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ELECTRON, CONFOCAL AND ATOMIC FORCE MICROSCOPIC ANALYSIS OF PLATELETS, FIBRIN AND ERYTHROCYTES IN ATOPIC ASTHMA

BY

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DECLARATION

I, Sajee Alummoottil, declare that:

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DEDICATION

Do not be anxious about anything,
but in every situation,
by prayer and petition,
with thanksgiving,
present your requests to God.

And the peace of God,
which transcends all understanding,
will guard your hearts and your minds endeavours.

- *Philippians 4:6-7*

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PUBLICATIONS

1. Alummoottil S. Phulukdaree A, Phasha M.N. Bester J. Grobbelaar C. van Rooy M. Pretorius E. Ultrastructural evaluation of clot properties and gene expression of clotting factors XII and XIII in asthma. Manuscript number: BJH-2018-00358 *(Original article, appendix 5)*

2. Alummoottil S. Phulukdaree A. Buys A. Bester J. van Rooy M. Grobbelaar C. Pretorius E. Analysis of the formed elements in blood: erythrocytes and platelets in asthma *(Original article)*

3. Alummoottil S. Phulukdaree A, van Rooy M. Pretorius E. Atopic Asthma, an allergic airway inflammatory disorder: a mechanistic insight *(Review article)*

PRESENTATIONS

The paper titled: Ultrastructural evaluation of clot properties and gene expression of clotting factor XIII in asthma. Alummoottil S. Phulukdaree A, Phasha M.N. Bester J. Grobbelaar C. van Rooy M. Pretorius E. was accepted for presentation at the following institutional, national and international conferences:

1. University of Pretoria, Faculty of Health Science: Faculty Day 22-23 August 2017, South Africa

2. 45th Conference Of The Physiology Society Of Southern Africa, 27th – 30th August 2017, Groenkloof Campus Auditorium, Pretoria, South Africa

3. 25th Fibrinogen and 3rd FXIII Workshop, June 3 - 7, 2018 at the Graylyn International Conference Centre of Wake Forest University, North Carolina, USA (appendix 4)

ABSTRACT

Background: The underlying risk factor of non-communicable diseases is inflammation. The pathophysiology of asthma and/or allergic asthma is also, no surprisingly, also linked to chronic inflammation. The prevalence and incidence of asthma is on the increase, despite of a variety of treatment modalities. Asthma therefore is a growing burden on healthcare system and on society.

Asthma pathophysiology is not only linked to inflammation, but also with activation of coagulation and reduced fibrinolysis due to plasma and locally derived factors in the airways. Alterations to these factors may thus influence the biophysical and mechanical properties of the coagulation, fibrin deposition followed by asthma pathology.

Methodology: The objective of this study was to evaluate changes of blood components in asthma patients (n=30) compared to controls (n=30) using routine haematology screening using a haematology analyser; structural changes and axial ratio analysis using light microscopy (erythrocytes), elasticity using atomic force microscopy (erythrocytes); ultrastructural changes using scanning electron microscopy (erythrocytes, platelets and fibrin), transmission electron microscopy (platelets) and confocal microscopy (platelets) as well as the viscoelastic properties of the fibrin clot using thromboelastography; and mRNA levels of FXIII-A in whole blood by quantitative PCR.

Results: The haematology findings of controls and asthma patients were within normal clinical ranges. There was, however, a significantly higher level of circulating monocytes ($p=0.0066$), erythrocytes ($p=0.0004$), haemoglobin ($p=0.0057$) and haematocrit ($p=0.0049$).

The analysis of eosin stained erythrocytes by light microscopy showed more echinocytes, acanthocytes and ovalocytes compared to controls and the axial ratio was also significantly higher (controls: 1.2±0.22nm vs. asthma: 1.165±0.16nm, $p<0.0001$). Atomic force microscopy findings showed significantly reduced erythrocyte membrane elasticity ($p=0.001$). Morphological changes of erythrocytes were also noted from scanning electron microscopy.

Platelet morphology and ultrastructure were qualitatively assessed using scanning electron microscopy, transmission electron microscopy and confocal microscopy and showed morphological changes indicative of platelet activation in asthma samples.

In addition, the assessment of clot kinetics by thromboelastography also showed alteration with a tendency to produce stronger fibrin clots in asthma samples. The reaction time was higher ($p<0.0001$), alpha-angle was lower ($p<0.0001$), maximum rate of clot formation was higher ($p<0.0001$) – all indicative of a longer time for clotting to occur but the maximal amplitude which is indicative of clot strength and stability was significantly higher in asthma samples ($p=0.0478$). As the aggregation and cross-linking of fibrin fibres depends on factor XIII (FXIII), its excessive production may lead to alterations in fibrin polymerisation and crosslinking resulting in stronger fibrin clot formation and resistance to fibrinolysis. Densitometry analysis of scanning electron microscopy images of fibrin fibres showed a significantly lower variance in asthma samples ($p<0.0001$) indicative of more matt like structures compared to normal fibrin fibres in controls.

The analysis of gene expression by qPCR revealed a significant 17.34-fold higher FXIII-A mRNA level in whole blood of asthma patients compared to controls indicating greater potential for *de novo* production of FXIII-A compared to the control group. These results would facilitate further research possibilities for identifying potential biomarkers in allergic asthma.

These findings support the hypothesis of this study: enhanced coagulation may be attributed to the altered morphology and activation of platelets, erythrocyte and fibrin networks in asthma.

Conclusion: In conclusion, altered erythrocyte and platelet morphology, excess production of FXIII-A, altered fibrin architecture and clot properties affects the coagulation profile in asthma, systemically. Further research is needed to extrapolate exact mechanism by which increased systemic coagulation contributes to the pathophysiology of the disease locally.

LIST OF ABBREVIATIONS

α_2AP	A2-antiplasmin
µl	microliter
ADAM33	associated with disintegrin and metalloproteinase domain 33 gene
AFM	atomic force microscopy
AIDS	acquired immunodeficiency syndrome
CD	cluster of differentiation
cDNA	complimentary deoxyribose nucleic acid
cFXIII	cytoplasmic factor thirteen
CLCA1	calcium-activated chloride channel regulator 1
CM	confocal Microscopy
CV	coefficient of variation
DALY	disability-adjusted life year
DNA	deoxyribose nucleic acid
FI	Fibrinogen
FII	Prothrombin
FIII	tissue factor
FIV	Calcium
FV	Proacclerin, labile factor
FVI	Unassigned
FVII	Stable factor, proconvertin
FVIII	Antihaemophilic factor A
FIX	Antihaemophilic factor B
FX	Stuart-Prower factor
FXI	Plasma thromboplastin antecedent
FXII	Hageman factor
FXIII	Fibrin-stabilising factor/ factor thirteen
FXIIIa	activated factor thirteen
FXIII-A	factor thirteen sub unit A
FXIII-B	factor thirteen sub unit B
FXIV	Pre-kallikerin
FXV	HMWK

FXVI	vWF
FXVII	Anti-thrombin III
FXVIII	Heparin cofactor II
FXIX	Protein C
FXX	Protein S
GINA	Global Initiative of Asthma
Gp	glycoprotein
H_0	null hypothesis
H_1	alternate hypothesis
Hb	haemoglobin
HCT	haematocrit
HIV	human immunodeficiency virus
HMDS	hexamethyldisilazane
Ig	immunoglobulin
IgE	Immunoglobulin E
IL	interleukin
K	Kinetics
LM	light microscopy
LT	leukotriene
MA	Maximum amplitude
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
Min	minutes
ml	millilitre
MPV	mean platelet volume
mRNA	messenger ribonucleic acid
MRTG	Maximum rate of thrombus generation
nm	nanometre
OCS	open canalicular system
OsO_4	osmium tetraoxide
PAF	platelet activating factor
PAI-I	plasminogen activator inhibitor-I

PAR	protease activated receptors
PBS	phosphate buffer saline
PCT	plateletcrit
PDW	platelet distribution width
PG	prostaglandin
pFXIII	plasma factor thirteen
POSTN	periostin
PPP	platelet poor plasma
PRP	platelet rich plasma
qPCR	quantitative polymerase chain reaction
R	Reaction time
RDW	red blood cell distribution width
RNA	ribose nucleic acid
Sec	seconds
SEM	scanning electron microscopy
SERPINB2	serpin peptidase inhibitor, clad B member 2
TAFI	thrombin activated fibrinolysis inhibitor
TEG	thromboelastography
TEM	transmission electron microscopy
TF	tissue factor
TFPI	tissue factor pathway inhibitor
Th	T helper
TIM1	T-cell immunoglobulin domain
TMRTG	Time to maximum rate of thrombus generation
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TTG	Total thrombus generation
uPA	urokinase plasminogen activator
vWf	von Willebrand factor
WBCC	white blood cell count
X	times
Xg	centrifugal force

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CHAPTER 1
INTRODUCTION

According to the Global Asthma Report 2014, asthma is a highly prevalent chronic airway disease that affects 334 million people of all ages worldwide and is expected to increase by another 100 million by 2025 due to urbanization. Asthma therefore imposes a substantial burden on healthcare system throughout the world and also on society where individuals are affected through loss of quality of life. Although asthma is common among children, the burden of asthma impact increases with age, especially in elderly women.

Despite of a variety of treatment regimens, the prevalence of asthma is on the increase. Thus, there is a need to better understand the pathophysiology associated with asthma to translate scientific knowledge into improved treatment and patient management. Microscopy is an emerging technique that allows for the investigation of the ultrastructural properties of components of blood. It can unfold a category of information regarding structural and mechanical properties of the cells, aiding in the understanding of the functional and physiological properties of the components associated with asthma.

Asthma is a complex heterogeneous disease affecting the respiratory airways, characterised by airway inflammation, bronchoconstriction, airway remodelling and bronchial hyper reactivity. One of the major risk factor for asthma is allergy or atopy [a predisposition for immunoglobulin E (IgE) to allergens]. Both environmental and genetic factors play an important role in the pathogenesis of allergic asthma. A central feature of asthma pathology is airway inflammation. Similar to a chronic wound, a plethora of biochemical mechanisms initiate and propagate allergic inflammatory responses in asthma due to a wide range of environmental insults such as allergens, pathogens, pollutants, certain drugs and chemicals.

Inflammation is also a multifaceted and complex process, which is well controlled and critical for the normal physiology of the body to maintain homeostasis. Uncontrolled inflammation, however, can have detrimental consequences. A complex network of mediators and pathways orchestrate allergic inflammatory cascade in allergic asthma. These mechanisms cause the attraction and accumulation of immune cells and also the extravasation of plasma from the small blood vessels into the interstitial space in inflamed tissue. Altered coagulation cascade is one of the mechanisms that are potentiated due to inflammatory conditions. Evidence suggests a relationship exists between disturbed haemostasis and asthma pathogenesis.

Haemostasis is a normal physiological process that maintains blood-fluidity and has the potential to induce the formation of a clot to prevent bleeding. Blood is a fluid, composed of erythrocytes (red blood cells), leukocytes (white blood cells), platelets and plasma proteins such as fibrinogen suspended in plasma. The functions of blood include: transporting oxygen and carbon dioxide, nutrients, signalling molecules such as hormones and waste materials. Coagulation, another function of blood during injury is to prevent excessive bleeding and reduce blood loss by forming a clot at the site of injury. Asthma is a chronic inflammatory disease in which abnormalities in coagulation and fibrinolysis feature prominently among multiple mechanisms that have been implicated in the pathogenesis of asthma.

Coagulation occurs through a cascade, involving the interactions of various blood components leading to the conversion of individual molecules of the soluble protein fibrinogen into insoluble fibrin fibres in a complex network of fibrin, erythrocytes and platelets. Blood components such as platelets, erythrocytes and fibrin networks are sensitive to inflammation, and changes in these components can influence the coagulation process. Therefore the coagulation profile and the resultant clot as well as assessment of the blood components can be used as indicators of disease status of an individual as well as progression and severity of disease. Changes in the structure and function of blood components manifest in many inflammatory related diseases such as alzheimer’s disease, parkinson’s disease, lupus, thrombotic diseases,

rheumatoid arthritis and human immunodeficiency virus infection and acquired immunodeficiency syndrome (HIV/AIDS). Pathological changes in erythrocytes, platelets and fibrin have been studied extensively, however, little is known about the altered coagulation system leading to morphological change of blood components in asthma.

The effect of inflammation is not isolated to platelets and erythrocytes but also influences non-cellular components such as fibrin formation. Fibrin is a key component of blood clots and several factors influences its formation through the mode of polymerisation and cross-linking, consequently influencing the architecture of the resulting fibrin network and stability and persistence of the blood clot. Coagulation factor XIII (FXIII) is a unique coagulation protein that stabilizes the clot by determining its mechanical strength or stiffness, thus affecting fibrinolysis and ultimately, thrombolysis. The role of FXIII has recently come under the spotlight drawing much interest among researchers who study haemostasis. Therefore this study also investigated the messenger ribonucleic acid (mRNA) levels of FXIII subunit A (FXIII-A) in circulation to assess the potential for *de novo* synthesis and influence on clot properties.

Ultrastructural and mechanical properties of cellular blood components involved in the formation of clots have yet to be explored in asthma. The overarching aim of this study was therefore to study plasma, erythrocytes and platelets in allergic asthma, as little is known about how inflammation affects these formed elements in atopic asthma. To the best of our knowledge this is the first study to evaluate the ultrastructural properties of blood, platelets and fibrin in allergic asthma patients not only in a South African cohort, but globally. Asthma is an incurable disease but its symptoms can be controlled with proper treatment. Therefore, this research can provide insight on the pathophysiology of the disease, which can shed light towards additional treatment options to support the available therapies.

This study hypothesis is therefore:

H_0 Enhanced coagulation and inflammation does not alter the morphology of fibrin networks, platelet and erythrocyte, as well as the degree of platelet activation in atopic asthma.

H_1 Enhanced coagulation and inflammation alters the morphology of fibrin networks, platelet and erythrocyte, as well as the degree of platelet activation in atopic asthma.

Following from the hypothesis, the research questions/objectives as described below, directed the preparation of this thesis:

OBJECTIVE 1: The study of biochemical parameters linked to coagulation was done to compare the haematology parameters between atopic asthma and healthy controls.

OBJECTIVE 2: The qualitative evaluation of the differences between the ultrastructure, axial ratio and elasticity of erythrocytes using scanning electron microscopy, light microscopy and atomic force microscopy, respectively, in atopic asthma and healthy controls.

OBJECTIVE 3: The qualitative evaluation of the differences between the ultrastructure and activation of platelets between atopic asthma and healthy controls, using scanning electron microscopy, transmission electron microscopy and confocal microscopy.

OBJECTIVE 4: The quantitative assessment of the kinetics of coagulation in platelet poor plasma to determine the reaction time, speed at which clot formation occurs, rate of cross-linking and clot strength between atopic asthma and healthy controls using thromboelastography.

OBJECTIVE 5: The qualitative assessment of the differences between the ultrastructure of fibrin using scanning electron microscopy and densitometry analysis; and quantitative evaluation of the amount of factor XIII-A mRNA in whole blood.

This thesis includes a review of the literature pertaining to atopic asthma providing a summary of the available research findings that supports the inclusion of each of the research objectives. The outcome of the different objectives of the study will be discussed in the subsequent chapters.

The research chapters include patient demographics, haematological parameters, ultrastructural and mechanical properties of erythrocytes, platelets, fibrin, clot kinetics and FXIII-A mRNA levels in asthma. Furthermore, ultrastructural changes of erythrocytes and platelets of allergic asthma patients were assessed in this study and discussed. The ultrastructural changes include shape changes of erythrocytes and aggregation and activation of platelets. The changes of the blood components seen in asthmatic patients were compared with those of healthy controls.

The techniques used in this study include light microscopy (LM) (also used to measure axial ratios of erythrocytes) scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal microscopy (CM), atomic force microscopy (AFM), viscoelastic tests [thromboelastography (TEG)] and quantitative polymerase chain reaction (qPCR), to assess FXIII-A mRNA. Microscopy can be employed to study the pathological changes of blood components. It is a simple, non-invasive process that yields crucial information.

CHAPTER 2
LITERATURE REVIEW

2.1 Overview of Asthma

Asthma, a chronic inflammatory disorder (1), affects more than 300 million individuals worldwide with prevalence rates ranging from 1% to 18% in different countries and is expected to increase up to 400 million by 2025 (2-4). Considering the prevalence, extent and duration of the episodes, asthma is the fourteenth most significant chronic disease in the world (5). Currently 5.4 million people receive treatment for asthma and three people die from asthma on average per day (6). Asthma is characterised by airway eosinophilic inflammation and structural changes (remodelling), which are associated with an irreversible loss in lung function (7, 8).

Asthma is known as a heterogeneous inflammatory syndrome with a series of complex, overlapping individual diseases characterised by distinct genetic components, environmental causes and immune-pathological signatures (9, 10). Atopy is a strong predisposing risk factor for asthma (11) thus a model of asthma has evolved in recent decades to describe asthma as an allergic disease, in which allergen exposure causes allergic sensitisation. As asthma is much more multifaceted than just allergic asthma, asthma phenotypes have been developed to address the complexities of the disease to provide personalised asthma management. The term 'phenotype' has been used to denote a wide array of similar clinically observable characteristics by cluster analysis based on demographic, clinical and pathophysiology of asthmatic patients (6, 12). In this literature review the epidemiology of the condition, its classification and why it may be referred to as an inflammatory condition is discussed. As the focus of this thesis is the effects of asthma on the haematological system, emphasis and attention will be placed on what is known about one of the important hallmarks of inflammation, namely (pathological) coagulation. See figure 2.1 for the layout of this chapter.

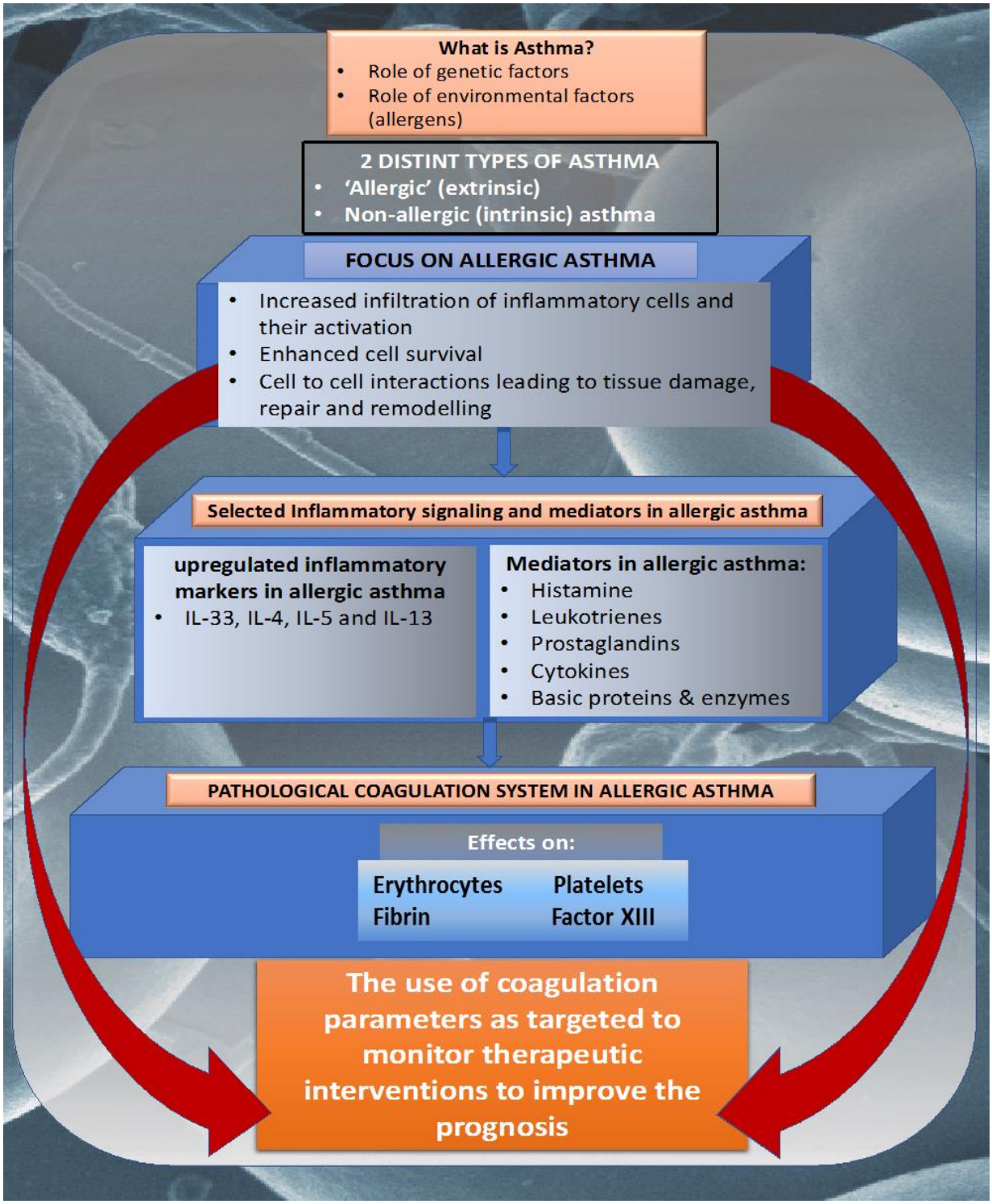

Figure 2.1: Layout of this chapter: Asthma as an inflammatory condition and its effects on the haematological system

2.2 Epidemiology of Asthma

The prevalence and the severity of asthma have increased over the last century and it is one of several non-communicable medical burdens on the health systems of a country, yet the pathogenesis of this disease is not fully understood. Asthma is the fourteenth most important contributor to disability in the world in terms of extent and duration of the disorder. According to the world health report 2014, 334 million people have asthma. This includes 14% of the world's children and 8.6% of young adults (aged 18-45 years) that experience asthma symptoms while 4.5% of young adults have been diagnosed with asthma and/or are on asthma treatment. (13). The disability-adjusted life year (DALY) is a measure of overall disease burden, expressed as the number of years lost due to ill-health, disability or early death, and figure 2.2 shows the significant effect of asthma in African countries(13).

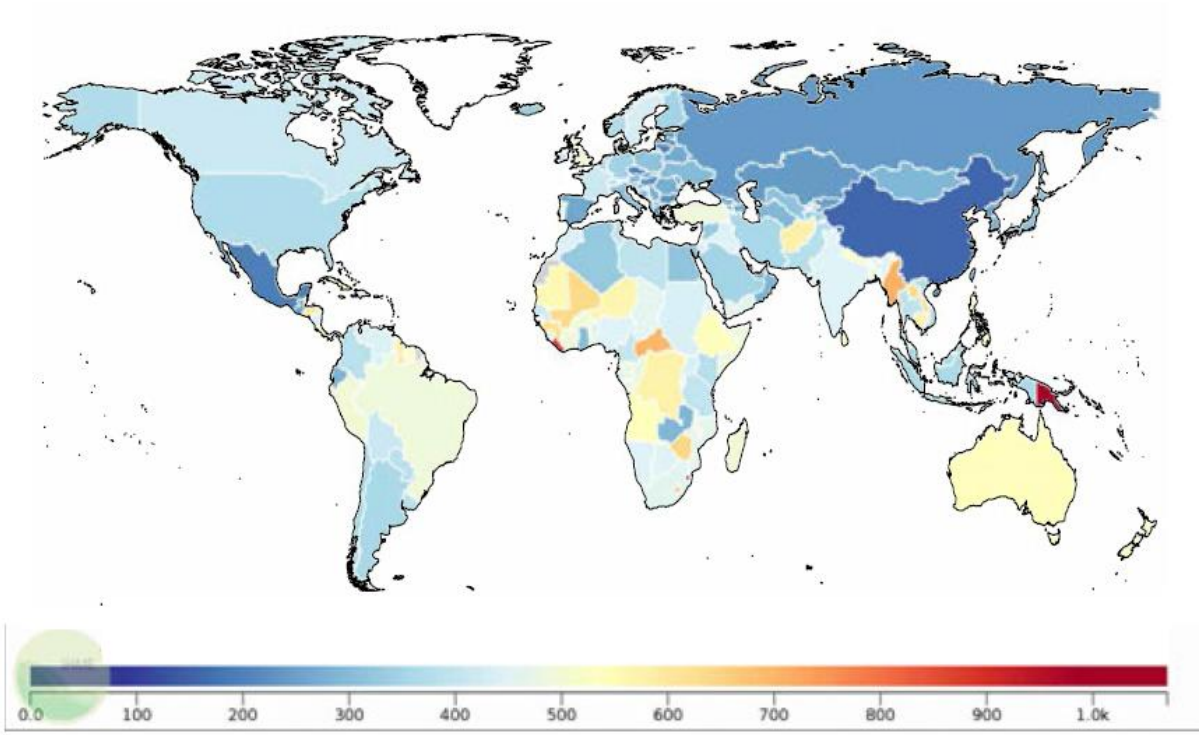

Figure 2.2: Disability adjusted life years per 100,000 population (heat map data from 2010) attributed to asthma showing the significant effect in African countries (13)

2.3 Classification of Asthma

The concept of asthma classification evolves as our understanding regarding its pathophysiology increases.

Table 2.1: Linking essential pathogenic mechanisms with asthma phenotypes (reproduced from Agache *et al.* 2012) (14)

Asthma phenotypes and corresponding endotypes				
Allergic asthma	**Intrinsic asthma**	**Neutrophilic asthma**	**Aspirin intolerant asthma**	**Extensive remodelling asthma**
Eosinophilic	Eosinophilic	Activation of innate immune response	Eosinophilic	Lack of inflammation/extensive remodelling
Th2 mediated inflammation	Neutrophilic	Histone Deacetylase C2 (HDAC2) abnormal recruitment	Alteration in the eicosanoid metabolism/sensitivity to leukotrienes C4,D4 and E4	Abnormal epithelial-mesenchymal trophic unit activation
Steroid - responsive	Associated with antibodies/antigens	Increased neutrophil survival	Steroid-responsive	Abnormalities of airway smooth muscle (ASM)
Responsive to allergen specific immunotherapy	Steroid-responsive	Responsive to antioxidants/ antibiotics	leukotriene receptor antagonist - responsive	Defective repair mechanisms
Anti IgE responsive	Steroid-resistant	Anti TNF-α responsive		Steroid resistant
Anti IL-5 responsive		Responsive to HDAC regulators		ASM-targeted treatment responsive
Anti IL-4/IL-13 responsive				Matrix metalloprotease-targeted treatment responsive

Th: T helper 2, Ig: immunoglobulin, TNF: tumour necrosis factor, IL: interleukin

Asthma is, however, classically divided into two distinct phenotypes: 'allergic' (atopic/extrinsic) and non-allergic (intrinsic) asthma. A distinction is also made between 'eosinophilic ('type 2 high') and non-eosinophilic ('type 2 low') asthma (6, 15, 16). People with extrinsic asthma, are atopic with an IgE mediated sensitization to aeroallergens (17, 18). Intrinsic asthma is prevalent in adulthood with no clinical or family history of allergy (16, 19-21). There is a genetic predisposition in atopic asthma which develops in childhood and is considered to be more responsive to treatment than intrinsic asthma (14). Asthma phenotypes (without insights into the pathological mechanisms) are clinically relevant; however, asthma endotypes describe subtypes of the disease based on cellular and molecular mechanisms (Table 2.1) (14, 22).

2.4 Genetic/Hereditary factors

The basis of human disease is the genotype-phenotype linkages (23). In asthma, interaction between genes and environmental factors affects the immune response. Population studies have shown the role played by cluster of differentiation 14 (CD14) gene and the innate-immunity genes and their significance on the susceptibility to asthma and allergy (24). Overexpression of several genes has been implicated in the pathogenesis of atopy and asthma (25). Although there is an appreciable genetic component associated with the disease, external factors significantly influence and regulate the immune system in asthma (26). Inflammation, together with genetic predisposition therefore forms the basis for development of asthma.

Allergic asthma is a heterogeneous disease which involves the intricate interplay between gene and environmental factors leading to an altered immune response (24, 27, 28). The genetic complexity is evident from the number of genes identified (35% - 95%) as contributors to the symptomatic manifestations in asthma (29, 30). The strongest risk factor for developing asthma is atopy (11, 31). Several genes of multiple loci that are associated with asthma have been identified. These include associated with disintegrin and metalloproteinase domain 33 gene *(ADAM33), DPP10, PHF11, GPRA,* T-cell immunoglobulin domain, mucin like domain *(TIM-1), PDE4D, OPN3, ORMDL3* and *GSDML* which have been implicated in the pathogenesis of allergic

asthma and other inflammatory diseases (25). The genetic variation in the genes encoding *ORMDL3* and *GSDML* on chromosome 17q21 locus has been specifically associated with the susceptibility to asthma (29, 32-34).

The development of asthma and disease progression is *ADAM33* found on chromosome 20p13 (35, 36). The gene *ADAM33* is expressed in airway smooth muscle cells, coding for disintegrin and metalloproteinase glycoprotein involved in intercellular and cell-matrix interactions. These are key molecules implicated for the mechanisms of smooth muscle remodelling and airway hyperresponsivenes (37).

Animal models provide evidence that the *TIM-1* gene is associated with allergic airway inflammation (38). Genetic and epidemiologic studies show that asthma and atopic asthma is associated with polymorphism in the *OPN3* gene on 1qter hence a putative asthma susceptibility gene (39). In asthma, this gene plays an important role in immune modulation (5). Other studies, however, argue that there is no one specific gene or environmental factor that solely accounts for asthma (39, 40). Expression of three signature genes: periostin *(POSTN)*, calcium-activated chloride channel regulator 1 (*CLCA1)* and serpin peptidase inhibitor, clad B member 2 (*SERPINB2*) are upregulated by the T helper 2 (Th2) cytokine, interleukin-13 (IL-13). Levels of IL-13 in the airway is used to identify “Th2 high” individuals who have been diagnosed as asthmatic (16).

2.5 Non-genetic (environmental and behavioural) risk factors

When considering non-genetic risk factors of asthma, it is important to distinguish between the triggers of asthma attacks and the causes of the underlying pathological process. Both groups of factors contribute to the severity and persistence of asthma.

Environmental factors include tobacco smoke (including second hand smoke and prenatal exposure), allergens, microorganisms, air pollutants, medication, food allergens (food additives and preservatives)(13). These factors play a pivotal role in

the induction and exacerbation of allergic asthma (25, 41-43). The inflammatory and allergic responses in asthma can also be attributed to many environmental factors such as the presence or inhalation of dust mites, domestic animals, cockroaches and moulds (44). Exposure to air pollutants and tobacco smoke promotes IgE synthesis resulting in allergen sensitization and increases the risk of developing asthma (45, 46). In atopic asthma, allergen exposure is an aggravating factor in both children and adults (47).

Currently there is no cure for atopic asthma, only symptomatic treatment and the progress in the development of new effective medication for atopic asthma is also very slow (31). Despite the availability of treatment, the incidence of atopic asthma has doubled in the past decades due to frequent exacerbations and steroid resistance hence the economic burden of asthma has escalated (48, 49). Due to the inflammatory nature of the condition, the next paragraphs will discuss inflammation, specific pro-inflammatory mediators in asthma, and how it affects haemostasis.

2.6 Inflammation

Inflammation is a normal response to injury and is beneficial to the host during the first phases of wound healing (50); however, an abnormal immune response to stimuli leads to chronic inflammation (31). The initiation and perpetuation of allergen-driven inflammation is considered to be the central feature among many factors that play a role in the pathophysiology of allergic asthma (51). An altered immune response initiates and drives chronic inflammation in allergic asthma and involves an orchestrated interplay between the airway epithelium, innate immune system and adaptive immune system (50, 52).

Airway inflammation is a hypersensitive reaction which involves various cell types and multiple mediators and pathways (22, 25, 26). The development of chronic inflammation in allergic asthma depends on the increased influx of inflammatory cells such as eosinophils, mast cells, T lymphocytes and platelets; furthermore the subsequent activation of platelets, enhanced cell survival and cell-to-cell interactions

in the airways also plays a role (53-55). The first phase of an allergic response involves allergen sensitization which is the ability of the airways to recognise the allergen by processing it by dendritic cells and then presenting them to lymphocytes to evoke Th2 cytokine response (56). These inflammatory cells and their mediators orchestrate the pathogenic features of asthma. Accumulating evidence indicates that many cellular and molecular mediators regulate chronic allergic inflammation in asthma.

2.7 Specific pro-inflammatory mediators in asthma

A complex network of pro-inflammatory mediators is linked to allergic inflammation in asthma. The inflammatory cells release mediators such as lymphokines (immunomodulatory cytokines released by T cells), proinflammatory cytokines (cytokines that promote and amplify the inflammatory response), chemokines (cytokines that are chemoattractants for leukocytes), growth factors (factors that promote cells survival) and eicosanoids (lipid mediators that have multiple effects in the airway), which promote allergic inflammation in asthma (31, 57, 58). The eicosanoids, end-products of arachidonic acid metabolism, are recognized as key mediators in inducing the inflammatory response in asthma and include leukotrienes (LTs), prostaglandins (PGs) and thromboxane (5). These mediators are capable of inducing inflammation by the accumulation of immune cells and plasma extravasation from small vessels into the interstitial space in inflamed tissue and bronchoconstriction (59). Leukotrienes are involved in the progression of allergic diseases such as asthma, cardiovascular disease and rheumatoid arthritis (60). Leukotrienes also play an important role in pathophysiology of asthma by mediating inflammation and bronchoconstriction (5).

Antigen-specific Th2 helper cell cytokines, such as IL-4, IL-5 and IL-13 mediate allergic inflammation in asthma (6, 61). IL-13 plays a pivotal role in regulating allergic inflammation (62) by stimulating the differentiation of naïve T cells into Th2 helper cells (63-65). Furthermore, IL-13 contributes to airway remodelling by promoting the deposition of extracellular matrix in the asthmatic airways (66). High levels of Th2

cytokines promote allergic inflammation in asthma by increasing IgE synthesis and facilitating the infiltration of eosinophils resulting in hyper responsiveness in airways (17, 21, 67). The humoral antibody IgE is bound to high-affinity receptors on the surface of the basophils and mast cells present in the subepithelial layer of airways resulting in their activation and the release of inflammatory mediators such as leukotrienes, prostaglandins and histamines (figure 2.3). Furthermore these mediators are implicated in inducing airway smooth muscle contraction and stimulate mucous hypersecretion by goblet cells resulting in the narrowing of airways in asthma (25, 68, 69). Pro-inflammatory molecules that are upregulated in asthma are also known to be upregulated in coagulopathies (70).

