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The effect of the *ex vivo* addition of vitamin D on the prothrombotic and the fibrinolytic potential in prostate cancer patients' blood

Submitted in fulfilment of the requirements for the degree

Master of Science (Human Physiology)

by

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Abstract

Cancer and its consequences are amongst the major causes of death worldwide. Prostate cancer (PCa) is the most frequent non-cutaneous malignancy in men globally, with South Africa having the fourth highest prostate cancer mortality rate in the world. Patients diagnosed with PCa have been found to have a hypercoagulable state as a result of the elevated systemic inflammation that is a hallmark of cancer.

Vitamin D supplementation has been shown in recent studies to improve the survival rates of individuals with PCa due to its anti-tumorigenesis properties. Given the link between chronic inflammation, hypercoagulation and PCa survival rates, improving the thrombotic status of PCa patients could be beneficial in the management of these patients.

This study investigated the hypercoagulable state of both metastatic and non-metastatic PCa patients, in comparison to a reference group, before and after the *ex vivo* supplementation with vitamin D. The morphological and viscoelastic properties of whole blood and platelet poor plasma were investigated using light microscopy, scanning electron microscopy and thromboelastography®.

This study indicated that vitamin D supplementation might potentially aid the pathology caused by abnormal RBC shape, albeit caution should be used in metastatic PCa populations since supplementation was seen to result in a tendency to create stronger, more rigid clots at a faster pace. Although vitamin D supplementation shows potential as a more cost-effective therapy regime for the elevated thrombotic risk commonly seen in individuals with PCa, additional research is needed to determine the impact of vitamin D supplementation on hypercoagulability *in vivo*.

Keywords: Prostate cancer, hypercoagulable, vitamin D supplementation, morphology, viscoelasticity, platelet poor plasma, whole blood, ex vivo

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Declaration of Originality and Authorship

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DECLARATION

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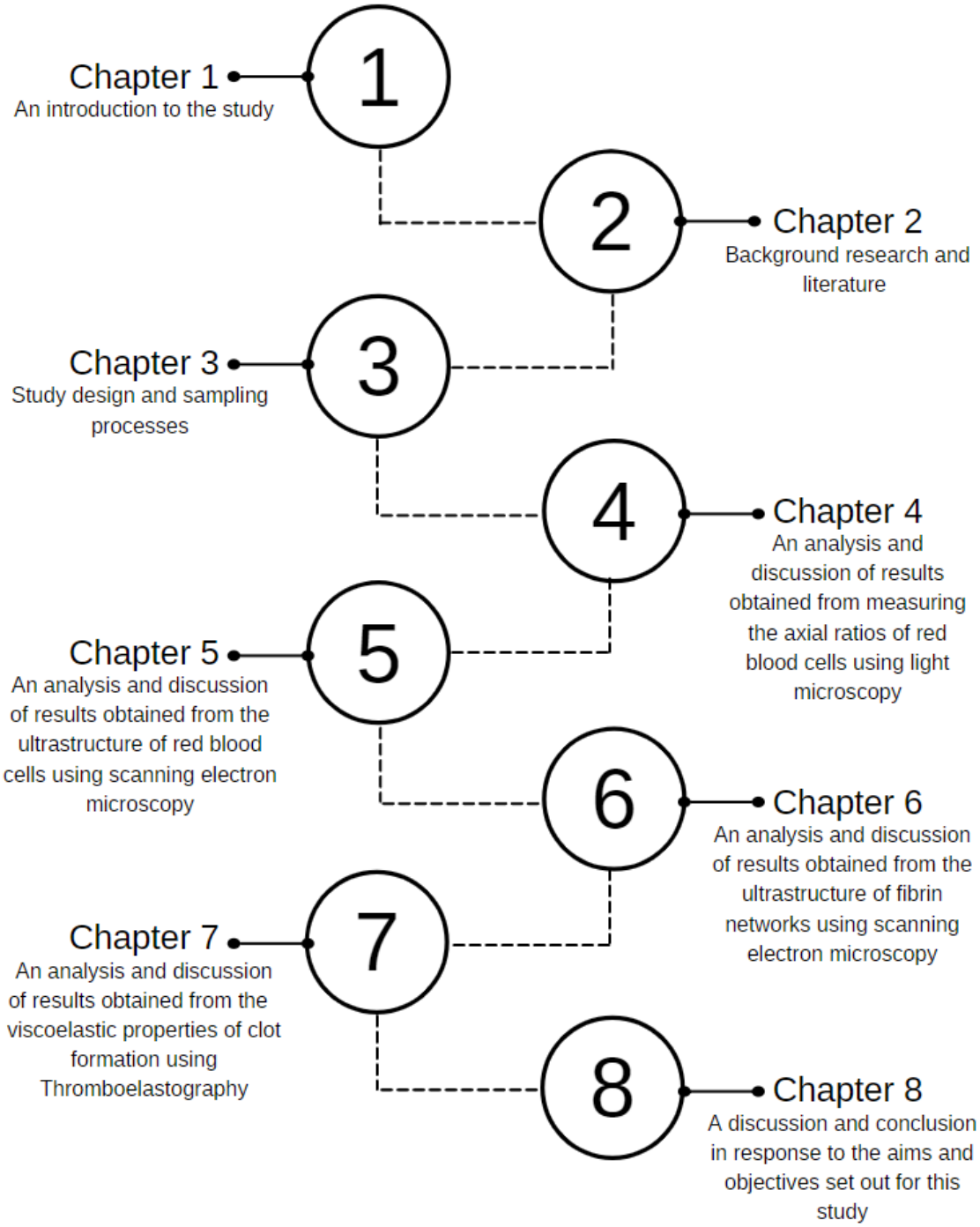
Abbreviations

DBP	-	Vitamin D Binding Protein
HRP	-	Horseradish peroxidase
HMDS	-	Hexamethyldisilazane
K	-	Kinetics
LM	-	Light Microscopy
MA	-	Maximal Amplitude
MRTG	-	Maximum Rate of Thrombin Generation
PARs	-	Protease Activated Receptors
PBS	-	Phosphate Buffered Saline
PCa	-	Prostate Cancer
PPP	-	Platelet poor plasma
PRP	-	Platelet rich plasma
PSA	-	Prostate specific antigen
R	-	Reaction Time
SBAH	-	Steve Biko Academic Hospital
SEM	-	Scanning electron microscopy
TAFI	-	Thrombin activatable fibrinolysis inhibitor
TEE	-	Thromboembolic event
TEG®	-	Thromboelastography
TF	-	Tissue Factor
TM	-	Thrombomodulin
TMRTG	-	Time to Maximum Rate of Thrombus Generation

tPA	-	Tissue plasminogen activator
TTG	-	Total Thrombus Generation
WB	-	Whole blood
VDR	-	Vitamin D receptor
VWF	-	Von Willebrand Factor

Site Map

This dissertation is structured as follows:



Chapter 1: Introduction

Prostate cancer (PCa) is the second most prevalent cancer in males worldwide and has become the fifth leading cause of death due to its progression to metastasis and the rise of treatment resistant illness.[1-2] The leading risk factors for PCa include increasing age and genetics, and management of the disease is dependent on whether the cancer is confined to the prostate gland (localized), invading nearby structures (locally advanced) or metastatic.[1]

Terminally ill patients, particularly those with cancer, are often at risk of developing multiple complications. Coagulation is a typical haemostatic response mechanism that reduces blood loss by generating clots that slow and eventually block blood flow at sites of injury. In inflammatory conditions, such as PCa, a hypercoagulable state occurs in the body, increasing the likelihood for clots to develop in the circulatory system.[3] Tumour cells can activate the coagulation system, increasing the risk of embolic or thromboembolic disorders.[4] Thromboembolic events (TEE) are not only a leading cause of death in cancer patients, but also have an impact on quality of life.[5]

Vitamin D deficiency has been linked to an increased risk of cardiovascular events, and according to findings, vitamin D supplementation appears to decrease a prothrombotic profile.[6] Given the link between hypercoagulation and PCa survival rates[7], improving the thrombotic status of PCa patients could be beneficial in the management of these patients.

This aim of this study was to investigate the morphological and viscoelastic profiles in PCa patients before and after the *ex vivo* addition of vitamin D. This was a lab-based, experimental study which looked at the changes observed in blood supplemented with vitamin D. In order to assess these changes, the red blood cell deformability, ultrastructure of clotting components and viscoelastic properties of clot formation were investigated.

The red blood cell deformability before and after treatment was assessed through whole blood (WB) smears visualised by light microscopy (LM), the ultrastructure of red blood cells (RBCs) and fibrin networks were investigated using scanning electron microscopy (SEM) and viscoelastic properties of clot formation using thromboelastography® (TEG®). The results from the above-mentioned techniques

were used to evaluate the changes in hypercoagulability before and after vitamin D supplementation in comparison to results obtained from a reference population.

All techniques were performed on blood obtained from PCa patients at the Steve Biko Academic Hospital (SBAH), which were then treated with vitamin D *ex vivo.* The experimental group (n= 60) was divided into two groups, namely metastatic (n= 27) and non-metastatic (n= 33), which were then compared to a reference group (n= 11). The results show that following vitamin D supplementation, the incidence of abnormally shaped RBCs was reduced and fibrin networks tended to form with fewer instances of dense, matted deposits. The viscoelastic properties of blood from the metastatic group after treatment showed that vitamin D supplementation resulted in the tendency to form stiffer, more rigid clots that form more quickly. The results further showed that vitamin D supplementation may potentially aid in the pathology caused by abnormal RBC morphology, albeit caution should be exercised in metastatic PCa patients.

Chapter 2: Background and literature review

2.1 Chapter objective

The literature relevant to the current study is reviewed in this chapter.

2.2 Introduction to prostate cancer

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy in men worldwide,[8] with South Africa having the fourth highest PCa mortality rate globally.[9] South African men have a 1:19 chance of being diagnosed in their lifetime, according to the National Cancer Registry, and research indicates that the risk for aggressive PCa is higher in African males.[10] This increased risk gap is caused by socioeconomic, educational, cultural and genetic variables, as well as differences in care delivery and treatment selection.[11] Currently, PCa treatment has resulted in a significant economic burden and thus more effective treatment methods are needed to improve the survival rate in PCa patients.[8]

The prostate is a small gland that plays an important role in the male reproductive system and is located inferiorly to the bladder. Prostate cancer often develops very slowly and may not cause significant damage; however, some forms are more aggressive and, without treatment, may spread rapidly. Patients may be asymptotic in the early stages or may present with symptoms of urinary voiding and/or storage related to PCa, involving decreased urinary stream, pushing, frequency, urgency, and vesical tenesmus.[12] In the advanced stages, PCa may cause bone pain, renal failure, haematuria, pathological bone fractures, physical exhaustion and weight loss.[12]

Risk factors for PCa include increasing age, African ancestry, altered androgen metabolism, family history, obesity, and some dietary factors, with ageing being the leading risk factor for developing PCa.[13] Prostate Specific Antigen (PSA) is a common first line test for PCa diagnosis.[12,14]

The epithelial cells of the prostate gland express PSA, and the quantification of serum levels is used as a diagnostic tool for the detection of PCa.[15] Serum PSA levels may be higher in males with PCa as PSA synthesis is increased within the cancerous tissue, moreover, tissue barriers between the prostate gland lumen and capillaries are disturbed, allowing additional PSA to enter the blood stream.[14] However, many other factors may also lead to an incline in PSA that are unrelated to PCa, such as trauma,

inflammation, and infection, and thus at least two measurements taken three weeks apart are required to confirm a PCa diagnosis.[12]

The Gleason scale (see **Table 2.1**) of histological differentiation is used to classify PCa.[12] This scale is imperative for determining the stage and prognosis of patients with PCa. The scale is used additively, with the first number representing the primary histologic grade and the second number representing the secondary histological grade.[12]

Table 2.1: Histological definition of Gleason scores and corresponding grade groups.[16]

Gleason Score	Grade Group	Definition
2-6	1	Only individual, well-formed glands
7 (3+4)	2	Predominantly well-formed glands with lessor component of poorly formed glands
7 (4+3)	3	Predominantly poorly formed glands with lessor component of well-formed glands
8 (4+4 or 3+5 or 5+3)	4	Only poorly formed glands *or* predominantly well-formed glands and lessor component lacking glands *or* predominantly lacking glands with lessor component of well-formed glands
9-10	5	Lack of gland formation (or with necrosis) with or without poorly formed glands

For both prognostic and therapeutic purposes, the stage of the disease is critical. Generally, the Tumour-Node-Metastasis (TNM) scale is used to classify clinical and pathological stages in PCa.[12] The scale considers PSA levels and the Gleason score to classify PCa patients as low risk, intermediate risk and high risk.[12] The importance of this classification stems from the fact that each group has different ten year survival rates: 83% for low-risk, 46% for intermediate risk and 29% for high risk.[12] Roughly 40% of patients with non-localized disease develop metastases to distant locations, significantly decreasing survival rates.[17] In 80% of individuals with advanced disease, PCa has a preference for bone, which is often the predominant location for metastasis.[17]

In PCa, the bone is an organ of utmost importance, with bone metastases being a common cause of patient mortality.[17] The PCa cells disrupt the homeostatic balance between bone-forming osteoblasts and bone-resorbing osteoclasts once they have colonized the bone, stimulating osteolysis and osteoblasts to produce weak, woven bone.[17]

Prostate cancer is a disease that progresses gradually, and patients can die from other causes including diabetes, cardiovascular disorders, and stroke, depending on the age at which they are diagnosed. An individualized evaluation is therefore needed to decide which therapeutic modalities are the most suitable in each case.[12] Treatment options range from observation to more radical treatments, such as radical prostatectomy and radiation.[12] The body of evidence demonstrating that cancer-related inflammation is a key factor in patient prognosis is growing and becoming more consistant,[18] and inflammatory mediators have been identified as active participants in the development, surveillance and progression of PCa.[19]

2.3 Inflammation in cancer

The connection between cancer and inflammation is one of the most important areas for both clinical and translational research.[20] Inflammation is a system of reactions involving cytokines, neutrophils, adhesion molecules, complements and immunoglobulin G, that together orchestrate an intricate, localized response to non-self substances, such as bacteria, and in response to tissue injury in the body.[21] In response to an injury, immune system components are activated to protect the site of injury and repair damage, resulting in inflammation.

There is a large body of evidence indicating the link between immunity, inflammation, and cancer, with these interactions playing an important role in patient prognosis.[22] A small fraction of cancers develop as a result of germline mutations, while the vast majority are caused by the interactions between somatic mutations and environmental factors.[23] Chronic inflammation is thought to play a role in 15-20% of cancers, as illustrated by the links between stomach cancer and Helicobacter pylori infection, liver cancer caused by hepatitis B and C infection, colon cancer caused by inflammatory bowel disease and bladder cancer caused by schistosomiasis infection.[24]

Previous research has suggested the theory that inflammatory signal molecules have a direct pro-oncogenic effect, along with oxidative stress and prolonged growth signalling, as a possible mechanism underlying the link between chronic inflammation and cancer.[25] The severity of PCa has been shown to be associated with elevated pre-diagnostic inflammation markers. In addition, a pre-diagnostic cytokine profile indicative of a type 1 T-helper cell shifted immune response has been seen to be inversely associated with PCa risk.[25]

Despite numerous studies, the role of inflammation in PCa has not yet been fully clarified. Nevertheless, inflammation remains an integral component of prostatic diseases and can help to shift the balance towards tumour growth.[20] The presence of inflammatory infiltrate in benign as well as malignant lesions can be observed. The presence of chronic inflammation in benign prostate tissues classifies such lesions as prostatitis.[20] Chronic inflammation is believed to play a role in the development of various different types of cancer. Chronic inflammation often leads to a state of hypercoagulation.

2.4 Hypercoagulation and cancer

Hypercoagulation is a state marked by the increased tendency to form blood clots within a blood vessel. Cancer favours the activation of blood coagulation, with the appearance of a hypercoagulable state being profound in cancer patients.[26]

Hypercoagulability of the blood is a typical systemic change in cancer patients that predisposes them to thrombosis, which has a negative impact on their quality of life and survival.[27] Cancer cells are directly linked in the pathophysiology of thrombosis through a number of pathways, including thrombin triggering and generation, as well as a procoagulant-anticoagulant force imbalance.[27] Tissue factor (TF), a transmembrane receptor protein, which not only initiates coagulation but also promotes tumour development, angiogenesis and metastasis has received significant attention. Cancer patients are found to have increased levels of circulating procoagulant microparticles, which are correlated with an increased thrombosis risk.[27]

2.4.1 Hemostasis and the coagulation cascade.

During normal clotting, at vessel injury sites the body responds with physiological processes to maintain homeostasis and bleeding is reduced by the formation of a hemostatic plug. The aim of this coordinated process is to form a stable blood clot utilizing blood components such as platelets and clotting factors.[28] Hemostasis is achieved by a careful balance of pro- and anticoagulant substances that inhibit bleeding while also preventing pathological thrombi formation.[28]

Platelet plugs form rapidly after vascular injury as a result of interactions between platelets, sub-endothelium and adhesive proteins. Platelet adhesion, platelet activation and platelet aggregation/plug formation are the three key processes in primary hemostasis.[28] Von Willebrand factor (vWF) binds to specific sites on exposed collagen following vessel injury and mediates the adhesion of platelets to the exposed endothelial matrix. Platelet activation is then triggered, which then recruits and activates additional platelets to the injured site.[28]

Secondary hemostasis occurs simultaneously with primary hemostasis, the initiation phase is triggered by the extrinsic pathway and is then further amplified by the intrinsic pathway.[26] The key components involved in the extrinsic pathway are the transmembrane receptor TF and plasma FVII/FVIIa factors, and the intrinsic pathway are plasma FXI, FIX and FVIII factors.[26]

The coagulation cascade consists of a series of reactions in which zymogens are cleaved into their active forms, and is initiated when TF is exposed to blood components following vascular injury.[29] The TF then binds to its ligand FVII, resulting in FVII activation (FVIIa). This binding activates the downstream conversion of FX to active FX (FXa), which may also happen through the alternative reaction in which the FVII/FVII-TF complex converts FIX to FIXa, further activating FX in conjunction with its cofactor FVIIIa. Prothrombin is then converted to thrombin by the combination of FXa, FVa, phospholipid and calcium, further converting soluble plasma fibrinogen to insoluble fibrin, resulting in clot or thrombus generation. Thrombin activates FXIII to FXIIIa which covalently cross-links and stabilises the fibrin clot.[30] A summary of the coagulation pathway is illustrated in **figure 2.1**.

