:<br>DD / MM / YYYY | Allocated Study ID (e.g. C1):<br>*(Will be allocated by Investigator)* |
|---|---|
| **Personal Information** | |
| Age: | Hospital number: |
| **Medical Information & History** | |
| Do you smoke tobacco or any related product?<br>(If yes, for whole long?) | ☐ Yes *Length of time:*<br>☐ No |
| Would you say that you have consumed 5/more drinks on the same occasion in the past 30 days? | ☐ Yes<br>☐ No |
| Do you make use of contraceptives? | ☐ Yes<br>☐ No |
| Do you have or have you ever had any of the following conditions? | |
| ☐ Diabetes<br>☐ High Blood Pressure<br>☐ Heart Problems<br>☐ Heart attacks<br>☐ Inflammatory conditions | ☐ Stroke<br>☐ Arthritis<br>☐ Other *Specify:* ___________<br>☐ Allergies *Specify:* ___________ |
| *HIV Status:* | ☐Positive ☐Negative ☐Unknown |
| Medication history: | |
| ☐Yes<br>☐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 | |

| | |
|---|---|
| ☐Yes ☐No<br>☐Yes ☐No | Corticosteroids<br>Anti-inflammatories (drugs or substances that reduces inflammation (redness, swelling and pain) in the body.<br>Anti-coagulants<br>Other: ___________________________ |

**Experimental Test Results**

**Thromboelastography**

| *R* | *K* | *Angle* | *MA* | *G* | *MRTTG* | *TMRTTG* | *TTG* |
|---|---|---|---|---|---|---|---|
| | | | | | | | |

**FBC parameters**

| RBC count | WBC count | PLT count | MCV | MCH | MCHC | Haemoglobin | Haematocrit |
|---|---|---|---|---|---|---|---|
| | | | | | | | |

**Scanning Electron Microscopy**

| Features of Interest | Reference Image |
|---|---|
| | |
| | |

**Light Microscopy**

| Average Axial Ratio | |
|---|---|

# Addendum 4: 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: **S Ferreira**, Student No: **18008306** studying at the University of Pretoria discussed the project with the title; **Investigating hypercoagulability in patients with intracranial haemorrhage by studying the viscoelastic and ultrastructural properties of whole blood** with me.

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

Yours sincerely

______________________

Prof Pieter WA Meyer

Ass. Professor and HoD

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

Room 5-40, Level 5, Pathology Building
University of Pretoria, Private Bag X323
Pretoria 0001, South Africa
Tel +27 (0)12 319-2977
Fax +27 (0) 12 323 0732
Email name.pieter.meyer@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Departement Immunologie
Lefapha la Disaense tša Maphelo
Kgoro ya Immunolotši

## Addendum 5 : Turnit in report

Shene Ferreira 18008306 Thesis.docx

ORIGINALITY REPORT

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