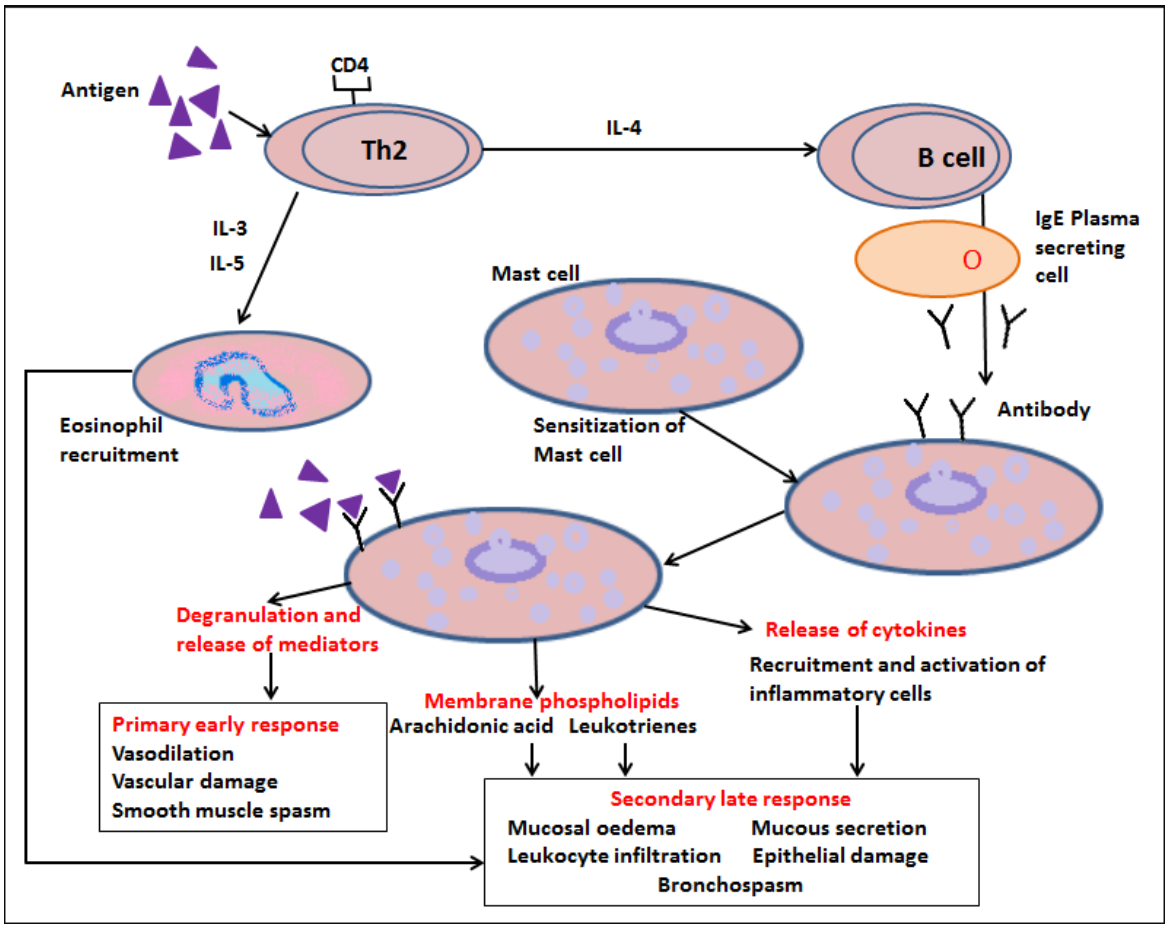

Figure 2.3: Shows various immune cells and mediators that are involved in the inflammatory process [adapted from (71)]. IL: interleukin, CD: Cell differentiation, IgE: immunoglobulin E, Th: T helper

2.8 Haemostasis

Haemostasis is a physiological mechanism of the body that functions to maintain blood fluidity (72). Primary haemostasis (platelet plug formation), coagulation (formation of a fibrin-rich thrombus) and fibrinolysis (the breakdown of the formed thrombus) are the three phases of haemostatic process which are closely linked to each other, tightly controlled and overlap to a large degree in order to maintain vessel patency while preventing blood loss due to endothelial injury (73).

A dynamic balance exits between the procoagulant (pro-thrombotic) pathway that initiates thrombus formation and the anticoagulant (anti-thrombotic) mechanisms that prevents clot formation (74), in normal physiology this balance is slightly in favour of the anti-thrombotic factors (75). Naturally present anticoagulant mechanisms in the body include: tissue factor pathway inhibitor (TFPI), protein C and S and antithrombin (75). An imbalance of the coagulation system could result in thrombosis (the pathological formation of a thrombus) or haemorrhage (a bleeding tendency), characteristic of numerous pathologies such as myocardial infarction and thromboembolic- and haemorrhagic stroke. This balance between procoagulant and anticoagulant pathways therefore need to be tightly controlled (75, 76). Factors such as trauma, pro-inflammatory cytokines, environmental products and infectious agents may disturb this delicate balance in haemostasis (76, 77).

In normal physiology thrombus formation is limited to the site of endothelial injury, but in pathological conditions thrombus formation is not restricted to this site (72). In haemostasis the thrombus is necessary for a transient period to allow adequate wound healing to take place after which the thrombus is resorbed by fibrinolysis (76). Should fibrinolysis not be adequate or thrombus formation altered, the clot could rupture leading to emboli in the circulation. Adequate and efficient fibrinolysis is therefore vital for the maintenance of haemostasis (76). The overall biochemical environment including clot structure and the reactivity of thrombus-associated cells such as platelets influence the efficacy of fibrinolysis (78)

Coagulation and fibrinolysis, and consequently haemostasis, involves complex interactions between three major components, the endothelium, platelets and coagulation proteins, ultimately leading to the formation of a thrombus (77).

2.9 Endothelium

The endothelium has numerous functions of which one of the most important is that it forms a protective barrier protecting the underlying tissue against vascular insults as a result of shear or endothelial injury (79). Other functions include the regulation of vascular tone, cellular adhesion, smooth muscle cell proliferation, and inflammation (79).

Healthy endothelial cells exhibit anti-thrombotic properties due to the expression of antiplatelet and anticoagulant agents that prevent platelet activation and aggregation as well as fibrin formation (75, 79). When an endothelial injury occurs the endothelium becomes highly thrombogenic by releasing a number of factors that promote thrombus formation to prevent blood loss from the injury site (80). Tissue factor (TF /factor III) and von Willebrand factor (vWf) are the main thrombogenic factors that initiate the first phase of platelet activation and consequently coagulation (80).

Tissue factor is a membrane bound glycoprotein found in the subendothelial tissue and will not come in contact with blood unless it is exposed by the disruption of the vascular endothelium (75). The exposure of TF initiates the formation of a thrombus via the extrinsic pathway of coagulation. TF binds factor VII to form active factor VII. FVIIa directly or indirectly (through the activation of FIX) activates FX essential for thrombin generation leading ultimately to thrombus formation through the conversion of fibrinogen to fibrin (87,89)(74). Certain allergens (e.g. derived from dust mites) can facilitate contact between the blood and TF by degrading the tight junctions of the airway epithelial cell and endothelial cells, exposing TF, and stimulating coagulation in the lungs (81)**.** The presence of pro-inflammatory cytokines (such as IL-1) may lead to the exposure of TF (79). Since asthma leads to proinflammatory cytokines, this will

result in the expression of TF and the activation of coagulation in asthmatic individuals (82).

Von Willebrand factor is a glycoprotein that is essential to the activation of coagulation (75). Von Willerbrand factor mediates platelet adhesion and binds to FVIII leading to platelet adhesion to an injury site and consequently the formation of a platelet plug (75, 83).

2.10 Coagulation proteins

The majority of coagulation proteins (also known as factors) circulate as zymogens (proteolytic proenzymes) classified as vitamin K serine proteases (e.g. factors II, VII, IX, X). The active conformation is usually denoted by letter ‘a’ as a suffix to the factor number, i.e. FIIa) (75). Table 2.2 summarises the factors involved and the function of the factors in coagulation.

Table 2.2: Summary of coagulation factors Adapted from (75)

Clotting factor	Clotting factor name	Function
I	Fibrinogen	Clot formation
II	Prothrombin	Activation of I, V, VII, VIII, XI, XIII, protein C, platelets
III	Tissue factor	Co-factor of VIIa
IV	Calcium	Facilitates coagulation factor binding to phospholipids
V	Proacclerin, labile factor	Co-factor of IX-tenase complex
VI	Unassigned	Activates X: forms tenase complex with factor VIII
VII	Stable factor, proconvertin	Activates factors IX, X
VIII	Antihaemophilic factor A	Co-factor of IX-tenase complex
IX	Antihaemophilic factor B	Activates X: Forms tenase complex with factor VIII
X	Stuart-Prower factor	Prothrombinase complex with factor V: Activates factor II
XI	Plasma thromboplastin antecedent	Activate factor IX
XII	Hageman factor	Activates XI, VII and prekallikrein
XIII	Fibrin-stabilising factor	Crosslinks fibrin
XIV	Pre-kallikerin	Serine protease zymogen
XV	HMWK	Co-factor
XVI	vWF	Binds to VIII, mediates platelets adhesion
XVII	Anti-thrombin III	Inhibits IIa, Xa, and other proteases
XVIII	Heparin cofactor II	Inhibits IIa
XIX	Protein C	Inactivates Va and VIIIa
XX	Protein S	Co-factor for activated protein C

2.11 Coagulation

The classic coagulation cascade was first introduced by Macfarlane and Davie & Ratnoff in 1964. For many years, this was accepted as the most accurate model to describe coagulation. According to the classic coagulation cascade two independent pathways, an intrinsic (involving factors XII, XI, IX and VIII) and extrinsic pathway involving FVII activated by tissue factor, that converge on a common pathway, leading to the conversion of fibrinogen to fibrin and the formation of a thrombus (72, 80, 84), The classic coagulation cascade, however, proposed that coagulation occurs independent of platelets (74). Recently it has come to light that platelets are not only involved in the coagulation cascade, but that they play an integral role in the formation of a thrombus (75) (74). This gave rise to a new, cell–based model of coagulation that emphasises the involvement of platelets (72, 74, 75). According to this model, the coagulation process can be divided into four phases (and is depicted in figure 2.4):

2.11.1 Phase 1: Initiation

During the initiation TF is expressed due to endothelial disruption causing factor VII to rapidly bind to TF forming the active TF-factor VII complex. The formation of this complex causes the activation of factors IX and X. Factor X, can either be directly or indirectly activated via factor IX. Factor X converts prothrombin to thrombin in one of two ways: it either cleaves prothrombin itself or activates factor V which then cleaves prothrombin to its active form. A small amount of thrombin is formed that plays an essential role in the subsequent phases of thrombus formation (72, 75).

2.11.2 Phase 2: Amplification

The exposure of TF and collagen by the damaged endothelium results in the adhesion of platelets to the injury site as a result of the interaction of glycoprotein (Gp) Ia/IIa (present on the surface of the platelet) and vWf. The formed thrombin, in the presence of increased levels of calcium in the blood, leads to the activation of factors V, VIII, IX and XI, leading to the generation of more thrombin. These factors contribute to increased platelet adhesion and further activation. Platelets release inactive FV from

their alpha granules when they are activated further contributing to the cleavage of prothrombin to form thrombin (72, 84).

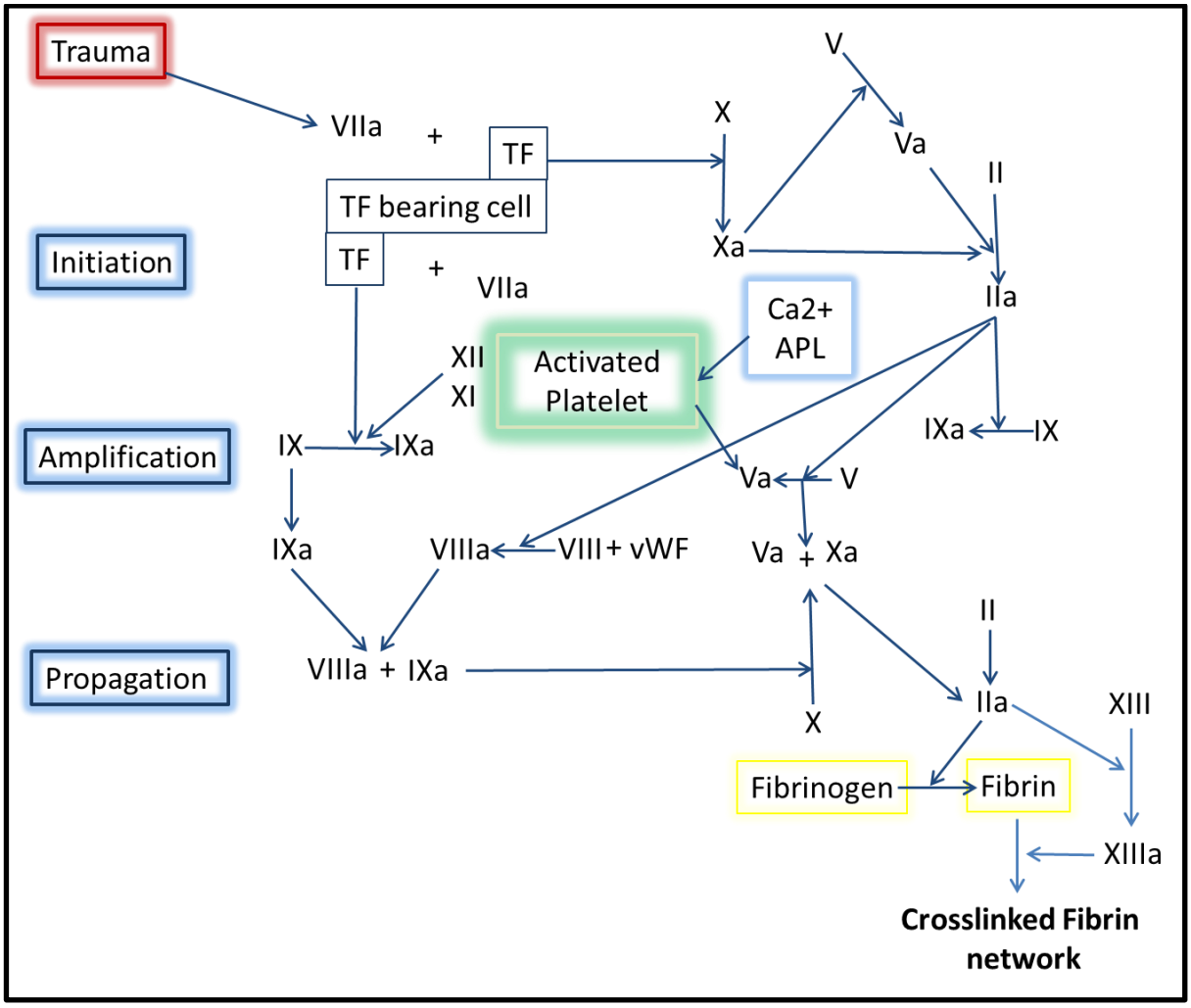

Figure 2.4: Schematic representation of the phases of the coagulation cascade discussed in detail in 2.11.1 to 2.11.4 (85).

2.11.3 Phase 3: Propagation

The surface of activated platelets form the substrate for the steps involved in propagation phase. The propagation phase involves the formation of the tenase complex (FVIIIa and FIXa) which functions to form tenase and prothrombinase complex (FVa and FXa). The tenase complex consists of activated factors VIII and IX

and activates the responsible for the generation of large amount of thrombin. The formed thrombin then converts fibrinogen to fibrin, forming the mechanical framework for the thrombus (72, 84).

2.11.4 Phase 4: Stabilization

The thrombin during the first three phases of coagulation activates FXIII. FXIII is responsible for the covalent linking of fibrin polymers to form a stable and mechanically sound thrombus that can withstand the pressure exerted by the flowing blood (75). In addition, the generation of thrombin also protects the formed thrombus from fibrinolysis by activating thrombin activated fibrinolysis inhibitor (TAFI) (73).

2.12 Fibrinolysis

Fibrinolysis is the enzymatic process that is responsible for the dissolution of the formed thrombus once wound healing as taken place. Fibrinolysis involves the conversion of the proenzyme plasminogen to plasmin (75). This conversion catalysed by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) which is released from vascular endothelium once the injury has been healed (76).

Plasminogen activator inhibitor-I (PAI-I), inhibits premature fibrinolysis by preventing the breakdown of the clot prior to the completion of wound healing (76). Both A2-antiplasmin (α_2AP) and TAFI further inhibits fibrinolysis by preventing the conversion of fibrin into fibrin degradation products and preventing the conversion of plasminogen to plasmin respectively (80). This process is summarised in figure 2.5.

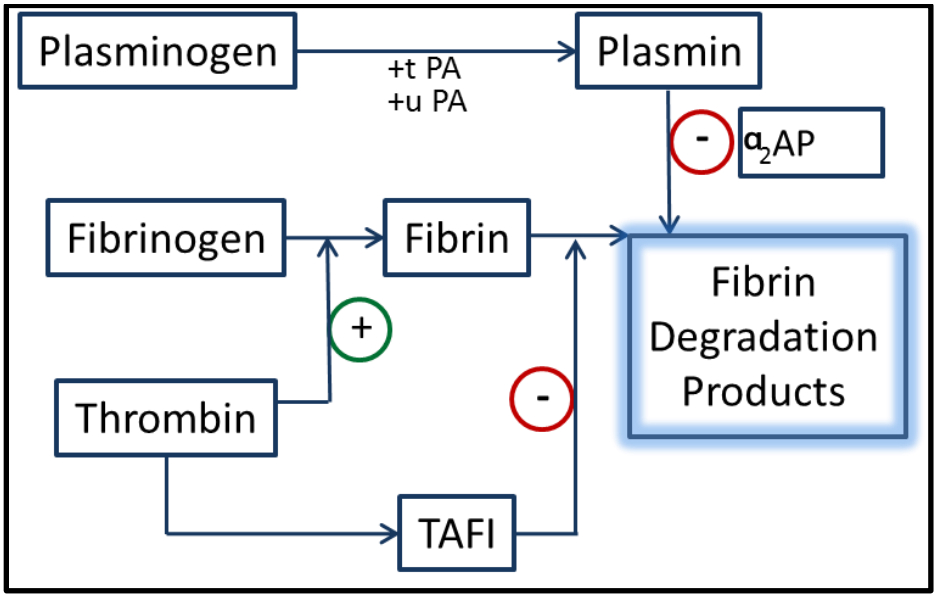

Figure 2.5: Clot stability is influenced by several factors which influence fibrinolysis as depicted diagrammatically. +: stimulates, - inhibits, tPA: thrombin plasminogen activator, uPA urokinase plasminogen activator, α_2AP: A2-antiplasmin, TAFI: thrombin activated fibrinolysis inhibitor [adapted from(75)].

2.13 Altered haemostasis in allergic asthma

Haemostasis is one of the most complex physiological self-defence systems, not only involved in control of blood fluidity but also interfering in major physiopathological processes (73). Any pathological scenario can tilt the balance that exits in haemostasis resulting in abnormalities (75).

Hypercoagulability, coagulation and inflammation are interlinked whereby inflammation leads to the activation of the coagulation cascade and coagulation is also able to induce and modulate inflammation (figure 2.6) (70, 77). Wagers *et al.* (2004) postulated that impaired coagulation and fibrinolysis resulting in fibrin deposition in asthmatic airways attributed to airway hyper responsiveness and airway closure in asthma patients (86).

Both plasma and locally derived factors contribute to the activation of coagulation cascade in asthmatic airways (87). Altered local activities of the anticoagulant protein

C / protein S, antithrombin III system down regulates fibrinolysis, resulting in a procoagulant state in the bronchoalveolar space (88, 89) (90). Coagulation and fibrinolysis have been considered as processes that take place in the vascular compartment, however, the airways also represent a body compartment in which coagulation and fibrinolysis mechanisms can be initiated and regulated (77, 91).

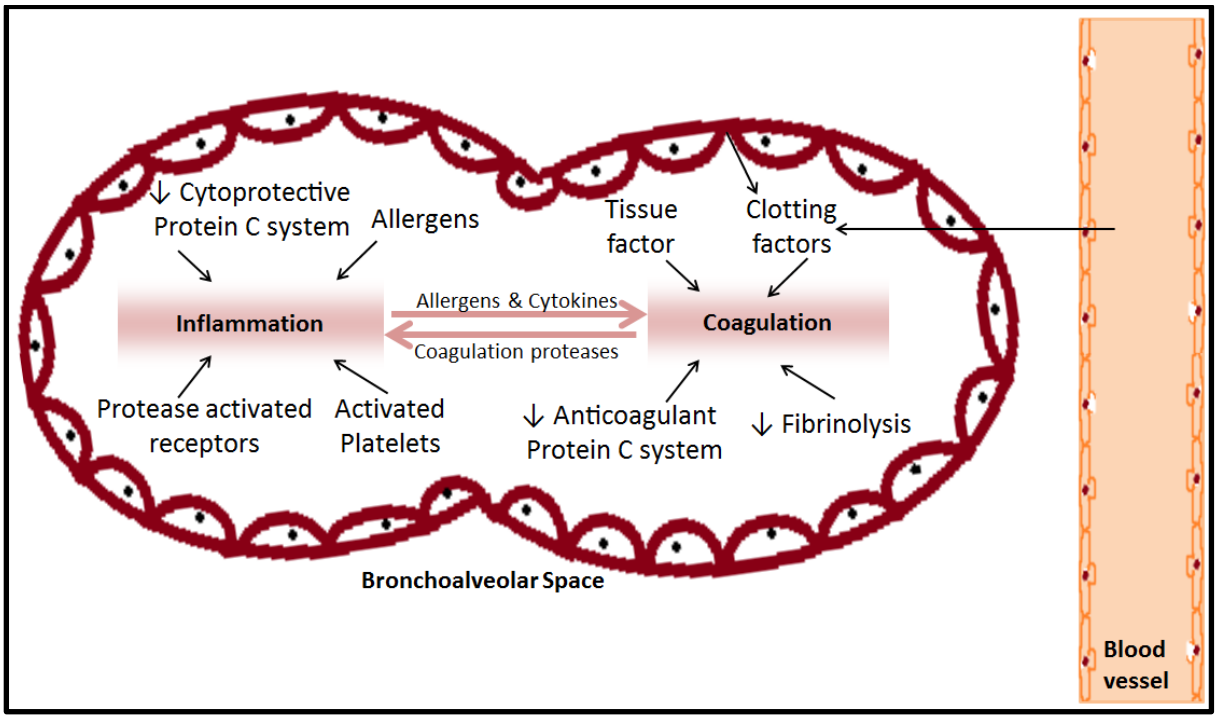

Figure 2.6: The link between inflammation and coagulation in asthma (redrawn from de Boer *et al.*, 2012)

Research findings indicate that the extravasation of plasma from capillaries results in activation of extravascular coagulation in the lung interstitium and bronchoalveolar compartment upon allergen provocation (92). High levels of PAI-1 inhibit fibrinolysis resulting in the deposition of extracellular matrix in the asthmatic airways (93-96). Atelectasis due to formation of bronchial plugs, composed of predominantly fibrin, has been found in patients with severe refractory asthma (86, 97).

Allergens (e.g. dust mites) can facilitate contact between plasma and TF by degrading the tight junctions of the airway epithelial cell and endothelial cells thereby increasing pulmonary coagulation (81). Coagulation factor Xa modulates airway remodelling by

stimulating mucin production (by regulating the amphiregulin), smooth muscle cell proliferation and collagen deposition (98, 99). Mucous hypersecretion by airway mucosa results in mucous plugs is also found in other chronic airway diseases such as cystic fibrosis and chronic bronchitis (100). Mucin production from nasal cells and bronchial epithelial cells is also attributed to the elevated levels of thrombin that has been detected in asthmatic airways after allergen exposure (101, 102).

Proinflammatory cytokines (IL-3, IL-4, IL-5 and IL-13) activate coagulation proteases and modulate inflammatory response through specific protease activated receptors [(PAR1 and PAR2)] on capillary endothelium and inflammatory cells (70). Activation of PAR1 and PAR2 by coagulation proteases is the key link between coagulation and inflammation (77). PAR2 can also be activated by other proteases derived from both the host and from allergens (103, 104).

2.14 Role of platelets in Asthma

In normal physiology platelets are involved in the maintenance of haemostasis as well as the initiation of repair process after tissue injury in disorders of the cardiovascular system (105). Due to the key role of platelets in coagulation, abnormalities in the structure and function of platelets can influence coagulopathies. Alterations in the ultrastructure and morphology of platelets are involved in pathophysiology of many diseases such as diabetes mellitus, cancer, HIV/AIDS, neurological diseases and asthma (106, 107). A change in platelet behaviour and function during and after allergen exposure is associated with asthma, allergic rhinitis and allergic dermatitis (108, 109). Platelets participate in the inflammatory processes by acting as inflammatory cells and by releasing mediators which are involved in coagulation cascade (figure 2.4) (107, 110, 111).

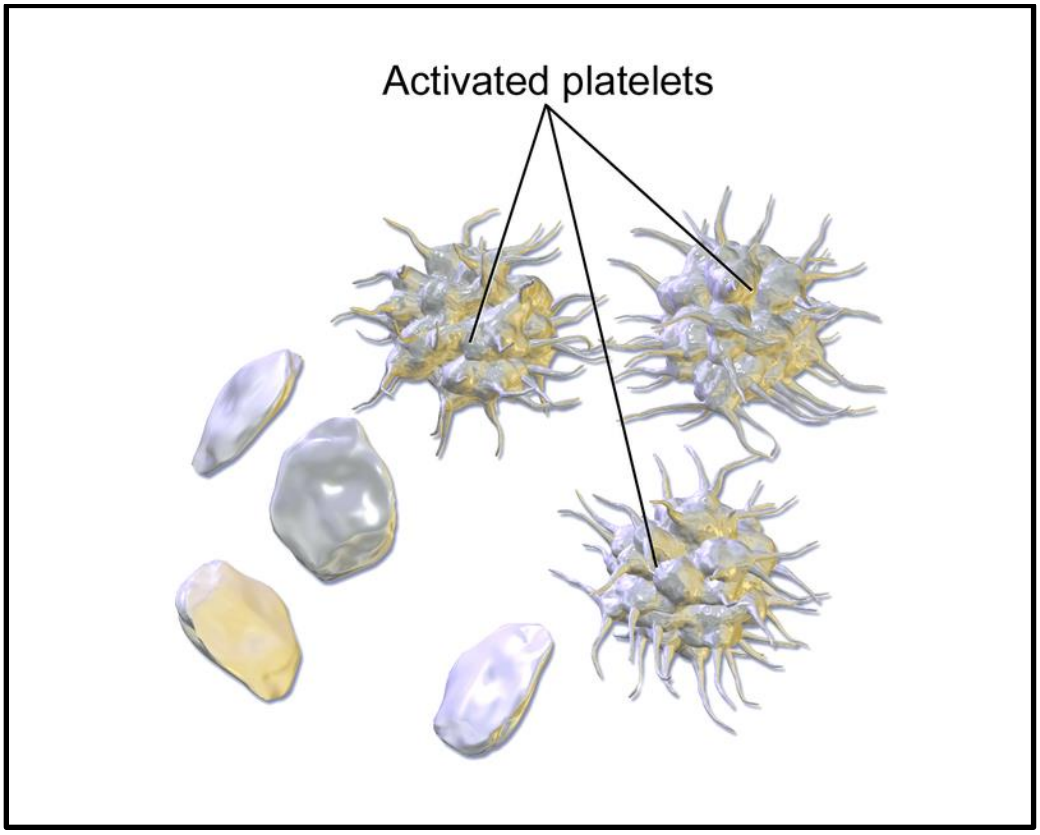

Figure 2.7: Schematic representation of inactive (round and smooth) and activated platelets (112).

Previously published research shows that a change in the platelet morphology from a tight round aggregate to a loosely connected granular appearance with pseudopodia is associated with asthma (figure 2.7) (107, 112, 113). Platelets participate in allergic airway inflammation via neutrophil, eosinophil and lymphocyte recruitment (form platelet aggregates in allergic asthma) and by releasing mitogenic, proinflammatory and coagulation mediators (114). The hyper activation of platelets is associated with many disease conditions and may be due to the damage of the vessel wall or activation of endothelium by chemicals, cytokines and inflammatory processes involved in conditions like allergic asthma (107, 115).

The increased activation of platelets is attributed to bronchial hyperresponsivenes, bronchoconstriction, airway inflammation and airway remodelling in atopic asthma (figure 2.8) (77, 108, 116, 117). Platelets are activated by various stimuli leading to the expression of surface receptors [high- affinity IgE receptor (Fc epsilonRI)], the

secretion of vasoactive substances and proinflammatory and procoagulant mediators [thromboxanes, histamines, serotonin and platelet activating factor (PAF)], adhesion of the activated platelets, platelet aggregation and finally thrombus formation (107, 118). This is followed by airway remodelling such as epithelial and smooth muscle thickening and subepithelial reticular fibre deposition resulting bronchoconstriction (119, 120).

The adhesive characteristic of platelets are key for platelet aggregation to each other, to the damaged endothelium and to leukocytes in order to form a stable platelet plug (121). Pitchford *et al.* postulated that platelets cause airway remodelling in asthmatics by their adhesion to the vasculature of the bronchi resulting in smooth muscle contraction which leads to an asthma attack (119). Platelet aggregates found in pulmonary arterioles, bronchoalveolar lavage and airway epithelium further indicates their involvement in allergic asthma (120, 122).

Platelets contain preformed granules filled with proinflammatory and procoagulant mediators such as thromboxanes, histamines, serotonin and PAF that links inflammatory and coagulation processes. These mediators have been implicated in bronchoconstriction in atopic asthma (77, 123). PAF, a lipid derivative of phosphorylcholine has been implicated by initiating airway hyper responsiveness in allergic asthma (124, 125).

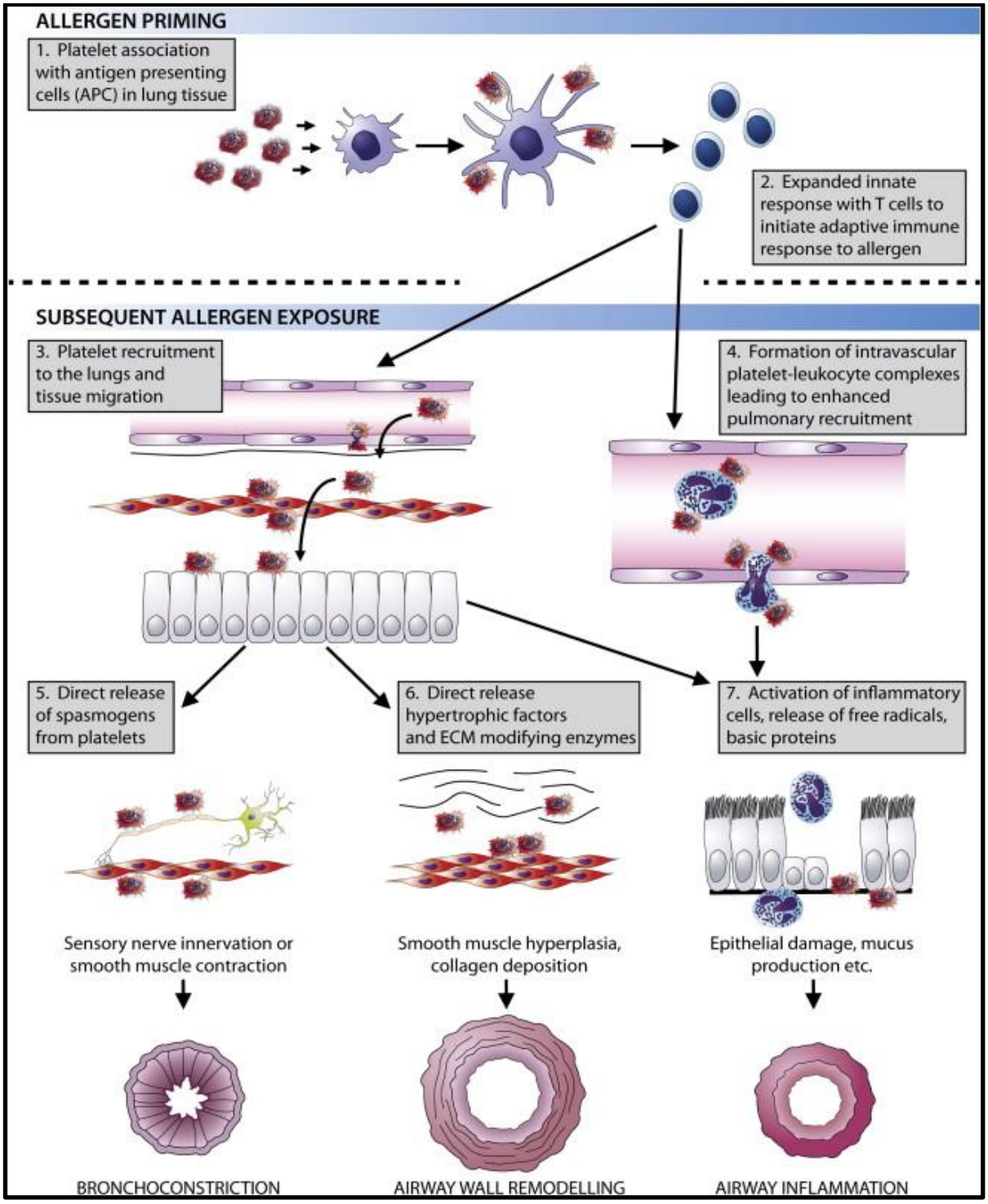

Figure 2.8: A direct role for platelets in asthma pathogenesis, ECM: extracellular matrix (109).

Evidence shows that the injection of PAF into guinea pigs caused a dose related bronchoconstriction and a prolonged increase in airway hyper responsiveness (123, 126). During asthma, activated platelets release PAF which causes bronchial hypersensitivity, inflammatory cell infiltration and mucous hypersecretion (77, 126, 127).

2.15 Role of fibrin and factor XIII in asthma

Fibrin is the end product of coagulation which is formed from fibrinogen by thrombin. The assembly of fibrin fibres takes place in a highly ordered fashion and fibrinogen (the precursor of fibrin) plays an important role in haemostasis and high levels are associated with thrombotic complications (128). Thrombin cleaves the fibrinogen molecule consisting of fibrinopeptides into fibrin monomers and these monomers assemble to form a fibrin network, which forms the core matrix of a thrombus (129). Airway fibrinogen has the potential to interact with immune cell receptors and promote coagulation, inflammation and fibrin clot formation (130, 131).

Bronchial hyper responsiveness in asthma is attributed to the fibrin deposition in distal airways (132). Fibrin is generated in the pulmonary compartments by bronchial epithelial cells and is essential for the normal airway repair after airway epithelial damage (133). However, the abnormal leakage of plasma proteins into the bronchoalveolar space that swings the physiological equilibrium towards a net procoagulant state leads to the accumulation of extravascular fibrin that contributes to airway hyper responsiveness and smooth muscle contraction resulting in airway closure in asthmatics (86, 134, 135). The detection of massive fibrin deposition in the alveoli and distal airways of an asthma patient who died from severe asthma attack shows evidence that exaggerated intra-alveolar fibrin production is associated with severe asthma. (86). Enhanced fibrin formation and turnover was also found in asthma by measuring D-dimer (a breakdown product of cross-linked fibrin) levels in sputum, where D-dimer levels were elevated. (87).

Evidence generated from a mouse model suggests increased PAI-1 in lung homogenates promotes fibrin deposition by suppressing fibrinolysis (86, 134). Fibrin affects vascular permeability, providing a matrix for fibroblast proliferation and the neutralisation of surfactant and altering surface tension (136, 137). Airway obstruction in allergen induced exacerbations of asthma can therefore also be attributed to surfactant dysfunction.

Factor XIII, a coagulation protein plays a pivotal role in the cross-linking of fibrin molecules thus stabilizing fibrin clots and is upregulated in asthma (138). Factor XIII is a transglutaminase present in the plasma (pFXIII) and also in the cytoplasm of cells such as platelets or monocytes (cFXIII) (139, 140). Factor XIII is a 326 kDa tetrameric complex with two A chains and two B chains and about half of the total Factor XIII-A in blood arises from platelets, which contain dimers consisting of only two A chain (76) Thrombin activates FXIII, which further stabilise the clot by a transglutaminase reaction results in the formation of multiple cross-links within fibrin chains and form a highly complex branched structure between neighbouring fibrin molecules (141)

Activated FXIII (FXIIIa) functions to stabilize the clot by covalently cross–linking fibrin monomers and incorporating α-2-antiplasmin into the clot structure (142). This determines the strength of the clot by critically influencing the architecture of fibrin network, thus providing protection of fibrin against fibrinolysis (138, 142, 143). The presence of FXIII-A mRNA and cFXIII in platelets and monocytes suggests the potential for production of FXIII-A *de novo* from these cell types (figure 2.9) (144, 145).