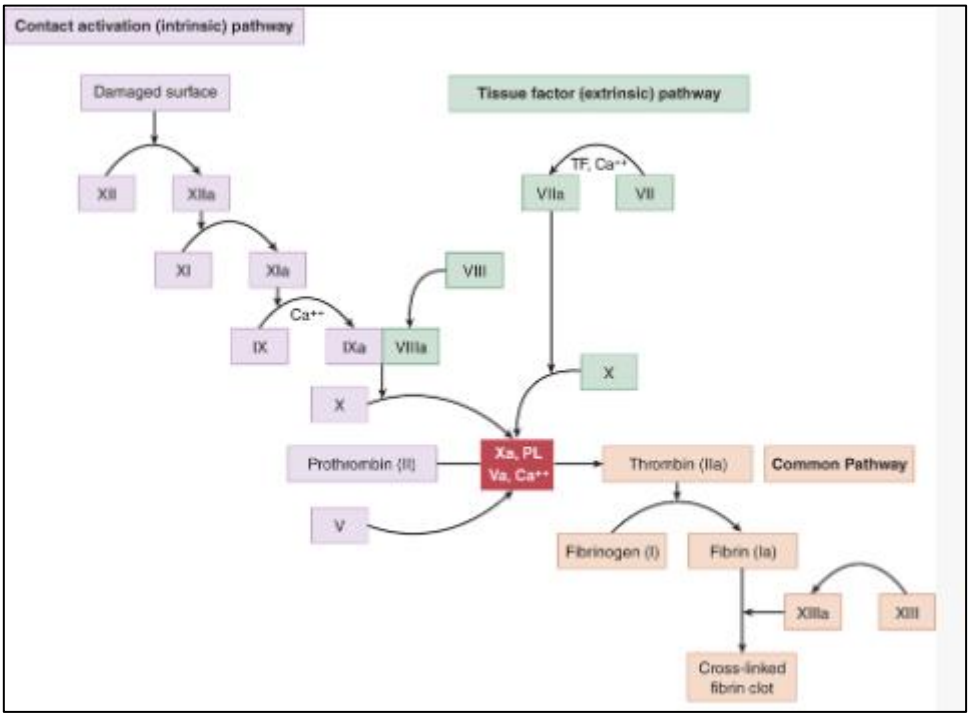


Figure 2.1: Summary of the coagulation pathway.[19] The coagulation cascade consists of a cascade of events that lead to haemostasis. The intricate process allows spontaneous bleeding to be rapidly repaired and prevented. Two pathways, intrinsic and extrinsic, originate separately but converge at a specific point, leading to fibrin activation. In the end, the aim is to stabilize the platelet plug with fibrin mesh.

2.4.1.1 Cell-based model of hemostasis

The cell-based model of hemostasis, described by Hoffman and Monroe (2003), reflects the importance of cells and cell surfaces during hemostasis and is more representative of *in vivo* coagulation than the traditional cascade model. The model describes three main overlapping phases: initiation, amplification and propagation.

The initiation phase occurs on the TF-bearing cell when the cell is exposed to flowing blood and plasma-based VIIa following injury.[28] A small amount of FIXa, Xa and thrombin is then produced by the TF-VIIa complex. During the amplification phase, the small amount of thrombin activates platelets, releases vWF and leads to the formation of FVa, FVIIa and FXIa.[28] The migration of a large number of platelets to the site of injury, as well as the creation of the tenase complex, which results in FXa generation on the platelet surface, characterize the propagation phase.[28] The FXa generated on platelets binds to Va rapidly and converts prothrombin to thrombin. Thrombin is then produced in large quantities, converting fibrinogen to fibrin. The activation of thrombin-activatable fibrinolysis inhibitor (TAFI) and FXIII stabilizes the clot.[28] The cell-based model of hemostasis is summarised in **figure 2.2**.

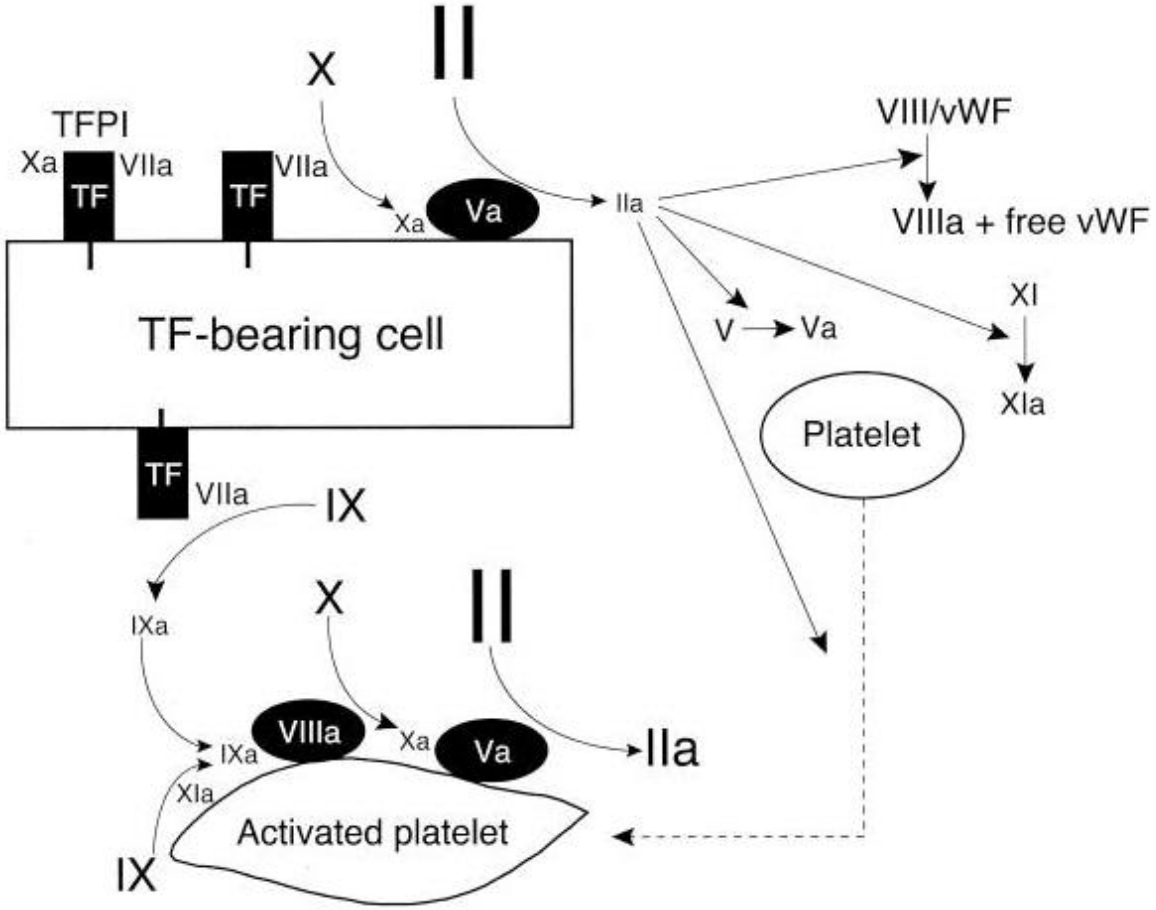


Figure 2.2: Summary of the cell-based model of coagulation.[26]

A procoagulant condition occurs in diseases where inflammation is unregulated, such as in sepsis and cancer.[29] This involves high platelet counts and reactivity, decreased regulation of natural anticoagulant pathways, increased coagulation, and thrombotic

disease risk, and impaired fibrinolysis.[29] Haemostasis dysregulation is a common feature in all cancers, with most cancer patients experiencing observable haemostatic changes even though significant clinical symptoms, such as thromboembolisms, do not arise.

Inflammation being a hallmark of cancer that causes an increase in inflammatory system-induced coagulation molecules, primarily TF, thrombin, and protease-activated receptors (PARs), has an advantageous effect on tumour growth and progression.[29] The implication of increased TF levels associated with inflammation has been shown, resulting in the activation of the extrinsic coagulation pathway alongside the suppression of anticoagulant mechanisms.[26] The expression of TF on cancer cells has been found to be a major contributor to the hypercoagulable status of cancer patients.[31]

Recent studies have demonstrated the potential benefits of vitamin D supplementation as an anticoagulant.[32] Vitamin D binds to a nuclear vitamin D receptor (VDR), and may potentially exert anticoagulant effects by upregulating the expression of the anticoagulant glycoprotein thrombomodulin (TM), as well as downregulating the expression of TF.[32]

It has been found that PCa patients tend to be vitamin D deficient.[33] In a study by Nyame et al., (2016) it was found that men with PCa demonstrated lower median serum levels of vitamin D compared to healthy counterparts.[33] The association between vitamin D and PCa has been investigated worldwide, although it is yet to be confirmed within a South African context.

2.5 Vitamin D

2.5.1 Synthesis and mechanism of action

Vitamin D refers to a group of fat-soluble prohormones that exist in two main isoforms, namely vitamin D2 and 1α,25-dihydroxyvitamin D3 (1,25(OH)$_2$D3), with the latter being the active form.[34] Bioavailability may be derived through the diet, supplement use as well as the action of ultraviolet B radiation on 7-dehydrocholesterol, a precursor of cholesterol found in the skin, to yield the vitamin D hormone precursor cholecalciferol (vitamin D3). Vitamin D3 is then further metabolized by the liver to yield 25(OH)D3, and then again in the proximal tubules of the kidneys to yield the most active hormone form of these compounds,1,25(OH)2D3 (Calcitriol) (**Figure 2.3**: Summary of biological vitamin D synthesis.[23] The production of vitamin D requires the action of sunlight on the skin and then hydroxylation, first in the liver and then in the kidneys.). Vitamin D hormones are transported in the blood bound to vitamin D-binding protein (DBP or Gc-MAF)[35] and then act on target tissues via the nuclear VDR.[34]

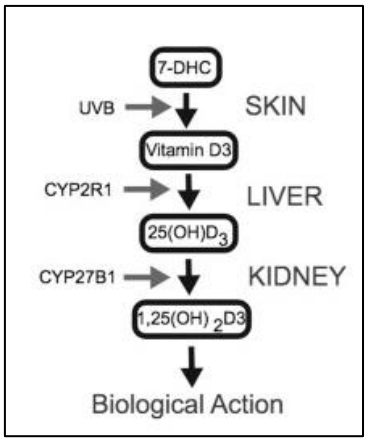


Figure 2.3: Summary of biological vitamin D synthesis.[23] The production of vitamin D requires the action of sunlight on the skin and then hydroxylation, first in the liver and then in the kidneys.

The role of vitamin D in calcium homeostasis and bone health is well known, however, vitamin D metabolites alter the physiology of various non-skeletal tissues and consequently control a wide range of biological functions. Through the VDR, 1,25(OH)$_2$ D3 activates a variety of signalling pathways that are important for a variety of cellular processes such as cell proliferation and differentiation, membrane transport, redox balance, adhesion and haemostasis.[36] The role of vitamin D and the VDR is emerging as a vital regulator of the pathogenic cycle of a number of non-skeletal diseases, such as pigment disorders, neurological, renal, infectious, autoimmune diseases and a number of cancers.[34,37]

Moreover, several laboratory studies have shown that vitamin D also has many associated anticarcinogenic effects including anti-inflammation, anti-angiogenesis, and pro-apoptosis. These effects have been established in numerous cell types by influencing cell differentiation and decreasing cell proliferation, invasion, growth, adhesion, migration and metastasis.[37-38] Vitamin D has also been found to induce transcriptional activation and repression of target genes via binding to the VDR, an intracellular receptor that upon activation results in the regulations of numerous genes binding to vitamin D response elements in DNA.[37]

2.5.2 Vitamin D and cancer risk/mortality

Sunlight is the principal provider of vitamin D for humans, and ecological research suggests that individuals with greater exposure to sunlight and thus higher levels of vitamin D have lower cancer incidence and mortality rates.[39] Further studies have shown that survival rates of patients diagnosed with some cancers were higher if the diagnosis was made during summer compared to winter, and that low vitamin D status was associated with increased rates of various cancers in populations living at higher latitudes.[37-38]

The incidence rates of PCa in African individuals are significantly higher than in white individuals, while death rates in African males are more than twice as high as in white males.[39] It is well known that there are higher rates of total cancer mortality in regions with less UVB radiation as well as in African individuals, each correlated with vitamin D deficiency.[39] According to a review by Norval et al, where serum vitamin D status was measured in South African population groups, it was found that the majority of individuals aged above 65 years were deficient.[40] Thus, it has been hypothesized that circulating vitamin D levels are significantly associated with both PCa risk and mortality.

A higher vitamin D status in PCa patients at the time of diagnosis has been found to be associated with higher survival rates.[38] Moreover, in studies conducted in Europe and America, supplementation with vitamin D during treatment was found to be associated with a reduced risk of general mortality.[37]

Despite significant experimental evidence, human vitamin D and cancer studies have provided no consistent evidence of a protective relationship, and there are as yet no formal guidelines for cancer prevention and vitamin D supplementation.[34] Thus this

study was intended to investigate whether increasing vitamin D status would in turn possibly improve life quality by reducing the thrombotic risk and thus the survival rates within this population.

2.5.3 Vitamin D and Hypercoagulation

Vitamin D deficiency has been found to be associated with an increased risk of arterial and venous cardiovascular events.[6] In a study on thrombin generation and fibrin clot structure after vitamin D supplementation, Blondon et al., (2019) found that supplementation with high-dose vitamin D was associated with reduced *in vitro* thrombin generation and decreased clot density, suggesting that vitamin D supplementation decreases a prothrombotic profile.[6] Low vitamin D levels have been linked to the development of deep venous thromboembolic events in individuals with ischemic stroke, according to a study by Wu and He (2018).[41]

Improving the prothrombotic status of PCa patients could prove to be valuable in the management of these patients, given the relationship between chronic inflammation, hypercoagulation and PCa survival rates.

2.6 Aims and Objectives

The following aims and objectives will lead this study:

2.6.1 Aims

To investigate the morphological and viscoelastic properties of clot formation in PCa patients before and after the *ex vivo* addition of vitamin D and compare this to a reference group.

2.6.2 Objectives

To achieve this aim, the following objectives have been identified for this study:

Objective 1: To determine the patient's baseline levels of vitamin D and PSA using ELISA.

Objective 2: To measure red blood cell deformability by calculating axial ratios using light microscopy and imageJ.

Objective 3: To study the ultrastructure of red blood cells and fibrin networks using scanning electron microscopy.

Objective 4: To measure the fibrin thickness using scanning electron microscopy and imageJ.

Objective 5: To measure the viscoelastic properties of clot formation using Thromboelastography®.

Chapter 3: Study design, sampling and patient demographics

3.1 Study Design

This study was designed as a laboratory-based experimental study. Sample analysis was done on blood *ex vivo* obtained from a reference group presenting with benign prostatic hyperplasia, and an experimental group of 60 PCa patients at SBAH. Recruitment took place over a period of eight months.

Background information was included in the consent form to record the demographics of the population to ensure sampling from a homogenous population.

Blood was collected in 4.5 ml Vacucare® citrate tubes (1:9 3.2% sodium citrate anticoagulation) (Lasec, MGRV454334) by a qualified phlebotomist and left at room temperature for 30 min before experimentation. Treated samples were created by incubating whole blood (WB) and platelet poor plasma (PPP) samples with a dose of 0.5 µg/kg Calcitriol (Sigma-Aldrich, D1530-10UG) for 10 min before experimentation. All WB experiments were completed within five hours of collection. The PPP was prepared by centrifuging WB at 2000 x g for 20 min at room temperature and was stored at -80 ºC until the day of the experiment.

3.2 Ethical Considerations

Ethical approval for this study was obtained from the University of Pretoria Research Ethics Committee under protocol number 422/2020 (Addendum 2 and 7). National Health Research Database (NHRD) approval was also obtained to recruit patients from SBAH as well as to access patient records (NHRD Ref: GP_202010_029) (Addendum 6). Dr Marinka Hoek was the clinician that assisted with patient recruitment by identifying participants that qualified and were willing to participate. The patients were also aware that their decision to participate did not influence their treatment. All participants were fully briefed on what the study and their participation entailed. Only after informed consent (Addendum 3) was obtained blood samples were collected, and all methods were conducted according to all requirements of the ethics committee and in accordance with the Declaration of Helsinki.

3.3 Sampling criteria and procedures

3.3.1 Reference group

3.3.1.1 Recruitment

Willing participants who met the following criteria were recruited from SBAH to serve as a reference group. All participants signed a consent form prior to sample collection. These individuals were only individuals with benign prostatic hypertrophy in whom no suspicion of PCa was present. The recruitment of a reference group from patients of the same age range as the experimental group at SBAH ensured homogeneity between the groups, limiting confounding factors such as differing socioeconomic status. The following criteria was selected to best imitate the experimental group with respect to all conditions, aside from the PCa as described below:

Inclusion Criteria

- Gender: Male.
- Ages: Between 45 and 85 years.

Exclusion Criteria

- Smokes any tobacco or related product.
- The use of chronic medication.
- Had any condition which could present with chronic inflammation.
- Had a history of an immune-compromised status.
- Had used herbal or vitamin supplements, corticosteroids, anti-inflammatory, anti-platelet or anti-coagulative medication within two weeks prior to sample collection date.
- HIV positive (determined from patient files).
- Excessive consumption of alcohol (drinking five or more drinks on the same occasion on at least one day in the past 30 days).[42]

Once informed consent had been discussed and an informed consent form had been signed, blood was drawn into two 4.5 ml citrate tube and one 4.5 ml clot activator tube from each participant by a qualified nurse or phlebotomist.

3.3.2 Experimental group

3.3.2.1 Recruitment

Patients with PCa were recruited voluntarily from SBAH. These patients must have been diagnosed with PCa by a qualified medical doctor. Dr Marinka Hoek, facilitated patient consultation in collaboration with the principal investigator to explain the purpose of this study, obtain the necessary informed consent and obtain the blood samples. Samples from patients at any stage and subtype of PCa were obtained. These were separated according to metastatic status. Metastatic workup was done using either a 99mTC bone scan or a Ga-PSMA PET/CT or MRI. Patient medical records were accessed in order to obtain the necessary clinical information. All patient information was handled anonymously. As described in the criteria below, patients had no other sign of infection or inflammation not related to PCa.

Inclusion Criteria:

- Gender: Male.
- Age: Between 45 and 85 years.
- Diagnosed with PCa.

