CHAPTER 5

COMPARING THE ULTRASTRUCTURE OF PLATELETS AND FIBRIN NETWORKS OF THE ASTHMATIC BALB/c MICE TO THAT OF HUMAN CONTROL AND ASTHMATIC SUBJECTS

5.1 Introduction

Platelets play an important role in asthma and there is a vast amount of clinical data that reveal that platelet activation accompanies allergen-induced bronchoconstriction in humans (Pitchford *et al*., 2003; Pitchford *et al*., 2008). According to Pitchford in 2007, the role of platelets in asthma are distinct from their classically known actions performed during thrombosis and haemostasis; and include the expression of adhesion molecules and contact-dependent activation of leucocytes, the release of a plethora of inflammatory mediators, activation in cells of the adaptive immune response and the ability to migrate and undergo chemotaxis (Pitchford, 2007). The author also mentions that clinical data from patients suffering from asthma, allergic rhinitis and allergic dermatitis reveal changes in platelet behaviour and function during or after allergen exposure. It is therefore useful to use platelets and fibrin to study a disease such as asthma.

Fibrinogen itself is also widely recognised as a marker for systemic inflammation as it is considered an acute phase protein.

The murine BALB/c asthmatic animal model is a widely used animal model and it has previously been used successfully for a number of *in vivo* immunological applications and for testing novel therapeutics. Pretorius and co-workers in 2007 were the first to use this model to study ultrastructure and changes in platelet ultrastructure during asthma were noted (Pretorius *et al*., 2007a). Bronchial lavage and white blood cell

counts, additionally confirmed an asthmatic state in the animals. This model was therefore proven to be a reliable, clinically relevant facsimile of human asthma. In the study, the ultrastructure of fibrin networks and platelets of control mice were compared to asthmatic mice treated with hydrocortisone and a plant material (Pretorius *et al*., 2007a). Results indicated that control mice possess major, thick fibers and minor thin fibers as well as tight round platelet aggregates with typical pseudopodia formation. Minor fibers of asthmatic mice have a netlike appearance covering the major fibers, while the platelets seem to form loosely connected, granular aggregates.

The question that arises is whether platelets and fibrin networks of humans with asthma will have the same ultrastructure as seen in the BALB/c asthmatic model. In order to answer this question, ultrastructure of platelets and fibrin networks from asthmatic subjects were studied and compared to that of human controls as well as the animals from the asthmatic BALB/c mouse model.

5.2 Materials and Methods

5.2.1 *Samples*

Blood was collected from two subjects: a controlled asthma subject and her 13 year old son, with severe, uncontrolled asthma. The female subject with controlled asthma has been suffering from asthma since childhood. However, the disease is kept under control with a strict diet for the past 10 years. She follows a diet rich in anti-oxidants, no grains or bread in any form, and at least seven different types of fruit and vegetables per day with very little meat and additional anti-oxidant supplements. This diet has been followed for the past 10 years, resulting in controlled asthma, without medication for the same number of years. However, a bronchodilator, Ventolin [salbutamol; Glaxo-SmithKline South Africa (Pty) Ltd, Johannesburg, South Africa] is used occasionally when an asthmatic response is triggered.

The second subject is a 13 year old male with chronic, severe asthma, treated with Seratide [salmeterol; Glaxo-SmithKline South Africa (Pty) Ltd, Johannesburg, South Africa] once a day and Ventolin once or twice per day. This subject does not follow a strict diet, where possible dietary triggers are excluded and do not include more than 2 vegetables and fruit daily, also no extra anti-oxidant supplementation is taken.

5.2.2 *Preparation of fibrin clots*

Blood was collected from two asthmatic subjects and three control subjects (Research Ethical Committee of the University of Pretoria, South Africa; ethical clearance number 115/2006). Fresh platelet-rich plasma (PRP) was prepared by drawing 40 ml of blood which was centrifuged at 1 000 rpm (maximum RCF = 17.523 x g; 1 250 g) for 2 minutes. Fibrin clots were prepared in order to obtain platelet aggregates as well as fibrin fibers from both the patients as well as the control groups. Human thrombin (provided by the South African National Blood Service) was used to prepare these fibrin clots from the asthmatic patients as well as the controls. Human thrombin is prepared from a single regular donor by calcium chloride activation of a euglobulin fraction of plasma obtained by apheresis. Each individual unit is tested and has to be non-reactive for hepatitis B surface antigen (HbsAg), HIV-1 antibody, HIV-2 antibody and HIV p-24 antigen, hepatitis V virus (HCV) antibody and antibodies to *Treponema pallidum*. These tests are performed by licensed assay methods. The thrombin solution is at a concentration of 20 U/ml and is made up in a biological buffer containing 0.2% human serum albumin.