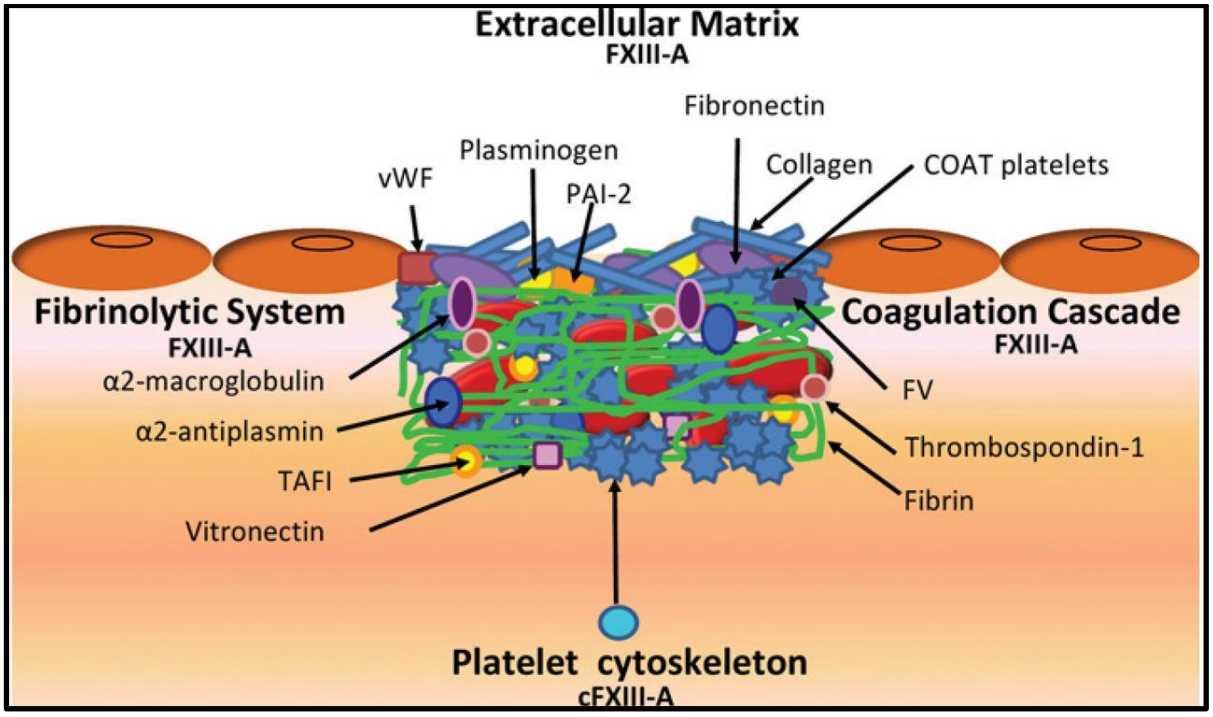

Figure 2.9: The role of FXIII-A and cFXIII-A in cross-linking in thrombus formation and extracellular matrix attachment. Following tissue damage, the coagulation cascade is activated to generate thrombin. Thrombin cleaves fibrinogen to form fibrin and activates platelets and plasma FXIII-A. Activated platelets undergo conformational changes due to the cross-linking of cytoskeleton proteins by cFXIII-A. Plasma FXIII-A cross-links fibrin producing the structural framework of the thrombus (146).

Factor XIIIa provides stability to the clot and thus plays a major role in protecting the fibrin from degradation due to the presence of the cross-links in fibrin γ- and α-chains (139). Thus FXIII regulates fibrinolysis by increasing clot stiffness. Studies also implicate Factor XIII as a key role player in the pathogenesis of thromboembolic diseases like myocardial infarction, ischemic stroke, deep vein thrombosis and pulmonary embolism by modulating clot structure and function (138, 139, 147).

Calcium binding sites on fibrinogen are important for both stability and polymerization. There are two binding sites in each of the γ-nodules (γ1 and γ2) and β-nodules (β1 and β2) of fibrinogen. Calcium influences fibrin polymerization by increasing the rate and extent of lateral aggregation thereby stabilising the thrombus (figure 2.10).

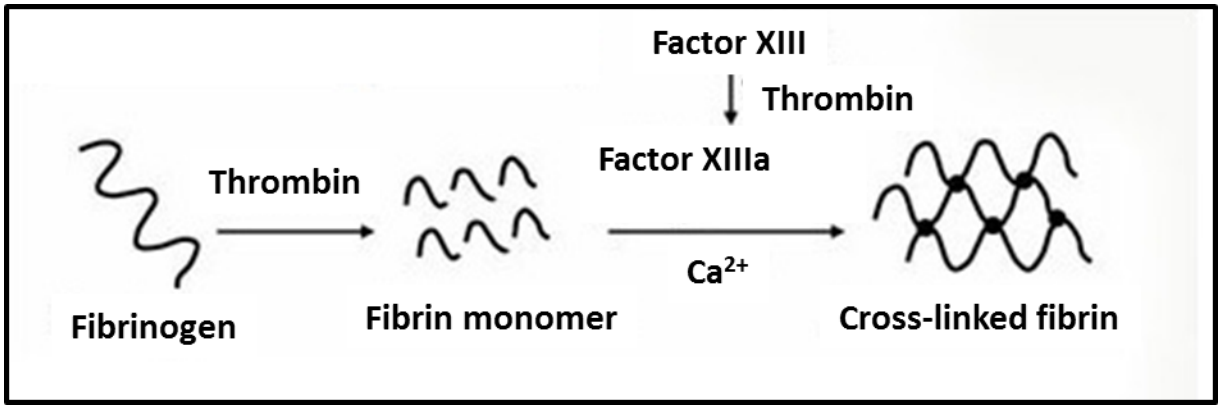

Figure 2.10: Schematic representation of the role of calcium in fibrin cross-linking.

2.16 Role of erythrocytes in asthma

Erythrocytes are complex cells which do not contain a nucleus or mitochondria and contain cytoplasm with 95% haemoglobin (Hb); a protein that carries oxygen to cells (148, 149). Erythrocytes play an important role in haemostasis. The flow property of the erythrocyte (haemorheology) depends on deformability which is the ability of erythrocytes to change its cellular morphology in the blood vessel and enable them to squeeze through the microvasculature in order to carry oxygen to the cells and return to the original discoid shape (150). Erythrocytes require a robust and highly deformable membrane to withstand shear stresses in the microvasculature (figure 2.11) (151, 152).

Deformability of erythrocytes is a determinant of blood viscosity which contributes significantly to blood flow (153) and is directly linked to the membrane properties such as elasticity, rigidity and stiffness and influences the dynamics of erythrocyte function (150, 154). The functional and physiological properties of the erythrocytes depend on their morphology and elastic properties (155). The elastic properties of erythrocytes are determined by structure of the membrane which allows the erythrocytes to undergo extensive deformability without cell fragmentation (156) Alterations in erythrocytes deformability due to change in structure contributes to the pathophysiology of many diseases (150, 157, 158).

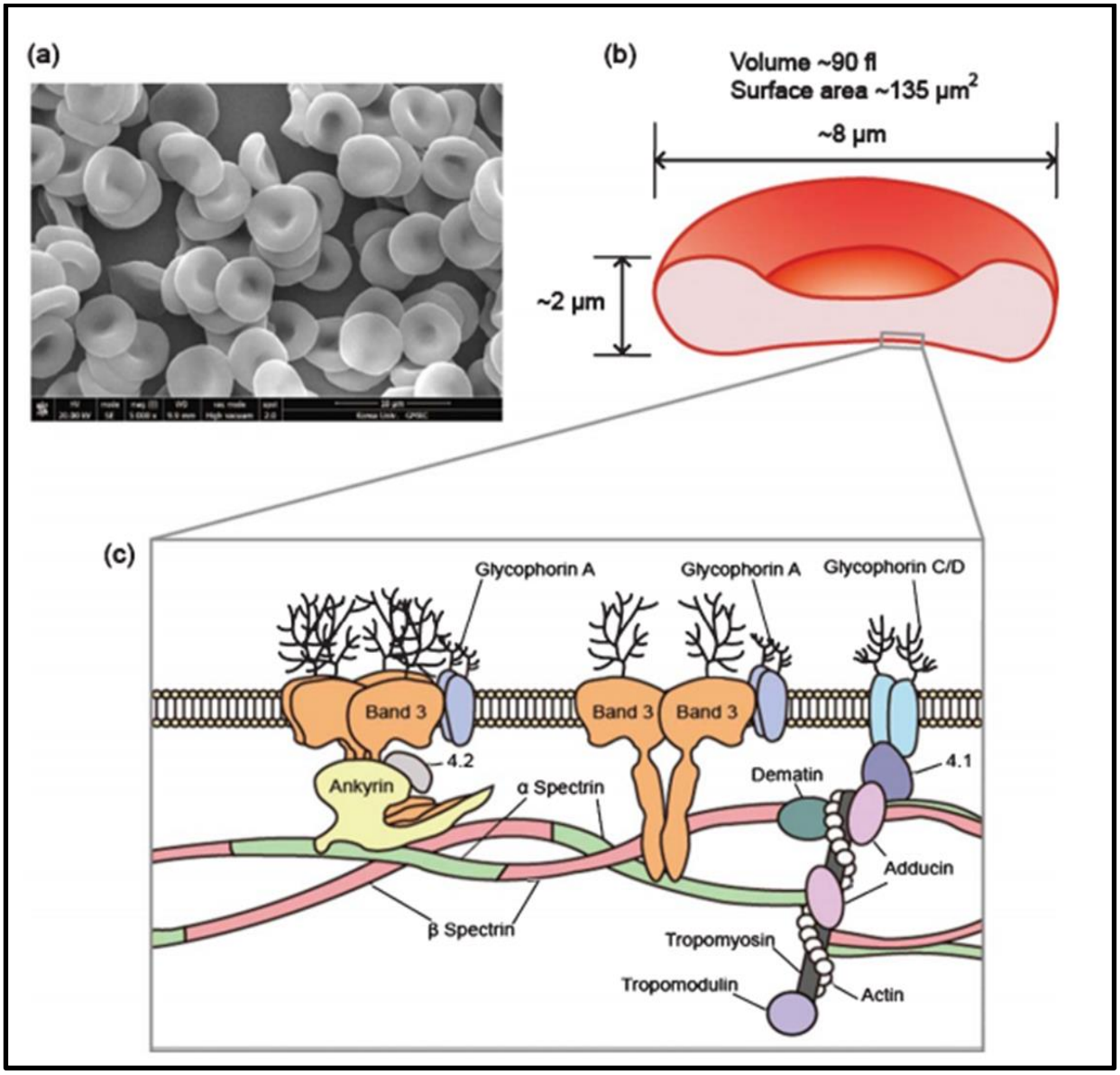

Figure 2.11: A: Erythrocyte image representative from a scanning electron micrograph, B: The shape of a human red blood cell with average geometric parameters, and C: A schematic representation of the organization of the erythrocyte membrane (152).

Pathological changes in the morphology of erythrocytes is associated with oxidative stress and inflammation (159, 160). Erythrocytes play a fundamental role in the inflammatory process by binding inflammatory mediators to surface receptors and having a changed deformability, rheology or sedimentation rate (161, 162). An increase in the concentration of inflammatory molecules in the blood can alter the

deforming capacity of the erythrocytes (159) thus influencing the pathophysiology of many diseases. During inflammation erythrocytes have an altered shape (loss of the characteristic biconcave shape) as well as inadequate flow properties and is thought to have a major influence in inflammatory diseases such as cardiovascular disease (150, 153).

Alterations in erythrocyte morphology and function due to inflammation in asthma are the result of the oxidation of membrane lipids and proteins (163, 164). Airway inflammation and some drugs used in asthma treatment can modify the structure and elastic properties of erythrocytes and may eventually cause haemolysis of the erythrocyte (155, 165). Altered erythrocyte integrity and function in asthma can also be due to oxidative stress caused by the release of reactive oxygen- and nitrogen species from the inflammatory airway cells as in chronic obstructive pulmonary disease (88, 163, 166).

As discussed in this chapter, one of the distinguishing features of allergic asthma is the presence of inflammation. This inflammation is not only chronic, but also systemic, and this results in an upregulation of inflammatory molecules that circulates throughout the body. One of the hallmarks of systemic inflammation is a pathological coagulation system that involves various parts of the haematological system. This results in changes to this haematological system, which is the the focus of this thesis, namely that, allergic asthma affects the haematological system, and that these alterations can be studied by using various novel research techniques. The hypothesis of this study is consequently is that coagulation is altered due to the altered morphology of fibrin networks, platelets and erythrocytes in asthma.

In order to study this hypothesis, the following research objectives direct this thesis:

1. To compare the serology and full blood count between asthma and controls.
2. To qualitatively evaluate the difference between the ultrastructure, axial ratio and elasticity of erythrocytes using SEM, LM and AFM, respectively.
3. To qualitatively evaluate the differences between the ultrastructure and activation of platelets using SEM, TEM and CM.
4. To assess the kinetics of coagulation in platelet poor plasma to determine the reaction time, speed at which clot formation occurs, rate of cross-linking and clot strength, quantitatively using TEG.
5. To qualitatively evaluate the differences between the ultrastructure of fibrin using SEM and to quantify the amount of factor XIII-A mRNA in whole blood.

The next chapters will discuss each individual research objective in more detail.

CHAPTER 3

PATIENTS DEMOGRAPHICS AND DESCRIPTIVE ANALYSIS OF HAEMATOLOGY AND MATERIALS AND METHODS

3. Introduction

This chapter discusses the sample population, study design and specific details of all the material and methods that was used in this study. The various techniques will then only be referred to briefly, in the actual research chapters.

3.1 Study Design

3.1.1 Patient recruitment

Whole blood samples were obtained from 60 voluntary participants. The sampling was non-random and on a “convenient” basis.

- 30 healthy individuals (control group)
- 30 patients (experimental group) diagnosed with asthma from Steve Biko Hospital, Lung Unit.
- Patients were categorised based on the severity of asthma into intermittent, mild persistent, moderate persistent and severe persistent by symptoms and lung function tests on the basis of patient information records.

i. Inclusion criteria:

Control Group -

Healthy individuals eligible for the inclusion in this study fulfilled all of the following criteria:

- Able to provide written informed consent (appendices 6 and 7).
- Males and females of any race who are aged ≥ 18 years.
- Individuals who were not suffering from any inflammatory diseases including asthma and were not on any chronic medications.

Experimental Group -

Patients eligible for the inclusion in this study fulfilled all of the following criteria:

- Able to provide written informed consent.
- Males and females of any race, aged ≥ 18 years.
- Patients with a diagnosis of persistent asthma [according to global initiative for asthma (GINA) 2011] for a period of at least 6 months prior to screening.
- Patients were atopic as diagnosed historically by either a skin prick test (≥3mm diameter above background) or a positive specific IgE test (≥0.35 IU eq./ml)
- Patients who were demonstrated to have reversible airway obstruction or airway hyper reactivity or have shown either of such responses in previous tests within the last year.

ii. Exclusion criteria:

- Patients who have smoked or inhaled tobacco products within the 6 month period prior to screening.
- Patients with a history of chronic lung diseases other than asthma, including chronic obstructive pulmonary disease, bronchiectasis, sarcoidosis, interstitial lung disease, cystic fibrosis and tuberculosis.
- Patients who have ischemic heart disease and hypo- and hyperthyroidism.
- Patients with a history of malignancy of any organ system.
- Patients with neurodegenerative diseases, rheumatoid arthritis, diabetes and other autoimmune diseases.
- Patients with acute illness other than asthma.
- Patients who are HIV positive, on the basis of patient information records.

3.1.2 Blood Sample Collection

Following approval from the Research Ethics Committee (Ethical clearance number: 463/2013, appendix 1), a statistician (Dr. L Debusho) (appendix 2) and doctoral committee of the Health Sciences Faculty of the University of Pretoria (appendix 3), blood samples (5ml) for the study were collected, via venipuncture, in citrate tubes

from participants. The blood was processed to obtain platelet poor plasma and an aliquot of whole blood was used for laboratory analysis.

3.2 Patient demographics and biochemical parameters

The healthy control group consisted of both males (n=19) and females (n=11) with an age range of 18 to 86 years. The healthy controls did not suffer from any inflammatory or chronic diseases and were not on any medication.

Thirty patients with allergic asthma were included in this study, of which 9 were male and 21 female (age range 19 to 87 years). All patients who participated in this study presented with allergic asthma. Common asthma treatment includes several medications that can be prescribed in the form of inhalers, oral medications, and the use of nebulisers or breathing machines. The medications mainly focus on decreasing airway inflammation which relieves the symptoms of the asthma attack. The types and doses of prescribed asthma medications depend on age, symptoms, and severity of the condition. (5)**.** Table 3.1 summarizes the prescribed medication of the recruited asthma patients. The medication included long-acting bronchodilators, corticosteroids, and various inhibitor medications (anti-LTs, anti-IgE and phosphodiesterase-4 inhibitors). A total of 28 patients were on corticosteroids and immunosuppressant medication. Corticosteroids and immunosuppressant drugs are a class of drugs that inhibit or reduce the activity of the immune system of the body, reducing pro-inflammatory cytokines. Several patients were on bronchodilators, 19 on class I and 22 on class II. Fourteen patients were on leukotriene receptor antagonist and 9 patients were on phosphodiesterase-4 inhibitors. Eicosinoids (LTs, PGs and thromboxane) are recognized as key mediators in inflammatory response associated with asthma (167)**.** A total of 15 patients were on anti-histamine medication. All these medications are the most effective drugs for asthma treatment that ameliorates airway inflammation by modulating immune responses and also by reducing ILs, thus relieving asthma symptoms and asthma attack. Reduced levels of cytokines

concomitantly reduce platelet activation (70) and thus reducing the pro-coagulant system in asthma patients.

Table 3.1: Demographic and medication usage of study participants

	Control	**Asthma**
Age [Mean (range)]	53 (17 - 86)	45 (19 - 87)
Gender (M/F)	19/11	9/21
Medication		
Corticosteroid Immunosuppressant	-	28
Bronchodilator, Class I B2 adrenergic R antagonist	-	19
Bronchodilator, Class II B2 adrenergic R antagonist	-	22
Leukotriene receptor antagonist	-	14
Anti-histamine	-	15
Competitive nonselective phosphodiesterase inhibitor	-	9

3.3 Haematology analysis

A full blood count was performed to determine whether variations in the number of blood cells may have affected coagulation in the asthmatic individuals (Table 3.2). Statistically, different results are further depicted by the use of dot plots to emphasise the varying distribution amongst asthma patients (figures 3.1 to 3.4). A blood count could indicate the presence of infection (white blood cell count), anaemia (red blood cell count) or platelet abnormalities (168). For this study, blood was drawn by

venipuncture and the test performed by using a haematology analyser (Samsung LabGeoOHC10, South Africa). Infection and inflammation may lead to alteration in endothelial cell function, cytokine production and fibrinogen and can therefore affect the coagulation profile of an individual (169).

Table 3.2: Whole blood analysis of controls and asthma patients

	Normal Range	Control (n=30)		Asthma (n=30)		
		Mean	Interquartile range	Mean	Interquartile range	p value
White Blood Cells	3.92 - 9.88	6.46	5.99 - 7.09	7.14	5.41 - 8.38	ns
Lymphocytes $*10^9$	1 - 4	2.30	1.93 - 2.57	2.04	1.42 - 2.79	ns
Monocytes $*10^9$	0.18 - 1	0.36	0.24 - 0.42	0.69	0.29 - 0.96	0.0066
Granulocytes $*10^9$	2 - 7.5	3.81	3.52 - 4.01	4.43	2.92 - 5.64	ns
Erythrocyte$*10^{12}$	4.7 - 6.1	4.72	4.42 - 5.02	5.72	4.79 - 6.41	0.0004
Haemoglobin	13.5 - 18	14.20	13.2 - 14.9	16.67	13.6 - 20.1	0.0057
Haematocrit	38.8 - 50	41.75	38.5 - 44.9	48.68	40.6 - 57.0	0.0049
Platelets $*10^9$	150 - 450	237	193 - 265	192	107 - 278	ns

Level of significance: $p<0.05$, Mann Whitney test. Where ns: not significantly different.

3.4 Red blood cell indices

3.4.1 Haemoglobin

Haemoglobin, an iron-containing conjugated protein present in erythrocytes, is responsible for the transportation of oxygen. Decreased Hb concentration may be an indication of anaemia, therefore it is important to determine Hb (168).

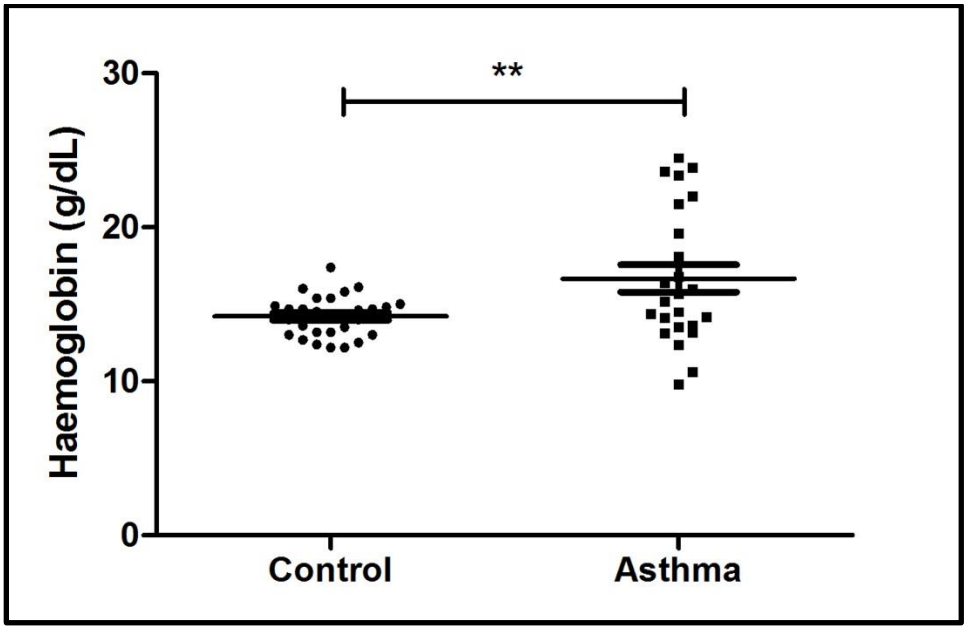

Figure 3.1: Haematology results showing the difference in haemoglobin levels between control and asthma patients. **p<0.01, Mann Whitney Test

Six asthma patients had abnormal Hb index. Three of the male patients had higher Hb index, between 23.4 g/dl and 23.9 g/dl and three females with higher Hb index ranging from 22-24.5 g/dl, figure 3.1. However, all the other patients had normal Hb index and are within the interquartile range (Table 3.2). High Hb levels have been shown to reduce platelet activity and coagulation (170).

3.4.2 Erythrocyte count

Erythrocytes are the cellular component of blood that contains Hb which transports oxygen to the rest of the body. The normal ranges are, in men 4.7 to 6.1 million cells/µl and in women ranging from 4.2 to 5.4 million cells/µl.

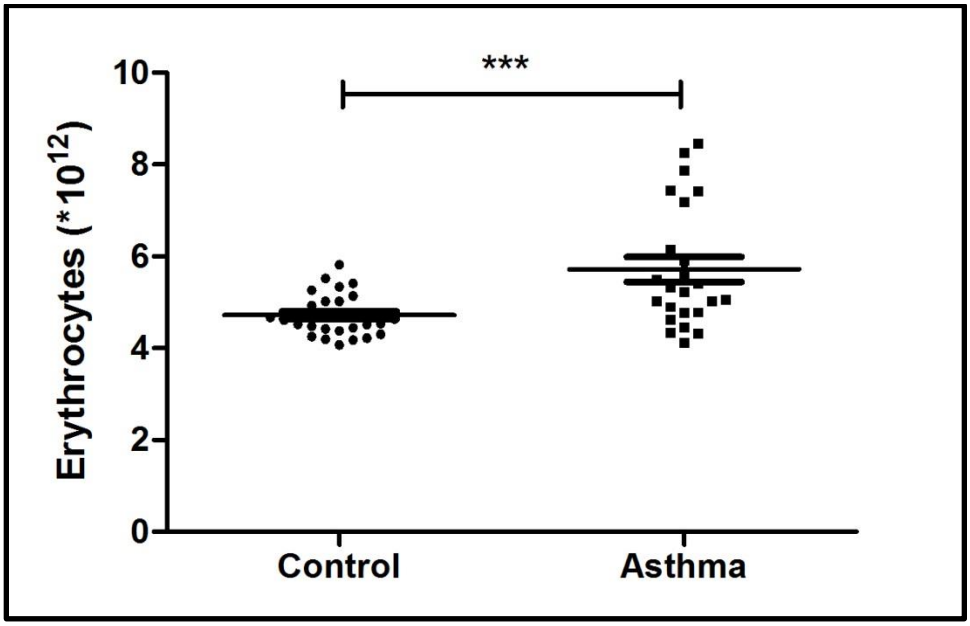

Figure 3.2: Haematology results showing the difference in erythrocyte count between control and asthma patients. ***p<0.001, Mann Whitney Test

Of the 30 asthma patients, six had an increased erythrocyte count ranging from 7.2 x 10^{12}/l to 8.46 x 10^{12}/l and occurred in conjunction with higher Hb index, which may influence the viscosity of blood and later, coagulation.

3.4.3 Haematocrit

Haematocrit (Hct) index indicates the percentage of the blood volume that is made up of blood cells, since erythrocytes are the most prevalent cell, Hct is mainly dependent on erythrocyte count (168). The Hct value changes for male (42-54%) and female (38-46%) and the normal range is between 35-50%. The majority of individuals in the experimental group had Hct index within the normal range except six. Three male patients had increased Hct index between 66% and 70% and three female patients with Hct index ranged between 60-70%. In all of these six patients, Hct index increased with high Hb index and erythrocyte count index.

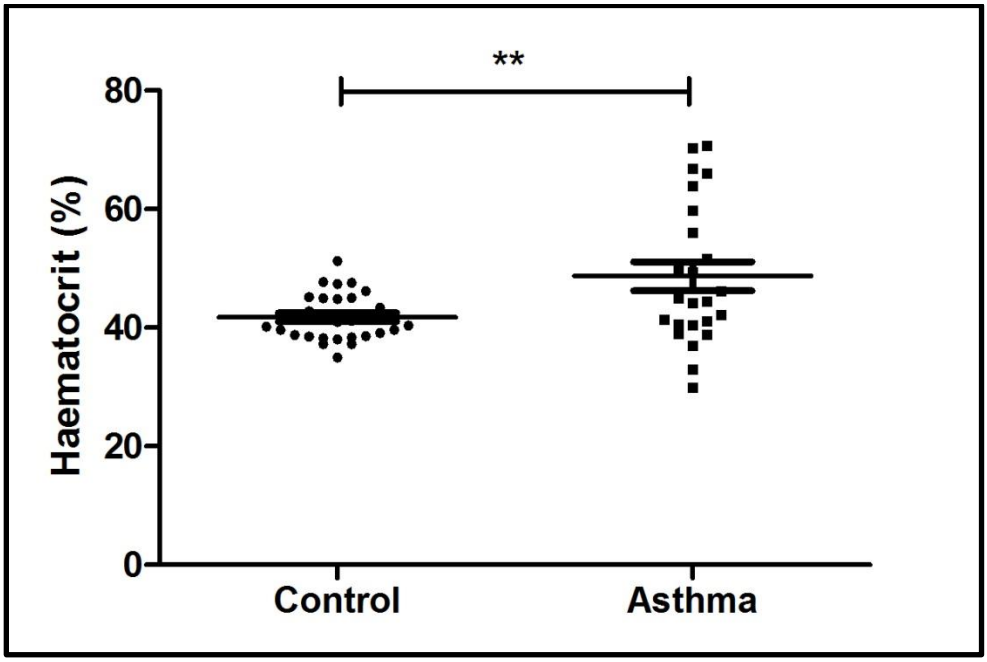

Figure 3.3: Haematology results showing the difference in haematocrit levels between control and asthma patients. **p<0.01, Mann Whitney Test

Three other tests were performed in conjunction with the above mentioned test. Those tests are: Mean Corpuscular Volume (MCV) to determine the volume of erythrocytes, Mean Corpuscular Hb (MCH) indicate the average amount of Hb in each red blood cell in the body, Mean corpuscular Hb concentration (MCHC) refers to the average amount of Hb in erythrocytes and red blood cell distribution width (RDW). All patients had MCV, MCH, MCHC and RDW levels within the normal range.

3.3.4 White blood cell count

White blood cell count (WBCC) is a test that measures the number of white blood cells in the blood (normal range: 4.5 to 11.0 x 10^9 per litre). The normal number of white blood cells ranges from 4 to 10x10^9 per microliter of blood, of which 40 to 80% is neutrophils, 20 to 40% contains lymphocytes, 2 to 10% monocytes, 0 to 6% eosinophils and 0 to 2% basophils.

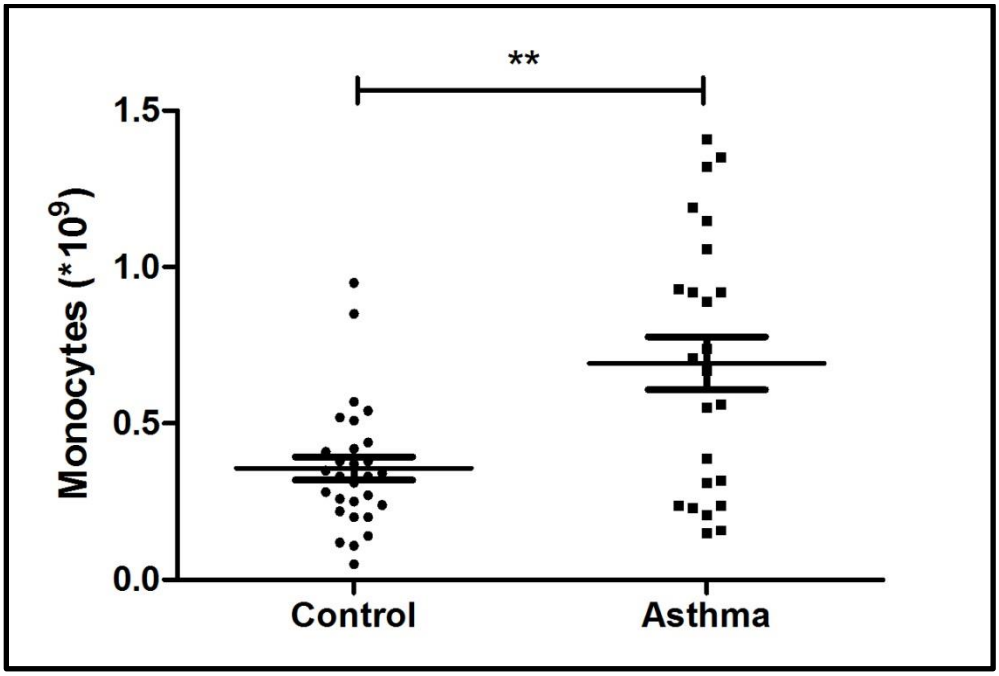

Figure 3.4: Haematology results showing the difference in monocyte count between control and asthma patients. **p<0.01, Mann Whitney Test

A high mean monocyte count was present in patients (figure 3.4), an indication of an activated immune response which is commonly observed in asthma as a result of the inflammatory component of the disease. Granulocytes are white blood cells that contain secretory granules and the secretions are released during infections, allergic reactions, and asthma.

3.3.5 Platelet Count

Platelet count is the number of platelets present in the blood. A normal platelet count ranges between 150 to 450x10^9/l. All individuals had platelet count within the normal reference range, indicating that alterations in coagulation were not a result of thrombocytopenia or thrombocytosis. The quantitation of plateletcrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW) were also measured and appeared normal in all the participants with no significant difference observed between the groups.

3.4 Experiments

3.4.1 Preparation of erythrocytes sample for Light Microscopy

After the blood was collected in a citrate vacutainer (BD Biosciences, South Africa), 10µl of the blood was placed on a glass slide (Labotec, South Africa) and a thin smear of the blood was made across the slide by using another glass slide. The sample was allowed to air dry and then placed in 100% methanol (Merck, South Africa) for 5 minutes (min) and allowed to air dry again. The sample were then stained with Methylene blue (Sigma-aldrich, South Africa), for 5min and then rinsed with running tap water until the water was clear of the stain and left to air dry again. The dried the sample was then placed in Eosin (Sigma-aldrich, South Africa), for 30 seconds (sec) and rinsed with tap water until the water was cleared of the stain and left to air dry again. A coverslip was mounted over the completely dried sample with Entellan (Merck, South Africa). Smears were viewed using a Nikon Trans Optiphot Light Microscope (Nikon, Japan). Micrographs of erythrocytes were obtained. These micrographs were used to determine the axial ratios (figure 3.5) with a Red Blood Cell Analyzer^{TM} program, and a comparison was made between the control and experimental groups. At least 3 images per patient was captured and fifty cells per image were used to determine the axial ratio.

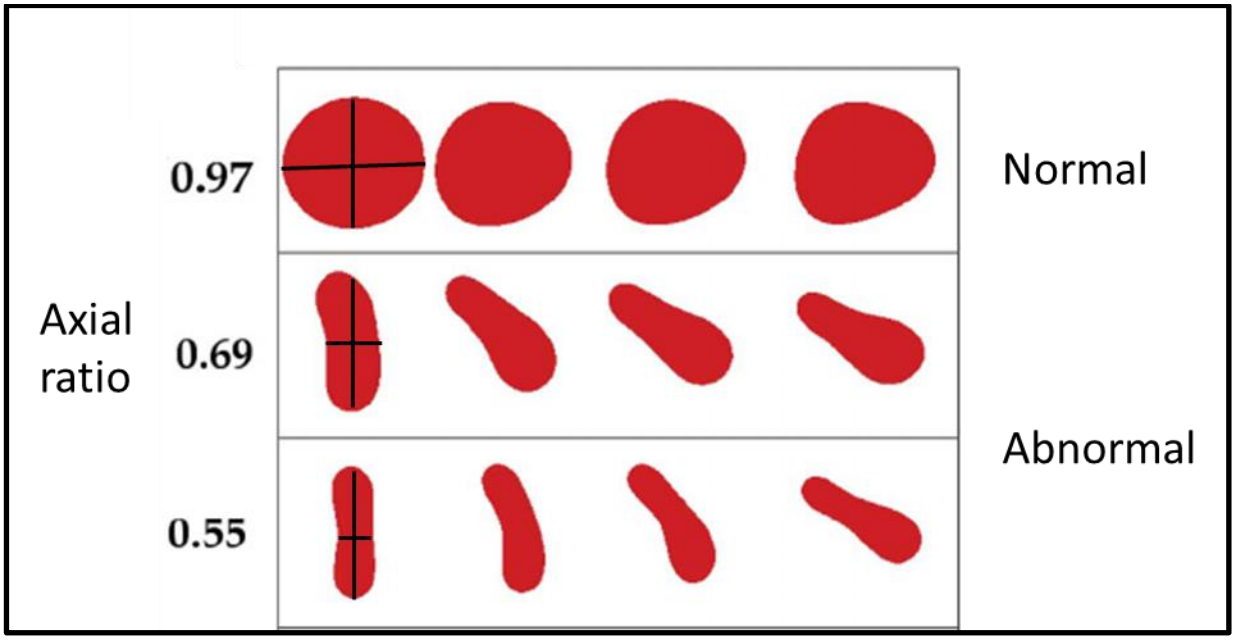

Figure 3.5: A diagrammatic representation of erythrocyte axial ratios in normal and abnormal cells. Adapted from (171)

3.4.2.1 Preparation of erythrocytes for Atomic Force Microscopy

Whole blood was centrifuged at 268xg for 30sec and the supernatant (plasma, platelets and white blood cells) was discarded in order to obtain the erythrocytes. 100µl of erythrocytes was collected in a 1.5ml tube and centrifuged again at 268xg for 8min in order to obtain an erythrocyte pellet. The remaining pellet containing erythrocytes was suspended in 2.5% gluteraldehyde (Merck, South Africa) for 30min. The sample was rinsed three times with 0.075M phosphate buffer saline (PBS, , Sigma-aldrich, South Africa) for 10min each (the mixture was centrifuged, the supernatant discarded and the pellet resuspended) before being fixed with 1% osmium tetraoxide (OsO_4, Sigma-aldrich, South Africa) for 30 min (to ensure the preservation of membrane phospholipids). The samples were rinsed three times with 0.075M PBS for 3min each (the mixture was centrifuged, discarded supernatant and resuspended the pellet). The samples were dehydrated with a series of ethanol (Merck, South Africa) 30%, 50%, 70%, 90%, and three times with 100% ethanol 3min each (the mixture was centrifuged, discarded supernatant and resuspended the pellet). The sample was centrifuged again, supernatant discarded and the pellet resuspended in hexamethyldisilazane (HMDS, Sigma-aldrich, South Africa), for 30min.

After 30min the sample was centrifuged, the supernatant was discarded and then resuspended again in HMDS. 10µl of the suspended material was placed onto a glass cover-slip in such a way to ensure an even distribution of cells. The sample was left to air dry. An Atomic Force Microscope (Dimension Icon, Bruker, USA) was used to study the morphology of erythrocytes. Data obtained from AFM as a summary of "Force Curves" used to determine the elasticity differences between experiment group and the control.