Exclusion Criteria

- Smokes any tobacco or uses any related product.
- Had any condition which could present with chronic inflammation.
- Had a history of an immune-compromised status.
- Had used herbal or vitamin supplements, corticosteroids, anti-inflammatory, anti-platelet or anti-coagulative medication within two weeks prior to sample collection date.
- HIV positive (determined from patient files).
- Excessive consumption of alcohol (drinking five or more drinks on the same occasion on at least one day in the past 30 days).[42]

Once informed consent (Addendum 4) had been discussed and an informed consent form had been signed, two 4.5 ml citrate tubes and one 4.5 ml clot activator tube of blood were drawn from each participant by a qualified nurse or phlebotomist. Blood samples were collected at a single interval during their consultation, thereby avoiding any interference with subsequent allocation of treatment regimes.

Division of experimental group

The experimental group was divided into two groups as non-metastatic and metastatic, as shown in **Figure 3.1**.

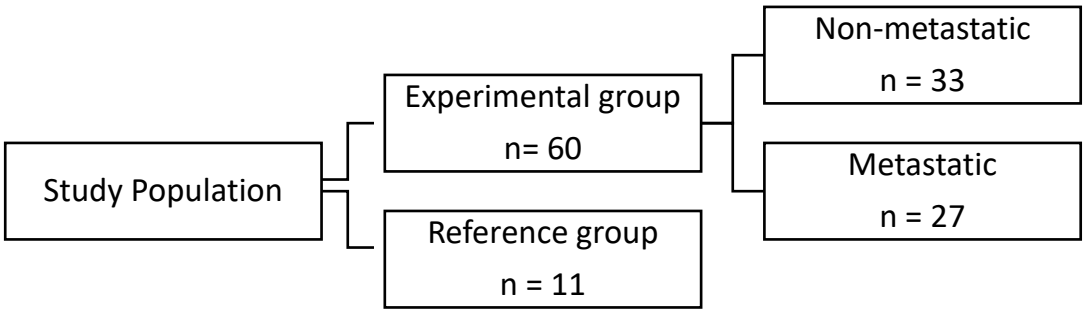


Figure 3.1: Diagram illustrating the study population grouping.

3.3.3 Patient data

The average age of the patients in the reference, non-metastatic and metastatic groups was 64, 67 and 71, respectively. A sample size of 33 were recruited for the non-metastatic experimental group and a size of 27 were recruited for the metastatic experimental group. A reference group of 11 patients were included.

A sample from each participant was sent to the NHLS for baseline vitamin D and PSA testing. The median baseline PSA level was 19.5 for the non-metastatic group and 75.4 for the metastatic group. The median baseline vitamin D level was 85.8 nmol/L, 90.1 nmol/L and 84.1 nmol/L for the reference, non-metastatic and metastatic groups, respectively. These are summarized in **Table 3.3.1**. A summary of the medications used, as well as the T-staging of the experimental group, can be seen in **Table 3.3.2**.

Table 3.3.1: Patient demographics.

	Description	Units	Control	Non-metastatic	Metastatic
Age		years	64.0 (48.0, 71)	67.0 (61.0, 74.0)	71.0 (68.0, 75.0)
PSA	Prostate specific antigen (baseline)	ug/L	NA	19.5 (0.5, 24.0)	75.4 (7.4, 113.9)
Vit D	Baseline vitamin D	nmol/L	85.8 (61.1, 104.1)	90.1 (72.7, 102,3)	84.1 (65.1, 97.2)
Data expressed as median and 25% - 75% quartile range of each group.					

Table 3.3.2: Patient staging and medication use.

	Controls (n=11)	Prostate Cancer (n=54)					
T-stage	N/A	T1c	T2a	T2b	T2c	T3b	T4
		n=14	n=8	n=14	n=13	n=2	n=9
Patients on medication(s)	n=6	n=45					
Ridaq[1]	n=1	n=3	n=3	n=3	n=4	n=0	n=0
Amlodipine[2]	n=1	n=0	n=0	n=1	n=0	n=0	n=2
Atenolol[3]	n=0	n=0	n=0	n=0	n=0	n=0	n=1
Uromax[4]	n=5	n=4	n=4	n=0	n=5	n=1	n=3
Calcium[5]	n=0	n=0	n=0	n=0	n=0	n=0	n=1
Zoladex[6]	n=0	n=2	n=2	n=2	n=4	n=0	n=4
Enalapril[7]	n=0	n=0	n=0	n=1	n=1	n=0	n=0
Simvastatin[8]	n=0	n=11	n=1	n=1	n=1	n=0	n=1
Paracetamol[9]	n=1	n=0	n=1	n=0	n=1	n=0	n=2
Tramal[10]	n=0	n=1	n=1	n=0	n=1	n=0	n=1
Androcur[11]	n=0	n=0	n=0	n=1	n=0	n=0	n=2
Nifedipine[12]	n=0	n=0	n=0	n=1	n=0	n=0	n=0

Three participants were excluded due to lacking baseline vitamin D levels and three more were excluded due to incomplete metastatic status.

[1] Diuretic used to treat hypertension: Chemical name: Hydrochlorothiazide; Trade name: Ridaq (Aspen Pharmacare)

[2] Ca^{2+} channel blocker used to treat hypertension: Chemical name: Amlodipine maleate; Trade name: Amloc (Pharma Dynamics)

[3] Selective beta-1 blocker used to treat hypertension. Chemical name: Atenolol. Trade name: Various, brand used not listed for patient

[4] Selective a_{1A} receptor antagonist used to treat lower urinary tract symptoms, by relaxing smooth muscle in the bladder neck, prostate and urethra. Chemical name: Tamsulosin hydrochloride. Trade name: Various, brand used not listed for patient

[5] Calcium supplement: Chemical name: N/A; Trade name: brand used not listed for patient

[6] Injectable Gonadotropin releasing Hormone Agonist, used to suppress LH, FSH and thus Testosterone production in the testes. Chemical name: Goserelin Acetate. Trade name: Zoladex

7 ACE inhibitor used to treat hypertension: Chemical name: Enalaprilat; Trade name: Various, brand used not listed for patient

8 HMG-CoA reductase inhibitor used to treat high cholesterol. Chemical name: Simvastatin. Trade name: Various, brand used not listed for patient

9 Analgesic for mild pain. Chemical name: Acetominophen. Trade name: Various, brand used not listed for patient

10 Opiod analgesic used to treat moderate to severe pain. Chemical name: Tramaldol-hydrochloride. Trade name: Various, brand used not listed for patient

11 Steroidal antiandrogen used to block androgen receptor and inhibits LH release. Chemical name: Cyproterone Acetate.Trade name: Androcur

Chapter 4: Calculation of the Axial Ratios of Red Blood Cells

4.1 Chapter Objectives

The chapter objective was to measure RBC deformability by calculating the axial ratios of the RBCs using LM.

4.2 Introduction

The optical microscope, also known as the light microscope, is used to study cells microscopically such as blood cells using visible light and a system of lenses. Normal light-sensitive cameras are able to capture the image from an optical microscope to create a micrograph that depicts what is seen through the microscope.

A commonly used method to examine the general morphology of cells is LM, particularly in pathology, and provides an accurate portrayal of the cell distribution in a sample. In addition to morphology, the LM can be used to calculate the axial ratios of RBCs to statistically determine variation in cell size and form, as well as cell deformability.

4.3 Materials and Methods

Blood was collected in a citrate tube from the study participants as outlined in Chapter 3. Samples of WB smears were prepared for LM using 10 µl of WB before and after treatment, smeared on to a glass microscopy slide. Thereafter the smears were allowed to dry completely on a slide warmer. After drying, the slides were fixed in 100% methanol for five minutes and allowed to completely dry again. This was followed by staining with methylene blue (Merck, 03978-250ML) for five minutes. After the staining period the samples were rinsed with running tap water and allowed to dry and then stained with eosin (Merck, 861006-10G) for one minute. After one minute had passed, the samples were again rinsed with running tap water and left to completely dry. After the smears had dried, a coverslip was mounted with Entellan (Merck, 1.07961.0500). Smears were then viewed with the Zeiss AXIO Imager.M2 on a 100 times magnification at the Laboratory for Microscopy and Microanalysis at the University of Pretoria.

At least five images of each individual were taken to ensure an even spread of the sample was viewed. The images were downloaded and processed with ImageJ.

ImageJ (a public domain, Java-based image processing program developed at the National Institute of Health: http://rsbweb.nih.gov/ij/) was used to determine axial ratios of the RBCs. Axial ratios will always be greater than or equal to one and were calculated by using the largest diameter of the RBC as the numerator, and the diameter perpendicular to the line used to provide the numerator as the denominator. **Figure 4.1** illustrates how the axial ratios were calculated. The length of line A (127 pixels) was used as the numerator where line B (96 pixels) was used as the denominator which gives a ratio of 1.32.

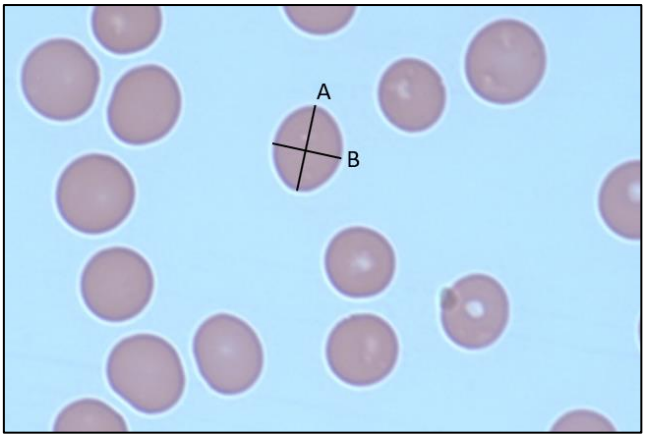


Figure 4.1: Illustrating how the axial ratios of the RBCs were measured in ImageJ.

4.4 Results

The data is presented in the form of micrographs that show the typical morphologies that were observed, as well as the statistical analyses of the measurements.

Figure 4.2 Micrographs A-D shows LM smears from the reference group. **Figure 4.3** shows the smears from both the metastatic on the right (A, C,) and non-metastatic on the left (B, D) experimental groups before treatment and **Figure 4.4** shows smears from the metastatic (A, C) and non-metastatic (B, D) experimental groups after treatment. The statistical analysis of the average RBC axial ratios determined from the reference and experimental groups before and after treatment can be seen in **table 4.1** and **4.2**.

The variation in cell size was determined using axial ratios, the deformability of the cells was calculated and the results were used to perform statistical analysis.

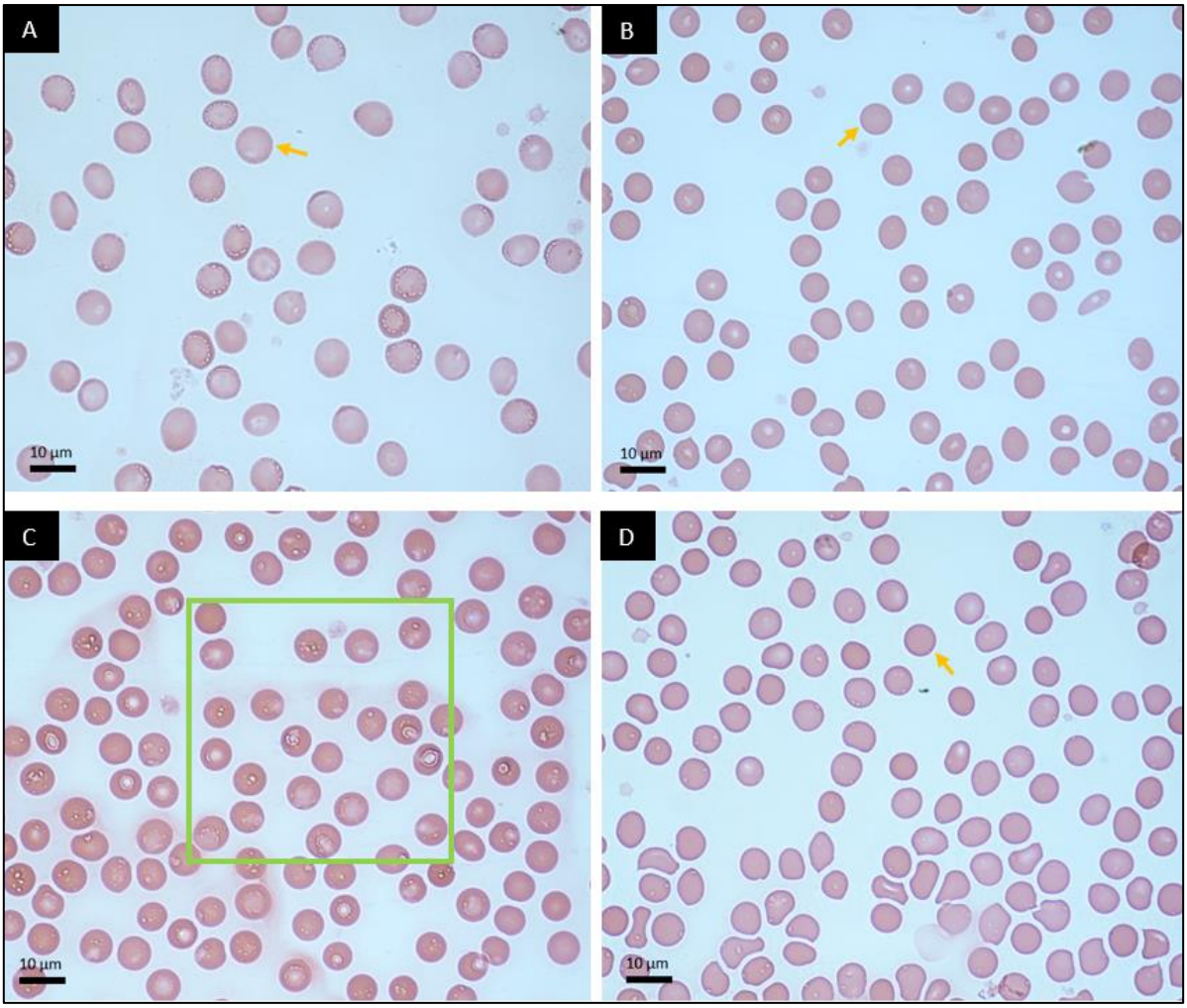


Figure 4.2: Micrographs of samples from the reference group. The expected normal discoid shape of erythrocytes is indicated by yellow arrows and the areas of cells with constant sizes is indicated in a green square. Scale bar = 10 µm.

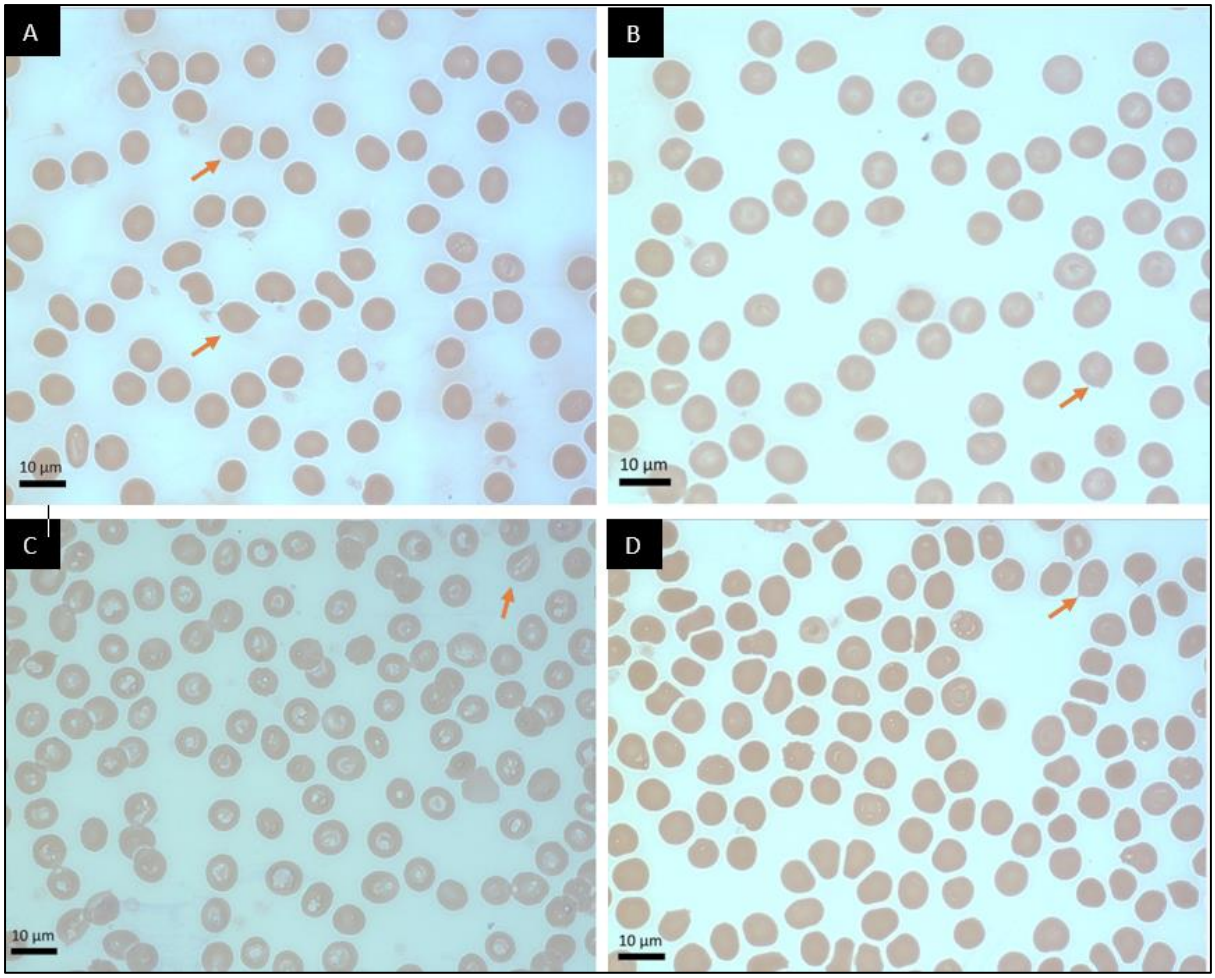


Figure 4.3: Micrographs of samples from the metastatic (A, C) and non-metastatic (B, D) experimental groups before treatment, where orange arrows indicate irregularly shaped cells. Scale bar = 10 µm