3.4.2.2 AFM measurements for membrane deformability of cells

The elasticity (deformability) measurements were performed on erythrocytes of healthy individuals and asthma patients to determine whether nano-mechanical property differences were present by using AFM. A rapid force-distance curve was recorded at each pixel. Calibration of the cantilever's deflection sensitivity and spring constant allowed the rapid quantitative analysis of these force-distance curves on a number of different areas on the sample. The curve obtained was used to calculate Young's modulus. Deformation is calculated using the variation between zero and the maximum force applied. Energy dissipation can be calculated by determining the area between the approach and the development of the retract curve (figure 3.6) (172).

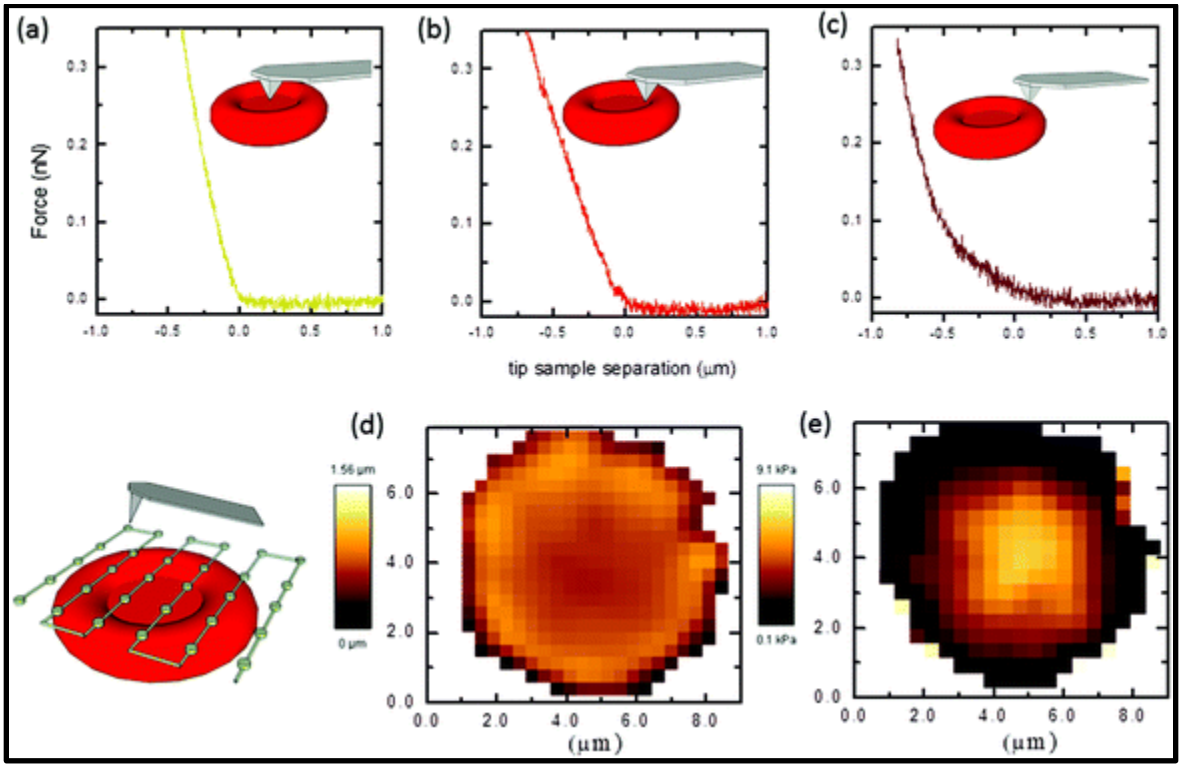

Figure 3.6: The force–distance curves measured at different distances from the centre of the erythrocyte is represented in A, B and C. Cantilever position is schematically represented in D: nanoscale zero force image of the entire erythrocyte; E: nanoscale Young's modulus map of the entire erythrocyte. Reproduced from (172)

3.4.2.3 Statistical Analysis of AFM data

The elasticity (deformability) measurements were performed on erythrocytes of healthy individuals and asthma patients by using AFM to determine whether nano-mechanical property differences were present. The Young's modulus of 50 randomly selected force-distance curves with good fit on each red blood cell, were compared between the two groups). A comparison between the two groups was performed for erythrocyte elasticity by using Statistical Analysis System (SAS) software and utilising the generalized mixed model for repeated measure function. A p-value of <0.05 was considered statistically significant.

3.5 Thromboelastography

Blood was centrifuged at 386xg for 10min and the supernatant (platelet-rich plasma) was collected in a 1.5ml tube and centrifuged again at 268xg for 8min in order to obtain platelet-poor plasma (PPP). (Since it has been found that erythrocytes and platelets influence the mechanical properties of clots, plasma was used to assess the functioning of the coagulation cascade in the absence of platelets). PPP was stored in 500µl aliquots in a -70° freezer. On the day of the experimentation, the aliquots were removed from the freezer and thawed at room temperature.

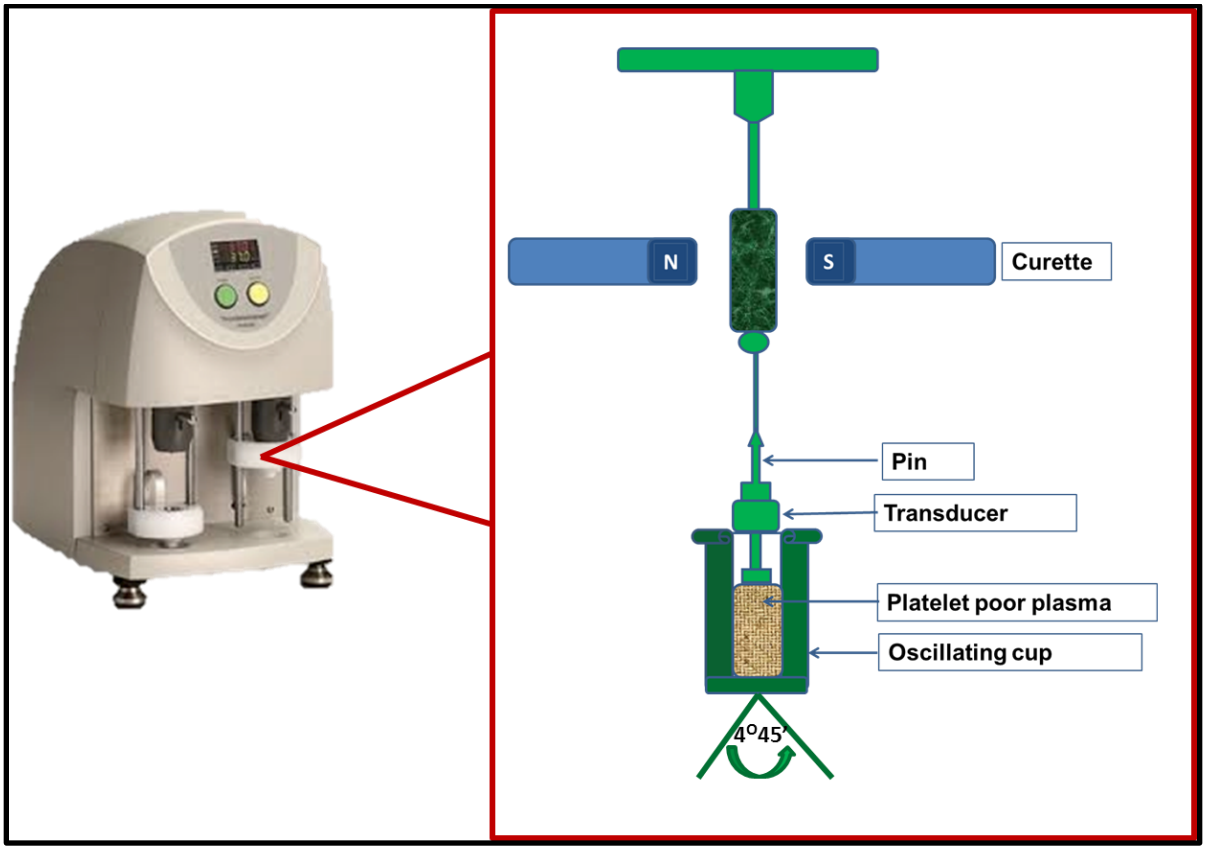

Figure 3.7: Image of a TEG instrument and diagrammatic magnification of the functional components adapted from (173)

For the TEG procedure, a small cup and a pin were mounted into the TEG instrument (figure 3.7, TEG®5S, Haemonetics, Switzerland). An aliquot of PPP (340µl) was added to the oscillating cup and then 20µl of calcium chloride (0.2M, Barker Medical, South Africa) was added to activate the coagulation process. The detector measures the

coagulation process as the sample oscillated. For the analysis of the clotting parameters a specialized TEG program (TEG® Manager Software, Switzerland) was used to monitor coagulation and clot kinetics (Table 3.3).

Table 3.3: Thromboelastography thrombus parameters [Taken from: (174)]

PARAMETER	PARMETER DESCRIPTION	EXPLANATION
R	Reaction time (minutes)	Latency time from the test initiation to the start of the clot (amplitude of 2mm); i.e. initial fibrin formation time
K	Kinetics (minutes)	Time taken to reach a certain level of strength for the clot (achieve amplitude of 20mm) i.e. amplification
A (Alpha Angle)	Angle (slope between the traces represented by R and K) (degrees)	A measure of the speed at which fibrin builds-up and cross-linking takes place, i.e. assessing the rate of clot formation
MA	Maximum amplitude (mm)	Maximum attainable strength or stiffness of the fibrin clot i.e., reflects ultimate strength of the clot
MRTG	Maximum rate of thrombus generation ($Dyne.cm^{-2}.s^{-1}$)	The maximum velocity of clot formation observed or the maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in dyne per cm^{-2}
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 ($Dyne.cm^{-2}$)	The clot strength: the amount of total resistance (to movement of the cup and pin) generated during clot formation. This is the total area under the velocity curve during clot growth, representing the amount of clot strength generated during clot growth

3.6 Preparation of fibrin clot for scanning electron microscopy

Fibrin clots were prepared by adding 5µl human thrombin (prepared in biological buffer containing 0.2% human serum albumin and is made to a concentration of 20 U/ml) provided by the South African National Blood Service (SANBS) to 10µl PPP on a coverslip. The cover slip was incubated for 10min at 37°C to create extensive fibrin fibres, followed by washing the clot in 0.075M PBS (pH 7.4) on a shaker for 20min. The washing process removed any blood proteins trapped within the fibrin network.

The washed cover slip with coagulum was fixed in a solution containing 2.5% gluteraldehyde in 0.075M PBS for 30 min. After fixation the clot was rinsed three times in 0.075M PBS for 3min in order to remove any residues. The clot fixed by a secondary fixative, 1% OsO_4 solution, for 15min. The clot was rinsed again three times with 0.075M PBS for 3 min each. The sample was dehydrated in series of 30%, 50%, 70%, 90% and three times in 100% ethanol. Each dehydration step lasts 3min and different concentrations of ethanol were used in the process to avoid osmotic shock of samples. After the dehydration step the samples were immersed in HMDS for 30min in order to dry the samples for viewing. The HMDS was removed and a drop of HMDS was then added to the sample and left to air dry, as summarized in figure 3.8.

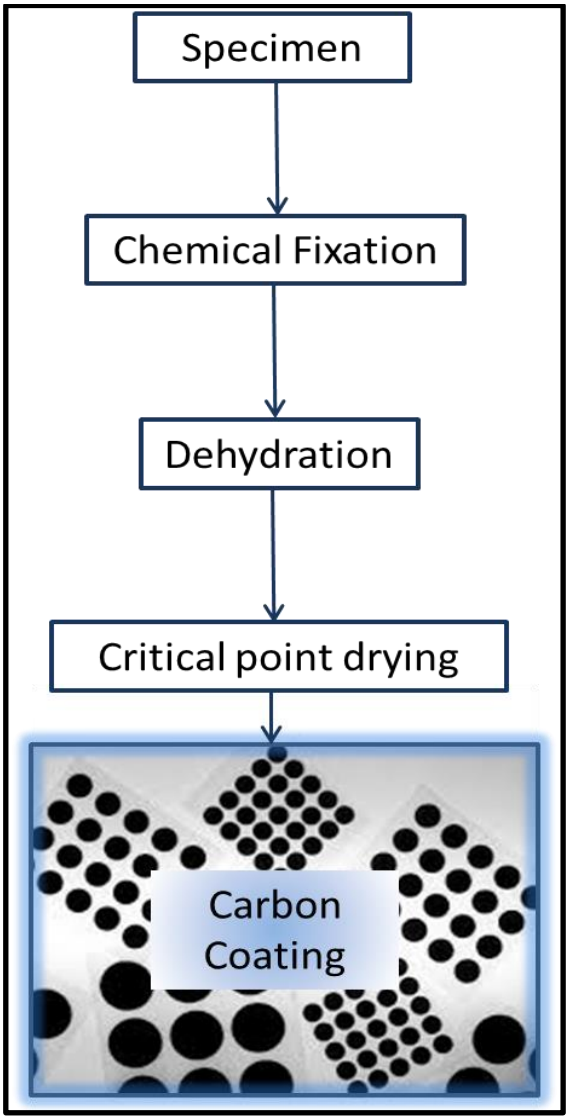

Figure 3.8: Brief summary of steps involved in the preparation of samples for scanning electron microscopy

The dried samples were mounted on metal plates and coated with carbon. The carbon coated fibrin clots were examined using a Zeiss ULTRA plus SEM (Zeiss, Germany) and the micrographs were taken at 1kV.

For erythrocytes morphology the same method was used except the PPP was replaced with whole blood. Two samples were analysed per patient and ten images were captured at increasing magnifications (10x, 20x and 40x). Two representative images from each individual were analysed using ImageJ software for quantitative

analysis of the density of the fibrin network. The histogram analysis function was selected in ImageJ. The mean and standard deviation from each histogram was used as our metric, to calculate the coefficient of variation (CV) of the 8-bit intensity of the fibrin fibres between the samples. The denser the clot, the less variation of color gradient is visible in the clot, compared to control clots where there is a more pronounce colour gradient between the “spaghetti-like” fibres and the darker background (175).

3.7 Preparation of platelets for Transmission Electron Microscopy

Blood was centrifuged at 386xg for 10min and the PRP (supernatant) was then collected in a 1.5ml tube. This PRP was used for TEM analysis.

Briefly, PRP was centrifuged at 2300xg for 8 min in order to obtain a platelet pellet which was fixed and dehydrated in preparation for SEM as described in section 3.6.

For all the ensuing steps, the sample was resuspended by using a vortex after the addition of the fluid and was centrifuged at 1000xg for 1min at the end of each step, before the supernatant was removed. Fixation was started with 2.5% gluteraldehyde for 30 min followed by three washes with 0.075M PBS buffer. Each wash step lasted for 3 min. After the third buffer wash, 1% OsO_4 was added to complete fixation. The sample was kept in the OsO_4 for 15min. The sample was again washed three times with 0.075M PBS, for 3min each. The sample was then dehydrated in a series of 30%, 50%, 70%, 90% and then three times in 100% ethanol. Each dehydration step lasted 3min.

After dehydration the TEM samples were infiltrated with resin. The infiltration process had two steps; starting with 50% EMBED 812 resin (Thermo-fisher Scientific, South Africa) was added to the 1.5ml tubes and kept it for 30 min and then 100% resin for 4 hours. The sample was resuspended in a vortex after the addition of the resin and then centrifuged at 1000xg for 1min before the resin was decanted.

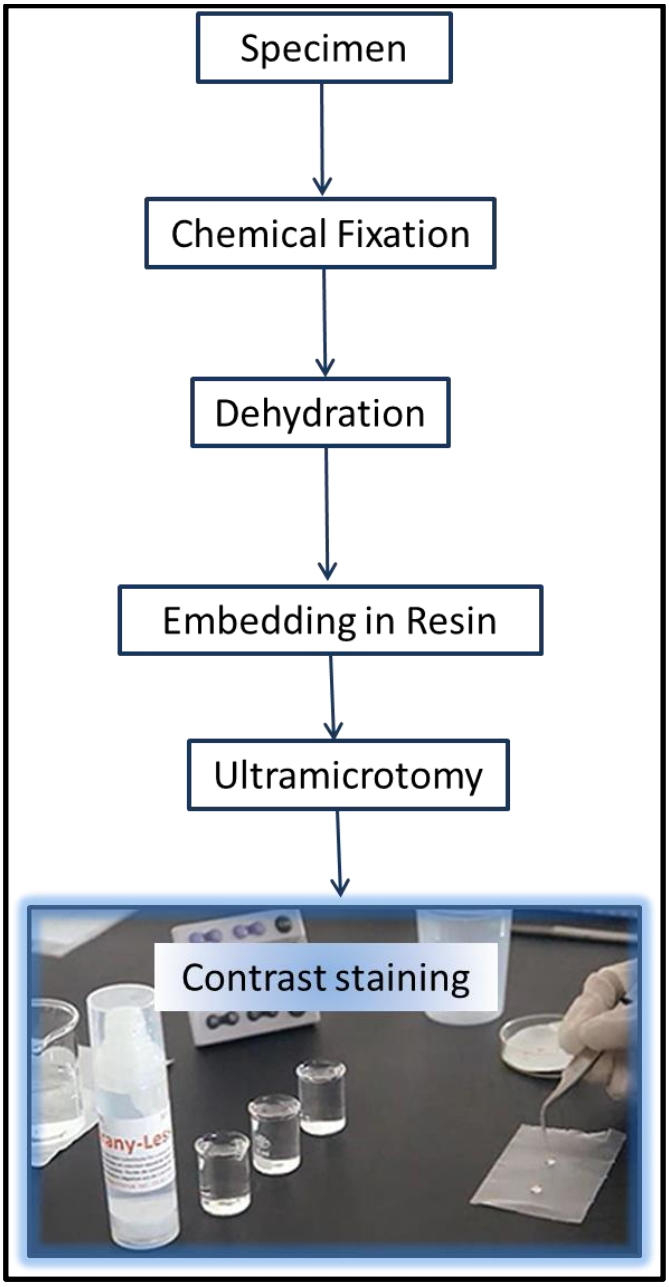

Figure 3.9: Brief summary of steps involved in the preparation of samples for transmission electron microscopy

The infiltrated samples was embedded with 100% resin with a sample number in a rubber mould and left to polymerise in an oven at 60°C for 48. The samples were sectioned using an ultra-microtome (Leica Ultracut E, South Carolina, USA). Contrast

staining was done with uranyl acetate (Sigma-aldrich, South Africa) and lead citrate (Sigma-aldrich, South Africa), as summarised in figure 3.9.

The stained samples were viewed using a transmission electron microscope (JEM 2100F, JEOL Ltd, Tokyo, Japan). TEM was used to analyse the internal structure of platelets.

3.8 Preparation of platelets for Confocal Microscopy

The blood was centrifuged at 386xg for 10 min to obtain platelet rich plasma (PRP). The supernatant was transferred to a 1.5ml tube and was centrifuged at 2300xg for 8min in order to obtain a platelet pellet. The excess plasma was decanted and 10µl of the plasma was used to resuspend the platelet pellet. 20µl CD41-FITC (Excitation: 488nm, Emission: 525nm, Beckman Coulter) was added to the resuspended sample. The sample was incubated at room temperature for 20min in the dark. After incubation 10µl of the sample was mounted on a glass slide with a cover slip. The sample was viewed with a confocal microscope (Zeiss LSM 510 META) with a Plan-Apochromat 63 X 1.4 Oil DIC objective. The filters that were used were: Ch3-1: BP 505 – 550 and Ch3-2: LP 560.

3.9 Quantitative Polymerase Chain Reaction

The principle of quantitative PCR is based on the amplification of a segment of DNA using specifically designed primer pairs, DNA template (in this case cDNA synthesised from RNA isolated from whole blood), the enzyme DNA polymerase, the relevant co-factors (magnesium chloride ($MgCl_2$)), SYBR green fluorescent dye and nucleic acids. There are three major steps involved in the PCR amplification reaction viz. denaturation, annealing and extension under thermocycling conditions (figure 3.10).

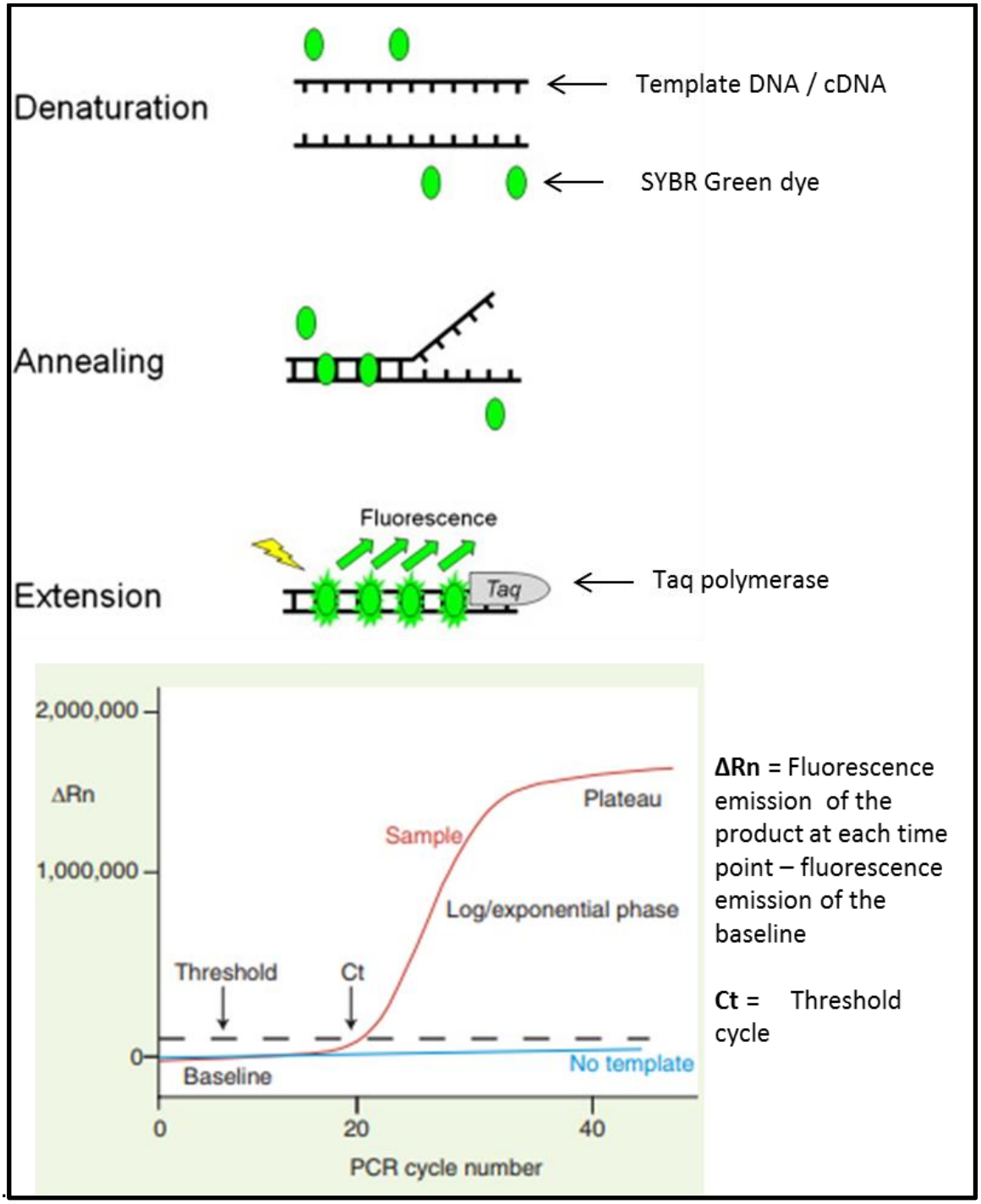

Figure 3.10: Diagrammatic representation of the principle of quantitative PCR (176)

DNA denaturation occurs at approximately 95^{o}C during which double stranded DNA forms single stranded templates. The process of annealing involves the primer sets to anneal to the 3' ends of each DNA template, a process which occurs at a lower temperature (between 54^{o}C and 60^{o}C) and is dependent on the guanine and cytosine content of the primer sequence. The extension of DNA occurs at 72^{o}C, the optimal temperature at which DNA polymerase [derived from *Thermus aquaticus* (Taq)] functions to extend the target DNA sequence from the 3' end of the annealed primer. The fluorescent dye docks into double stranded DNA thus enabling quantification. The fluorescence is captured at the end of the annealing step of each cycle and used to quantify the amount of cDNA from each samples, normalised against a housekeeping gene (18S) and relative to the control group.

Total RNA was extracted from whole blood using QIAzol® kit. QIAzol lysis reagent was added to an appropriate vessel for disruption and homogenization and subsequent centrifugation: 200µl QIAzol lysis reagent (Qiagen, South Africa) per 200µl whole blood. The tube containing the homogenized ruptured whole blood was placed on the bench top at room temperature for 5min. 0.2ml of chloroform per 1ml QIAzol lysis reagent was added to the tubes and shaken for 15s.

3.9.1 Extraction of RNA

The tubes were incubated at room temperature for 2-3min then centrifuged at 12 000xg for 15min at 4°C. The upper, aqueous phase was transferred into a new tube. 0.5ml isopropanol per 1ml QIAzol lysis reagent pipette earlier was added to the tubes and mixed thoroughly by vortex. Then the tubes were incubated at room temperature for 10min. Tubes were centrifuged at 12 000xg for 10min at 4°C. The supernatant from each tube was carefully aspirated and discarded.

At least 1ml of 75% ethanol per 1ml QIAzol lysis reagent pipetted in the beginning was added and centrifuged at 7500xg for 5min at 4°C. The supernatant was removed completely and the RNA pellet was briefly air-dried. Re-dissolving the RNA was done

in 10µl of RNase-free water. The quantification of the total RNA molecule from each sample was done with a Nano drop (Thermo-Fisher Scientific, South Africa) then the samples was kept at -80°C.

3.9.2 cDNA synthesis and amplification of FXIII-A mRNA

For each sample, cDNA was synthesised using the iScript cDNA Synthesis kit (BioRad, South Africa). RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using the iQ Superscript reagent (Bio-Rad). Specific PCR primers (500nM) (factor XIII-A sense: CCCAGAAACAGACACGTACATTCT, antisense: TCTCATTGTCCAGATACACAGCAT; housekeeping gene 18S: sense: ACACGGACAGGATTGACAGA, antisense: CAAATCGCTCCACCAACTAA, annealing temperature: 54^{o}C) were used to assess the mRNA levels of key molecules involved in the coagulopathy pathway using iTaq™ Universal SYBR® Green Supermix (BioRad, South Africa) on a thermocycler (Rotor-Gene Q, Qiagen). The fluorescence detected per well is directly proportional to the efficacy of the PCR and the relative number of template cDNA present. The mean cycle threshold was used to calculate the relative fold change in expression between groups using the method described by Livak and Schmittgen, 2001 (177).

3.10 Statistical Analysis

Differences between asthma clots and control clots were determined by using a two-tailed non-parametric t-test (Mann Whitney test), one-way ANOVA (Kruskal-Wallis test) followed by Dunn's Multiple Comparison Test and correlation (spearman) using Graphpad Prism software. The differences were considered to be significant when the P-value < 0.05.

CHAPTER 4
ANALYSIS OF THE FORMED ELEMENTS IN BLOOD: ERYTHROCYTES AND PLATELETS

4. Introduction

In this chapter the results from the analysis of the formed elements in blood is presented. Erythrocytes and platelets are particularly vulnerable to circulating and upregulated inflammatory molecules. As discussed in the literature chapter, these circulating molecules cause pathological changes to erythrocyte membranes and clumping, spreading and hyperactivation of platelets. Results presented in this chapter, show how erythrocytes and platelets deform and change in the presence of the circulating and upregulated inflammatory markers present in asthma. Various techniques were employed, including LM, AFM, SEM, TEM and CM to present evidence of the effect of the inflammatory environment on platelets and erythrocytes in asthma. The ultrastructural changes to the formed elements of blood, together with viscoelastic TEG results, AFM results and structural changes present in fibrin(ogen) in asthma patients, confirms the systemic inflammatory profiles of these individuals.

4.1 Erythrocytes

4.1.1 Light microscopy and axial ratio

Morphological analysis of the erythrocytes of participants was done by using LM. figure 4.1A represents the erythrocytes of the healthy individuals and figure 4.1B represents the erythrocytes of asthmatic patients.

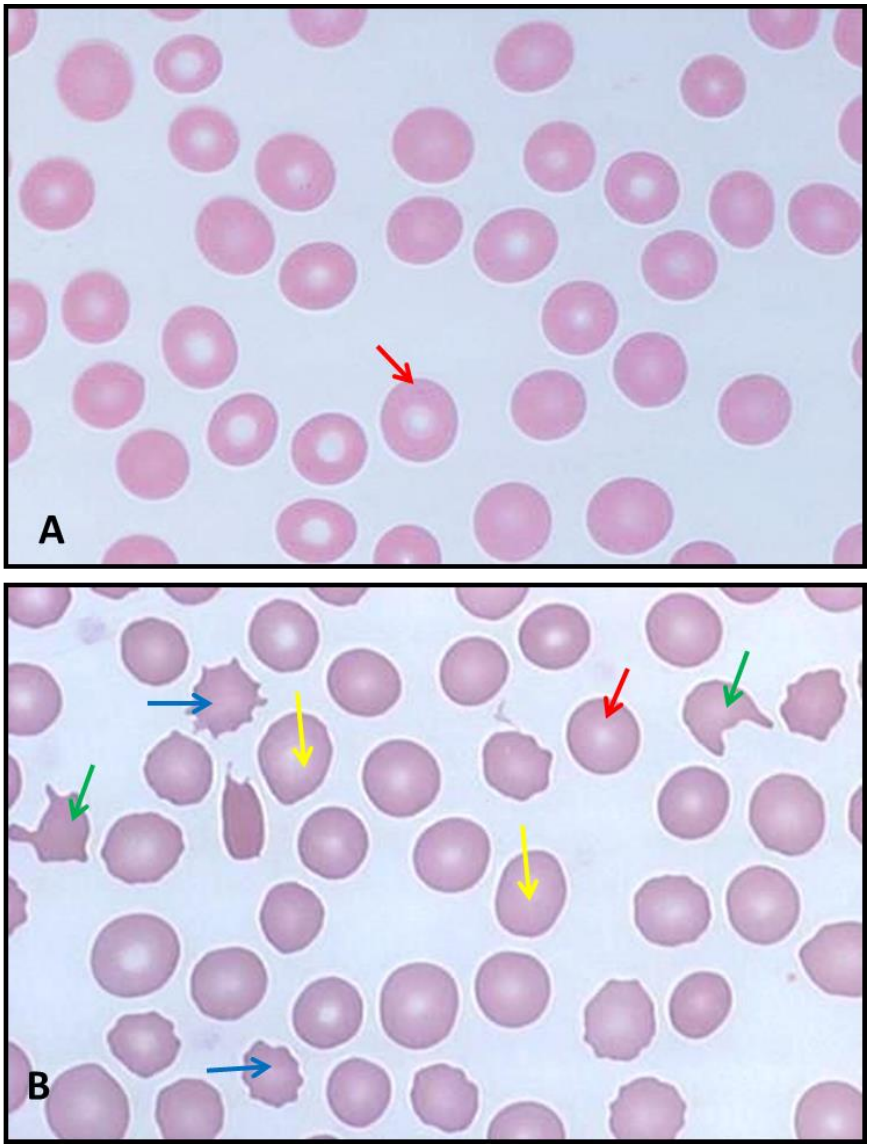

Figure 4.1: Light microscopy images of erythrocytes in healthy controls and in asthmatic patients. Magnification: x100. A: Healthy control group, B: Experimental group (patients with asthma) Red arrow: normal discoidal cell, Blue arrow: echinocyte, Green arrow: acanthocyte, Yellow arrow: ovalocyte

The erythrocytes of the healthy control group appear round and disc-shaped which is consistent with the normal discocyte morphology. Some morphological variations of erythrocytes were observed in the blood of asthma patients. The abnormal shaped erythrocytes (poikilocytes) found in the blood of asthma patients include: echinocytes , acanthocytes and ovalocytes and are indicated by arrows in the figure 4.1B. Echinocytes are abnormal shaped erythrocytes with evenly spaced rounded

projections on the edge of the cell membrane. Acanthocytes are erythrocytes demonstrating irregularly –spaced projections and ovalocytes have slightly elongated shapes.

The axial ratio of erythrocytes in both control and experimental groups were measured and compared using a program, Red Blood Cell Analyzer^{TM}. The maximum length of the individual cell and width of the individual cell perpendicular to the maximum length were measured and the axial ratio was calculated by dividing the maximal length of the individual red blood cell with the width of the cell. To compare the healthy controls to the patients with asthma, 50 cells of each individual were measured.

Statistical analysis (described in chapter 3, section 3.1) was done between the two groups to compare the axial ratio. The mean axial ratio for asthma patients was higher than controls (1.2±0.22nm vs. 1.165±0.16nm). A significant difference in the mean axial ratio of erythrocytes was found between the two groups and is indicated by a p-value of $p<0.0001$. The comparative results are plotted in the graph and shown in the figure 4.2. The graph clearly shows a much higher axial ratio value for asthma patients with distribution, outliers and density in data points indicating altered erythrocyte morphology.

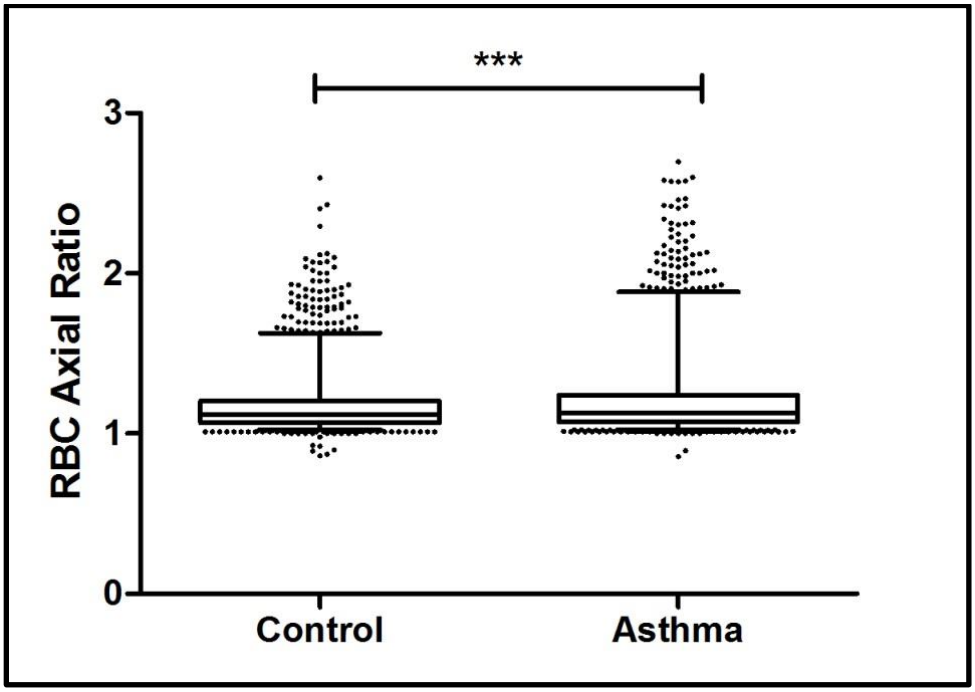

Figure 4.2: A comparison of erythrocyte axial ratio between control and asthma patients. Data is represented as a dot plot with the mean and standard error of the mean. Where *p<0.05, ***p<0.0001

4.1.2 Atomic force microscopy on erythrocyte membrane elasticity

The elasticity of erythrocytes was studied by using AFM. The elastic modulus of erythrocytes was determined by the use of Young's modulus. A comparison between the healthy individuals and asthmatic patients was made. By plotting the displacement on the erythrocyte membrane against the corresponding cantilever force, a set of force distance curves were obtained. Young's modulus values were calculated from the angle formed by the contact region slope of the retraction curve.