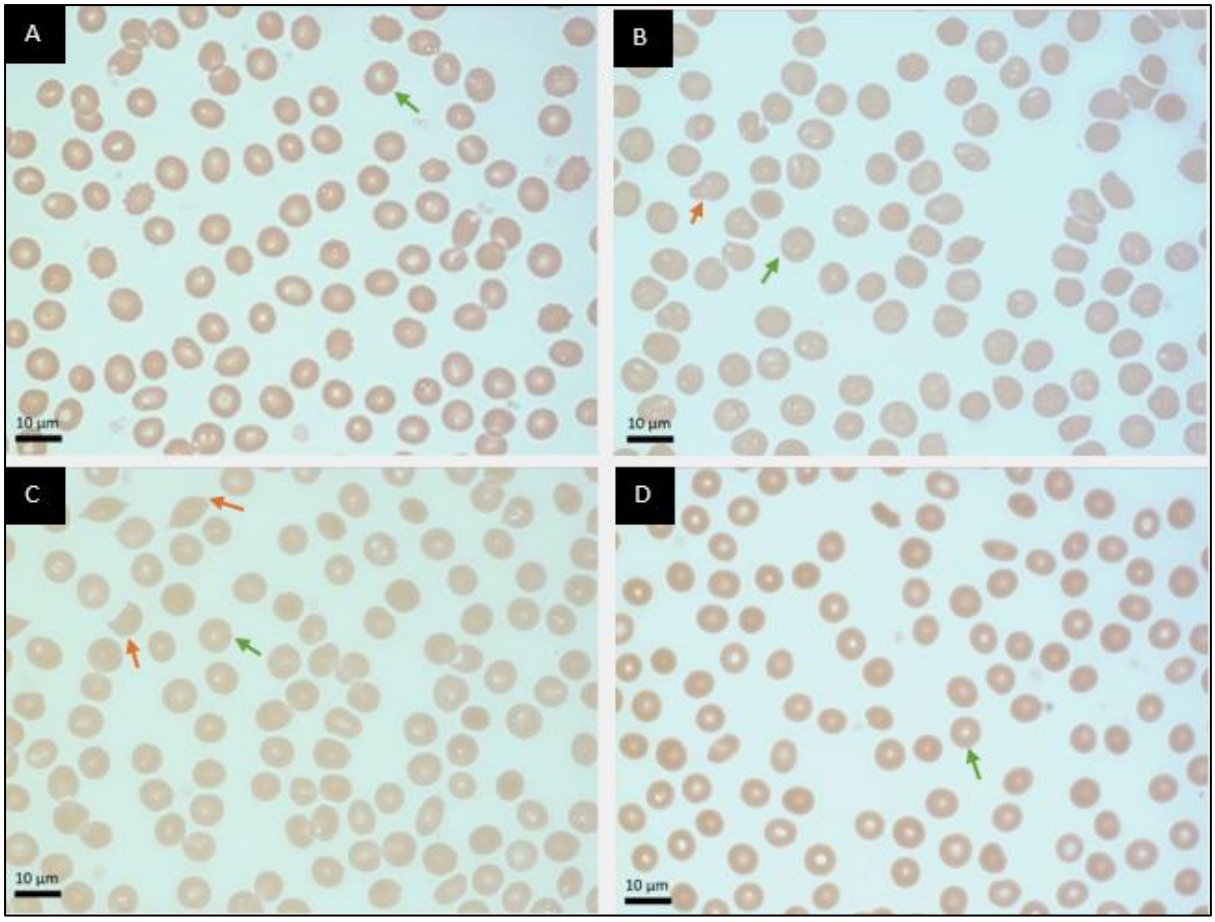


Figure 4.4: Micrographs of samples from the metastatic (A, C) and non-metastatic (B, D) experimental groups after treatment**,** where normally shaped blood cells are indicated by green arrows and irregularly shaped cells are indicated by orange arrows. Scale bar = 10 µm.

In **Figure 4.2**, the reference group shows the typical, normal discoid shape of RBCs. The cells appear to be mostly uniform in size and shape, with a few irregularly shaped cells. The size of the cells appears to be consistent throughout the samples in the reference group. In **Figure 4.3**, the experimental group before treatment, an overall morphology of irregularly shaped cells can be observed. In **Figure 4.4**, the experimental group after treatment, some regularly shaped cells as well as some irregularly shaped cells can be observed.

4.5 Statistical Analysis

The variation in cell deformability was measured using axial ratios. The deformability of the cells was determined and the data was then tested for normality using the Shapiro-Wilk normality test, whereafter a student t-test was performed to determine if the experimental groups (both before and after treatment) differed significantly from the reference group. A p-value <0.05 was considered statistically significant. The metastatic and non-metastatic group were compared to the control group and not with

each other since these experiments were used to determine if the treatment improved the profiles to be similar to the reference group.

Table 4.1: The mean and standard deviation for the axial ratios of RBCs.

Axial Ratios		**Mean**	**Std deviation**
Reference (n=11)		1.099	±0.03985
Non-metastatic (n=33)	**BTx**	1.142	±0.05448
	ATx	1.124	±0.04406
Metastatic (n=27)	**BTx**	1.142	±0.05116
	ATx	1.128	±0.04271
BTx = Before treatment. ATx = After treatment.			

Table 4.2: Summary of the results of a student t-test comparing each experimental group to the reference group. *Indicate a P-value < 0.05.

Student t-test		**P-value**
Non-metastatic vs. reference	**BTx**	0.0276*
	ATx	0.1167
Metastatic vs. reference	**BTx**	0.0277*
	ATx	0.0713
BTx = Before treatment. ATx = After treatment.		

The axial ratios of RBCs in aged, healthy individuals were close to 1, indicating that they have the normal, expected discoid shape. For the reference (n=11), metastatic (n=27) and non-metastatic (n=33) groups, 20 cells per individual were measured.

4.6 Discussion

The foundation for any microscopic investigation is LM, as it provides a clear viewpoint and a meaningful starting point. During the LM analysis, visible shape variations between the reference, metastatic and non-metastatic groups was observed. In the reference group, the overall morphology of the RBCs was that of the expected normal discoid shape, whereas micrographs from the experimental groups showed marked deviations in the form of irregularly shaped cells. This finding correlates with what can be seen in the literature: systemic inflammation in inflammatory conditions such as cancer causes shape alterations in RBCs.[43] These shape changes can be attributed to the generation of hydroxyl radicals in inflammatory illnesses, where these radicals cause such damage to the membrane and cellular cytoskeletal structures that causes the cell to easily deform.[44]

However, after treatment with vitamin D, the cells in the experimental group appeared to revert back to the expected shape, with areas of cells with constant sizes being more prevalent than in samples before supplementation. Vitamin D has been shown to have antioxidant properties in cell culture and experimental tests.[45] Vitamin D may help to reduce oxidative stress, and thus may be a possible mechanism by which treatment seemed to improve RBC shape.

It should be noted that the morphology observed during LM analysis alone is not indicative of pathology, and thus the statistical analysis of the quantitative data found during analysis of the axial ratios should be considered when the importance of morphological alterations is discussed.

No statistically significant differences were observed during comparison of the experimental groups after treatment, although a significant difference was indeed observed when comparing the experimental groups before treatment with the reference group. This indicates that RBCs from PCa patients defer from the typical discoid shape significantly, as appears to be the case in a range of inflammatory disorders[46], and that vitamin D supplementation appears to influence the roundness of the RBCs.

4.7 Conclusion

In conclusion, based on LM analysis, it can be assumed that supplementation with vitamin D may address the pathology related to abnormal RBC morphology in PCa patients. The findings in this chapter highlight the necessity of further investigating the morphological alterations found after vitamin D supplementation. The SEM will be utilized in the following chapter to analyse these changes in greater detail and to provide additional insight into the results obtained in this chapter.

Chapter 5: Ultrastructural analysis of red blood cells and clots

5.1 Chapter objectives

The chapter objective is to study the ultrastructure of RBCs and fibrin networks using SEM.

5.2 Introduction

The SEM is an ultrastructural method that can reveal detailed information on the morphology of in individual blood cells and fibrin fibers, both of which are involved in coagulation.[47] Inflammation is associated with aberrant RBC's, fibrin fiber production and fibrin structure.

A crucial function is played by RBCs in both the coagulation system and inflammation. Since they are subjected to shear forces as they travel through the vascular system, RBC's are extremely deformable and elastic.[48] The properties of the RBC are highly dependent on their membrane composition. These membranes consist of 3 layers: 1) carbohydrate-rich glycocalyx, 2) lipid bilayer containing trans-membrane proteins and 3) membrane skeleton containing a structural network of proteins, responsible for the flexibility, durability and deformability during rheology.[48] Biophysical shape changes are triggered by biochemical membrane alterations, such as oxidative stress and the an increase of inflammatory molecules. Cell deformability is thought to be a primary factor in poor perfusion, increased blood viscosity and microvascular blockage.[43] The roughness of the cell membrane can also be used to comment on the health of the cell, with a decrease in membrane roughness often seen in diseased individuals.[49] In this study, the ultrastructure of RBC's were analysed in order to gain a better understanding of the changes observed during the LM analysis.

5.3 Methods and Materials

Blood was collected in a citrate tube from participants as outlined in Chapter 3. For WB analysis before treatment, 10 µl of WB was smeared onto two separate glass coverslips. Thereafter, 5 µl of thrombin (6.66 IU/ml) (provided by the SANBS) was added to one coverslip to activate the fibrin networks. After the samples were treated with vitamin D, as was described in chapter 3, 10 µl of WB was then smeared onto two separate glass coverslips. Thereafter 5 µl of thrombin was added to one coverslip

to activate the fibrin networks. The coverslips were left to dry briefly for one minute at room temperature after which they were placed into individual wells of a 24-well plate.

The samples were then washed by gentle immersion in 1X phosphate buffered saline (PBS) solution (Sigma, P5493-1L) (pH 7.4) with 0.01 M phosphate buffer and 0.154 M sodium chloride. After washing, the smears were fixed with 4% formaldehyde (Sigma, F8775-500ML) for a minimum of 30 minutes, followed by washing three times in PBS for three minutes each. The samples were then further fixed by covering in 1% osmium tetroxide (OsO_4) (Sigma, 75632-10ML) for 15 minutes. Once again, the samples were washed three times with PBS for three minutes each. The samples were then gradually dehydrated by covering them for three minutes in progressively higher concentrations of ethanol (Sigma, 24102-4x2.5L), i.e., 30%, 50%, 70%, 90% and finally three times with 100% ethanol. After the dehydration step, the samples were immersed in hexamethyldisilazane (HMDS) (Sigma, 440191-1L) for 30 minutes after which the HMDS was removed and replaced by a single drop of HMDS. The samples were then left to dry overnight.

During the final step, the samples were mounted on metal plates and coated with carbon to provide conduction of the samples in the SEM. The Zeiss Crossbeam 540 FEG-SEM or Zeiss Ultra Plus FEG-SEM (Carl Zeiss Microscopy, Munich, Germany) was used to study the ultrastructural morphology of samples at 1 kV, using the InLens detector.

In order to analyse the ultrastructural changes to RBCs for each sample, the following properties were noted:

1. Overall shape of the RBCs
2. The texture of the cell membrane
3. Cell interactions

Sample analysis of SEM preparations consisted of a series of representative micrographs being taken of each sample. Following this, features of interest were noted based on the above-mentioned properties. Images obtained from the reference group were then compared to those obtained from the experimental group. The differences that were observed were then noted and discussed.

5.4 Results

5.4.1 Red Blood Cell Ultrastructure

Micrographs of WB smears from the reference group can be seen in **Figure 5.1**: SEM micrographs of samples of WB in the reference group. Scale bar = 1 µm. **Figure 5.3** and **Figure 5.4** indicates WB smears from the experimental group before treatment, where left (images A, C, E) shows WB from the metastatic group and right (B, D, F) shows WB from the non-metastatic group and **Figure 5.4** indicates WB smears with added thrombin. Samples of WB from both experimental groups after treatment, where left shows the non-metastatic group and right the metastatic group can be seen in **Figure 5.5**, where **Figure 5.6** shows WB smears with added thrombin.

In the reference group, smears of WB with added thrombin, seen in **Figure 5.2**, shows the formation of a fibrin net around the typical discoid shape normally observed in healthy RBCs. This is in contrast to what can be seen in smears from the experimental group, where the formation of fibres compromises the RBC structure, resulting in cells that contort easily.

5.4.1.1 Reference group:

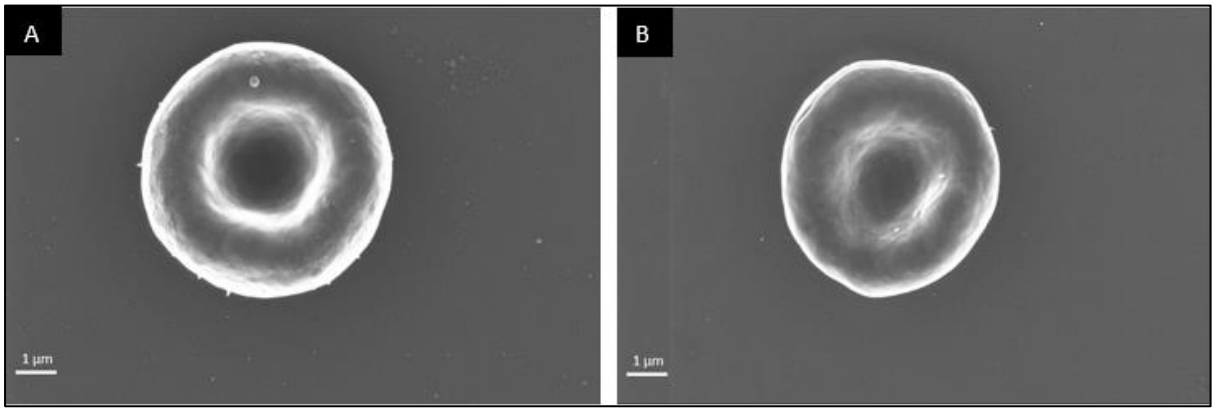


Figure 5.1: SEM micrographs of samples of WB in the reference group. Scale bar = 1 µm

Figure 5.1 depicts micrographs taken of individuals from the reference groups shows normally shaped discocytes.

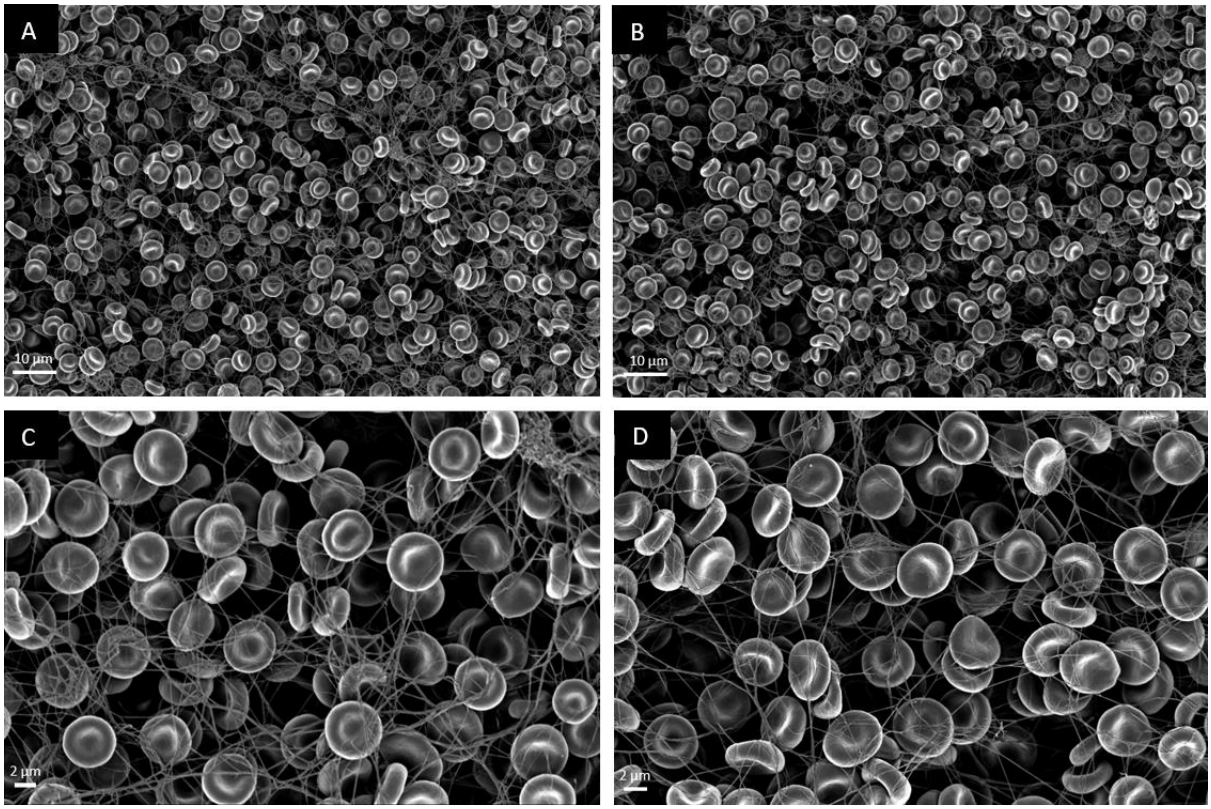


Figure 5.2: SEM micrographs of samples of WB with added thrombin to create an expansive fibrin network around RBCs in the reference group. Images A and B: Scale bar = 1 µm. Images C and D: Scale bar = 2 µm.

Figure 5.2 depicts WB from the reference group with added thrombin, which serves to form a fibrin network around RBC's. The characteristic shape of RBC's is maintained overall, with some samples showing a small number of cells twisting and folding in the presence of fibrin fibers.