AFM values representing the mean elasticity modulus (Young's modulus) on erythrocyte membrane surfaces assessed for asthma patients were higher than those obtained for healthy erythrocytes. A comparison of Young modulus values between the asthma patients and control individuals were performed by utilising one-way ANOVA (analysis of variance). The results of the ANOVA demonstrated significantly higher values for erythrocytes assessed from the asthma group when compared to those assessed from the control group (p-value <0.001). The table 4.1 provides a

summary of the results obtained from the analysis. Since the AFM values (Young's modulus values) are inversely proportional to degree of elasticity (178), higher Young's modulus values obtained for erythrocytes in asthma patients, indicate a reduction in membrane elasticity or deformability of the cells.

Table 4.1: Summary of erythrocyte elasticity in asthma and controls

	Control	**Asthma**
Mean	46711	50760
Standard Error	749	896
Median	34745	32749
Mode	13082	2689
Standard Deviation	39211	56216
Sample Variance	1537498620	3160222538
p-Value	**0.001**	

4.1.3 Scanning electron microscopy analysis of erythrocytes

The morphology of erythrocytes was studied using SEM. The erythrocytes in the control group showed normal morphology with a biconcave disc shaped structure as shown in figure 4.3 and figure 4.4. The erythrocyte membrane was also studied which appeared smooth and granular, depicted in figure 4.5 and figure 4.6 representative of the SEM images obtained to illustrate the erythrocytes of the asthma patients. In asthmatics, erythrocytes show shape changes as analogous to that of images obtained from light microscope. Abnormal erythrocytes identified in asthma patients include ovalocytes and echinocytes. Under higher magnification (100X), erythrocyte membranes appeared irregular and granular.

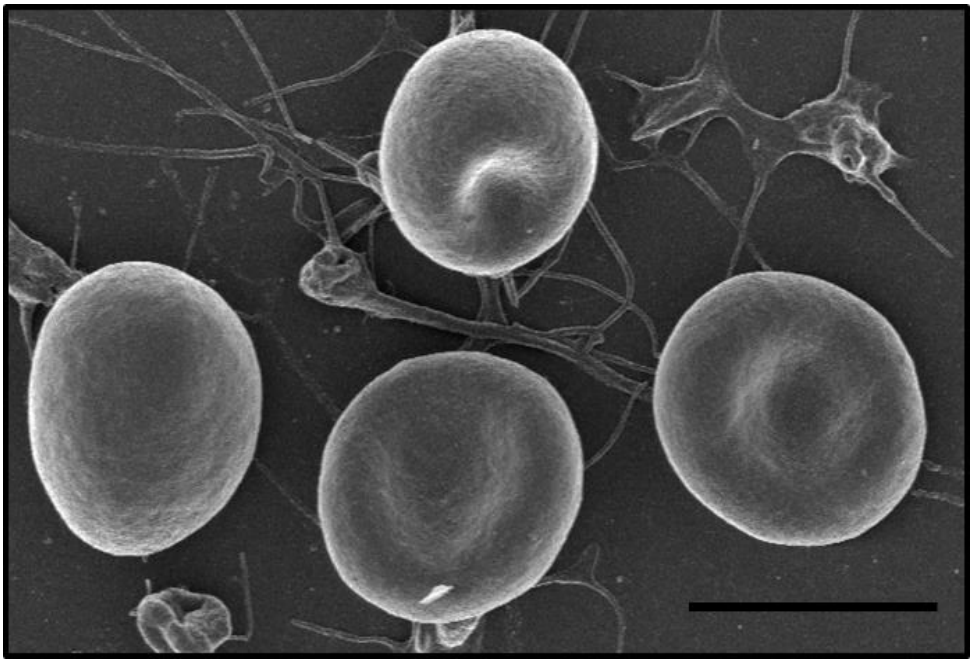

Figure 4.3: Scanning electron microscopy images erythrocytes of the healthy controls at 10K magnification. Scale bar: 2µm

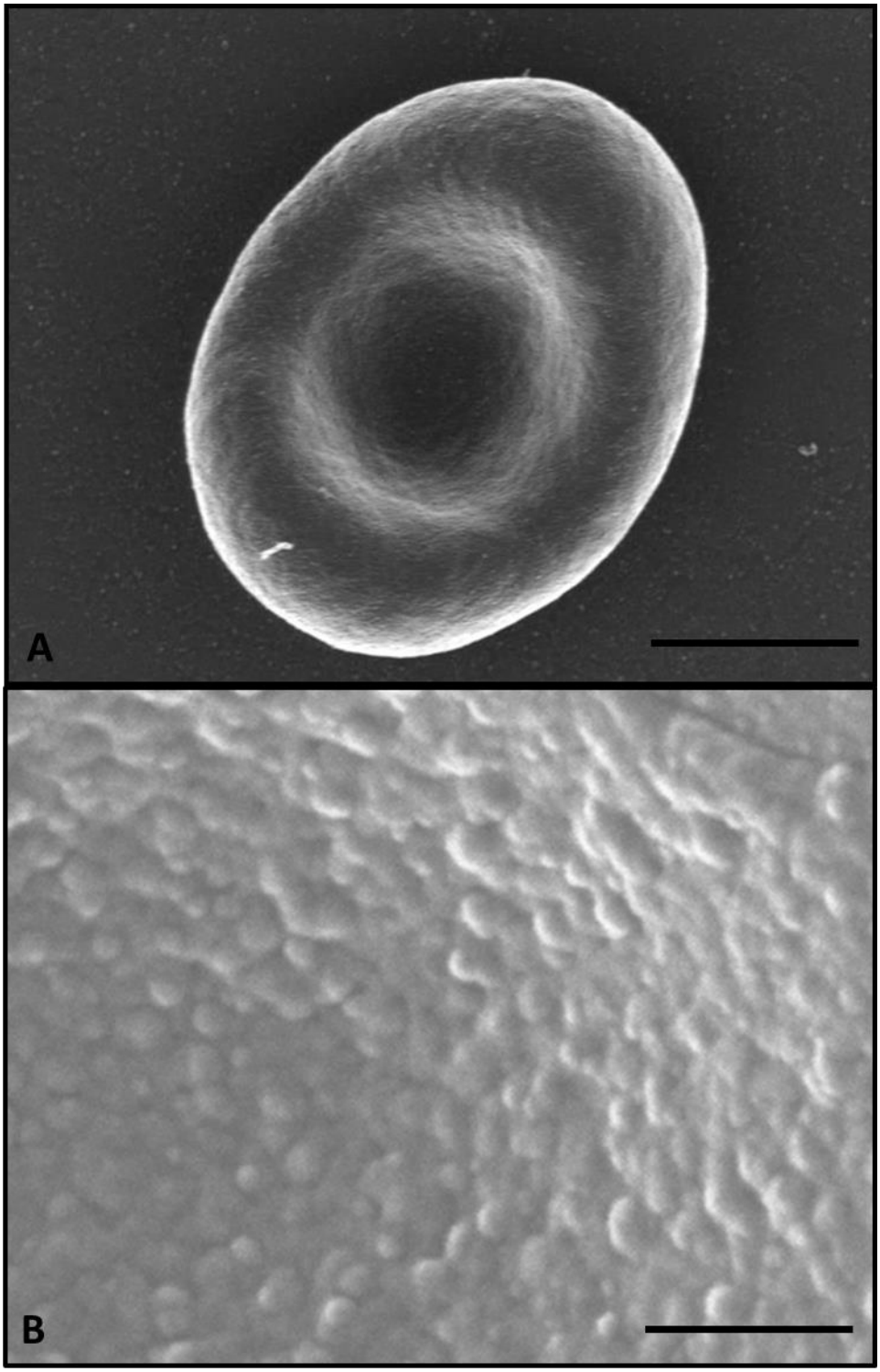

Figure 4.4: Scanning electron microscopy images erythrocytes of the healthy controls at 50k and 100k magnification. Scale bar: A: 6µm, B: 50nm

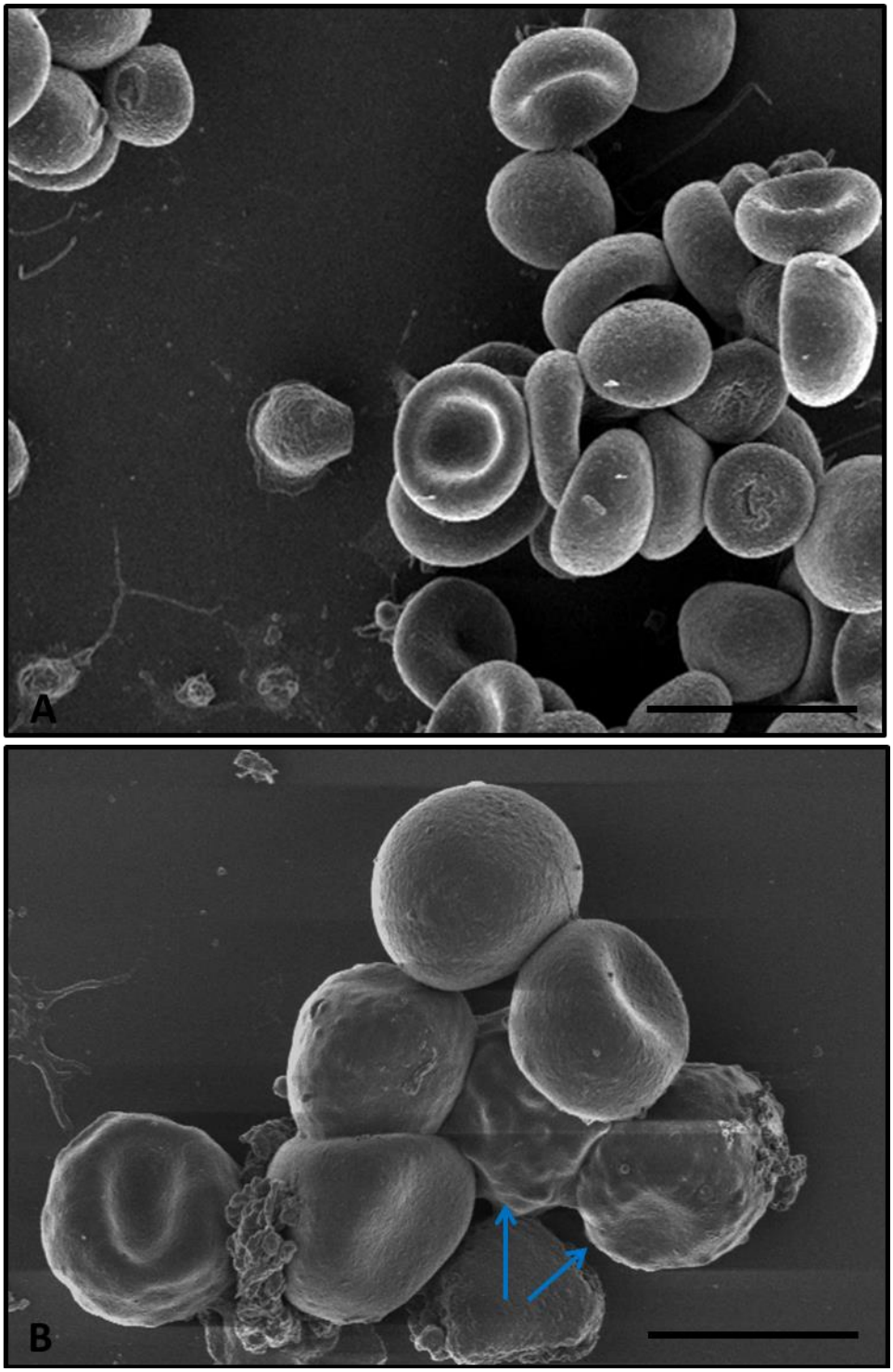

Figure 4.5: Scanning electron microscopy images erythrocytes of asthma patients at 5K and 10K magnification. Blue arrow: echinocytes. Scale bar: A: 1µm, B: 2µm

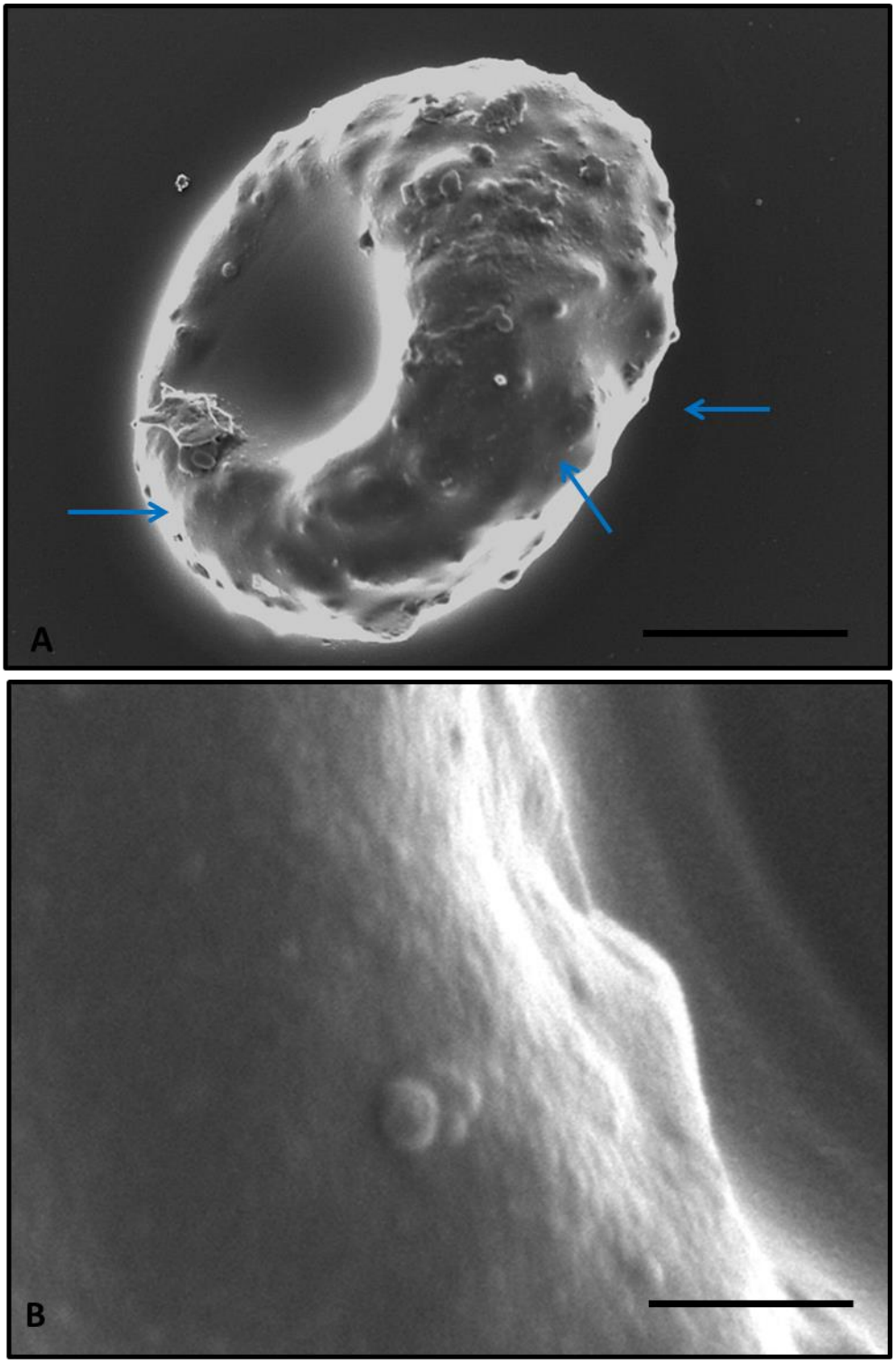

Figure 4.6: Scanning electron microscopy images erythrocytes of asthma patients' 50k and 100k magnification. Blue arrow: uneven cell membrane. Scale bar: A: 6µm, B: 50nm

4.2 Platelets

4.2.1 Scanning electron microscopy

Scanning electron microscopy was employed to analyse the morphology of platelets in asthmatic and control group. figure 4.7 (A and B) shows images of a platelet representative of a healthy individual. Healthy platelets are discoidal with small pseudopodia (indicated by green arrows), showing slight contact activation.

Under normal physiological conditions, platelets do not get activated and they are non-adherent which allow them to flow freely in the blood. Since, the healthy individuals are not suffering from any inflammatory conditions, the platelets in control group are relatively inert (inactive and non-adherent). However, inert platelets can be activated by contact during sample preparation and will be able to adhere to the coverslip used to create the platelet smears. This may be the reason for the slight activation seen in the platelets in the control group.

The contact activation observed in the healthy participants is consistent with the previous studies by Pretorius *et.al* (113, 179). Figure 4.8 represents a typical healthy platelet membrane at high magnification. The membrane morphology of these platelets was analysed under high magnification (figure 4.8) to determine whether there area any alterations when compared to healthy individuals. The platelet membrane has typical globular structure with open canalicular system (OCS) represented by white arrows. The platelet membrane appears granular.

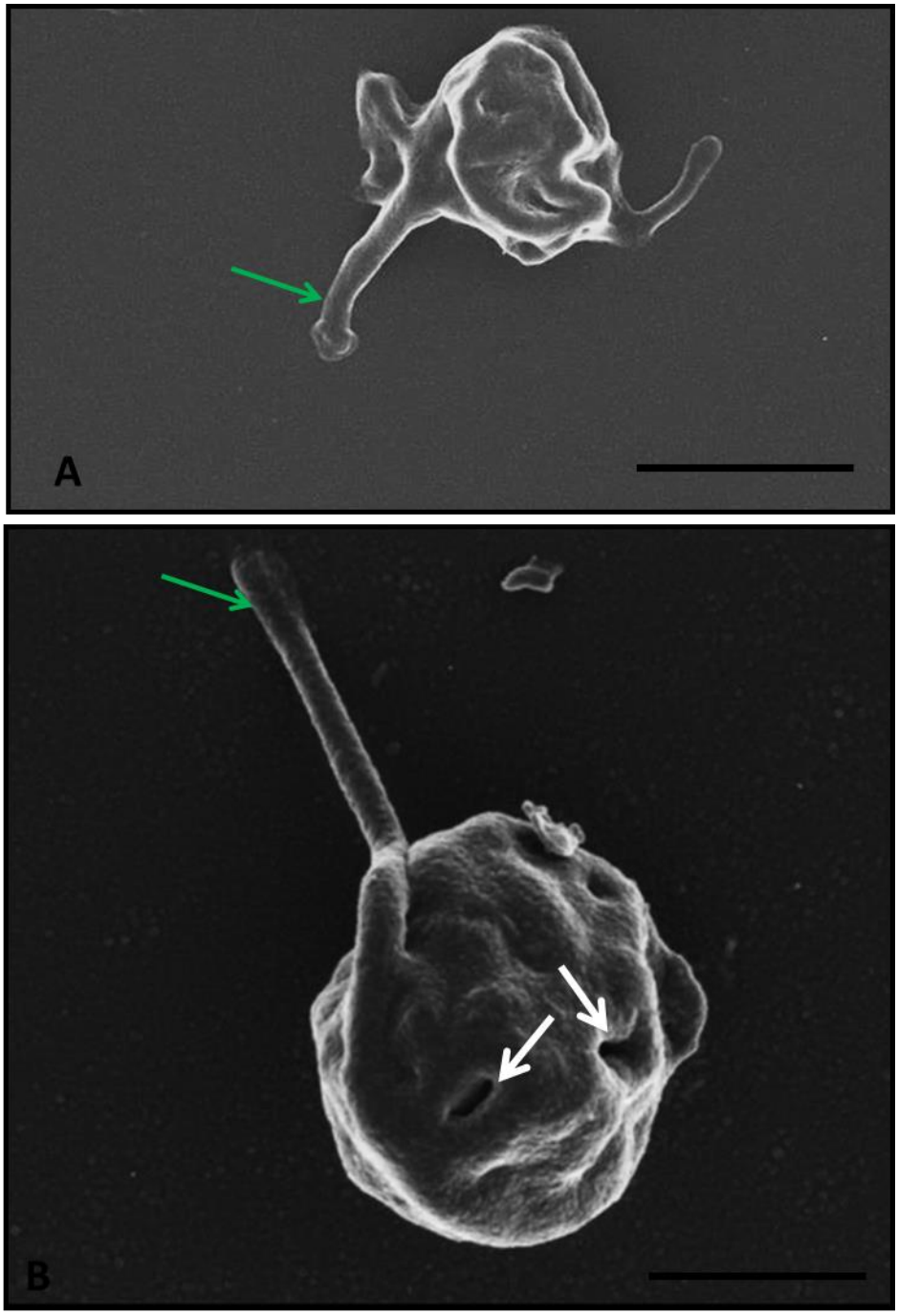

Figure 4.7: A-B Scanning electron microscopy images of platelets in healthy controls: Green arrow: Platelets of healthy individuals showing small pseudopodia. White arrows: Open canalicular system. Green arrows: pseudopodia. Scale bar: A: 6µm, B: 30µm

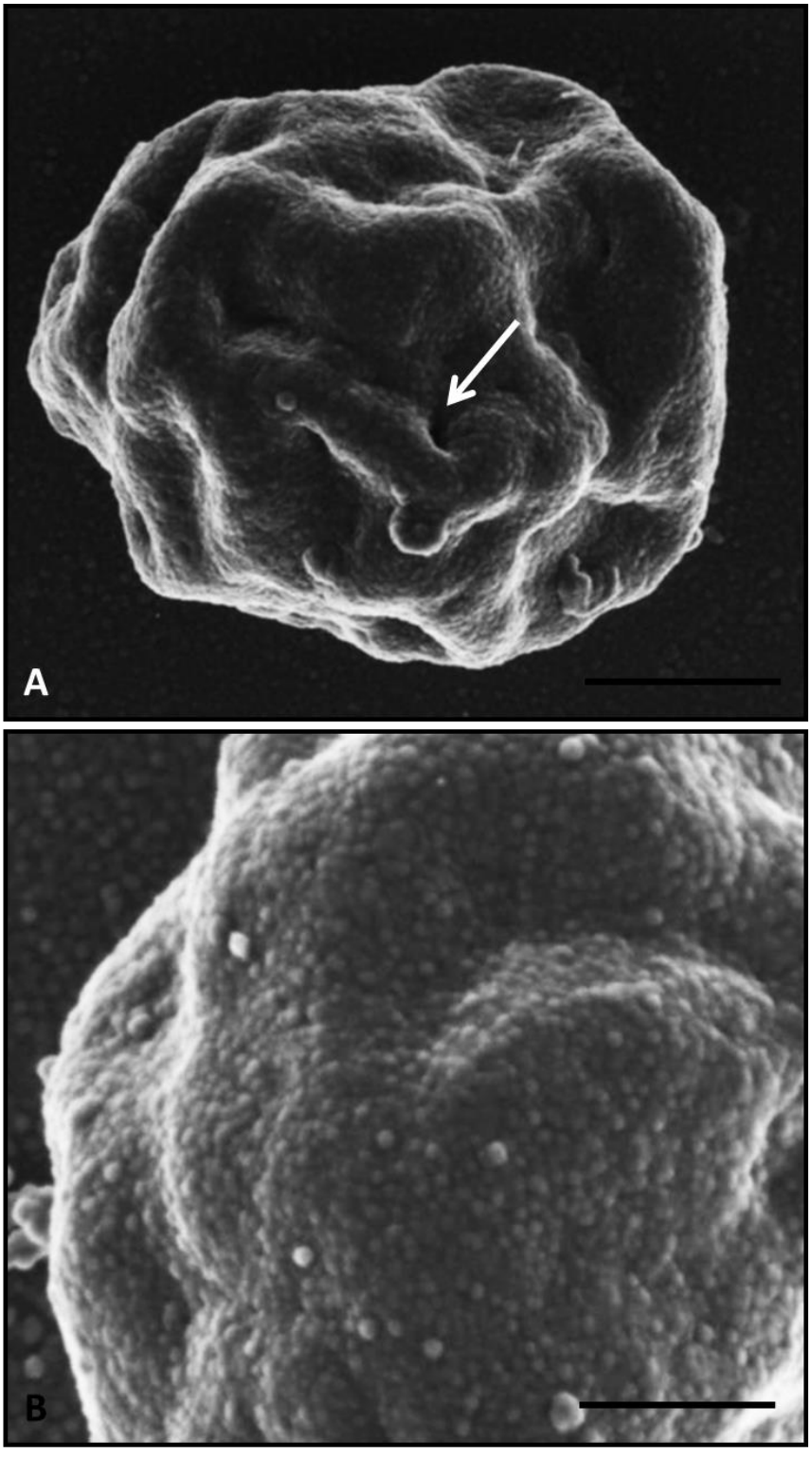

Figure 4.8: A-B Scanning electron microscopy images of platelets in healthy controls at a very high magnification. White arrows: Open canalicular system. Scale bar: A: 50µm, B: 75µm

Figure 4.9 and figure 4.10 represent the platelets from asthmatic patients. These platelets have changed morphology with more pronounced pseudopodia (Green arrow). In asthma patients transformation of platelets from a disc shape to an amorphous structure can be seen.

Asthmatic platelets also demonstrated different dynamics such as spreading (orange arrow in figure 4.9 & figure 4.10.) and platelet–platelet interactions (depicted in figure 4.9 A; and figure 4.10 A). The extensive pseudopodia formation, platelet spreading and platelet-platelet interactions are indications of platelet activation shown by platelets in asthma patients when compared to the control group.

The platelet membrane was also analysed under higher magnification. Figure 4.10 B represents the platelet membranes of asthma patients. In asthmatic platelets, the membranes appear more globular and granular. The membrane also has an open canalicular system. This change in morphology could be due to hyperactivation of platelets in asthma patients.

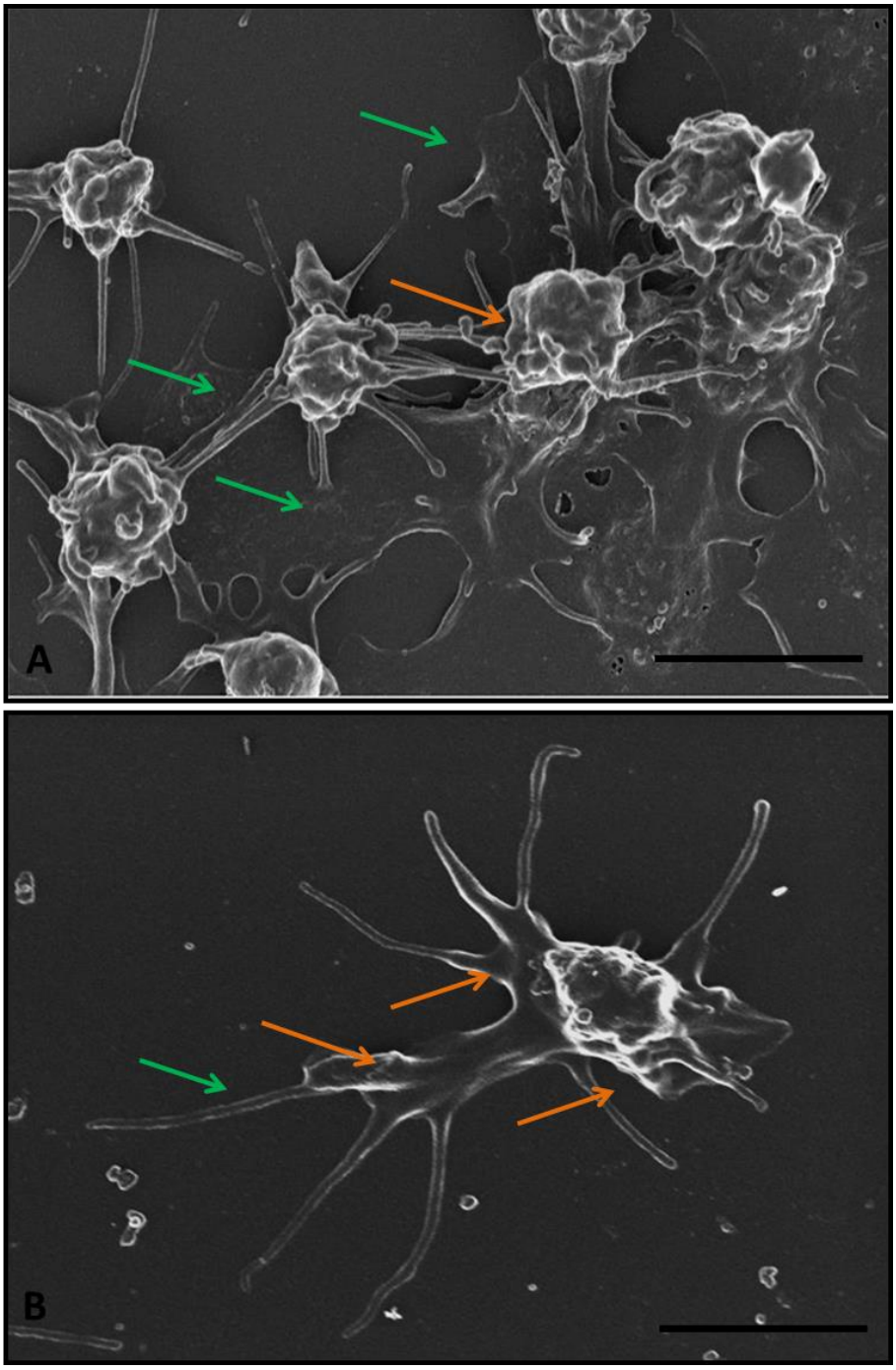

Figure 4.9: Scanning electron microscopy images of platelets in patients with asthma: Orange arrows: platelet spreading. Green arrows: pseudopodia and membrane spreading. Scale bar: A: 4µm, B: 6µm

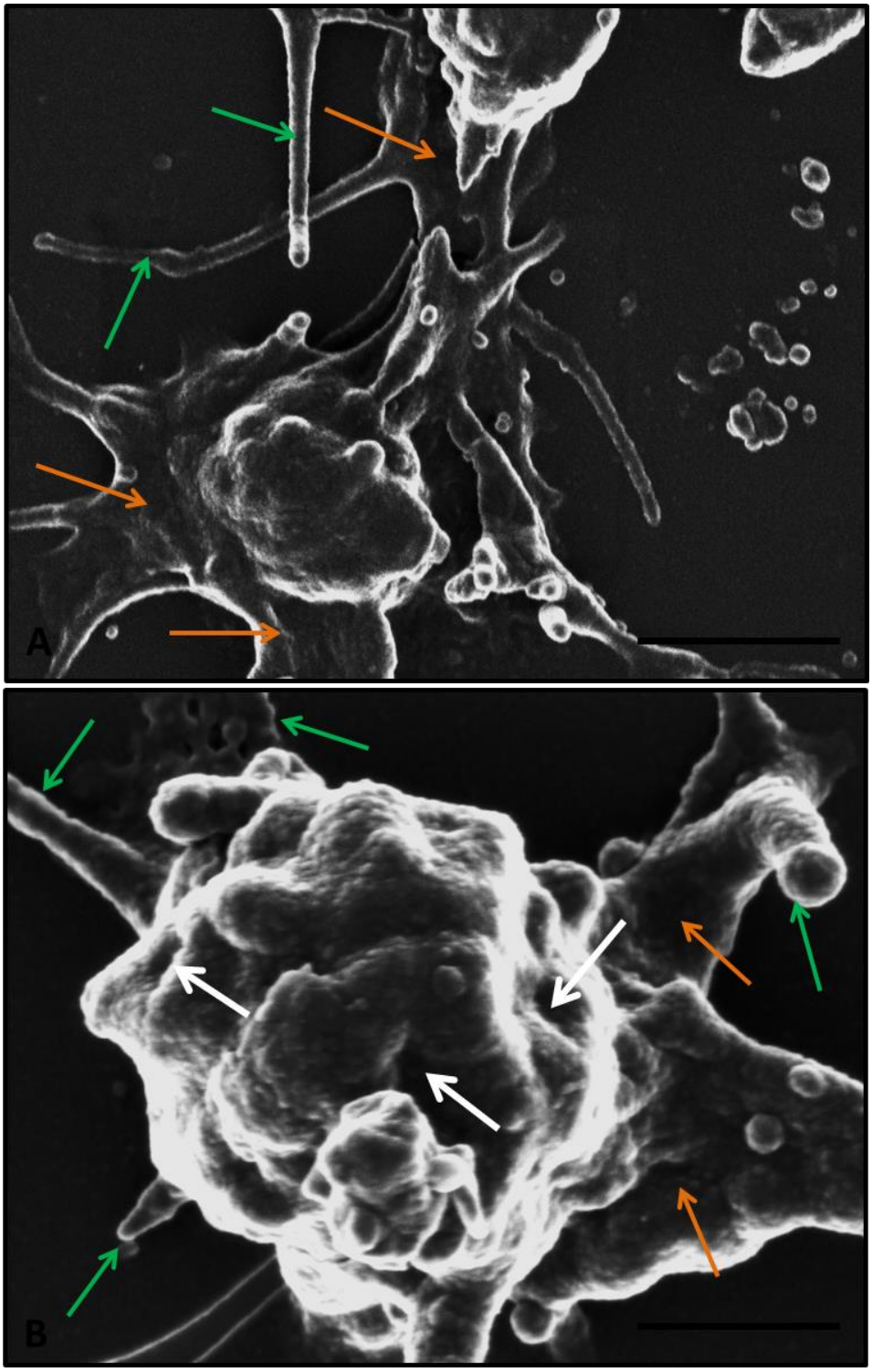

Figure 4.10: Scanning electron microscopy images of platelets in patients with asthma: Orange arrows: platelet spreading. White arrows: open canalicular system. Green arrows: pseudopodia and membrane spreading. Scale bar: A: 30µm, B: 50µm

4.2.2 Transmission electron microscopy

The internal morphology of the platelets was further studied using TEM. The results obtained are presented in figures 4.11 to figure 4.15. TEM can provide information regarding the internal structure of the platelets that are not visible with SEM. Figure 4.11 represents the platelets of healthy individuals and figure 4.12 to figure 4.15 represent platelet of asthma patients.

The platelet representative of the control group has a disc shape (typical of inactive platelets), while the platelet in asthma patients shows a change in shape with pseudopodia indicative of platelet activation which supports the findings of the SEM. The cytoplasm of platelets of control group contains platelet granules as well as organelles such as mitochondria (indicated by white arrow in figure 4.11)

Platelet granules include dense granules and alpha granules (180). Alpha granules are the most abundant granules in a human platelets and are round to oval in shape (denoted by orange arrows in figure 4.11). The dense granules are normally small with a dense core and a lighter surrounding space.

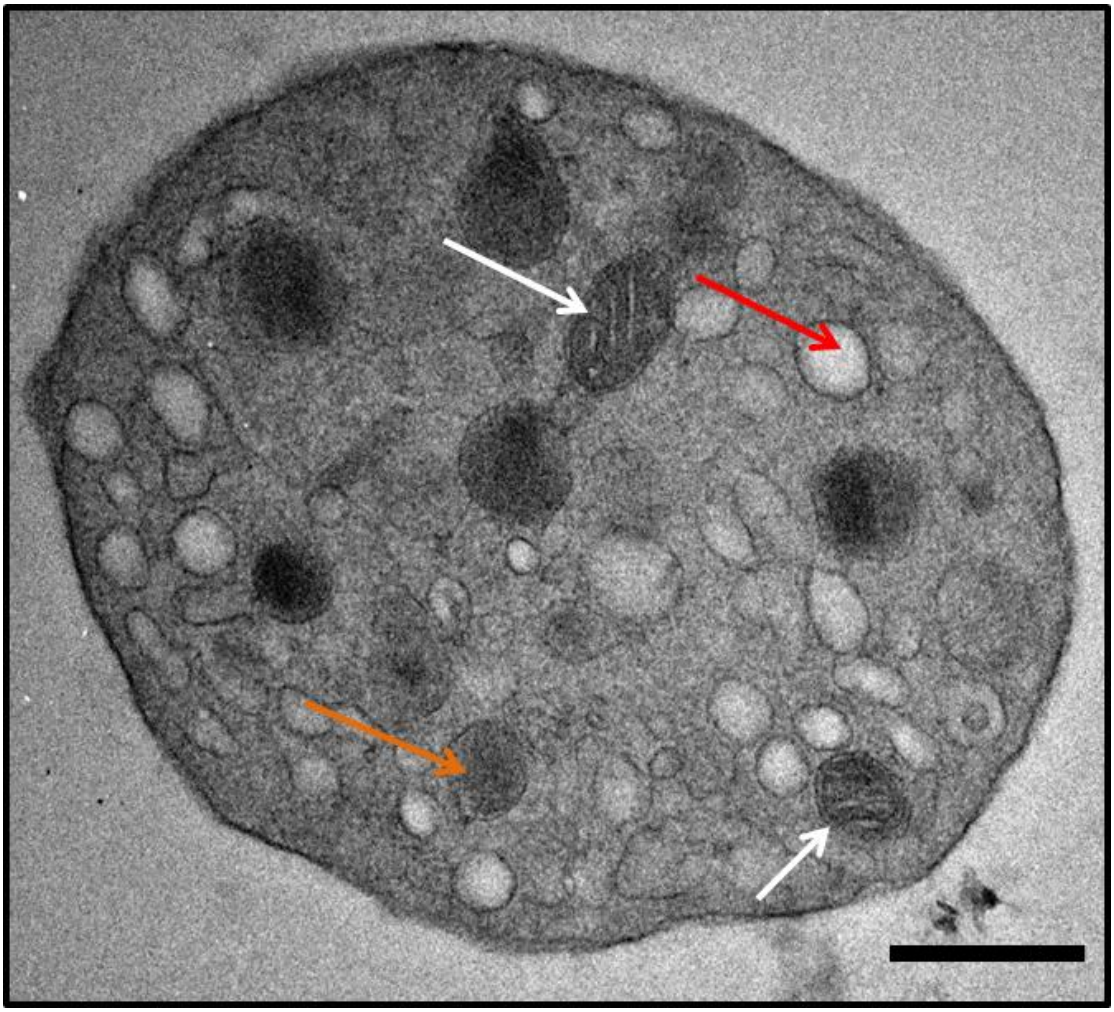

Figure 4.11: Transmission electron microscopy images of platelets in control. White arrows: mitochondria. Red arrows: Dense tubular system. Orange arrows: alpha granules. Scale bar: 2cm: 0.5µm

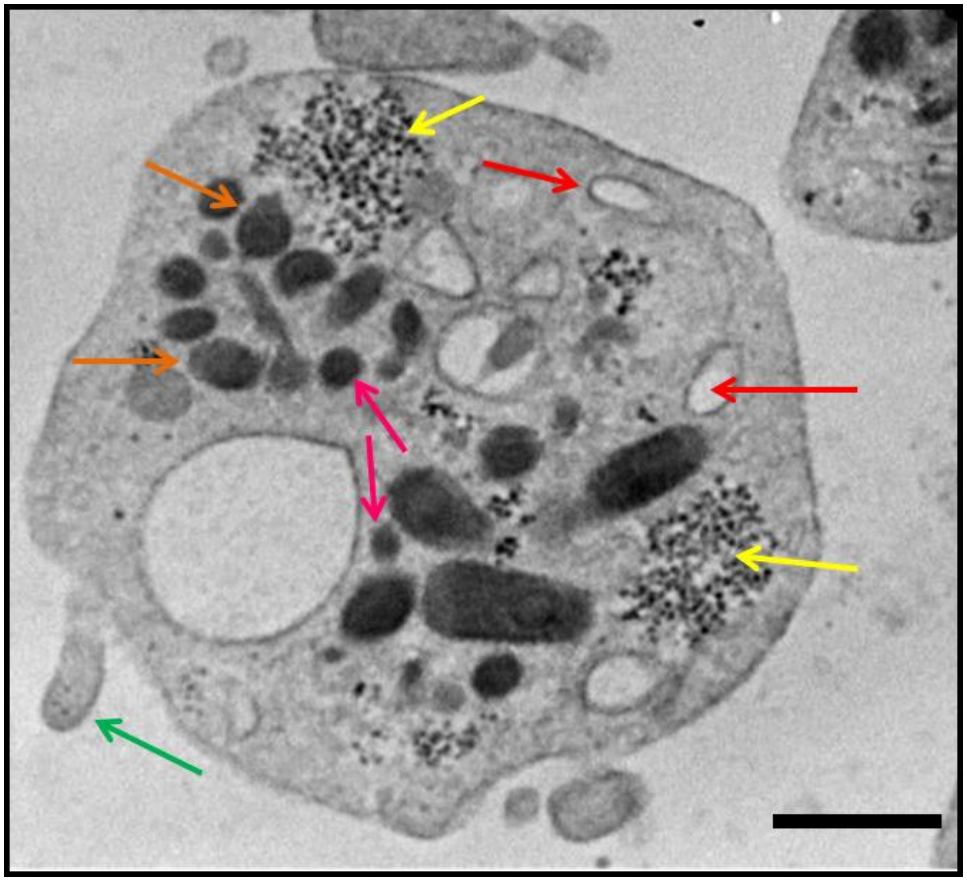

Figure 4.12: Transmission electron microscopy images of platelets in asthma. Red arrows: Dense tubular system. Green arrows: pseudopodia. Pink arrows: dense granules. Orange arrows: alpha granules. Yellow arrows: glycogen. Green arrow: pseudopodia

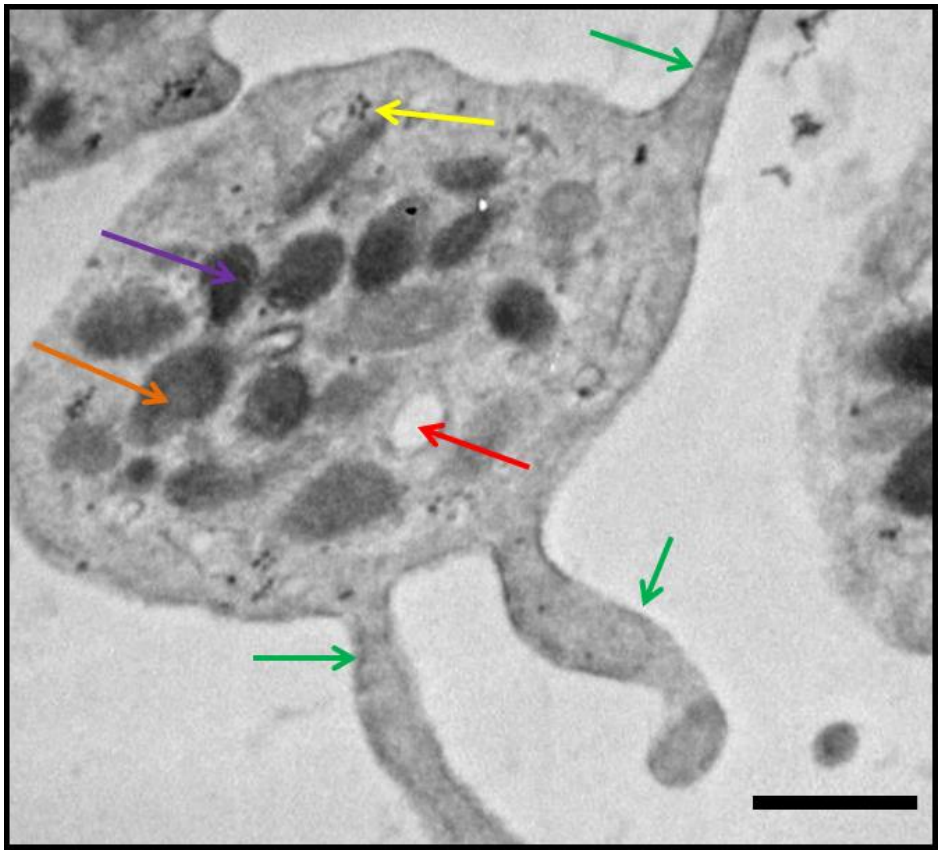

Figure 4.13: Transmission electron microscopy images of platelets in A: control and B-D: asthma patients. Red arrows: dense tubular system. Green arrows: pseudopodia, purple arrows: dense granules. Orange arrows: alpha granules. Yellow arrows: glycogen

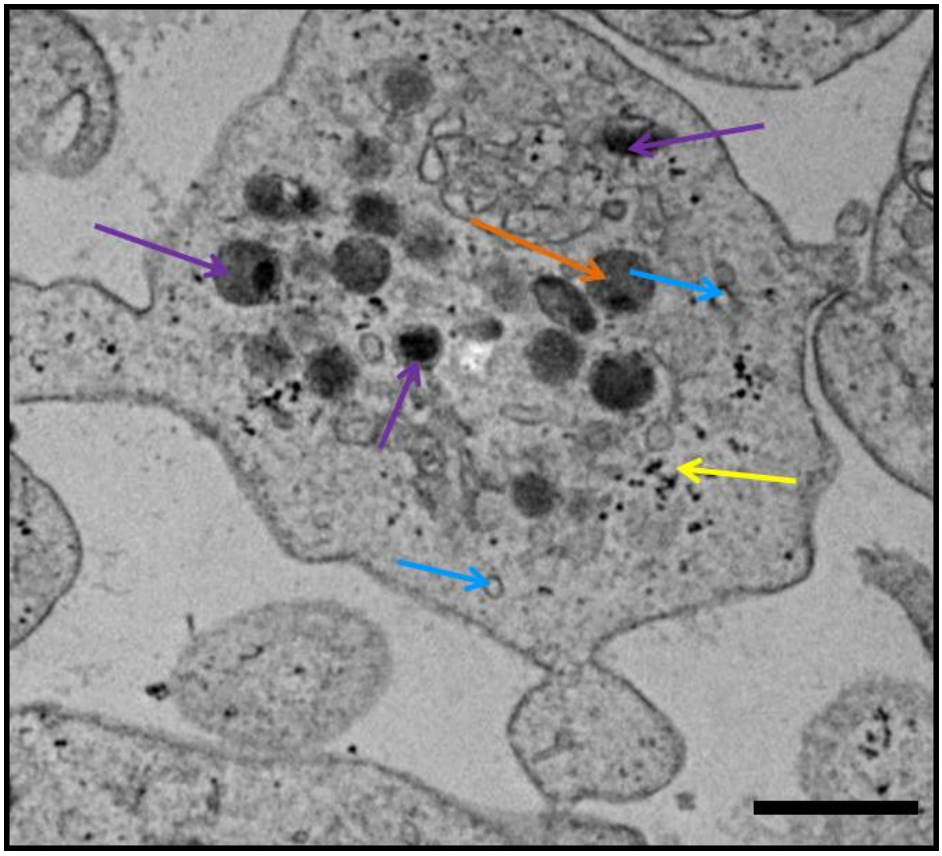

Figure 4.14: Transmission electron microscopy images of platelets in A: control and B-D: asthma patients. Purple arrows: dense granules. Orange arrows: alpha granules. Blue arrows: open canalicular system. Yellow arrows: glycogen

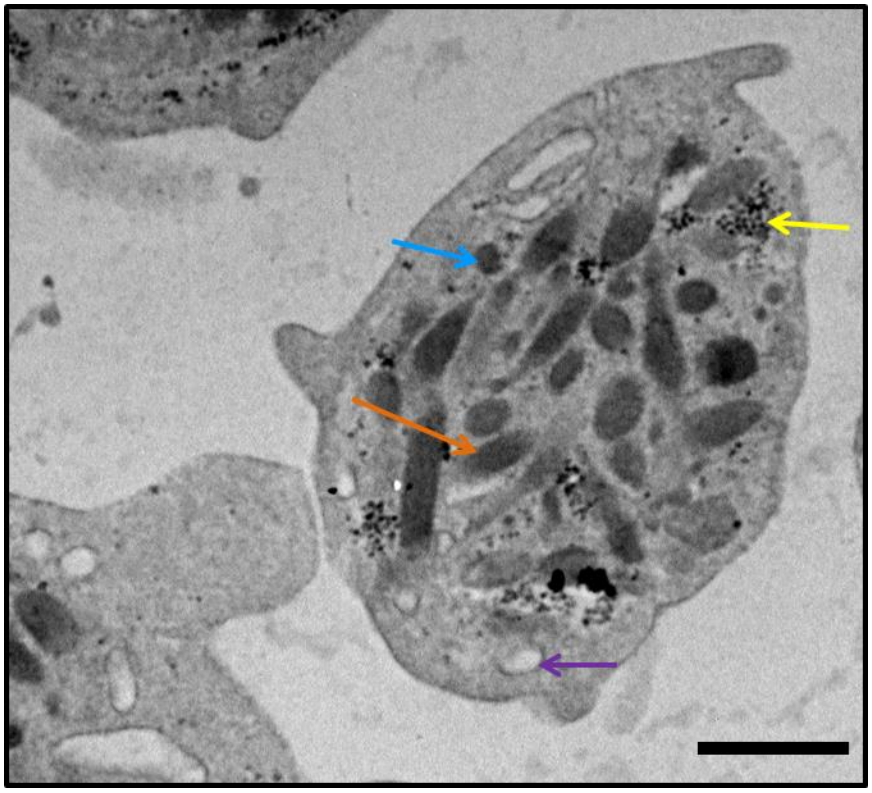

Figure 4.15: Transmission electron microscopy images of platelets in asthma patients. Purple arrows: dense tubular system. Orange arrows: alpha granules. Blue arrows: dense granules. Yellow arrows: glycogen

Figure 4.11 to figure 4.15 represent the platelets of asthmatic patients. These platelets have undergone a change in shape and are replete with numerous secretory granules in comparison with healthy controls. These secretory granules include both alpha and dense granules (denoted by orange and pink arrows in figure 4.12 to figure 4.15). Asthma platelets also have pseudopodia (indicated by green arrow in figure 4.13), indicative of platelet activation. The invaginations of the platelet cell membrane forms the elaborate systems of tunneling called open canalicular system indicated by blue arrow in figure 4.14 and dense tubular system indicated by red arrow in figure 4.12. The OCSs appear as clear regular channels with round shape whereas the DSTs are irregular and oblong. The DTS stores calcium and a variety of enzymes involved in platelet activation which reduce the contrast between these channels and the cytoplasm. The morphology of the OCS and DTS appeared similar in both groups. Organelles such as mitochondria (denoted by white arrow in figure 4.11) also

observed in the cytoplasm of these platelets. No structural differences were found in these organelles. Numerous widely distributed glycogen granules (yellow arrow in figure 4.12 to figure 4.15) were also found in asthma platelets. Glycogen is a readily mobilized, stored form of glucose and supply energy to the platelet for the process of activation

4.2.3 Confocal microscopy

The images representing the platelets of healthy and asthmatic patients are depicted in figure 4.16. These images were studied to elucidate whether platelet activation was present, where the fluorochrome (FITC)-bound antibody react with the transmembrane glycoprotein CD41 expressed by platelets. The CD41 antibody is not an activation marker, but can be used to study the structural morphology of the platelets in order to confirm the morphological alterations seen with SEM. The platelet alterations seen with SEM were confirmed by confocal microscopy seen with shape alterations present in the asthmatic individuals. The platelets in asthmatic patients are irregularly shaped with fluorescent pseudopodia visible due to increased activation. Platelet interaction can also be seen in figure 4.16 from asthma patients further confirming the activation of platelets in asthmatic individuals. These results support the findings of SEM and TEM.

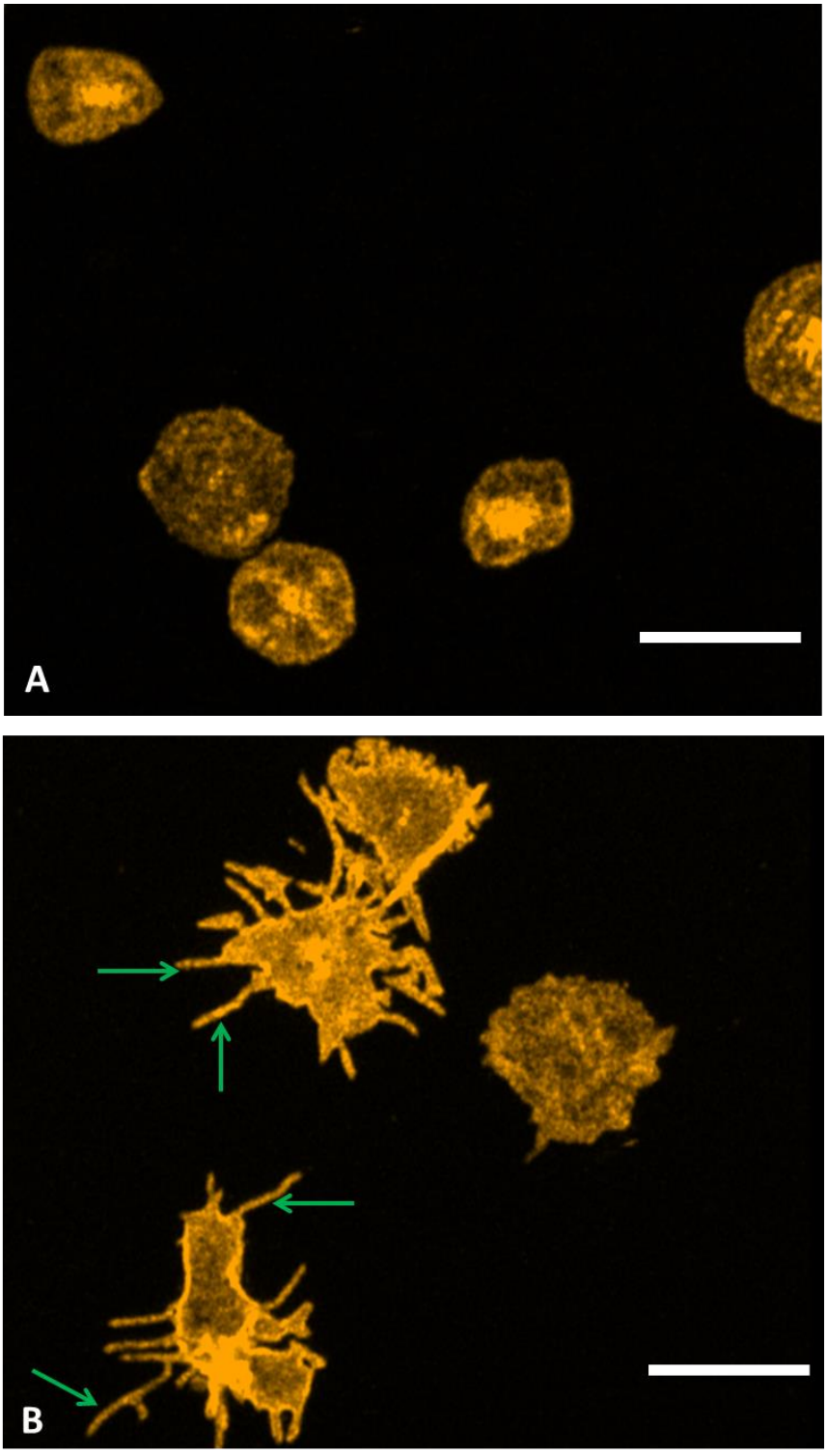

Figure 4.16: Confocal microscopy images of platelets in control and asthma patients: A: discoidal platelets in control group, B: activated platelets in asthma patients, Green arrow: pseudopodia. Scale bar: 2cm: 5µm

4.3 Discussion

4.3.1 Erythrocyte morphological changes observed

It has been always assumed that erythrocytes do not play an active role in blood coagulation, even though they make up the majority of the thrombus. However, recent evidence has indicated that a PS flip takes place on the membrane of the erythrocytes cell creating a prothrombotic surface for the conversion of fibrinogen to fibrin, thereby influencing coagulation (181). For this reason, the morphology of erythrocytes was studied to determine the role that morphological alterations could play in the formation of the clot in the presence of chronic inflammation in asthmatic patients.

Tissue perfusion depends on the rheological properties of the blood. Haemorheology is the study of the flow of blood in a blood vessel, mainly focused on the dynamics of erythrocytes in the vascular system (182). The erythrocytes have the capacity to alter their shape due to a flexible membrane allowing them to enter capillaries with a very small diameter (182). Any change in erythrocyte physiology influences the rheology of the blood. The dynamics of the erythrocytes especially their deformation and orientation have an impact on blood fluidity and subsequently on blood flow (154) influencing tissue perfusion (154). In several disease processes, blood flow may be severely impaired due to alterations in cell rheology by genes, parasites or by changes in the microenvironment (154). Erythrocytes have the ability to bind and transport oxygen to the cells of the body via Hb. This physiological function of erythrocytes depends on to its shape and biomechanical characteristics.

Erythrocytes morphology may influence the density of the clot thus altering the coagulation cascade by increasing thrombus strength and reducing thrombolysis (183). The morphological profile of erythrocytes of asthma patients detected via imaging studies using LM and SEM showed shape variations, from discocytes to poikilocytes. Poikilocytes are abnormally shaped erythrocytes found in blood. The abnormally shaped erythrocytes observed in the images of asthmatic patients mainly include echinocytes along with acanthocytes, and ovalocytes. SEM was employed to determine whether the shape changes were also visible under higher magnification.

SEM images of erythrocytes supported the findings obtained from LM. Poikilocytosis is a condition in which increased numbers of poikilocytes (more than 10% of the erythrocytes) are found in the blood. Since these abnormal cells do not make up more than 10% of the total population of erythrocytes among majority of the individuals with asthma, it doesn't indicate any pathology. However, presence of defective erythrocytes in the blood of patients with asthma can reduce the oxygen content in the blood leading to lethargy. The observed shape changes in some individuals with asthma could be the effect of the drug used for treatment (155).

Axial ratios of erythrocytes in each group were determined and found to differ in a statistically significant manner ($p<0.0001$). An increased axial ratio in asthmatic erythrocytes also supports the ultrastructural variations. The normal discocytic shape of erythrocytes gets transformed into echinocytic shape in which a spiculated membrane can be observed (181). Hence the morphopathology of erythrocytes may be attributed to cell membrane abnormalities. It has been reported that the formation of echinocytes or acanthocytes can alter the membrane bilayer leaflet induced by lysophopholipids, fatty acids or diverse chemical agents (184, 185).

The elasticity or deformability of erythrocytes can affect the viscoelastic properties of the clot (183). The results obtained from the study of erythrocytes show a clear relationship between elasticity and erythrocyte morphology. Research shows that normal shaped erythrocytes are more elastic when compared to poikilocytes (186). Statistical analysis of AFM data showed that erythrocytes in asthmatics possessed significantly reduced membrane elasticity relative to that of erythrocytes from healthy controls. Since Young's modulus is inversely proportional to the elasticity of the erythrocytes (178), the higher Young's modulus values obtained for erythrocytes in asthma patients indicate a decrease in membrane elasticity or deformability of the cells. This decrease in membrane elasticity may be the cause of the shape changes observed with LM and SEM. Reduced cellular life span is associated with decreased erythrocyte membrane deformability or elasticity (178). Since a decrease in elasticity

would reduce the ability of the red blood cell to return to its original shape once it has been deformed.

One of the goals of the study was to determine whether there are any ultrastructural changes in the erythrocyte morphology and of platelets in asthma patients. The results of this study indicate that erythrocytes in asthma show shape changes and reduced elasticity or deformability. The change in biophysical profile of erythrocytes detected in this study may be ascribed to the modulation of the cell membrane due to the presence of oxidative damage or inflammatory conditions present or may be the effects of drugs that are used in asthma treatment, which require further research. Since the oxygen carrying capacity is significantly compromised in asthma patients, this finding may enable new strategies for the treatment and the management of asthma.

4.3.2 Platelet morphology and activation

Platelets play a critical role in the maintenance of haemostasis by forming a platelet plug that is the first step in the prevention of blood loss when damage to a blood vessel occurs. The plug is then reinforced by the formation of fibrin fibres that provides stability to the clot. Platelets are also involved in several other functions in the body such as modulating inflammatory and immune responses also involving the coagulation process (180). Platelets get activated upon endothelial disruption or when endothelium is perturbed by inflammation (187). Previous research have indicated that platelets undergo activation in response to a variety of stimuli characterised by shape change, pseudopodia formation, spreading and the secretion of several platelet granules present in the cytoplasm of the platelets (188). Platelet activation is the first step in the activation of the coagulation cascade (189). This study, in addition to the study of erythrocytes, aimed to evaluate the overall morphology of platelets to determine their contribution to procoagulant environment present in asthma patients.

Kuwahara and co-workers reported that platelets undergo shape changes during activation and adhesion, and therefore these changes can be used to detect both the

presence and degree of platelet activation present in individuals (190). Platelet activation was observed in individuals with asthma by investigating the morphology of platelets, using SEM, TEM and CM. Upon activation, platelet undergo shape change, which is characterised by the presence of pseudopodia, spreading and platelet aggregation (190). These morphological changes indicative of platelet activation were observed in asthma. Since there was no addition of thrombin, these changes are present without any artificial activation of the coagulation. Since these shape changes were not present in healthy individuals it can be concluded that platelet activation is present in asthmatic individuals and are activated most likely by the presence of chronic inflammation. The finding of platelet activation in the experimental group is supported in the literature indicating that platelet activation is increased in asthmatic patients (117, 191).

The morphological changes found in platelets of individuals with asthma provide some insight into the physiological function of platelets in relation to thrombogenesis. Hemisphere-shaped platelet with extensive spreading observed in platelets of asthma patients found in SEM images provide evidence that activation and adhesion process are almost complete. Although slight activation is expected due to contact activation (seen in healthy controls) with the preparation of platelet smears, noticeable changes related to platelet activation are shown by these platelet in asthma patients. These changes include; spreading, numerous pseudopodia and platelet aggregation. Platelet spreading is the last step in platelet activation which forms a tight seal across the damaged area and also involves the recruitment and adherence of other platelets to the injury site. The spreading of platelet (demonstrated in asthma patients), may indicate that platelets are hyperactivated in asthmatic individuals. In addition, the platelet-platelet interactions and aggregation of platelets furthermore indicate the presence of platelet activation. These findings corroborate the evidence of platelet activation and its contribution to the pathogenesis of asthma (191). Platelet count was found to be normal in both the healthy and experimental groups, suggesting that platelet activation contributes to the procoagulant environment seen in asthmatic individuals, rather than thrombocytosis or thrombocytopenia.

The platelet activity in the experimental group corroborates the data in the literature, supporting the enhanced platelet activity in bronchial asthma (117, 192). The study of platelets can be useful both in the detection of various disease and in the study of disease progression. Platelets are normally in an inactive form when they circulate through the blood vessels. The ultrastructural evaluation of platelets in asthma shed light regarding the involvement of platelets in the asthma pathophysiology. It can be concluded that platelets might contribute to asthma pathogenesis through platelet activation and aggregation and also by releasing inflammatory mediators. Further research into platelet activation, pathways and potential biomarkers could open avenues towards new diagnostic and therapeutic possibilities for asthma.

CHAPTER 5
KINETICS OF COAGULATION, ULTRASTRUCTURE OF FIBRIN AND FACTOR XIII-A MRNA ANALYSIS

5. Introduction

Thromboelastography is an established laboratory tool which provides information regarding the dynamics of clot development, stabilization and dissolution reflecting overall *in vivo* haemostasis. Several parameters are used to study thrombus formation and to obtain information regarding the kinetics of the clot.

Recently, a study on the relationship between clot parameters measured by TEG and clot ultrastructure identified the importance of standardizing data interpretations using these two methods, particularly in the clinical setting (174), summarised in Table 5.1. These methods, together with the complimentary technique of quantifying of mRNA levels in whole blood of patients provide insight into the coagulation profiles of asthma patients and a potential for coagulation to be further influenced by *de novo* production of factor XIII-A as a key molecule involved in fibrin crosslinking (as described in the literature chapter).

5.1 Thromboelastography assessment of coagulation profile

Thromboelastography is a unique test that provides information regarding clot development and mechanical properties of the clot including the time taken before clot formation starts, the kinetics of the fibrin clot, the time taken to achieve maximum strength of the clot, and the final strength and stability of the clot (193). The parameters that are measured and the results obtained from TEG are shown in the Tables 5.2 and 5.3.

Table 5.1 Clot structure of platelet poor plasma as seen with SEM formed with addition of thrombin [reproduced from (174)]

Clot structure: SEM of platelet poor plasma with added thrombin: Healthy individuals	
Fibrin network	A net with clearly visible open spaces between individual branching elongated fibres (this relates to TTG)
Fibrin fibre thickness	The individual elongated fibres show branching; fibrin thickness: diameter ≈ 90 to 110 nm per fibre (this relates to MA)
Fibrin protein packaging structure	In unperturbed (human) fibrin contains 30 ± 3% α-helices, 37 ± 4% β-sheets, and 32 ± 3% turns, loops, and random coils, resulting in an extended, individual partially granular fibre appearance, due to the protein folding.

Platelet poor plasma clot ultrastructure compared to TEG clot parameters: In pathology

	Hypercoaguable	Hypocoaguable
R	↓ Dense matted deposits without any individual fibres formed	↑ Variable degrees of decreased coagulation: ranging from short branching fibres to no fibrin formation with only globular uncoagulated plasma proteins visible to only. This morphology relates to an R-time that can increase to >60 min resulting in no actual clot initiation.
MA and TTG	↑ Individual fibres are meshed with significantly less open spaces visible.	↓ Variable degrees of sparsely spaced, shorter branching meshed fibres with no or few elongated individual fibres visible between mesh.

Table 5.2 Individual measurement of TEG parameters of platelet poor plasma from asthma patients

Sample	R	K	Angle	MA	MRTGG	TMRTGG	TGG
A1	15.6	3.2	19.3	30	4.89	18.33	214.96
A2	11.8	3.2	48.7	28.5	3.57	14	200.09
A3	13.4	6.3	29.4	22.5	2.03	16.83	144.8
A4	7.6	2.4	56.6	37.7	10.86	10.58	300.68
A5	6.8	1.4	47.4	43.6	14.14	8.92	389.12
A6	11.1	2.4	33.2	34.7	6.1	13.5	266.87
A7	17.6	4.4	36.9	27.8	2.65	20.25	192.66
A8	10	3.9	44.5	30	6.94	13.25	214.92
A9	10.3	2.2	50.6	33.8	6.02	12.42	256.45
A10	9.8	7.8	21.7	38.4	5.76	17.92	314.18
A11	10.7	2.7	55	26.6	4.59	12.42	181.19
A12	12.3	5.8	44.4	52.9	8.19	20.58	562.77
A13	8.8	3.2	50.5	38.7	4.25	12.67	317.27
A14	15.2	4.1	0	35.1	5.58	20.25	227.24
A15	12.8	1.7	32.5	50.3	7.65	15.08	507.31
A16	8.8	3.3	51.4	33	4.46	12.83	248.93
A17	9.2	2.5	28.4	28.3	4.86	11.08	197.84
A18	18.5	2.9	44.6	56.2	9.13	23.5	642.99
A19	13.3	6.8	29.8	26.5	1.49	14.67	180.15
A20	13.6	3.4	49	31.4	3.76	16.83	228.79
A21	7.4	3.3	40.8	47.4	6.72	12.67	450.11
A22	7.9	2.8	29	30.1	4.37	9	216.12
A23	11.4	2.8	53.7	33.1	4.8	13.92	248.42
A24	12.8	2.4	55.9	44.2	8.51	15.58	397.08
A25	8.3	1.4	65	41.7	8.93	9.92	358.54
A26	11.6	1.8	30.8	38.9	7.64	13.42	318.81
A27	16.3	2.8	51	35.2	5.52	18.92	272.94
A28	10.7	3.5	46.4	33.3	4.41	14.25	250.32
A29	14.2	4.2	44.9	26	2.93	16.08	176.2
A30	11.4	1.8	62.7	44.2	6.68	13.42	398.08

Mean	**11.64**	**3.35**	**41.80**	**36.00**	**5.91**	**14.77**	**295.86**
SD	**3.03**	**1.56**	**14.18**	**8.47**	**2.70**	**3.60**	**121.22**
Median	**11.40**	**3.05**	**44.75**	**34.25**	**5.55**	**13.96**	**253.39**
IQR 1	**9.10**	**2.40**	**30.55**	**29.63**	**4.34**	**12.61**	**211.21**
IQR 3	**13.45**	**3.95**	**51.10**	**42.18**	**7.64**	**17.10**	**366.19**

TEG: thromboelastography, R: reaction, K: kinetics, MA: maximum amplitude, TMRTG: Time to maximum rate of thrombus, MRTG: Maximum rate of thrombus generation, TTG: Total Thrombus Generation, SD: Standard deviation, IQR: interquartile range

Table 5.3 Individual measurement of TEG parameters of platelet poor plasma from controls

Sample	R	K	Angle	MA	MRTGG	TMRTGG	TGG
C1	9.00	9.70	53.10	20.70	1.71	10.73	131.10
C2	2.50	6.10	49.10	26.00	1.53	12.08	142.49
C3	4.10	2.50	71.90	24.30	5.60	8.58	160.73
C4	4.90	3.60	67.30	37.90	3.89	8.33	305.44
C5	5.50	2.10	73.10	32.60	17.76	5.08	240.05
C6	5.60	1.50	74.70	32.90	7.50	5.00	247.21
C7	5.80	3.10	61.60	35.20	20.07	8.58	471.55
C8	6.20	4.80	49.50	30.50	2.64	14.33	219.26
C9	6.60	6.30	48.60	28.00	1.75	14.33	194.34
C10	6.80	2.80	60.60	32.80	5.74	10.25	244.45
C11	7.20	6.10	49.50	29.20	2.33	14.58	206.27
C12	7.30	4.60	61.30	28.20	2.66	9.25	195.46
C13	7.60	2.10	67.40	32.30	5.94	11.58	238.88
C14	7.70	6.80	47.50	25.00	2.36	13.58	166.75
C15	7.80	1.20	77.60	31.50	8.85	5.75	253.48
C16	7.80	4.30	52.30	26.20	3.69	10.42	179.32
C17	8.00	1.70	71.20	35.80	8.90	12.40	279.10
C18	8.20	2.70	61.80	40.10	4.66	12.00	336.05
C19	8.30	3.40	71.00	27.20	4.41	6.50	187.25

C20	9.20	2.50	66.10	27.50	4.63	10.67	190.74
C21	9.60	2.40	64.10	36.40	5.27	9.50	287.23
C22	9.80	2.00	68.90	35.00	5.44	9.75	269.89
C23	9.80	2.00	69.00	31.90	6.31	7.00	234.60
C24	10.80	2.40	65.30	35.20	4.83	9.42	273.12
C25	10.80	3.70	53.70	34.80	4.09	19.67	267.85
C26	11.20	1.70	71.80	39.90	10.37	8.67	332.96
C27	11.20	1.30	76.50	32.60	7.77	6.33	243.34
C28	11.20	2.20	67.60	28.30	5.67	8.08	198.04
C29	16.10	1.80	71.60	34.00	6.25	9.42	249.37
C30	8.20	2.70	61.80	40.10	4.66	12.00	336.05
Mean	**8.16**	**3.34**	**63.52**	**31.74**	**5.91**	**10.13**	**242.75**
SD	**2.65**	**1.98**	**9.19**	**4.94**	**4.18**	**3.23**	**69.72**
Median	**7.90**	**2.60**	**65.70**	**32.45**	**5.05**	**9.63**	**241.70**
IQR 1	**6.50**	**2.00**	**53.55**	**27.88**	**3.43**	**8.27**	**193.44**
IQR 3	**9.80**	**4.38**	**71.30**	**35.20**	**6.61**	**12.02**	**274.62**

TEG: thromboelastography, R: reaction, K: kinetics, MA: maximum amplitude, TMRTG: Time to maximum rate of thrombus, MRTG: Maximum rate of thrombus generation, TTG: Total Thrombus Generation, SD: Standard deviation, IQR: interquartile range

The statistical analysis of the data (Tables 5.2, 5.3 and 5.4) shows that patients with asthma demonstrated a significant increase in R time, the variable characterising the rate of initial fibrin clot formation in comparison with healthy controls ($p<0.0001$). The maximal amplitude (MA) of the clot was also slightly higher in asthma patients ($p<0.0478$). The α-angle, indicative of speed of clot formation, was significantly lower in asthmatics than the control group ($p<0.0001$). A significantly higher TMRTG that represent the time taken from clot initiation to maximum clot generation was shown ($p-<0.0001$) in asthma patients in comparison to control group. There were no significant differences between the asthma patients and controls in other parameters such as

kinetics (K), maximum rate of clot formation (MRTG) and the amount of resistance (TTG) of the formed thrombus.