5.4.1.2 Experimental group:

Before treatment

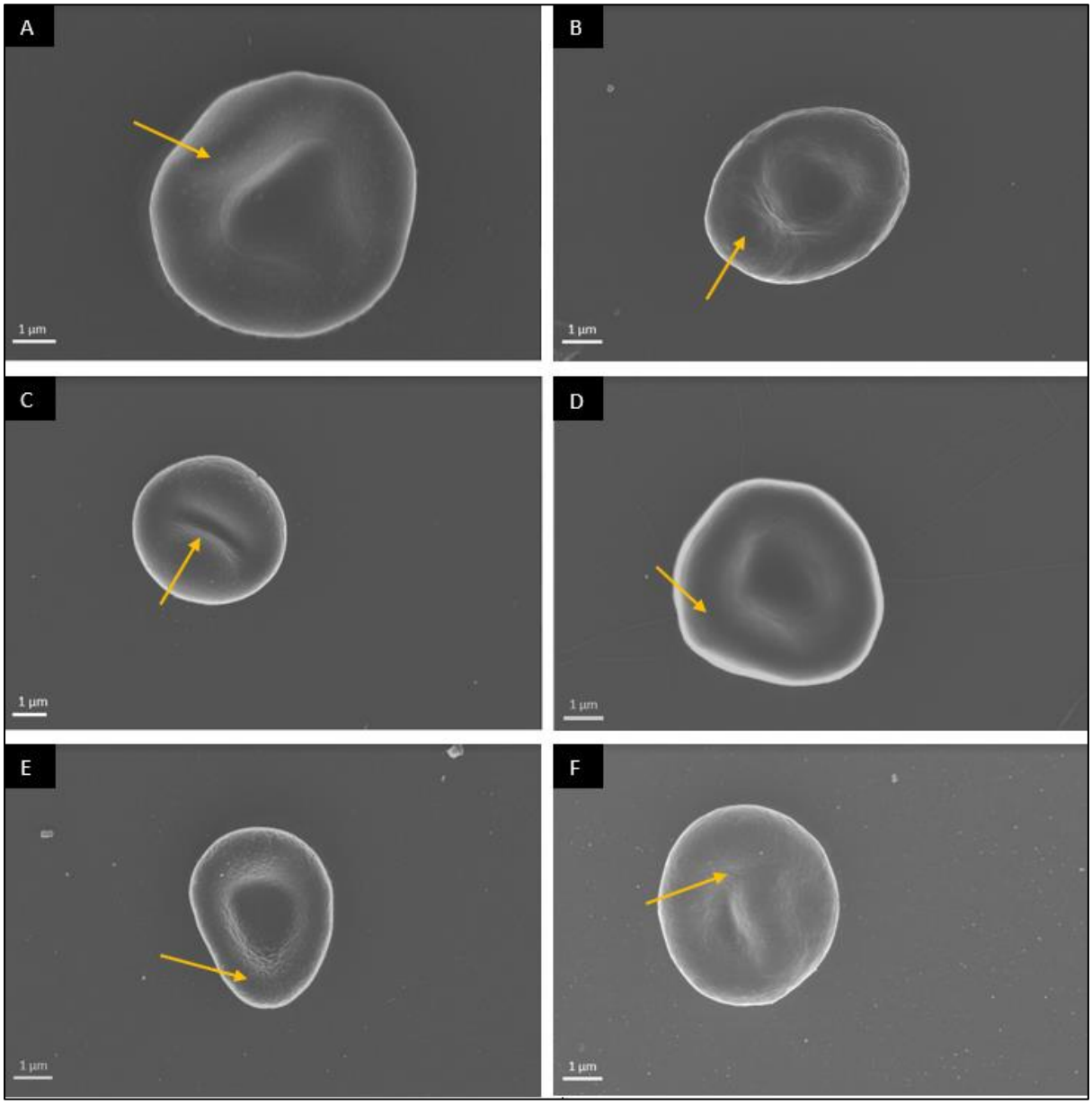


Figure 5.3: SEM micrographs of samples of WB in the metastatic (A, C, E) and non-metastatic (B, D, F) experimental groups before supplementation with vitamin D. Irregular shapes are indicated by yellow arrows. Scale bar = 1 µm

Figure 5.3 shows WB from both experimental groups before treatment, showing the metastatic group on the left (images A, C, E) and non-metastatic group on the right (B, D, F). Samples of WB in the experimental group showed cells with irregular shapes.

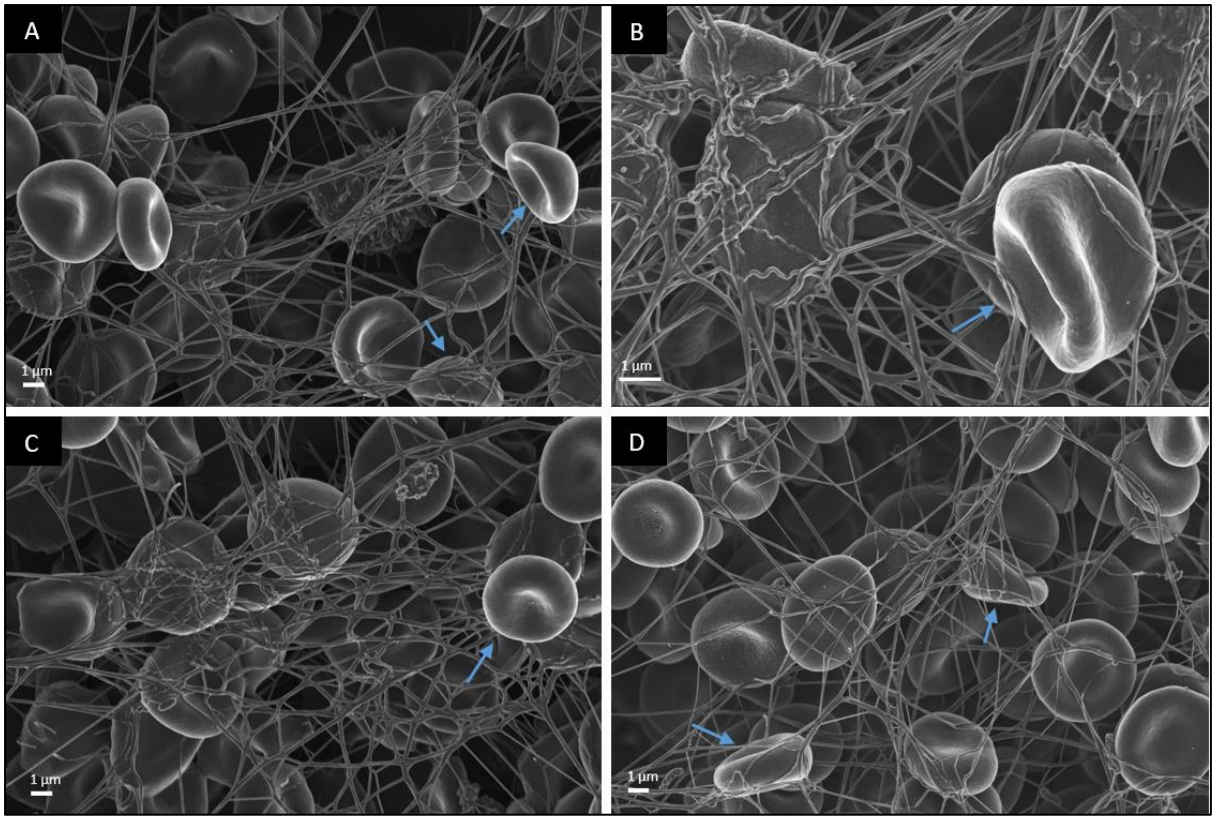


Figure 5.4: SEM micrographs of samples of WB with added thrombin to create an expansive fibrin network around RBCs in the metastatic (A, C) and non-metastatic (B, D) groups before supplementation with vitamin D. Irregularly shaped cells are depicted by blue arrows. Scale bar = 1 µm

Samples of WB with added thrombin from the metastatic (A, C) and non-metastatic (B, D) groups before treatment are depicted in **Figure 5.4**. The morphology of the RBCs is compromised and in majority of the samples the RBCs contort easily in the presence of fibrin fibers. The fibrin networks formed are dense and disorganized, and these features were further investigated in chapter 6.

After treatment

Samples of WB in the experimental group after treatment showed cells with irregular shapes, with both dacrocytes and stomatocytes being observed in the metastatic group and stomatocytes being observed in the non-metastatic group. **Figure 5.5** depicts micrographs of the abnormally shaped cells observed in both groups.

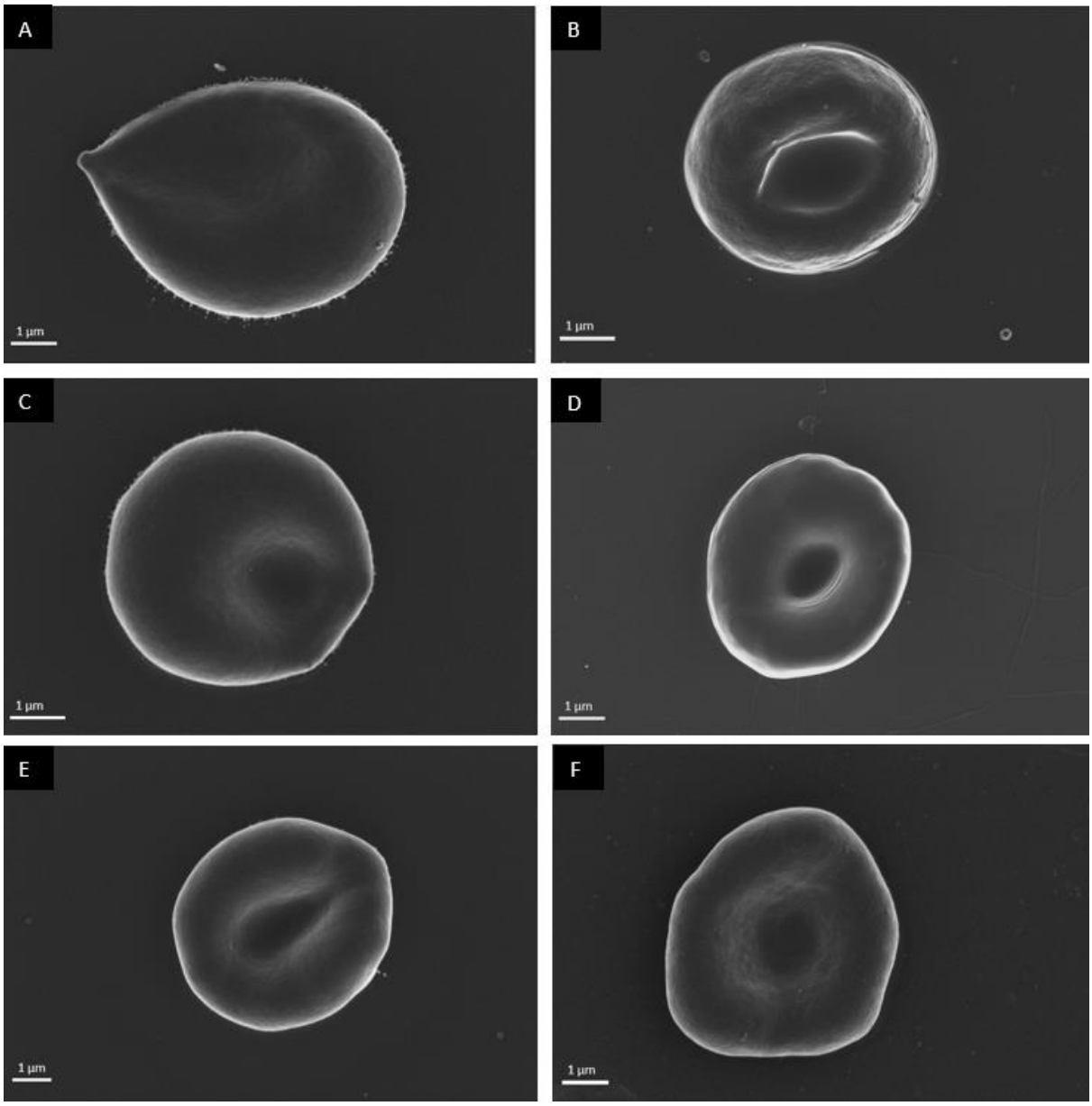


Figure 5.5: SEM micrographs of abnormally shaped cells observed in the metastatic (A, C, E) and non-metastatic (B, D, F) after supplementation with vitamin D. Scale bar = 1 µm.

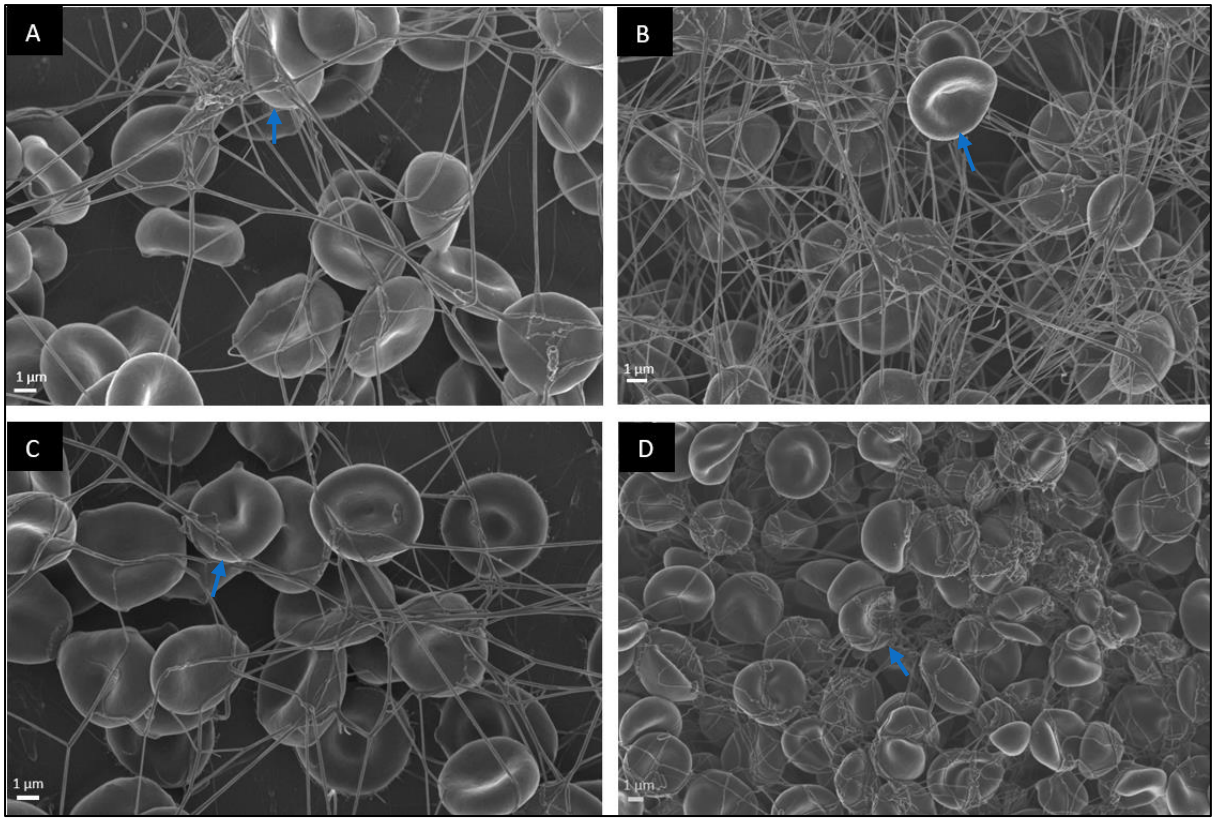


Figure 5.6: SEM micrographs of samples of WB with added thrombin to create an expansive fibrin network around red blood cells in the metastatic (A, C) and non-metastatic (B, D) groups after supplementation with vitamin D. Abnormally shaped cells are depicted by blue arrows. Scale bar = 1 µm.

Samples of WB with added thrombin from the metastatic (A, C) and non-metastatic (B, D) groups after treatment are depicted in **Figure 5.6**. Irregularly shaped RBCs that twist and fold easily in the presence of fibrin fibers were observed.

Ultrastructural analysis showed that RBC's in both the reference and experimental groups demonstrated irregularly shaped blood cells (poikilocytes).

5.5 Discussion

The major function of RBCs, which are found in practically all vertebrates, is to transport oxygen to the body's tissues. The shape of RBCs has a significant impact on their functionality, with RBCs assuming a disk like (discocyte) form in normal conditions, which improves their flow characteristics in arteries and capillaries.[50] During the ultrastructural analysis of RBCs, abnormally shaped cells (poikilocytes) were discovered in both the reference (**Figure 5.1**) and experimental groups before treatment (**Figure 5.3**), though the irregular shapes are present in the experimental groups to a greater extent and severity, particularly in the metastatic group. Due to the fact that both the reference and experimental group fall in the same age range, age-related inflammation could bear responsibility for the poikilocytes observed in the reference group.

In samples from the experimental group before treatment, RBC structure appears to be disrupted, causing the cells to lose their capability to sustain their characteristic discoid form. Dacryocytes (teardrop shaped RBCs) were also frequently noted in the metastatic group. Dacryocytes are most commonly found in patients with malignant infiltrative diseases of the bone marrow[51], which is often caused by PCa.[52]

Although the irregular shapes were still present after treatment with vitamin D (**Figure 5.5**) they had a lesser effect and extent.

The addition of thrombin to WB samples results in the generation of a fibrin mesh. When fibrin is generated around and over RBC's in healthy individuals, they do not deform substantially, and the usual discoid shape can still be observed.[44] This is in line with what was observed in micrographs of WB with added thrombin from the reference group. A shape change was observed in samples from the experimental group, where RBCs were significantly distorted in the presence of fibrin fibers. A study by E. Pretorius (2013) proposed that this ability to deform is caused by the fact that inflammatory diseases cause RBCs to be easily deformed to a pointed shape in smears, and that when thrombin is added they become ensnared in the fibrin mesh.[44] This causes significant shape changes due to the fibrin's pressure on the stressed cells.[44] Hypercoagulability in inflammatory diseases such as cancer is well established, and the RBC entrapment in the fibrin mesh may be an additional element in the formation of tight clots.[44] The addition of vitamin D did not have a noteworthy

change on the deformability of RBC's in the fibrin mesh, and cell deformability seen in these samples may be attributed to the stress exerted on RBCs during smearing.

5.6 Conclusion

In conclusion, there are structural changes visible in PCa patients, especially within the metastatic experimental group, when compared to samples from the reference group. These observed structural changes confirm what has been seen during LM analysis in chapter 4. Supplementation with vitamin D showed a lesser prevalence of abnormally shaped RBCs, although irregular shapes were also observed in the reference population and may be due to age related inflammation, thus more evidence is needed before a definite conclusion can be made. The formation of a fibrin network plays a significant role in clot formation, and fibrin fibers will be studied in greater detail using SEM in the following chapter.

Chapter 6:Ultrastructural analysis of fibrin networks

6.1 Chapter objectives

The chapter objective is to study the morphology of fibrin fibers and measure the fibrin thickness using scanning electron microscopy and imageJ.

6.2 Introduction

Thrombin and fibrinogen interact during blood clotting, with thrombin cleaving the fibrinogen molecule into two peptides, and eventually generating fibrin monomers. These fibrin monomers combine to form a fibrin network, which can be examined using ultrastructural methods.[53]

In this study, PPP samples were also analysed using SEM in order to achieve a more focussed view on fibrin fibre formation. The PPP samples have a concentrated amount of fibrinogen therefore allowing the study of the fibers during clot formation without the presence of other blood cells. Fibrin generates branched networks of double-stranded protofibrils that are laterally linked, which are crucial for blood clots' remarkable mechanical resiliency.[54] Changes in fibrin morphology is closely linked to inflammation in general, thought to be brought on by changed fibrin fiber biochemistry.[55] Atypically structured clots are formed during an inflammatory response, when the group of closely regulated coagulation factors are influenced.[55]

6.3 Methods and materials

Blood collected in a citrate tube was centrifuged at 2000 x g for 20 minutes at room temperature. For PPP analysis before treatment, 10 µl of PPP was smeared onto two separate glass coverslips with 5 µl of added thrombin supplied by the SANBS to activate the fibrin networks. After the samples were treated with vitamin D, 10 µl of PPP was then smeared onto two separate glass coverslips with 5 µl of added thrombin. The coverslips were left to dry briefly for one minute at room temperature after which they were placed into individual wells of a 24-well plate.

The samples were then further prepared for SEM using the method outlined in chapter 5.

A series of representative micrographs were taken of each sample where the appearance and arrangement of fibrin networks was noted. Images obtained from the

reference group were then compared to those obtained from the experimental group, where differences that were noticed were then discussed.

6.4 Results

Smears of PPP with added thrombin to create a fibrin network in the reference group can be seen in **Figure 6.1**. **Figure 6.2** depicts PPP smears from the non-metastatic experimental group (images A, C, E) and metastatic experimental group (images B, D, F) before treatment. PPP smears from the non-metastatic (images A, C, E) and metastatic (images B, D, F) after treatment are shown in **Figure 6.3.**

6.4.1 Reference group

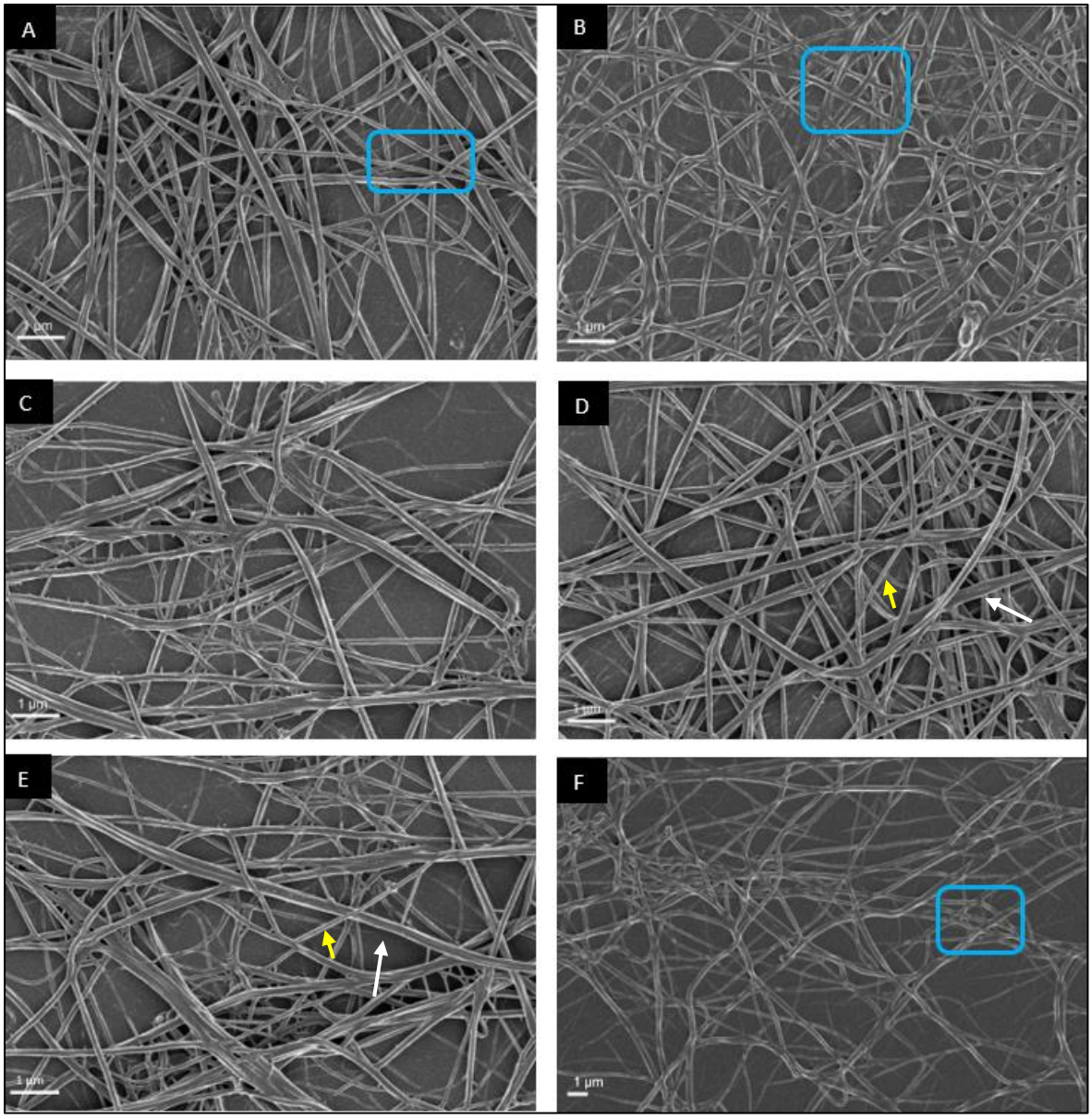


Figure 6.1: SEM micrographs of PPP samples with added thrombin in the reference group. The expected organized mesh is indicated by blue squares. White arrows indicate a thick, major fibrin fiber. Yellow arrows indicate a thin, minor fibrin fiber. Scale bar = 1 µm.

Figure 6.1 depicts PPP samples with added thrombin to form fibrin networks in the reference group. The fibrin formations in this group are fairly consistent, forming organized networks (indicated in blue) with a balance between the formation of thick, major fibers (indicated by white arrows) and thin, minor fibers (indicated by yellow arrows).

6.4.2 Experimental group

6.4.2.1 Before treatment

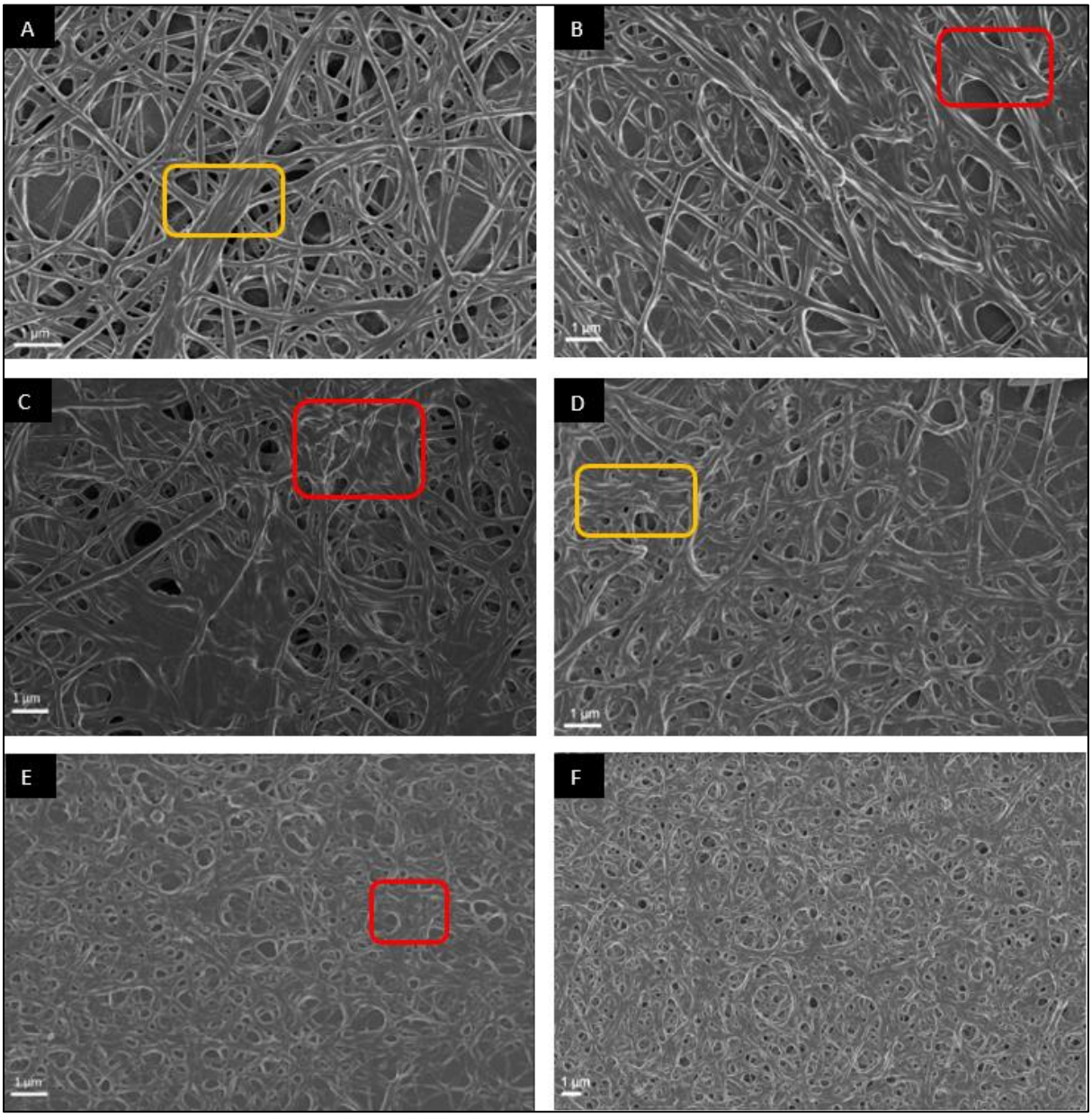


Figure 6.2: SEM micrographs of fibrin networks in the metastatic (A, C, E) and non-metastatic (B, D, F) groups before treatment. Dense matted deposits are indicated by red squares. Coiled fibers are indicated by orange squares. Scale bar = 1 µm.

Figure 6.2 depicts PPP samples with added thrombin to create fibrin networks in the metastatic (left) and non-metastatic (right) groups before supplementation with vitamin D. The fibrin networks formed in the group before treatment tend to form dense, matted deposits (DMDs) where strands are arranged in a disorganised mesh. Images B, C and E depict areas of DMDs (indicated in red) with few open spaces between fibers. Images A and D show coiled fibers (indicated in orange) that stick to other fibers to create a messy clump of disorganised strands.

6.4.2.2 *After treatment*

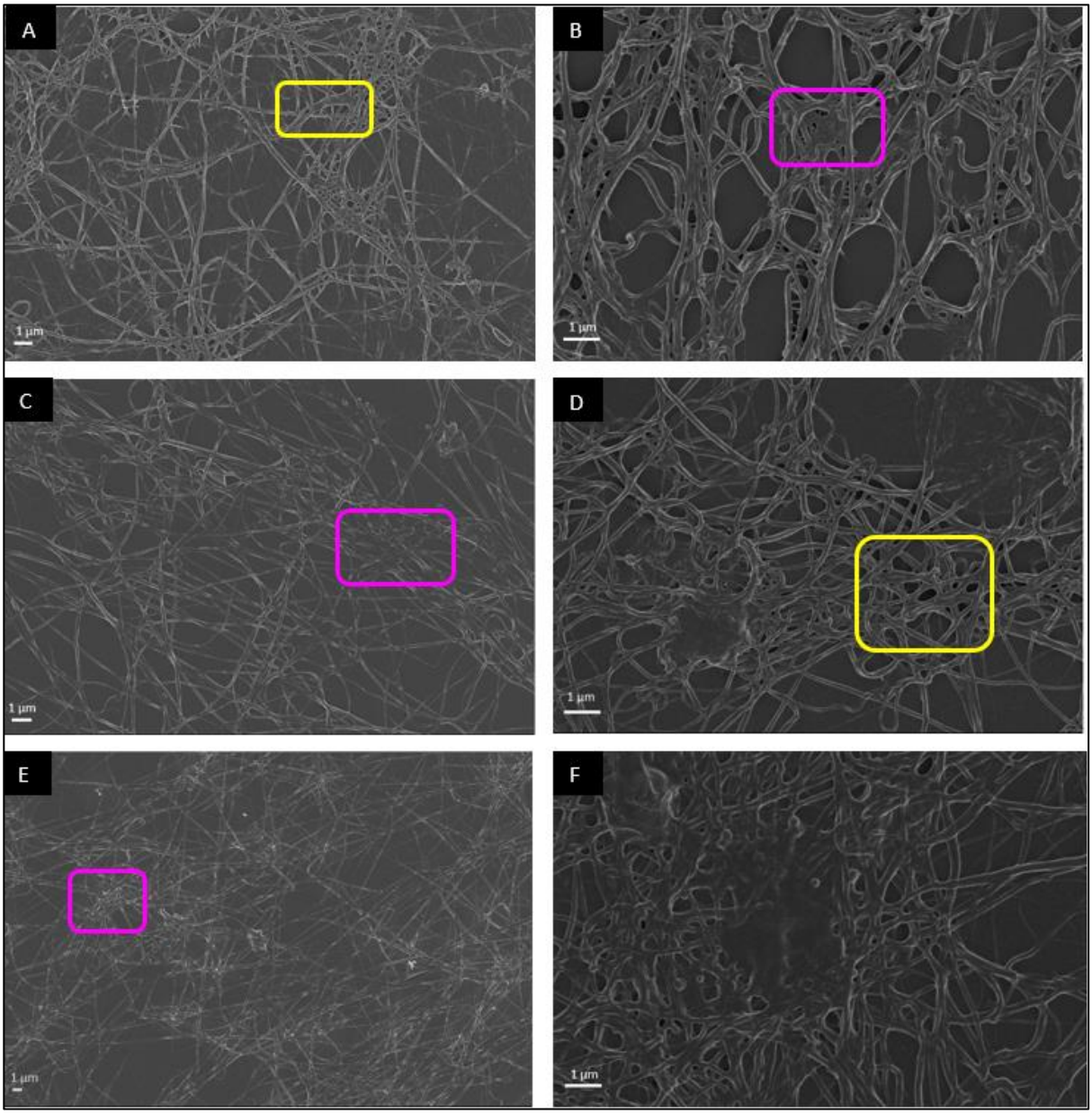


Figure 6.3: SEM micrographs of PPP with added thrombin in the metastatic (A, C, E) and non-metastatic (B, D, F) groups after supplementation with vitamin D. Areas with dense matted deposits are indicated by purple squares and coiled fibers are indicated by yellow squares. Scale bar = 1 µm.

Figure 6.3 represents the metastatic (A, C, E) and non-metastatic (B, D, F) groups after supplementation with vitamin D. The mesh appears to be less dense overall, although a few areas of DMDs are still apparent, as can be seen in images B, C and F. Morphologically, fibers appear to be slightly more organized with more distinct empty spaces between them when compared to the results obtained before treatment. Coiled, sticky fibers can be seen, creating distinct areas of disorganised networks where fibers tend to stick together.

6.5 Statistical Analysis

To establish quantitative data, the average fibrin fiber thickness was determined from SEM images taken. For each sample, 50 fibers were counted. GraphPad Prism was used for statistical analysis. A Shapiro-Wilk test was performed to test for normality, where it was found that values were not normally distributed. Mann-Whitney tests were then performed to establish differences between the experimental and reference groups. A p-value < 0.05 was considered statistically significant.

Table 6.1: The mean and standard deviation for the fibrin thickness data.

Fibrin Thickness		Mean (nm)	Std Deviation
Reference (n=11)		142.2	±54.78
Non-metastatic (n=33)	**BTx**	147.7	±50.50
	ATx	123.3	±53.23
Metastatic (n=27)	**BTx**	136.0	±46.83
	ATx	181.9	±60.32
BTx = Before Treatment ATx = After Treatment			

Table 6.2: Summary of the results of a Mann-Whitney test comparing each experimental group to the reference group. *Indicates a P-value < 0.05

Mann-Whitney		P-value
Non-metastatic vs. reference	**BTx**	0.9786
	ATx	0.1321
Metastatic vs. reference	**BTx**	0.7330
	ATx	0.2723
BTx = Before treatment ATx = After treatment		

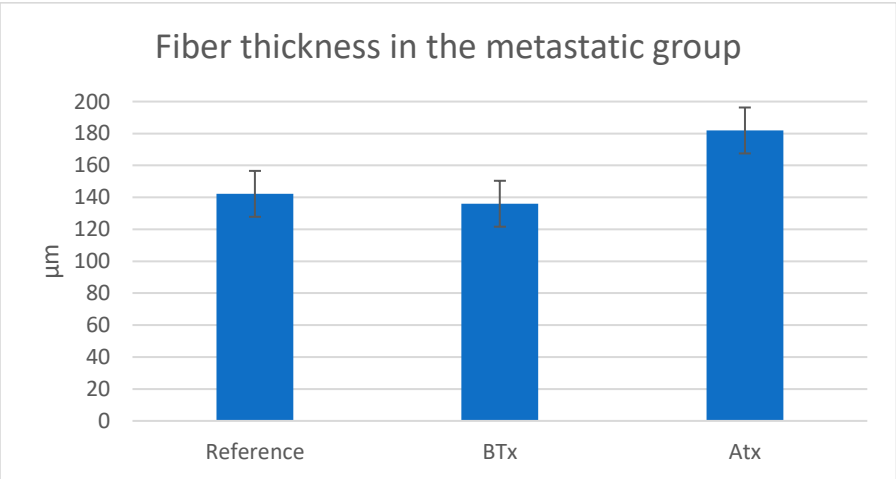


Figure 6.4: A graph to show the mean fiber thickness between the reference group and metastatic experimental group before and after treatment.

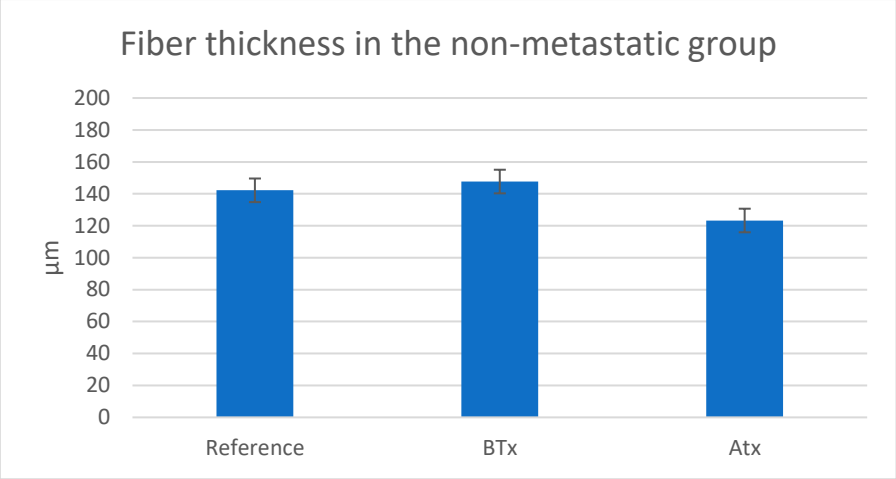


Figure 6.5: A graph to show the mean fiber thickness between the reference group and non-metastatic experimental group before and after treatment.