Table 5.4: Comparison of TEG parameters of controls and asthma patients

TEG Parameters	Control (n = 30)		Asthma (n = 30)		p value
	Median	Mean (SD)	Median	Mean (SD)	
R time (min)	8.16	7.8 (2.69)	11.64	11.4 (3.03)	<0.0001
K (min)	3.36	2.5 (2.01)	3.35	3.05 (1.56)	ns
α Angle (^{o})	63.58	66.1 (9.35)	41.8	44.75 (14.18)	<0.0001
MA (mm)	31.45	32.3 (4.76)	36	34.25 (8.47)	0.0478
MRTG (Dyne.cm^{-2}.s^{-1})	5.95	5.27 (4.25)	5.91	5.55 (2.7)	ns
TMRTG (min)	10.06	9.5 (3.26)	14.77	13.96 (3.6)	<0.0001
TTG (Dyne.cm^{-2})	239.53	240.05 (68.65)	295.86	253.39 (121.22)	ns

N: sample size, TEG: thromboelastography, R: reaction, K: kinetics, MA: maximum amplitude, TMRTG: Time to maximum rate of thrombus, MRTG: Maximum rate of thrombus generation, TTG: Total Thrombus Generation

Statistically different results are further depicted by the use of dot plots to emphasise the varying distribution of specific parameters amongst asthma patients (figure 5.1 – 5.4).

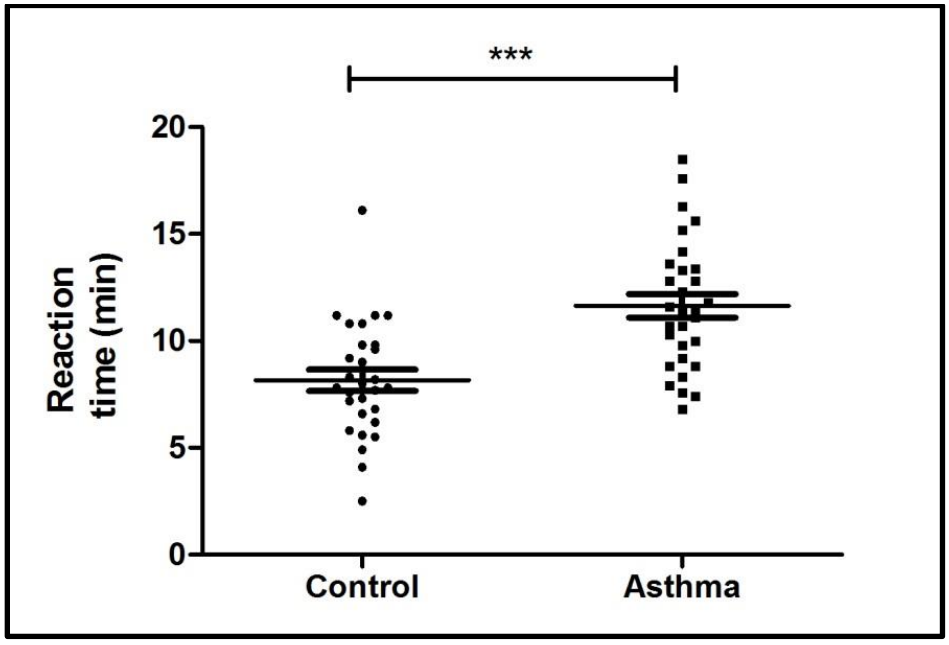

Figure 5.1: A comparison of reaction time control and asthma patients. Data is represented as a dot plot with the mean and standard error of the mean. Where ***p<0.0001

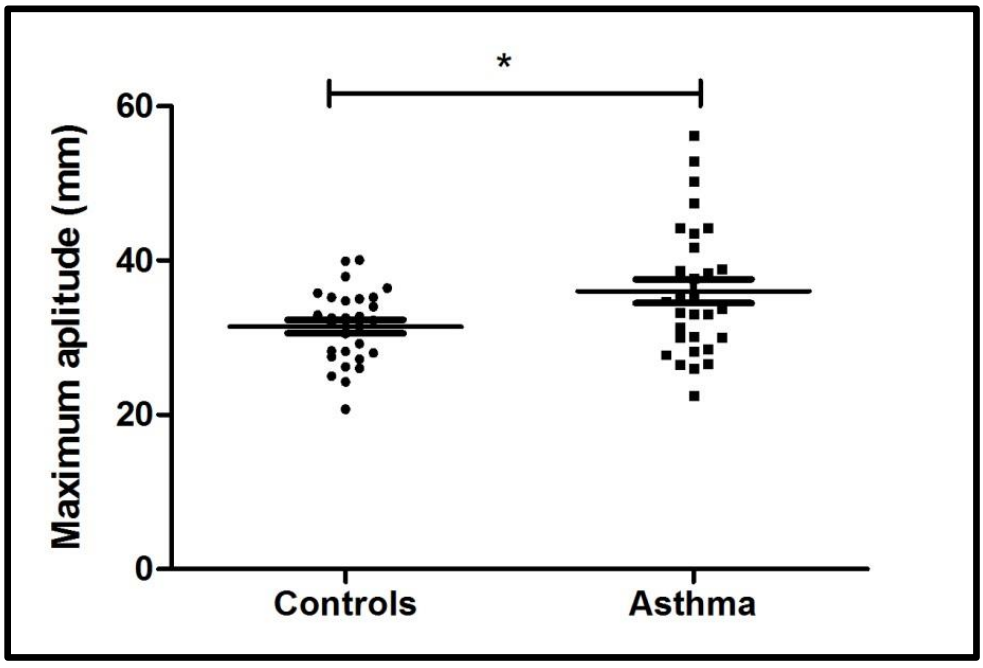

Figure 5.2: A comparison of maximum strength/stiffness of the clot between control and asthma patients. Data is represented as a dot plot with the mean and standard error of the mean. Where *p<0.05

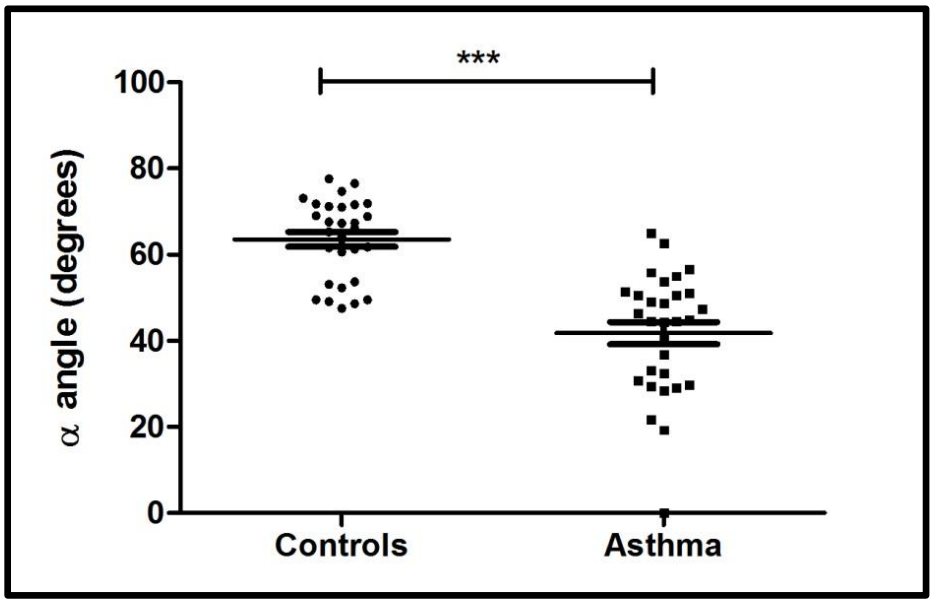

Figure 5.3: A comparison of the speed at which fibrin build up and cross linking takes place. Data is represented as a dot plot with the mean and standard error of the mean. Where ***p<0.0001

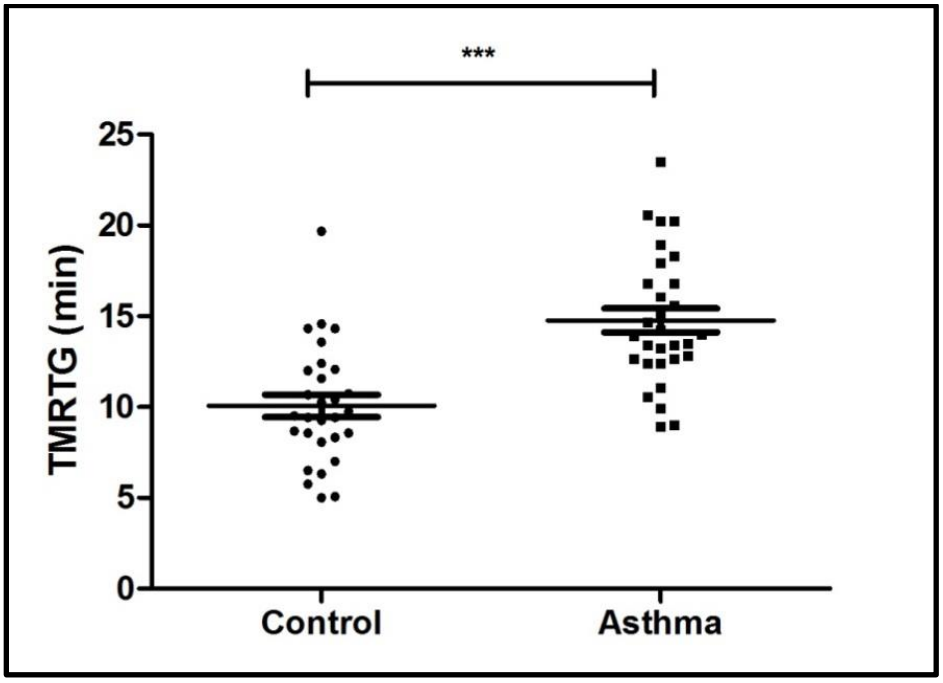

Figure 5.4: A comparison of the time interval observed before the maximum speed of the clot growth. Data is represented as a dot plot with the mean and standard error of the mean. Where ***p<0.0001

5.2 Ultrastructural changes of fibrin fibres and factor XIII mRNA levels

5.2.1 Scanning electron microscopy

SEM was employed to study the morphology of fibrin networks both in control and asthmatic individuals. The ultrastructure of fibrin formation was assessed, following exposure to thrombin. Figure 5.5 and figure 5.6 show representative examples of SEM micrographs of the healthy and asthma individuals.

The fibrin networks of healthy individuals consist of thick (major) fibres and thin (minor) fibres creating an organised mesh of straight fibres. Morphological alterations with less organised fibrin fibres were observed in the asthmatic patients. The arrangements of fibrin network of asthma patients represented in figure 5.6 appear as a thick matted net, with fibrin fibres adhering to each other that cover the surface of the formed thrombus.

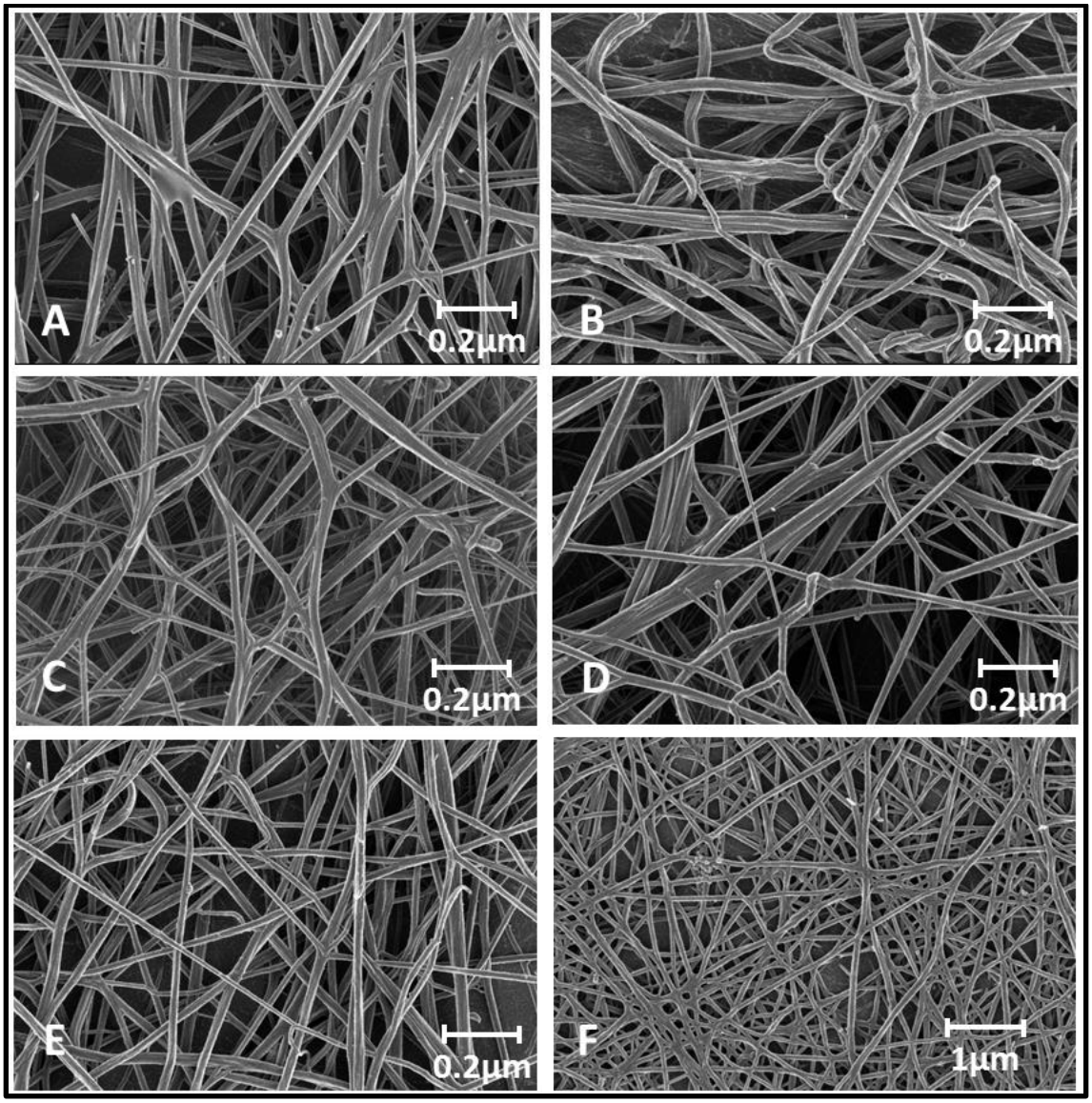

Figure 5.5: Scanning electron microscopy images showing the ultrastructure of fibrin in control (A-F)

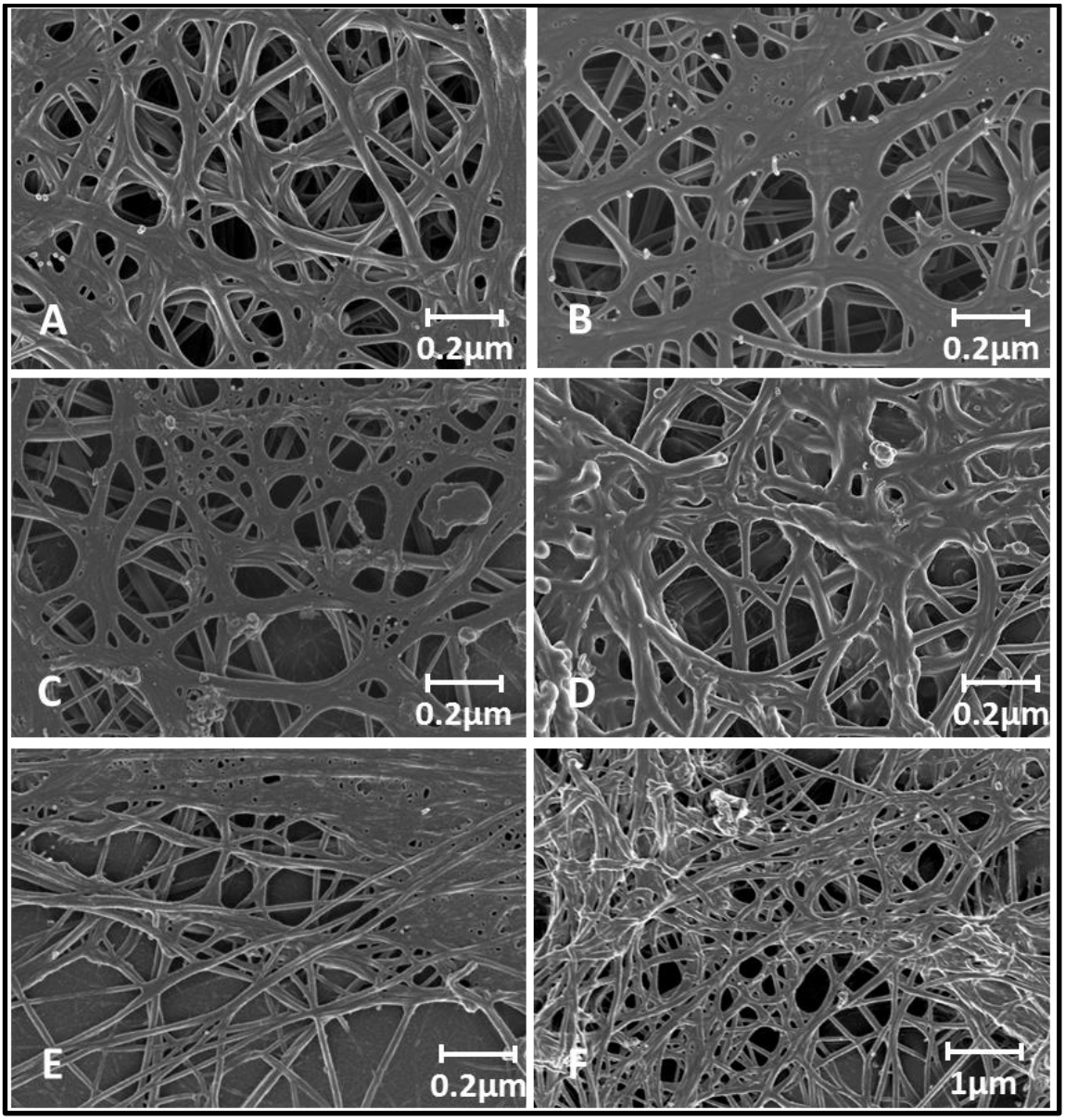

Figure 5.6: Scanning electron microscopy images showing the ultrastructure of fibrin in patients with asthma (A-F)

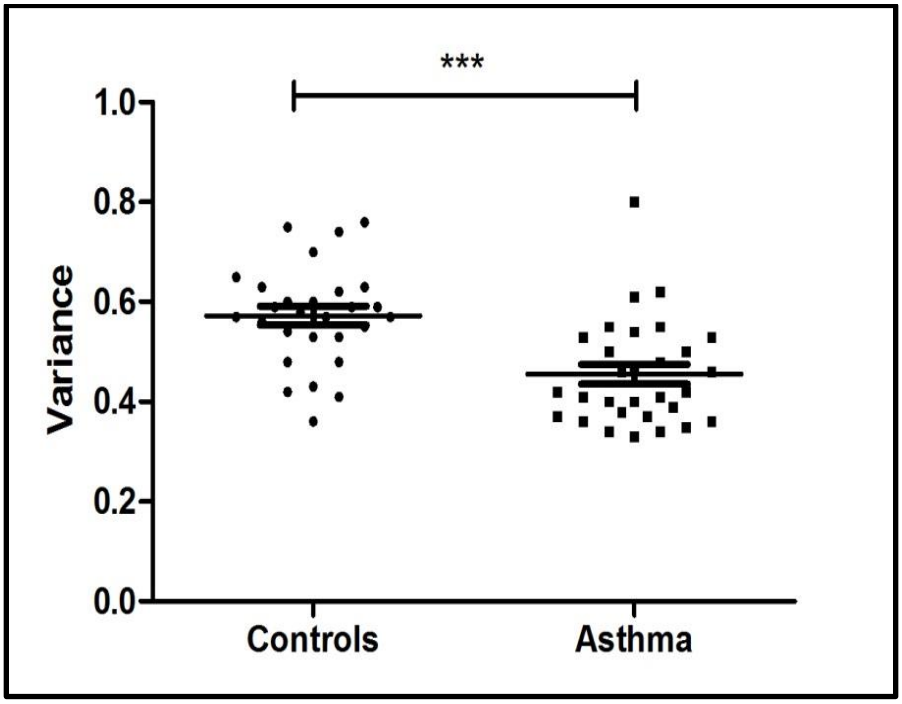

Figure 5.7: Densitometry analysis between scanning electron micrograph images of fibrin clots in controls and asthma patients. Where ***p<0.0001

The densitometry analysis between controls and asthma patients was also done and shown in the Figure 5.7. Density of fibrin networks was analysed using ImageJ software to calculate variance.

The denser the clot, the less variation of colour gradient is visible in the clot, compared to control clots where there is a more pronounce colour gradient between the “spaghetti-like” fibres and the darker background.

Figures 5.8 to 5.13 are randomly selected asthma samples for which the TEG graph (represented by the white line) shows a deviation from the norm compared to control TEG graphs (represented by the balck line) – a corresponding image from the samples SEM analysis accompanies each graph also depicting the matt like formation of fibrin as opposed to spaghetti-like fibres.

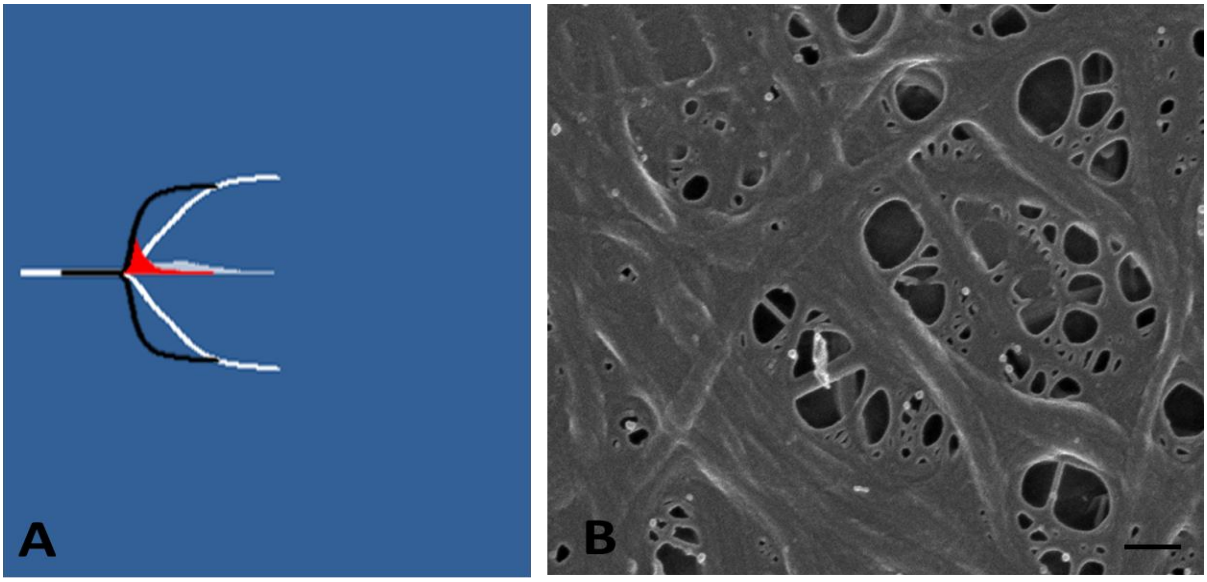

Figure 5.8: A: Control TEG graph represented by the black line and red area highlight and asthma sample 19 represented by the white line and grey highlight. B: Corresponding asthma sample 19 scanning electron microscopic image of fibrin clot. Scale: 1cm: 400nm

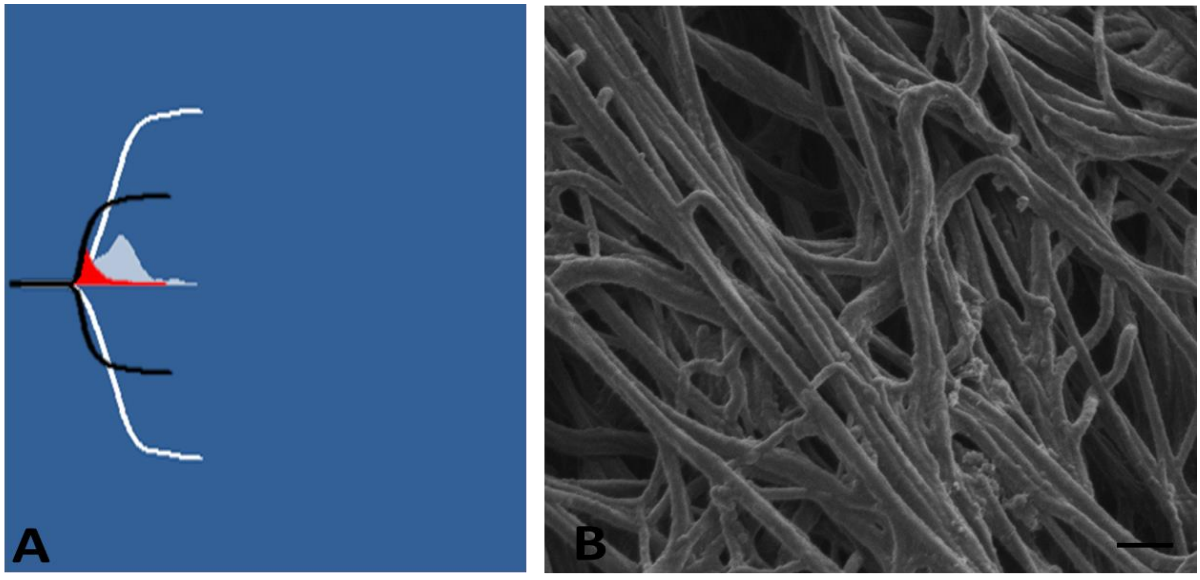

Figure 5.9: A: Control TEG graph represented by the black line and red area highlight and asthma sample 21 represented by the white line and grey highlight. B: Corresponding asthma sample 21 scanning electron microscopic image of fibrin clot. Scale: 1cm: 400nm

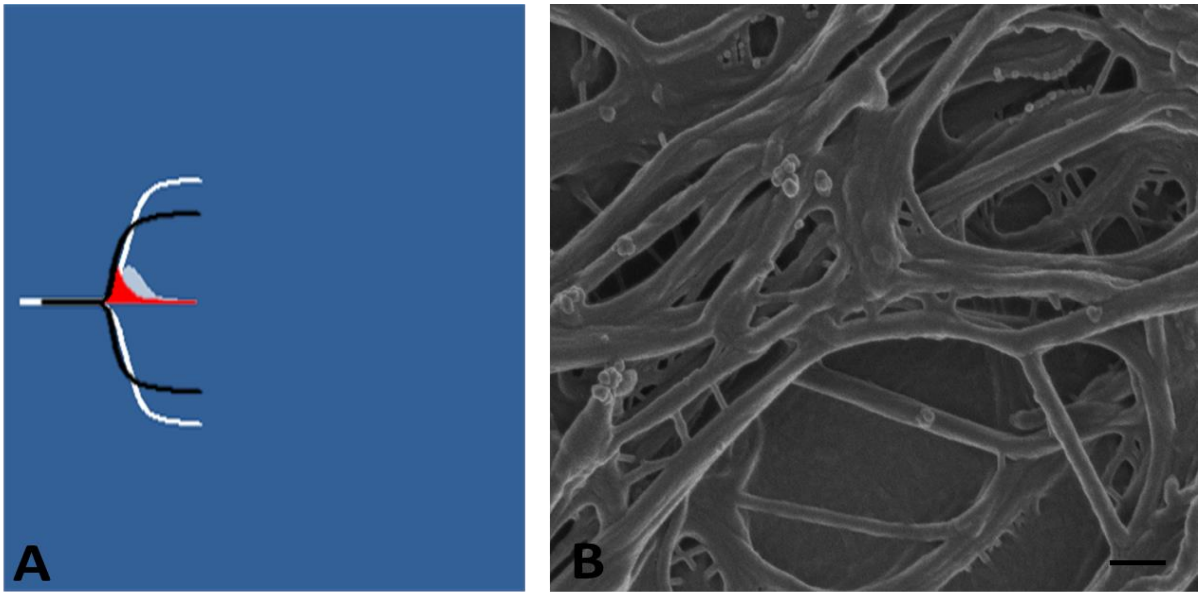

Figure 5.10: A: Control TEG graph represented by the black line and red area highlight and asthma sample 23 represented by the white line and grey highlight. B: Corresponding asthma sample 23 scanning electron microscopic image of fibrin clot. Scale: 1cm: 400nm

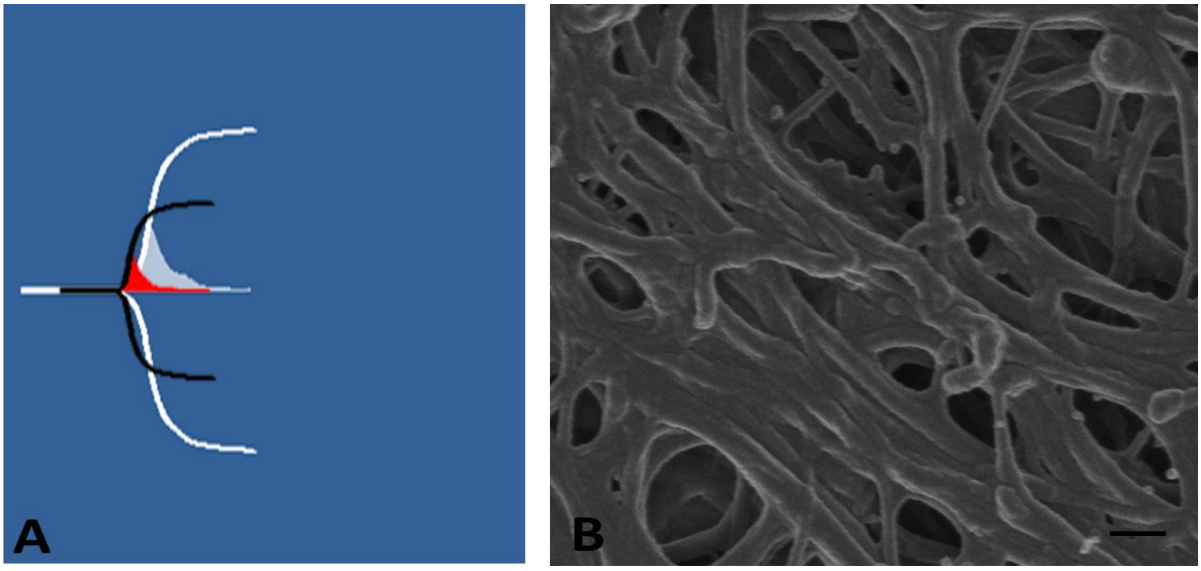

Figure 5.11: A: Control TEG graph represented by the black line and red area highlight and asthma sample 24 represented by the white line and grey highlight. B: Corresponding asthma sample 24 scanning electron microscopic image of fibrin clot. Scale: 1cm: 400nm

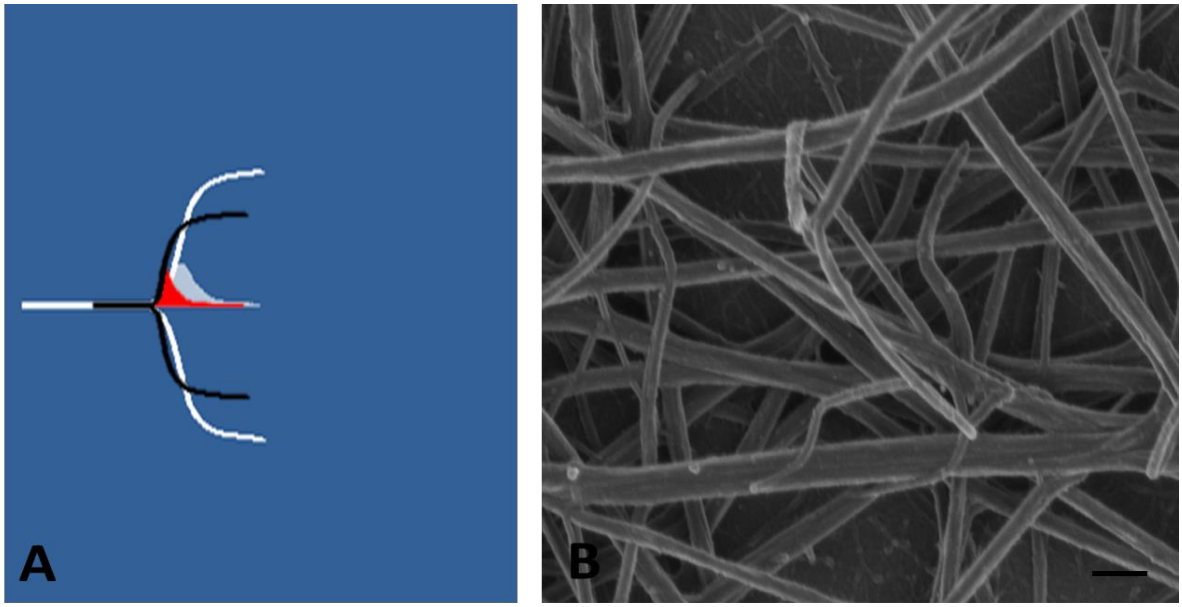

Figure 5.12: A: Control TEG graph represented by the black line and red area highlight and asthma sample 27 represented by the white line and grey highlight. B: Corresponding asthma sample 27 scanning electron microscopic image of fibrin clot. Scale: 1cm: 400nm

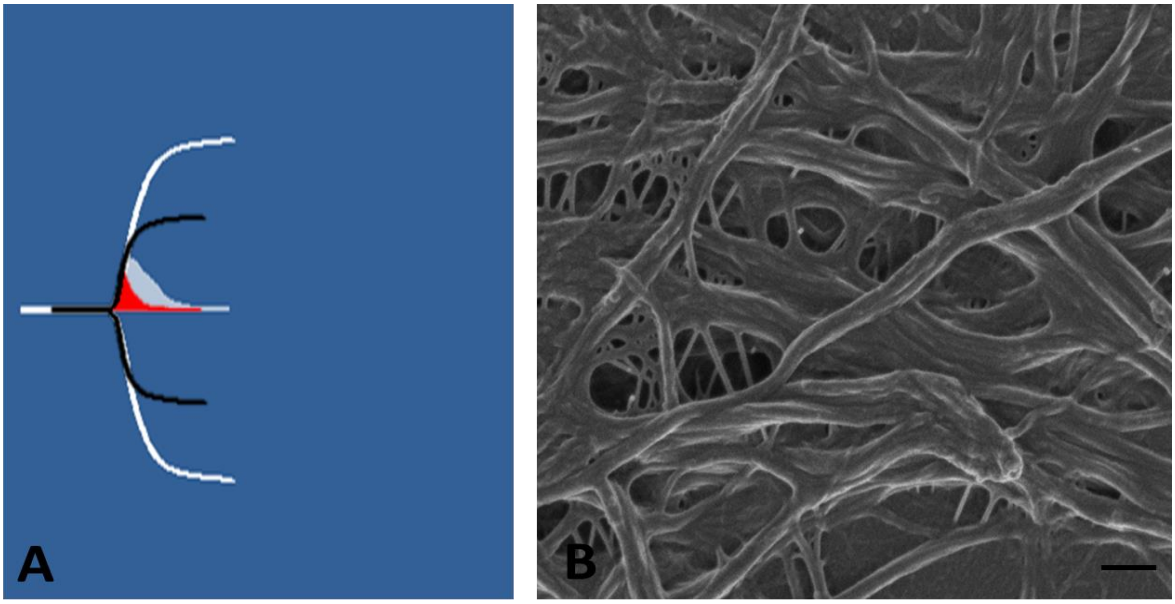

Figure 5.13: A: Control TEG graph represented by the black line and red area highlight and asthma sample 30 represented by the white line and grey highlight. B: Corresponding asthma sample 30 scanning electron microscopic image of fibrin clot. Scale: 1cm: 400nm

5.3 Quantitative polymerase chain reaction

The relative fold change of gene expression of FXIII-A between controls and asthma patients was assessed by qPCR and calculated using the mean cycle thresholds from controls and samples FXIII-A gene and housekeeping gene according to the equation described by Livak and Schmittgen, 2001 (177).

The mRNA levels detected in asthma patients than controls for FXIII-A (17.43±5.06-fold, $p<0.0001$, figure 5.14). The correlation analysis for Factor XIII-A and clot strength (MA) was conducted. There was a noted correlation between Factor XIII-A and clot strength (Spearman r: 0.50, $p=0.0334$, figure 5.15).