6.6 Discussion

Fibrinogen is a blood protein that, when activated by thrombin, polymerizes into a network of fibrin fibers.[56] The fibrin network is made up of major, thick fibers that make up the majority of the clot during normal fibrin network formation.[53,57] Typically, some minor, thin fibers are sparingly arranged among the thicker fibers in a net with clear open spaces visible between threads. Numerous studies done by Pretorius et al. has shown that fibrin morphology changes during inflammation and that these changes are especially noted in fibrin packaging.[58-60] In the presence of inflammation, such as is observed in PCa, fibrin clots abnormally, resulting in expansive, disorganized networks and, in some cases, a continuous layer with no visible individual fibers forms.

The reference group overall had an organised mesh profile, consisting of a balance of thick and thin fibers, with tacky or fused fibers apparent in some samples, which may be attributed to age-related inflammation. In contrast, as seen in **Figure 6.2**, patients with PCa create an expansive fibrin network with a changed morphology. In these cases, fibrin networks formed dense, matted layers, which is typically associated with hypercoagulable-type profiles seen in various thromboembolic diseases.[61] The twisted, tacky strands arranged in a tight formation observed in the experimental groups before treatment would be expected to form clots that are difficult to break down, as has been found to be the case with these types of fibrin formations.[62]

These changes in morphology are more prevalent in samples from the experimental group before treatment, with the after-treatment group presenting with slightly less predominant DMDs. There appears to be minor changes after treatment, with the

fibers appearing to be arranged in a less dense and disorderly manner, which suggests that vitamin D supplementation may promote fibrin networks to form with a more loose fibrin conformation, potentially decreasing the thrombotic risk in PCa patients, as clots of this nature have been found to be cleaved at a faster rate than those with a tight fibrin conformation due to the increased permeability allowing for accelerated fibrinolysis.[61-62]

Statistical analysis on the quantitative data obtained by measuring the cross-sectional diameter of fibrin fibers did not find any significant differences when comparing the experimental groups to the reference groups.

6.7 Conclusion

Fibrin network analysis showed an increased tendency to form disorganised and dense fibrin networks in samples from PCa patients, when compared to the organised fibrin networks observed in the reference group. This phenomenon was increased in the metastatic group, as is expected from the literature and what is known of inflammatory conditions. Vitamin D appeared to have a minor influence on fibrin network arrangements in the experimental group after treatment, where fibrin tended to form in a looser conformation with less predominant DMDs. Vitamin D treatment did not have a significant influence on the fibrin fibre thickness.

Both RBCs and fibrin networks play a critical role in clot formation, and the viscoelastic properties of blood both before and after treatment will be investigated in the following chapter.

Chapter 7: Analysis of the viscoelastic properties of clot formation

7.1 Chapter objectives

The chapter objective is to measure the viscoelastic properties of clot formation using TEG®.

7.2 Introduction

Clotting parameters and clot structure are useful indicators of the hypercoagulation status of an individual.[47] Thromboelastography® is currently used as a diagnostic and research tool for determining the viscoelasticity of WB or PPP.[47]

During the TEG® analysis, a standard disposable cuvette and pin is placed inside the machine with either PPP or WB, as was used in this study. The resistance of the developing clot is measured over time using a revolving torsion wire, which is then plotted on the y-axis of a graph, as depicted in **Figure 7.1**. The parameters observed after analysis can then be used to determine the viscoelastic properties of the resultant clot.

Seven different TEG® parameters can be measured during clot formation using PPP or WB, however, to show pathological coagulability, not all of these indicators need to change. The number of TEG® parameters altered is connected to the degree of pathological coagulability, thus it is important to examine each of the parameters individually.[47]

7.3 Methods and materials

Blood was collected in 4.5 ml citrate tubes from reference and experimental groups as described in Chapter 3. For each participant, four samples were prepared. For WB sample analysis before treatment, 340 µl of the WB was placed in a cup of the TEG® (TEG® 5000 computer-controlled device, Haemoscope Crop., Niles, IL, USA), together with 20 µl of 0.2 M calcium chloride ($CaCl_2$) (Barker Medical, 7003) to activate the coagulation process. The vitamin D dosage was added as discussed in chapter 3. After the incubation period, 340 µl of the WB was placed in a cup of the TEG® together with 20 µl of 0.2 M calcium chloride.

Samples of PPP were prepared as explained in chapter 6. For PPP analysis before treatment, 340 µl of PPP was placed in a cup of the TEG® together with 20 µl of 0.2 M calcium chloride. After the samples were treated with vitamin D, 340 µl of the PPP was placed in a cup of the TEG® together with 20 µl of 0.2 M calcium chloride.

The coagulation process was then allowed to run until MA was reached (**table 7.1** and **figure 7.1**), since only the rate and strength of clot formation is relevant to this study.

Table 7.1 outlines the TEG® clot parameters of both WB and PPP, with explanations for each parameter measurement obtained. The TEG® parameters obtained for an individual with a hypercoagulable profile for WB is explained in **Table 7.2** and PPP is explained in **Table 7.3**. **Figure 7.1** depicts an example trace with the various parameters labelled.

Table 7.1: TEG® parameters for WB and PPP.[47]

Parameter	Explanation
R: Reaction time (minutes)	Time of latency from start of test to initial fibrin formation (amplitude of 2 mm); i.e., initiation time.
K: Kinetics (minutes)	Time taken to achieve a certain level of clot strength (amplitude of 20 mm) i.e., amplification.
A(Alpha): Angle. The slope between the traces represented by R and K (degrees)	The angle measures the speed at which the fibrin build up and cross-linking takes place, assessing the rate of clot formation, i.e., thrombin burst.
MA: Maximal Amplitude (mm)	Maximum strength/stiffness of the clot. Reflects the ultimate strength of the fibrin clot, i.e., overall stability of the clot.
G-value: Elastic modulus strength of the thrombus (Dyn per cm^{-2})	Shear elastic modulus strength of the thrombus.
MRTG: Maximum rate of thrombus generation (Dyn $cm^{-2}s^{-1}$)	The maximum velocity of clot growth observed or maximum rate of thrombus generation using G.
TMRTG: Time to maximum rate of thrombus generation (minutes)	The time interval observed before the maximum speed of the clot growth.
TTG: Total thrombus generation (Dyn cm^{-2})	The amount of total resistance to the movement of the cup and pin generated during clot formation, i.e., the clot strength

Table 7.2: TEG® parameters explained for WB.[47]

WB TEG® Profile typically observed in hypercoagulable individuals		
R (min)	Increases	Clot forms faster.
K (min)	Decreases	Clot reaches a set (20 mm) thickness quicker.
Angle (degrees)	Increases	An increased thrombin burst resulting in more cross-linking of fibrin fibres.
MA (mm)	Increases	Increased platelet and/or fibrin interaction resulting in a denser, more rigid clot.
MRTG (Dyn $cm^{-2}s^{-1}$)	Increases	Increased clot growth.
TMRTG (min)	Decreases	Decreased time from clot initiation to maximum clot formation.
TTG (Dyn.cm^{-2})	Increases	Increased clot strength.

Table 7.3: TEG® parameters explained for PPP.[47]

PPP TEG® Profile typically observed in hypercoagulable individuals		
R (min)	Decreases	Dense, matted deposits without any individual fibers forms.
K (min)	Decreases	Clot reaches a set (20 mm) thickness quicker
Angle (degrees)	Increases	An increased thrombin burst resulting in more cross-linking of fibrin fibers.
MA (mm) and TTG (Dyn.cm^{-2})	Increases	Individual fibers are meshed with significantly fewer open spaces visible.
MRTG (Dyn cm^{-2}s^{-1})	Increases	Increased clot growth.
TMRTG (min)	Decreases	Decreased time from clot initiation to maximum clot formation.

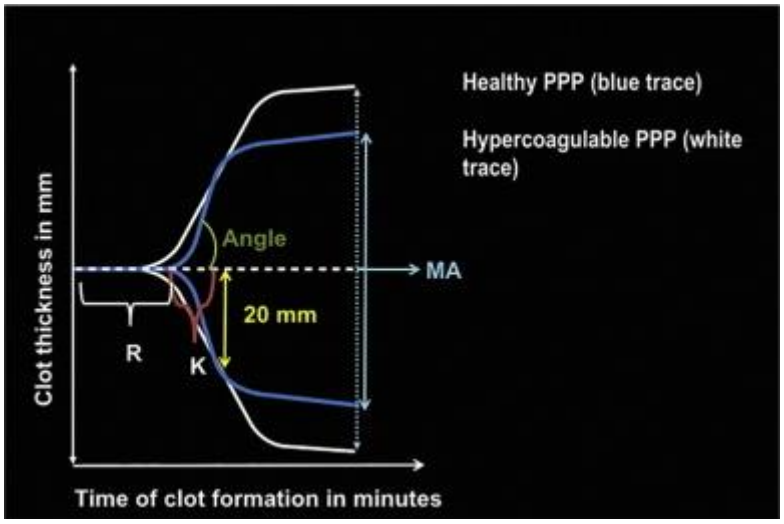


Figure 7.1: TEG® trace of the parameters measured in this study.[47]

7.4 Statistical Analysis

GraphPad Prism was used to analyse the results statistically. A Shapiro-Wilk test was performed to test for normality, where the results indicated that the values are not normally distributed. A Mann Whitney test was then performed to assess differences between the groups and to establish descriptive statistics. A P-value < 0.05 was considered statistically significant.

7.5 Results

Table 7.4 and **Table 7.5** shows the median of the TEG® parameters measured for WB and PPP, respectively, of the healthy reference group (n=11) and the non-metastatic experimental group (n=33) and metastatic experimental group (n=27) both before and after treatment *ex vivo* with vitamin D.

Table 7.4: Median and interquartile ranges of the TEG® parameters of WB samples from the reference and experimental groups (* indicates a P-value < 0.05)

Parameter	Units	Reference (n=11)	Non-metastatic (n=33)		Metastatic (n=27)	
			BTx	ATx	BTx	ATx
R	**minutes**	9.8 (5.6, 13.80)	8.2 (3.0, 15.3)	7.95 (3.8, 13.80)	9.8 (3.2, 19.3)	9.8 (1.0, 14.30)
P-value			0.3243	0.1024	0.8305	0.8928
K	**minutes**	3.8 (1.3, 6.9)	2.7 (1.2, 14.00)	2.95 (1.1, 5.7)	2.7 (1.4, 15.30)	2.3 (1.2, 5.2)
P-value			0.1815	0.0684	0.0512	0.0143*
Angle	**degrees**	54.1 (40.70, 75.30)	62.4 (38.60, 76.40)	62.7 (46.40, 77.60)	63.7 (39.0, 76.50)	64.4 (46.80, 78.50)
P-value			0.1361	0.0159*	0.0345*	0.0075*
MA	**mm**	59.5 (53.50, 72.50)	61 (31.50, 76)	62.75 (44.50, 83.00)	64 (37.0, 85.0)	66.5 (45.50, 68.00)
P-value			0.5226	0.2788	0.1515	0.0328*
G	**Dynes/cm^2**	7.4 (5.800, 13.20)	7.8 (2.300, 15.80)	8.4 (4.000, 24.40)	8.9 (2.900, 28.40)	9.9 (4.200, 40.50)
P-value			0.5226	0.2851	0.1515	0.0342*
MRTG	**Dynes/cm^{-2} s^{-1}**	4.04 (2.530, 12.48)	5.995 (0.8100, 16.67)	6.425 (2.180, 31.36)	7.17 (1.510, 31.40)	7.39 (2.580, 48.25)
P-value			0.1872	0.0424*	0.0050*	0.0035*
TMRTG	**minutes**	14.67 (7.830, 18.58)	11.54 (4.170, 22.83)	12.75 (5.250, 18.67)	14.25 (6.500, 25.58)	13.75 (5.920, 22.50)
P-value			0.1056	0.0910	0.5097	0.6512
TTG	**Dynes/cm^2**	748.3 (585.2, 1349)	788 (230.3, 1604)	859.1 (402.0, 2523)	910.7 (300.0, 2937)	992.4 (54.20, 4219)
P-value			0.5735	0.3860	0.0670	0.0882

BTx = Before supplementation with vitamin D.
ATx = After supplementation with vitamin D.

Table 7.5: Median and interquartile ranges of the TEG® parameters of PPP samples from the reference and experimental groups (*indicates a P value < 0.05)

Parameter	Units	Reference (n=11)	Non-metastatic (n=33)		Metastatic (n=27)	
			BTx	ATx	BTx	ATx
R	**minutes**	9.6 (4.200, 18.00)	12.45(5.400, 27.00)	11.8 (7.200, 21.20)	14.6 (6.900, 28.80)	14.85 (4.300, 25.20)
P-value			0.1912	0.3690	0.0154*	0.0405*
K	**minutes**	3.1 (0.800, 5.900)	3.00 (0.8000, 22.20)	2.3 (0.8000, 5.200)	3.15 (1.000, 14.50)	3.95 (1.200, 14.00)
P-value			0.7940	0.5644	0.3937	0.0214*
Angle	**degrees**	60.20 (46.60, 79.10)	62.2 (34.70, 82.70)	64.1 (46.20, 85.70)	54.8 (34.10, 78.60)	54.75 (36.30, 76.40)
P-value			0.9508	0.5870	0.2664	0.0795
MA	**mm**	38 (29.00, 56.60)	42.25 (19.00, 77.50)	42 (16.50, 71.00)	46 (4.500, 68.00)	41.8 (12.50, 80.20)
P-value			0.2433	0.2473	0.3617	0.4163
G	**Dynes/cm^2**	3.1 (2.000, 6.500)	3.65 (1.200, 17.20)	3.6 (1.000, 7.200)	4.25 (0.2000, 10.90)	3.6 (0.7000, 20.30)
P-value			0.2488	0.2837	0.3111	0.3780
MRTG	**Dynes/cm^{-2} s^{-1}**	5.29 (2.950, 20.88)	5.225 (0.5000, 37.67)	6.33 (1.210, 37.18)	4.78 (0.5800, 12.72)	4.36 (1.660, 26.82)
P-value			0.6077	0.9281	0.3796	0.0752
TMRTG	**minutes**	11.67 (5.080, 23.67)	16.38 (7.250, 37.58)	14.83 (8.170, 24.92)	19.67 (8.580, 36.25)	18.92 (6.500, 31.50)
P-value			0.1865	0.4591	0.0063*	0.0326*
TTG	**Dynes/cm^2**	312.8 (207.3, 777.0)	369.6 (119.4, 1726)	346.4 (101.4, 1242)	378.4 (25.08, 1113)	368 (72.03, 1991)
P-value			0.2407	0.2919	0.5615	0.3159

BTx = Before supplementation with vitamin D.
ATx = After supplementation with vitamin D.

7.6 Discussion

The viscoelastic characteristics of clot formation in the experimental groups, both before and after treatment, and reference group were shown to differ significantly in TEG® results reported in this chapter. The results will be discussed in two parts: first, the results obtained during WB analysis, and second, the results obtained during PPP analysis.

Whole Blood

After WB TEG® analysis where both the experimental groups before and after the *ex vivo* supplementation with vitamin D were compared to the reference group, five of the

eight parameters measured were found to be significantly different (P-value < 0.05). No significant differences were observed in the reaction time (R), time to maximum rate of thrombus generation (TMRTG) and total thrombus generation (TTG).

The amplification is measured by K, and comparison between the reference group and non-metastatic experimental group before and after treatment did not show significant differences. However, the median in the metastatic PCa group after treatment showed a K value significantly lower than that of the reference group (P-value = 0.0143). The low value of K in this group suggests that a clot forms at a faster rate in metastatic PCa patients after the *ex vivo* supplementation of vitamin D. The significant increase in the α-angle (p-value = 0.0075) in this group substantiates this, as this alludes to an increase in the cross linking of fibers in response to an increased thrombin burst. The lack of a significant difference in the reaction time suggests that while the *ex vivo* addition of vitamin D to metastatic patients' blood does not impact the activity of clot initiation factors, it does affect the rate at which clots form. A significant difference was also observed between the metastatic group after treatment and reference group when the considering the maximum amplitude (MA) and elastic modulus strength (G), with p-values of 0.0328 and 0.0342, respectively. These increased parameters suggest that there is an increase in platelet and fibrin interaction in this population after treatment, resulting in a denser, more rigid clot being formed.

Furthermore, a significant increase in the maximum rate of thrombus generation (MRTG) was observed in the non-metastatic group after treatment, as well as in the metastatic group both before and after treatment. This is indicative of increased clot growth which suggests a smaller period of time for clot formation to occur, although this is not substantiated by a significantly decreased reaction time.

According to a study by Toukh et al. (2014), the majority of hypercoagulable PCa patients showed abnormal parameters with respect to R and MA.[63] In this study, it was seen that PCa patients showed abnormalities with respect to K, α-angle, MA, MRTG and G following treatment. The TEG® results obtained from WB analysis indicate that the *ex vivo* supplementation with vitamin D does not have a significant positive effect on the hypercoagulable profile of non-metastatic PCa patients, and treatment caused stronger, more rigid clots to form more quickly in the metastatic group.

Platelet Poor Plasma

After TEG® analysis of the experimental and reference groups, some variation in the results was observed, which is to be expected when working with older patients and PPP, although only three of the eight measured parameters were found to differ significantly.

A significant increase (P-value = 0.0157) in R observed in the metastatic group both before and after treatment suggests that clots form at a slower rate than that of the controls, possibly indicating that fewer DMDs with more individual fibers form. This is in contrast to both what is expected from the literature[63] as well as what was observed from the SEM micrographs in chapter 5.

A significant increase (P-value = 0.0214) in K was observed in the metastatic experimental group after treatment, indicating that the clots in this group tend to take longer to reach a set length of 20 mm, forming at a slower rate than in the reference and before treatment groups. This could be explained by the fact that K indicates clot firmness, and PPP is unlikely to form a stabilized fibrin clot due to the fact that platelets and RBCs have been removed from the sample.

A significant increase in the maximum rate of thrombus generation (TMRTG) was observed in the metastatic group both before and after treatment, with p-values of 0.0063 and 0.0326, respectively. This indicates that more time is taken from clot initiation to maximum clot formation, showing a lower hypercoagulable risk than that of the reference group.

No significant differences were observed in the α-angle, MA, G, MRTG, TMRTG and TTG.

Based on these results, it is evident that PCa patients present with an overall hypercoagulable profile, which is consistent with what has been seen in the literature.[63]

7.7 Conclusion

The viscoelastic parameters of clot formation measured using TEG® revealed that clots develop at a quicker rate in metastatic PCa patients following *ex vivo* vitamin D supplementation with an increase in fiber cross-linking in response to an increased thrombin burst, resulting in clots that are more robust. Both the metastatic and non-metastatic groups' MRTG parameter increased significantly following treatment,

indicative of increased clot growth. Thus, caution should be exercised when supplementing vitamin D in this population, especially in metastatic patients.

The lack of significant results observed after PPP analysis suggests that supplementation with vitamin D is unlikely to affect the fibrinogen component of hypercoagulation, although further investigation using more sensitive methods may be needed to make any conclusions.

Chapter 8: Conclusion

Despite recent advancements in the medical field and the global effort to find effective treatment strategies, cancer diagnoses are still particularly prevalent across the world. PCa, in particular, is a reality that most men of advancing age face in South Africa. Focused, individualised treatment protocols seem to be the most effective approach to obtain a positive outcome. This brings about the necessity to focus increasing attention to the comorbidities and complications that may occur as a result of cancer and the management of patients on treatment, with a particular focus on quality of life and survival rates. Due to the prevalence of PCa in South Africa, where the majority of the population is affected by poverty, a more cost-effective treatment regime is needed to improve the quality of life of patients living with this disease.

The purpose of this study was to investigate whether vitamin D, a readily available supplement, could be a potential treatment for the increased tendency for thrombotic events due to the hypercoagulable state often observed in cancer patients. As discussed in the introduction, improving the haematological status of PCa patients could be beneficial in their treatment, given the link between chronic inflammation, hypercoagulation and cancer.

It is important to note that this study was designed as a laboratory-based, experimental study on blood obtained *ex vivo*, and thus more evidence is needed *in vivo* to confirm these results from future studies.

According to the Endocrine Society, vitamin D deficiency is defined as a serum concentration lower than 50 nmol/L[64], and thus it can be concluded from the results obtained from NHLS testing in chapter 3 that none of the participants recruited for this study were vitamin D deficient at the time of recruitment.

To investigate the effect of *ex vivo* vitamin D supplementation in blood obtained from PCa patients, samples from a reference group, non-metastatic and metastatic group were obtained and evaluated both before and after treatment using the techniques described in chapters 4 to 7. Properties of interest that were investigated include the viscoelastic properties of blood and the ultrastructural characteristic of blood components, namely RBCs and fibrin fibers. The hypercoagulability profiles of PCa patients both before and after treatment with vitamin D were measured, as well as for the reference group using different techniques performed on WB as well as PPP.

The results from SEM and LM analysis clearly show that the ultrastructure of RBCs in PCa patients is altered. The cornerstone of every microscopic investigation is LM since it offers a distinct point of view and useful starting point for the remainder of the study. During the LM analysis, there were evident shape differences between the reference, metastatic and non-metastatic groups before treatment with vitamin D. The axial ratios of RBCs were quantified using LM to statistically determine variation in cell size and shape, in addition to morphology, where it was found that axial ratios differed significantly between the reference and experimental group before vitamin D supplementation. This correlates with what could be seen in the micrographs, as well as what is expected to be seen in patients suffering from an inflammatory disease. After supplementation, there were no significant difference in the axial ratios between the reference and experimental groups, indicating that vitamin D supplementation may act directly on RBCs by improving their morphology.

Another microscopy technique that was utilized in this study was SEM, during which high resolution images allowed for the analysis of morphological changes to specific blood components, namely RBC and fibrin networks. During analysis it was seen that vitamin D supplementation reduced the incidence of irregularly shaped RBCs. In PCa samples before treatment, fibrin network analysis revealed an increased tendency to develop disorganised and dense fibrin networks, which appeared to be more prominent in the metastatic group. The addition of vitamin D to these samples had no discernible effect on the fibrin fiber thickness of the fibrin networks formed.

The viscoelastic properties of blood were investigated using TEG®, a method used in hospitals and clinical laboratories to examine the characteristics of a blood clot. After the *ex vivo* supplementation of vitamin D, the viscoelastic characteristics assessed indicated substantial changes in numerous parameters between the reference and experimental groups. After treatment the median K value in the metastatic PCa group was significantly lower than in the control group, suggesting that clots form more quickly in metastatic PCa patients after *ex vivo* vitamin D supplementation. This is substantiated by the groups considerable increase in α-angle, which shows increased fiber cross-linking in response to an increased thrombin burst. The considerable increase in MA and G parameters seen after treatment in the metastatic group compared to the reference group indicates that platelet and fibrin interaction has increased, resulting in a denser, more rigid clot. Based on this, it was observed that

following *ex vivo* vitamin D addition, clot formation in metastatic patients happened faster and produced stronger clots. The MRTG value increased considerably in both the metastatic and non-metastatic groups after therapy, indicating enhanced clot growth. The absence of significant results following PPP analysis shows that vitamin D supplementation is unlikely to impact the fibrinogen component of hypercoagulation, while additional research using more sensitive methodologies may be required before any conclusions can be drawn.

Through this study, it was determined that supplementation with vitamin D could potentially aid in the pathology experienced due to irregular RBC morphology, although caution should be exercised in metastatic PCa populations as it was found that treatment resulted in a tendency to produce stronger clots at a more rapid rate. It should also be noted that in these experiments a single dose of vitamin D was added to the blood samples and that more frequent dosages might have a bigger effect on the coagulation of these patients.

This study, however, is subject to some limitations, within which the findings need to be interpreted carefully. Firstly, this study was performed on blood obtained *ex vivo*, and thus the vastly more complex variables of vitamin D metabolism when ingested could not be taken into account, and caution should be exercised against directly translating the findings obtained in this study to *in vivo* cases. Secondly, a standard vitamin D dosage was added to all samples, and thus factors such as bodyweight were not taken into account, as would be the case when supplementation would be prescribed in a clinical setting. Thirdly, results of this study may not be completely generalizable due to the restricted sample size, as well as the unequal samples sizes between the reference and experimental groups. The low number of individuals in the reference group will have an impact on the statistical power of the study. Lastly, the exclusion criteria excluding patients if excessive alcohol consumption was present is based on self-reported data and should be noted as a limiting factor.

Further research is warranted to investigate the effects of vitamin D supplementation on hypercoagulability *in vivo*, although vitamin D supplementation does show promise as a more cost-effective treatment regime for the increased thrombotic risk normally observed in patients with PCa.

Additionally, future work could include the relationship between PCa treatment regimens and the coagulation properties of patients' blood. For decades, androgen deprivation therapy (ADT) has been the standard of care for advanced PCa.[65] PCa most likely causes hypercoagulability, which ADT may aggravate, potentially explaining why PCa studies have observed an increase in non-cancer related deaths and VTE is linked to an increased risk of death in patients with PCa.[65]

Addendum 1: MSc Research Committee approval for Protocol 422/2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

18 June 2020

Dr J Bester
Department of Physiology
Faculty of Health Sciences

Dear Dr,

Ms M Schültz, Student no 14005914

Please receive the following comments with reference to the MSc Committee submission of the abovementioned student:

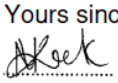
Student name	Michelle Schültz	**Student number**	14005914
Name of study leader	Dr Janette Bester		
Department	Human Physiology		
Title of MSc	**New title: The effect of the *ex vivo* addition of vitamin D on the prothrombotic and the fibrinolytic potential in prostate cancer patients' blood** An *ex vivo* study on the effect of vitamin D supplementation on the prothrombotic risk and fibrinolytic potential of prostate cancer patients		
Date of first submission	May 2020		
Comments to study leader May 2020	• Please consider revising the title, as it does not reflect what you will be doing. • Please revise the structure of the protocol. • Please define which vitamin D level you will be looking for. • Please elaborate in your protocol on the inclusion of patients with growth but not cancerous. • Please revise the flow diagram. • Please correct typographical errors and grammatical errors. • Revise the objectives and elaborate on what will be achieved and how will it be accomplished. • Please substantiate in your protocol the statement stating "…population of South Africa is prone to vitamin D deficiency". • Please add a section in the protocol describing how the candidate will comply with the new UP research data management system. • Please do not include the person's name on the data capturing sheet. The patient's details and identity must be anonymous. • **Please correct references. Please include the full list of the authors.** • Correct formatting errors such as page numbers. • Sentences should not be begin with numbers. • The committee requests that an additional co-supervisor be appointed for the academic support of the student. Please submit the CV of the co-supervisor.		

MSc Committee, School of Medicine
Faculty of Health Sciences
University of Pretoria,
Private Bag X323
Pretoria 0001, South Africa
Tel +27 (0)12 319 2325
Fax +27 (0)12 323 0732

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

	• **Exclusion criteria includes smoking of tobacco or tobacco related products. This should be tested for using the cotinine test.**
June 2020	• Thank you for submitting the revised protocol with a new proposed title. • Please correct references. Please include the full list of the authors. • Check overall formatting of the protocol, correct all typographical and grammatical errors. • Do not capitalise when it is not required (as for name of person, institution, etc.) • Figure 5 depicts a dilution series. Please correct. • Sentences should not be begin with numbers or abbreviations. • Exclusion criteria includes smoking of tobacco or tobacco related products. This should be tested for using the cotinine test. • Please update the authors list. • Please separate and revise the data management (including data capturing, storage and analysis) and the statistical analysis section. • Please revise statistics section.
	• Thank you for submitting the revised protocol and amended MSc form.
Decision	This protocol has been provisionally approved. Please submit the revised protocol to ethics, and supply the MSc committee with proof of acceptance. The internal and external examiners can be nominated and submitted to the MSc Committee six months prior to submission of the dissertation. Please ensure that the CV of the examiners includes: supervision, examination and publication records.

Yours sincerely



Prof Marleen Kock
Chair: MSc Committee

Addendum 2: Ethical Approval for Protocol 422/2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

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

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

26 August 2020

Approval Certificate
New Application

Ethics Reference No.: 422/2020
Title: The effect of the ex vivo addition of vitamin D on the prothrombotic and the fibrinolytic potential in prostate cancer patients' blood

Dear Ms M Schültz

The **New Application** as supported by documents received between 2020-07-09 and 2020-08-26 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2020-08-26 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 2021-08-26.
- Please remember to use your protocol number (422/2020) 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

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 3: Informed Consent – Reference Group

Information leaflet and informed consent form (Reference Group)

Study Title: *The effect of the* ex vivo *addition of vitamin D on the prothrombotic and the fibrinolytic potential in prostate cancer patients' blood*

Sponsor: Thuthuka Trust

Principal Investigator: Miss. Michelle Schültz
Department of Physiology, University of Pretoria
078 160 6538

Ethical clearance number: 422/2020

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

Date and time

Dear prospective participant

Dear Mr. .....

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 the association of vitamin D and prostate cancer with regards to increased clot formation (hypercoagulation), compared to individuals without prostate cancer. This will allow us to understand if prostate cancer patients in South Africa have low vitamin D levels and have changed blood clotting properties. We also want to investigate if the addition of vitamin D to blood samples will improve these clotting properties in prostate cancer patients. To do this research, we will use

specialized microscopes (scanning electron microscope and light microscope) to look at the structure of the RBCs; as well as equipment that tests blood clotting properties (called a thromboelastograph and turbidimetry) to determine the degree to which clotting is changed in the blood. We will then add vitamin D to that sample and test it again to see if the clotting and blood cell structure improves. We will compare the results to those of participants who have prostate cancer. **Your blood sample will be used as part of a reference group of healthy controls, meaning that you do NOT have prostate cancer, but will be compared to participants who do have prostate cancer.**

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

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

2 tubes of blood will be drawn by a qualified nurse from the urology department into a citrate tube, each containing 5ml of blood or the equivalent of one teaspoon.

The samples will be used to do scanning electron microscopy, light microscopy, turbidimetry, thromboelastography as well as measuring vitamin D levels.

We will then add vitamin D to one of the samples and do the tests again.

The blood drawl process will only be done once, and no follow-up tests will be required. Also, vitamin D will be added to the blood samples AFTER the blood is drawn and NOT to the patient directly.

4) 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. The University of Pretoria has limited insurance for research-related injuries.

5) Possible benefits of this study.

Although you may not benefit directly, the study results may help us to improve the treatment and understanding of prostate cancer 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.

6) Compensation

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

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

9) Ethics approval.

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

10) Information

If you have any questions concerning this study, please contact Miss Michelle Schültz tel: 078 160 6538

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. This will ensure the confidentiality of information so collected. Only the researcher will be able to identify you as a participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of all

your records will be kept in a locked facility at the Department of Urology at The University of Pretoria**.**

12) Consent to participate in this study

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

……. ……..

Participant's name and signature Date

……. ……..

Investigators name and signature. Date

……. ……..

Witness name and signature. Date

Participant code……

Verbal 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 and signature. Date

Addendum 4: Informed Consent – Experimental Group

Information leaflet and informed consent form (Prostate cancer patients)

Study Title: *The effect of the* ex vivo *addition of vitamin D on the prothrombotic and the fibrinolytic potential in prostate cancer patients' blood*

Sponsor: NRF Thuthuka

Principal Investigator: Miss. Michelle Schültz
Department of Physiology, University of Pretoria
078 160 6538

Ethical clearance number: 422/2020

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

Date and time

Dear prospective participant

Dear Mr. .....

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 the association of vitamin D and prostate cancer with regards to increased clot formation (hypercoagulation), compared to individuals without prostate cancer. This will allow us to understand if prostate cancer patients in South Africa have low vitamin D levels and have changed blood clotting properties. We also want to investigate if the addition of vitamin D to blood samples will improve these clotting properties in prostate cancer patients. To do this research, we will use specialized microscopes (scanning electron microscope and light microscope) to look

at the structure of the RBCs and fibrin fibres; as well as equipment that tests blood clotting properties (called a thromboelastograph and turbidimetry) to determine the degree to which clotting is changed in the blood. We will then add vitamin D to that sample and test it again to see if the clotting and blood cell structure improves. We will compare the results to those of participants who do not have prostate cancer.

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

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

2 tubes of blood will be drawn by a qualified nurse from the Urology department into a citrate tube, each containing 5ml of blood or the equivalent of one teaspoon.

The samples will be used to do scanning electron microscopy, light microscopy, turbidimetry, thromboelastography as well as measuring vitamin D levels.

We will then add vitamin D to one of the samples and do the tests again.

The blood drawl process will only be done once, and no follow-up tests will be required. Also, the vitamin D will be added to the blood samples AFTER the blood is drawn and NOT to the patient directly.

4) 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. The University of Pretoria has limited insurance for research-related injuries.

5) Possible benefits of this study.

Although you may not benefit directly, the study results may help us to improve the treatment and understanding of prostate cancer 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.

6) Compensation

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

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

9) Ethics approval.

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

10) Information

If you have any questions concerning this study, please contact Miss Michelle Schültz tel: 078 160 6538

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. This will ensure the confidentiality of information so collected. Only the researcher will be able to identify you as a participant. Results will be published or presented in such a fashion that patients remain unidentifiable. The hard copies of all your records will be kept in a locked facility at the Department of Urology at The University of Pretoria**.**

12) Consent to participate in this study

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

…… ……..

Participant's name and signature Date

…… ……..

Investigators name and signature. Date

…… ……..

Witness name and signature. Date

Participant code…….

Verbal 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 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 and signature. Date

Addendum 5: Letter of Statistical Support

BIOSTATISTICS UNIT

27 April 2020

LETTER OF STATISTICAL SUPPORT

This letter confirms that **M. Schultz** from the **Department of Human Physiology, Faculty of Health Sciences of the University of Pretoria** discussed her project: **"An ex vivo study on the effect of vitamin D supplementation on the prothrombotic risk and fibrinolytic potential of prostate cancer patients"** with me. I confirm that I will assist with the statistical analysis of the study data.

Data analysis

Descriptive statistics mean, median, standard deviations and interquartile ranges (with 95% confidence intervals where appropriate) will be used to describe TEG parameters and other continuous variables. Categorical variables will be described using frequencies and proportions. Associations between categorical variables will be tested for using Fisher's exact test. Analysis of covariance (ANCOVA) will be used to compare the difference in mean TEG parameters, as well as axial ratios, before and after the Vitamin D addition to samples, and baseline Vitamin D levels. Appropriate contrasts will be tested post-hoc. The correlation between change in TEG parameters and baseline vitamin D levels will be calculated using Pearson's and Spearman Rank correlations. Where appropriate log transformations of variables will be considered. Non-parametric techniques will be considered in the case of non-normality. All tests will be conducted at a 5% level of significance. All tests will be done using STATA 15.

Sample size

The researcher will recruit 20-30 patient samples in each of the 3 groups, resulting in a sample of 60 – 90.

Name: C Janse van Rensburg
Biostatistics Unit
MRC Pretoria
012 339 8529
Charl.JansevanRensburg@mrc.ac.za

Addendum 6: NHRD Approval

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

STEVE BIKO ACADEMIC HOSPITAL

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

For attention: janette Bester

NHRD Ref Number: GP_202010_029

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

TITLE: The effect of the ex vivo addition of vitamin D on the prothrombotic and the fibrinolytic potential in prostate cancer patients' blood

Permission is hereby granted for the above-mentioned research to be conducted at Steve Biko Academic Hospital.
This is done in accordance to the "Promotion of access to information act No 2 of 2000".
Please note that in addition to receiving approval from Hospital Research Committee, the researcher is expected to seek permission from all relevant department.
Furthermore, collection of data and consent for participation remain the responsibility of the researcher.
The hospital will not incur extra cost as a result of the research being conducted within the hospital.
You are also required to submit your final report or summary of your findings and recommendations to the office of the CEO.

Approved

Comment: _____

Date: 2020-10-26

Dr. J S. Mangwane
Manager: Medical Service

Addendum 7: Ethical Approval - Annual Submission for Protocol 422/2020

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

16 September 2021

Approval Certificate
Annual Renewal

Dear Ms M Schültz

Ethics Reference No.: 422/2020
Title: The effect of the ex vivo addition of vitamin D on the prothrombotic and the fibrinolytic potential in prostate cancer patients' blood

The **Annual Renewal** as supported by documents received between 2021-08-18 and 2021-09-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-09-15 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 2022-09-16.
- Please remember to use your protocol number (422/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

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

We wish you the best with your research.

Yours sincerely

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

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

Research Ethics Committee
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Addendum 8: Turnitin Submission Report

MSc Thesis - Michelle Schultz

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