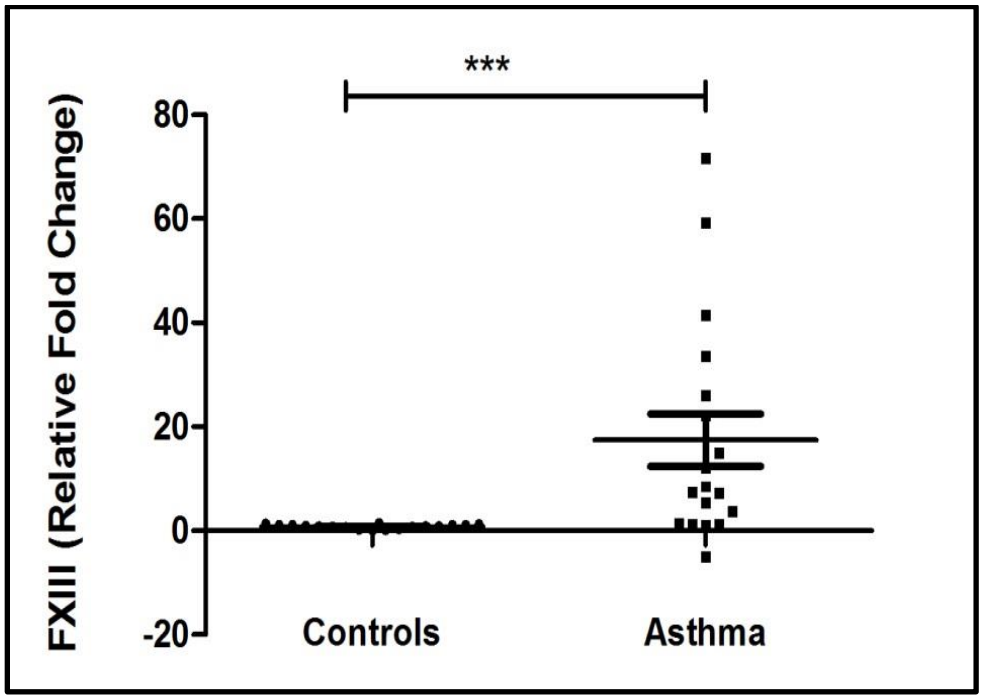

Figure 5.14: Levels of FXIII-A mRNA in whole blood of control and asthma patients. Data is represented as a dot plot with the mean and standard error of the mean (SEM). Where ***p<0.0001

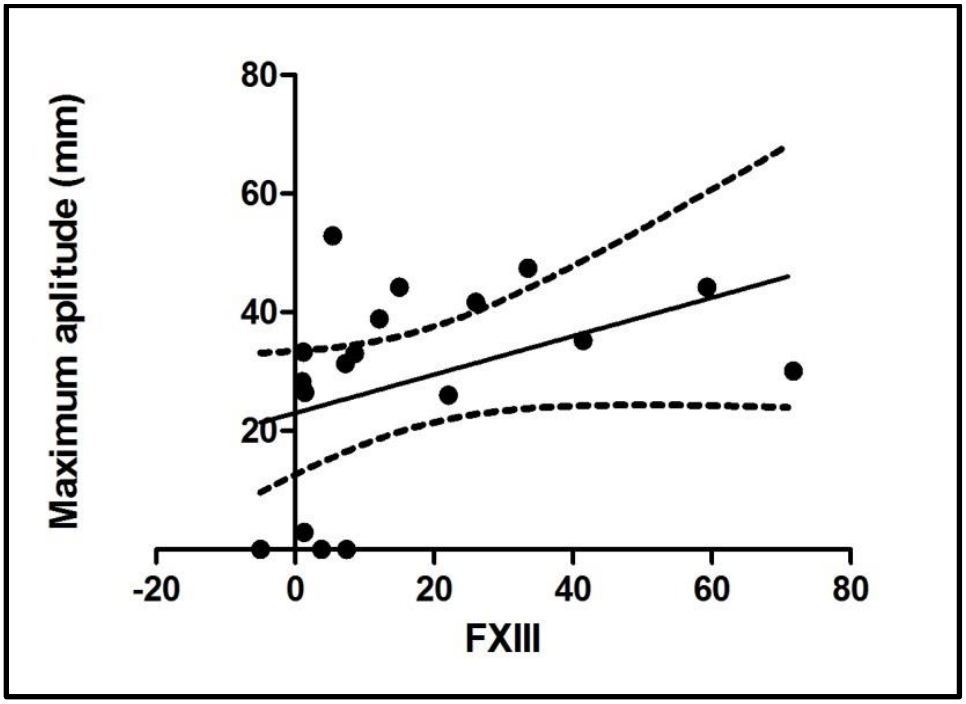

Figure 5.15: Graphical representation of spearman correlation analysis between FXIII-A and maximal amplitude for asthma patients. Data is represented as a dot plot with the 95% residual from the linear regression plot

5.4 Discussion

5.4.1 Coagulation profiles

An important part of clinical and laboratory medicine is the ability to assess haemostasis accurately**.** Clot formation and consequently haemostasis depends on the mechanical properties of the components of the coagulation system especially in diseased states (194, 195). The mechanical properties of the thrombus depended on the ultrastructural and nano-mechanical properties such as elasticity or stiffness of the individual constituents. Since the morphological alterations of coagulation components were found in individuals with asthma, the kinetics of the thrombus formation in these individuals were assessed to determine whether the viscoelastic properties, hence haemostatic process are also affected. TEG was used to measure the kinetics of the clot on platelet poor plasma. The structural features of the clot were correlated with the viscoelastic properties of the fibrin clot.

There are seven different parameters within which the kinetics of the clot formation can be assessed and be related to the pathological coagulation. However, not all of these parameters show variations in a pathological coagulation system analysis (174). Each variable has an impact on the system separately, therefore individual assessment of these variables is important for the evaluation of the system itself and for the treatment approach(174). In patients with asthma variations were detected among some of the parameters measured with TEG, when compared with the healthy control group. Asthmatics showed a prolonged R time for the initiation of fibrin formation. This indicates the time from the start of the test until the start of the fibrin formation is longer, in asthma patients. An alpha angle of less than 45º implies that there is a low association between fibrin and platelets, and a slow rate at which the fibrin formation takes place (193). In the experimental population the alpha angle is less than 45º which, as a result of the fact that no platelets are present in the used plasma, supports previous research. Even though, these parameters (R time and alpha angle) in asthmatics indicate an overall slower rate at which fibrin formation takes place, high MA shows a relatively stronger clot in asthma patients.

5.4.2 Fibrin and factor XIII

Clot stability can also be modulated by molecular mechanisms that control the fibrin network structure. Since the absence of platelets in the plasma, the reason for stronger clot in asthma could be due to the effect of excess FXIII, that promote cross-linking of the fibrin monomers at the end of the coagulation cascade. Evidence shows that altered haemostasis in asthma presents as reduced fibrinolytic activity, abnormal clot architecture, and decrease clot retraction rate (196). The increased resistance of fibrin clot to fibrinolysis found in asthmatics, can be attributed to the excessive cross-linking of fibrin to form high molecular weight alpha-polymer chains (197). The mechanism by which FXIII cross-linking influences fibrinolysis is the result of tightening the coupling between the protofibrils within fibrin fibres thus making the fibre stiff, less porous, increasing rigidity, elasticity and decreasing solubility (198-200). Furthermore, it alters the fibrin polymerization kinetics which results over cross-linked fibrin, resistant to fibrinolysis, leading to undesired prolonged persistence of thrombi (139). An early experimental study reported that both fibrin-fibrin and alpha2-antiplasmin–fibrin cross-links by factor XIII caused the resistance of pulmonary embolism to fibrinolysis (201).

Fibrin fibres and platelets are the main determinants of the clot mechanics since they make up the majority of the structure of the clot (195). The mechanical property of the clot depends on the mechanical behaviour of the fibrin fibres. This study investigated the ultrastructural properties of the fibrin fibres in both control and experimental groups to determine whether there are any alterations in fibrin network. From the SEM images obtained it was clear that the fibrin network is altered in patients with asthma when compared to the morphology of the control group. The findings indicate distinct differences in the ultrastructure of the fibrin network where the fibrin fibres appear thicker and/or matted in asthmatics. In control group fibrin fibres were more organised and consists of thick and thin fibres.

Table 5.5: Platelet poor plasma clot ultrastructure compared to TEG clot parameters and FXIII-A mRNA in asthma

MICRO-SCOPY	Clot ultrastructure	Altered	Morphological alterations with less organised fibrin fibres were observed. The arrangements of fibrin network appear as a thick matted net, with fibrin fibres adhering to each other.
TEG	R	↑	Variable degrees of decreased coagulation: ranging from short branching fibres to no fibrin formation with only globular uncoagulated plasma proteins visible.
	Alpha angle	↓	An alpha angle of less than 45º implies that there is a low association between fibrin and platelets, and a slow rate at which the fibrin formation takes place.
	TMRTG	↑	A significantly higher TMRTG represents the time taken from clot initiation to maximum clot generation was greater in Asthma samples.
	MA	↑	Individual fibres are meshed with significantly less open spaces visible, indicative of a hypercoaguable fibrin clot with greater clot strength.
qPCR	FXIII-A	↑	The mRNA of FXIII-A was higher in asthma samples. A significant association between Factor XIII-A mRNA levels and clot strength (MA) was noted.

The fibrin networks in patients with asthma appear denser, less organised with sticky fibres that clump together to form a thick mass. In asthma, fibres form an irregular net that covers larger part of the clot. Asthma platelet has a significantly thicker and tighter fibrin network than those of the healthy individuals. This changed fibrin morphology could therefore affect fibrinolysis. The rate of clot lysis depends on the fibrin network architecture that modulates the distribution of lytic enzymes (202, 203). It can therefore be concluded that a tighter fibrin network as seen in the asthmatic patients

with small open spaces and greater clot strength may affect the clot lysis since it would be more difficult of lytic proteins to enter the formed clot.

The architecture of a fibrin network can be governed by the kinetics of fibrin polymerisation. Levels of FXIII-A mRNA from asthma samples were significantly higher when compared to controls. This indicates greater production of FXIII-A in asthma. The ultrastructural difference observed in fibrin structure could therefore be due to the cross-linking of fibrin fibres during clot formation. Previous studies postulate that FXIII plays a key role in altered haemostasis observed in asthmatics (137, 138, 144, 145). These findings were observed in platelets, tissue macrophages and bronchoalveolar lavage samples, this is the first study to observe similar finding in whole blood from circulation among asthma patients.

A previous ultrastructural study using BALB/c asthma model that shows evidence of alterations in clot structure in asthma (204) which is similar to findings from this study of defective fibrin formation. This study found alterations in clot structure with less organized fibrin fibres forming an irregular web with a matted appearance in asthmatics. The architecture of the fibrin network is governed by the kinetics of fibrin polymerisation through FXIIIa–catalysed cross-linking which augments the clot rigidity by stiffening the existing fibres (205).

The findings summarised in table 5.5 show higher clot rigidity and altered network morphology in asthma patients, most likely due to the stiffening of the fibres by excess FXIII-A. The structural variability of the clot with more tightly packed fibres can cause lower permeability and resistance to fibrinolysis. The dense fibrin meshwork might be the result of uncontrolled cross-linking of fibrin by excessive activity of high levels of FXIII-A in asthma patients (139). Excessive cross-linking of fibres increase the resistance of fibrin clot to fibrinolysis (197). Evidence indicates accumulation of fibrin in the asthmatic airways that enhance the airway obstruction (86), that might be due to the defective fibrin formation by excessive production of FXIII-A and its resistance to fibrinolysis. The results from this study suggest that fibrin fibres are thicker and stronger in asthmatics.

CHAPTER 6
CONCLUDING DISCUSSION

Allergy related diseases such as asthma have been on the increase for the past 50 years (206). Asthma is identified as the 14th most significant chronic disease due to its rise in prevalence and incidence (5). Asthma affects more than 300 million people worldwide of all ages in which about 10% of the people are suffering from severe or uncontrolled asthma leading to 250,000 deaths annually (5). It is expected to affect another 100 million by 2050 if this trend continues (207). Since the prevalence and severity of asthma is on the increase, it imposes a high disease burden to both health care system and to society (13). Therefore, to curtail the economic burden of asthma, a better understanding of the disease pathogenesis and additional evidence–based treatment therapies are required. Even though our knowledge regarding asthma is increasing, the full pathophysiology is yet to be understood. Further research is required to understand the different mechanisms linked to the inflammatory process in asthma.

There is no cure for asthma; however, its symptoms can be controlled. Despite a variety of treatment strategies, the asthma prevalence is on the increase. Since current treatment modalities are not sufficient to overcome the disease, new research is required, to provide insight for alternate treatment methods. Currently importance has been given to the development of new treatment methods that inhibit different steps within the inflammatory process that is central to asthma pathophysiology (5, 167). A relationship exists between inflammation and coagulation in asthma. Abnormalities in coagulation and fibrinolysis feature prominently among multiple mechanisms that have been implicated in the pathogenesis of inflammatory diseases such as asthma (134). Evidence also shows that impaired coagulation and fibrinolysis resulting extravascular fibrin deposition contributes to airway hyper responsiveness and airway closure in asthma (86). Therefore the question arises as to whether inflammatory processes in asthma affect the structural and functional integrity of the

components of the coagulation. Hence, the aim of the study was to evaluate the coagulation process and ultrastructural properties of components of the clot, focussing on erythrocytes, platelets and fibrin by using LM, SEM, AFM and CM. The kinetics of the clot formation was also assessed by viscoelastic tests. In addition, to assess the potential *de novo* production of FXIII-A in asthma, the level of FXIII-A mRNA was determined by qPCR.

The sample population and the study design were discussed in chapter 3. This study measured blood parameters in both groups to determine whether there are variations in asthma patients and all were within range.

Chapter 4 of the thesis explains the evaluation of cellular components of the clot: erythrocytes and platelets. The LM images demonstrate some shape changes of erythrocytes in the experimental group. These variations are further supported by a measure of the axial ratio of erythrocytes in asthma patients, which was significantly higher. In addition to this, evaluation of the micromechanical property (viscoelastic property) of the erythrocyte membrane assessed by AFM; indicated significant reduced elasticity or deformability. Reduced elasticity can be due to increased stiffness by change in membrane integrity in erythrocytes of asthma patients. Biophysical shape changes in erythrocytes due to membrane changes can be caused by upregulated inflammatory molecules and oxidative stress (185).These pathological erythrocytes with altered elasticity can also influence the coagulation process by affecting the clot stability and its viscoelastic properties which may lead to thrombosis (183, 185).

Although the visible structural changes of erythrocytes in asthma seem minor, the structural differences were observed when measuring the mechanical and morphological properties, suggesting that even small structural differences can have a major influence on pathophysiology. In conclusion, as an active participant in clot structure, architectural abnormalities of erythrocytes may directly influence the coagulation profile. However, the exact contribution of erythrocytes towards asthma

pathology warrants further investigation. Furthermore, this study demonstrated that erythrocyte cell stiffness, measured quantitatively by AFM, is a reliable method. Since the oxygen carrying capacity is significantly compromised in asthma patients (155), these result also suggests that the increased stiffness due to decreased elasticity found in asthmatic erythrocyte might reduce the ability of the cell to bind oxygen. Therefore, this finding may be taken into consideration for alternate treatment strategies. The effect of shape changes and membrane alterations of erythrocytes is an important factor to consider when extrapolating the mechanisms contributing the pathogenesis of asthma.

The second objective of chapter 4 was to evaluate the ultrastructure of platelets and platelet activation to determine their contribution to the procoagulant environment in asthma. Activation of platelets is a complex process, and is accompanied by dramatic shape change in response to injury of vessels or foreign substances (208). Platelet morphology was studied using SEM, TEM and CM. The images of platelets illustrate structural changes that indicate platelet activation in asthma when compared to control platelets. This finding is in line with the previous research which showed that platelets react to stimuli by undergoing activation which is indicated by asymmetrical shapes changes, extensive pseudopodia formation, spreading and aggregation (188). These morphological changes observed in asthma are indicative of platelet activation. Since platelets activation is associated with pathology (189), these activated platelets are an indication of their involvement in the pathophysiology of this allergic disease and supports the view that platelet morphology may be used as a potential tool in the study of pathophysiology of different diseases.

The acellular component of clots includes fibrin (following activation and cross-linking of fibrinogen) which is dependent on factor XIII. An evaluation of these parameters was presented in chapter 5. This study investigated the ultrastructural properties of the fibrin fibres in both control and experimental groups to determine whether there are any alterations in fibrin network. From the SEM images and variance found in densitometry analysis obtained it was clear that the fibrin network is altered in patients

with asthma. This study employed the TEG technique to derive information regarding the clot kinetics of coagulation process and fibrin clot formation to determine any alterations in the asthma group. In addition, fibre arrangement and the ultrastructure of the fibrin clot formation were assessed by SEM, while FXIII-A mRNA was quantified using qPCR. The TEG results showed a prolonged R-time and TMTRG -time, and an alpha angle of less than 45º. This suggested a slow rate at which the fibrin formation occurred in asthma patients when compared to the control (193). However, MA, the width of the tracing representing the maximum clot strength or the stiffness of the clot was high in asthma patients, indicating the formation of a strong clot. The level of FXIII-A mRNA was also higher in asthma patients indicating a greater propensity for *de novo* synthesis of FXIII-A, which may explain the greater clot strength observed in the asthma group.

Inflammatory conditions, not only affect clotting, but also fibrin structure (185). The measurement of the mechanical property of the clot reflects its behaviour as a whole, while microscopy is best suited for the characterisation of fibrin clot structure. SEM was employed to evaluate the structural modifications of fibrin clot. Ultrastructural studies of fibrin network showed heterogeneity of fibre density with a tightly woven network. Aggregation of fibres indicates abnormal packaging in asthma, and composed of thick fibres bifurcating and sticking together, demonstrating a dense matted morphology. A continuous layer forms without any visible individual fibrin fibres in some cases. These findings corroborate research showing that fibrin nets appear thickened and matted in inflammatory disease conditions such as ischemic stroke, diabetes and rheumatoid arthritis (209). This suggests that changed morphology of fibrin network in asthma may also be due to inflammation.

The fibrin network structure and stability are greatly modulated by clot stabilizing factor, FXIII through cross-linking of fibrin fibres (203). FXIII also supports platelet activation by promoting adhesion and spreading, thereby stabilizing platelet- fibrin clots(140). The strong clot in asthma was further supported by significantly higher mRNA levels of FXIII-A in asthma patients when compare to control group, that

indicates a potential production of FXIII-A in asthma patients. Activity of high levels of FXIII-A leading to strong clot formation in asthma patients may contribute to reduced fibrinolysis in asthma. These findings provide further insight into the molecular mechanism of altered coagulation in asthma as well as therapeutic possibilities that can modulate fibrin structure and stability, which could prevent the accumulation of fibrin in asthmatic airways. The clinical relevance of these findings lie in the fact that the molecular mechanism of altered fibrin cross-linking may be better understood and could potentially be a therapeutic target – treated systemically and thus reduce the deposition of these molecules locally (in the lung) – reducing the symptoms associated with asthma.

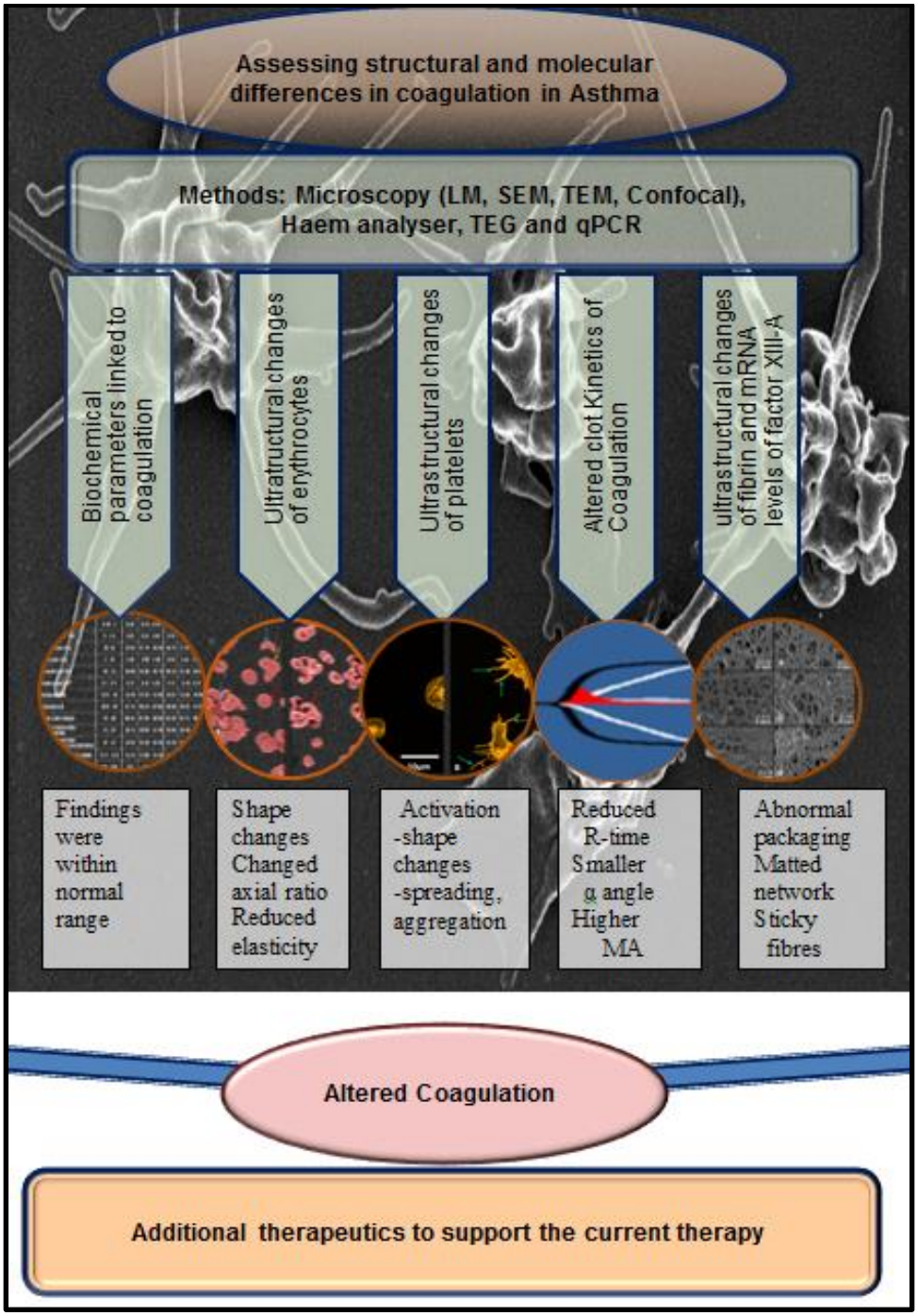

Figure 6.1 Summary of haematology, structural and ultrastructural changes observed asthma patients

In conclusion, higher levels of FXIII-A mRNA and the potential for *de novo* synthesis of FXIII-A, altered fibrin architecture and clot properties indicate that haemostasis is altered in asthma patients. Excess FXIII-A resulting greatly cross-linked stiffer fibres

that may lead to the accumulation of fibrin in asthmatic airways may contribute to the symptoms of asthma such as airway narrowing. The pathological clot with altered fibrin network morphology can also be attributed to the activation of platelets which consequently activates the rest of the coagulation cascade. Since there is a link between coagulation and inflammation, these pathological changes to cellular structure not only affect the coagulation profile in asthma patients but also may influence the inflammatory activity, hence the pathophysiology of the disease.

Furthermore, the findings of this study proved that enhanced coagulation and inflammation alters the morphology of fibrin networks and erythrocytes, as well as the degree of platelet activation in asthma, validating the alternate hypothesis (figure 6.1).

The technique used in this study such as LM, SEM, AFM, CM and TEG are reliable and non-invasive, compared to other routine tests available in the clinical settings. Further research incorporating the use of techniques discussed in this thesis can expand the body of knowledge regarding the pathophysiology of the disease as well as disease progression and possibly improve diagnostic and treatment strategies. Furthermore, this study has demonstrated that AFM could be considered as a possible diagnostic method for the evaluation and characterisation of micromechanical properties of the cellular systems, thereby helping us to understand physiological properties of the cells.

A limitation of the study is that results of this study showed the structural changes of the blood components in the systemic circulation. It did not, however, explain the exact contribution to the asthma pathophysiology as well as the effect of these components locally in the airways.

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APPENDIX 1

ETHICS APPROVAL LETTER

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 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

19/05/2014

Approval Certificate
New Application

Ethics Reference No.: 463/2013

Title: Electron, Confocal and Atomic Force Microscopic Analysis of Platelets, Fibrin and Erythrocytes in Atopic Asthma

Dear Ms Sajee Alummoottil

The **New Application** as supported by documents specified in your cover letter for your research received on the 29/10/2013, was approved, by the Faculty of Health Sciences Research Ethics Committee on the 19/05/2014.

Please note the following about your ethics approval:
- Ethics Approval is valid for 4 years
- Please remember to use your protocol number (**463/2013**) 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, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- 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); MPharMed.
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 2004 (Department of Health).

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♦ Web: //www.healthethics-up.co.za ♦ H W Snyman Bld (South) Level 2-34 ♦ Private Bag x 323, Arcadia, Pta, S.A., 0007

APPENDIX 2

STATISTICS APPROVAL LETTER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Denkleiers • Leading Minds • Dikgopolo tša Dihlalefi

DEPARTMENT OF STATISTICS

LETTER OF STATISTICAL SUPPORT

Date: 18 October 2013

This letter is to confirm that **Mrs S Alummoottil**, studying at the University of Pretoria, discussed the project with the title **Electron, Confocal and Atomic force Microscopic analysis of Platelets, Fibrin and Erythrocytes in Asthma** with me.

I hereby confirm that I am aware of the project and also undertake to assist with the statistical analysis of the data generated from the project.

The sample will consist of measurements obtained from the Light, Electron, Confocal and Atomic Force Microscopes for 1) an experimental group of 30 patients older than 18 years who meet the criteria for allergic asthma as set by the Lung Unit of Steve Biko Academic Hospital and 2) a control group consisting of healthy persons older than 18 years, not suffering from any inflammatory diseases and who are not on any chronic medication.

The data analysis will consist of descriptive statistics such as summary statistics and graphs to explore the data, while statistical techniques such as means / proportions comparison between healthy individuals versus asthma patients with respect to the study variables will also be performed. If the data does not meet the assumptions required for the statistical inferences an alternative method, e.g. non-parametric approach, will be considered.

Dr L Debusho
Department of Statistics
Internal Consultation Service
Tel 012 420 4643

APPENDIX 3

PhD COMMITTEE LETTER

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Kantoor van die Voorsitter
Skool vir Geneeskunde
Fakulteit Gesondheidswetenskappe

5 August 2014

Prof E Pretorius
Department Physiology
BMS Building
Room 9-21
UNIVERSITEIT VAN PRETORIA

Prof Pretorius

STUDENT: S ALUMMOOTTIL (PhD PHYSIOLOGY)
"Electron, Confocal and Atomic Force Microscopic Analysis of Platelets, Fibrin and Erythrocytes in Atopic Asthma" (New Title)

Mentioned student's protocol with new title has been approved by the committee meeting held on the 15th of April 2014.

Kind regards

PROF BG LINDEQUE
VOORSITTER: PhD KOMITEE

c.c. Me A. Strauss

APPENDIX 4

CONFERENCE: ABSTRACT ACCEPTANCE

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Alisa Phulukdaree <u04830670@up.ac.za>

Alummottil: Abstract Accepted for Poster Presentation; Fibrinogen and Factor XIII workshop

5 messages

Workshop, Fibrinogen <ifrs2018@wfu.edu> 15 March 2018 at 22:57
To: Alisa Phulukdaree <alisa.phulukdaree@up.ac.za>
Cc: Gloria Stickney <stickngd@wfu.edu>

Dear Alisa,

Congratulations! Your abstract has been selected for a poster presentation at the 25th Fibrinogen and 3rd FXIII Workshop! Poster are displayed throughout the duration of the workshop, with the main poster session scheduled for Wednesday, June 6 in the afternoon. Abstract title: Ultrastructural evaluation of clot properties and mRNA levels of clotting factor XIII in asthma patients.

All abstracts were reviewed by a panel of 15 experts, each abstract was evaluated and scored by three independent reviewers.

The workshop will be held June 3 - 7, 2018 at the Graylyn International Conference Center of Wake Forest University. Details can be found at the Workshop websites. The program will be posted in the next few weeks.

Please note that the early bird registration deadline is Wednesday, March 21, 2018.

We are looking forward to seeing you at the Workshop!

Best regards,

Martin Guthold

Alisa Phulukdaree <alisa.phulukdaree@up.ac.za> 15 March 2018 at 23:00
To: Sajee Alummoottil <sajee.alummoottil@up.ac.za>, Resia Pretorius <resia.pretorius@up.ac.za>

Congratulations Sajee!

You can start preparing for your trip 😊

[Quoted text hidden]

Mail Delivery Subsystem <mailer-daemon@googlemail.com> 15 March 2018 at 23:00
To: u04830670@up.ac.za

Address not found

Your message wasn't delivered to **resia.pretorius@up.ac.za** because the address couldn't be found or is unable to receive email.

LEARN MORE

The response was:

APPENDIX 5

RESEARCH ARTICLE: PROOF OF SUBMISSION

BRITISH JOURNAL OF HAEMATOLOGY

Impact factor: 4.711 (2014)

ISO 4 abbreviation: Br. J. Haematol

Editor: Finbarr E. Cotter

Discipline: Hematology

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ISSN: 0007-1048 (print); 1365-2141 (web)

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Alisa Phulukdaree <u04830670@up.ac.za>

Manuscript submitted - BJH-2018-00358

1 message

British Journal of Haematology <onbehalfof@manuscriptcentral.com> 22 February 2018 at 15:57
Reply-To: lorna@bjhaem.co.uk
To: sajee.alumoottil@up.ac.za, maphasha97@gmail.com, janette.bester@up.ac.za, resiap@sun.ac.za, alisa.phulukdaree@up.ac.za

Dear Dr. Alisa Phulukdaree,

Ultrastructural evaluation of fibrin, clot properties and mRNA levels of clotting factor XIII in asthma patients

Thank you for submitting your manuscript to the British Journal of Haematology. If you have any queries, please be ready to quote our manuscript reference number, BJH-2018-00358. "The British Journal of Haematology requires the submitting author (only) to provide an ORCID iD when submitting his or her manuscript."

The journal to which you are submitting your manuscript employs a plagiarism detection system. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published works.

Yours sincerely,

FINBARR COTTER
British Journal of Haematology

Any conflict of interest/funding source should be printed in the Acknowledgement section of the paper. If there are none, the author should clearly state that this is the case and we should include the statement "the authors have no competing interests", in the Acknowledgement section.

APPENDIX 6

INFORMED CONSENT TEMPLATE

CONTROLS

PARTICIPANT'S INFORMATION LEAFLET & INFORMED CONSENT FORM FOR A NON-INTERVENTION STUDY

STUDY TITLE:

Electron, Confocal and Atomic Force Microscopic Analysis of Platelets, Fibrin and Erythrocytes in Atopic Asthma

SPONSOR: Mrs S Alummoottil

Principal Investigators: Mrs S Alummoottil

Institution: University Of Pretoria

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S):

Daytime numbers: 012 3192239

Afterhours: 0835606688

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

				:
dd	**mm**	**yy**		**Time**

Dear Mr. / Mrs. .....

1) INTRODUCTION

You are invited to volunteer for a research study. This information leaflet 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 leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. In the best interests of your health, it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study, wherever possible.

2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of this study is to evaluate Asthma. By doing so we wish to learn more about how this condition develops and the effect that it has on the airways.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

If you decide to take part you will be one of approximately 30 healthy volunteers. This study will involve the taking of blood samples. Blood will be taken from a vein in the arm. It will take few minutes to draw blood. A total of 10ml of blood (i.e. 1 tablespoon) will be collected over the course of the entire study.

4) RISK AND DISCOMFORT INVOLVED.

The only possible risk and discomfort involved is the taking of blood from a vein. Drawing blood may result in pain and a bruise at the puncture site, or infection and bleeding from the site. The University of Pretoria has limited insurance for research related injuries, however, your protection is that the procedures are performed under sterile conditions by experienced personnel

5) POSSIBLE BENEFITS OF THIS STUDY.

Your support of this study will benefit Asthma patients.

6) I may at any time withdraw from this study.

7) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 3541677 / 012 3541330 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2008), 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.

8) INFORMATION If I have any questions concerning this study, I should contact:

Mrs S Alummoottil……Tel: 012 3192239…or Cell: 0835606688……

9) CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a fashion that patients remain unidentifiable.

10) CONSENT TO PARTICIPATE IN THIS STUDY.

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I hereby volunteer to take part in this study.

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

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

VERBAL PARTICIPANT INFORMED CONSENT (applicable when participants cannot read or write)

I, the undersigned, Mrs S Alummoottil, have read and have explained fully to the participant, named …….. and/or his/her relative, the participant information leaflet, which has indicated the nature and purpose of the study in which I have asked the participant to participate. The explanation I have given has mentioned both the possible risks and benefits of the study. The participant indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason.

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

Participant's Name _____
(Please print)

Participant's Signature _____ Date _____

Investigator's Name _____
(Please print)

Investigator's Signature _____ Date _____

Witness's Name _____

Witness's Signature _____ Date _____
(Please print)

(Witness - sign that he/she has witnessed the process of informed consent)

APPENDIX 7

INFORMED CONSENT TEMPLATE

PATIENTS

PATIENT'S INFORMATION LEAFLET & INFORMED CONSENT FORM FOR A NON-INTERVENTION STUDY

STUDY TITLE:

Electron, Confocal and Atomic Force Microscopic Analysis of Platelets, Fibrin and Erythrocytes in Atopic Asthma

SPONSOR: Mrs S Alummoottil

Principal Investigators: Mrs S Alummoottil

Institution: University Of Pretoria

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S):

Daytime numbers: 012 3192239

Afterhours: 0835606688

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

			:	
dd	**mm**	**yy**	**Time**	

Dear Mr. / Mrs. .....

1) INTRODUCTION

You are invited to volunteer for a research study. This information leaflet 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 leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures involved. In the best interests of your health, it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study, wherever possible.

2) THE NATURE AND PURPOSE OF THIS STUDY

You are invited to take part in a research study. The aim of this study is to evaluate Asthma. By doing so we wish to learn more about how this condition develops and the effect that it has on the airways. Some problems could be serious and if identified early could save you from having problems later on.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

If you decide to take part you will be one of approximately 30 patients. This study will involve the taking of blood samples. Blood will be taken from a vein in the arm. It will take few minutes to draw blood. A total of 10ml of blood (i.e. 1 tablespoon) will be collected over the course of the entire study.

4) RISK AND DISCOMFORT INVOLVED.

The only possible risk and discomfort involved is the taking of blood from a vein. Drawing blood may result in pain and a bruise at the puncture site, or infection and bleeding from the site. The University of Pretoria has limited insurance for research related injuries, however, your protection is that the procedures are performed under sterile conditions by experienced personnel

5) POSSIBLE BENEFITS OF THIS STUDY.

Many of these tests are done routinely on patients. It will enable us to treat you if you should have problems.

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

7) I may at any time withdraw from this study.

8) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 3541677 / 012 3541330 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2008), 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.

9) INFORMATION

If I have any questions concerning this study, I should contact:

Mrs S Alummoottil Tel: 012 3192239or Cell: 0835606688

10) CONFIDENTIALITY

All records obtained whilst in this study will be regarded as confidential. Results will be published or presented in such a fashion that patients remain unidentifiable.

11) CONSENT TO PARTICIPATE IN THIS STUDY.

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management in any way. I hereby volunteer to take part in this study.

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

.....	.....
Patient name	Date
.....	.....
Patient signature	Date
.....	.....
Investigator's name	Date
.....	.....
Investigator's signature	Date
.....	.....
Witness name and signature	Date

VERBAL PATIENT INFORMED CONSENT (applicable when patients cannot read or write)

I, the undersigned, Mrs S Alummoottil, 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/her 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.

Patient's Name _____
(Please print)

Patient's Signature _____ Date _____

Investigator's Name _____
(Please print)

Investigator's Signature _____ Date _____

Witness's Name _____

Witness's Signature _____ Date _____
(Please print)

(Witness - sign that he/she has witnessed the process of informed consent)