When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components, e.g. transforming growth factor, platelet-derived growth factor and fibroblastic growth factor are released into the coagulum. 20 µl of the PRP was mixed with 20 µl of human thrombin on a 0.2 µm millipore membrane to form the coagulum (fibrin clot). This millipore membrane was then placed in a Petri dish on

filter paper dampened with phosphate buffered saline (PBS) to create a humid environment and placed at 37 ºC for 10 minutes (Pretorius *et al*., 2007a). A washing process where the millipore membranes with the coagula were placed in PBS and magnetically stirred for 1hr followed this. This was done to remove any blood proteins trapped within the fibrin network (Pretorius *et al*., 2006).

5.2.3 *Preparation of washed fibrin clot for SEM*

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1hr. Each fibrin clot was rinsed thrice in phosphate buffer for 5 minutes before being fixed for 1hr with 1% osmium tetraoxide (OsO_4.) The samples were rinsed thrice with distilled water for 5 minutes and were dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting, coating with ruthenium tetraoxide (SPI Supplies, West Chester USA) and examining the tissue with a ZEISS ULTRA plus FEG scanning electron microscope. A series of micrographs were taken over the filter to ensure that the morphology seen is representative of the whole clot area.

5.3 Results

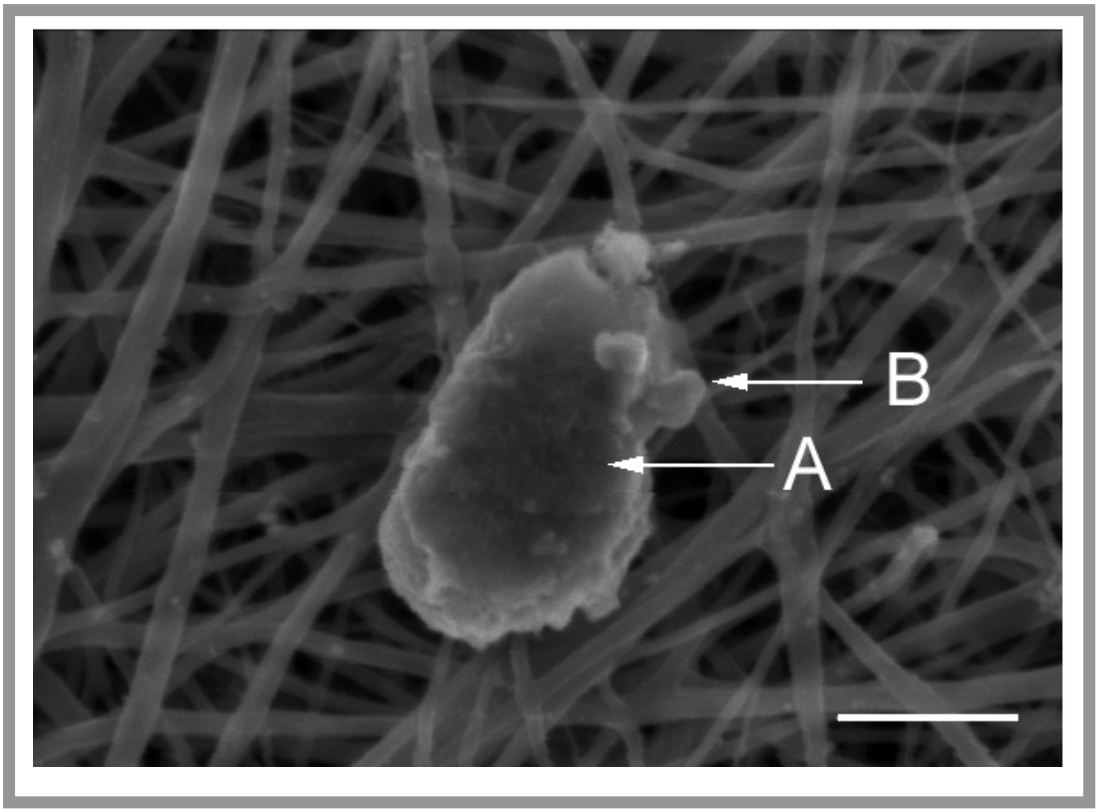

Figure 5.1a: Control platelet aggregate forming dense, round aggregate with pseudopodia. **Label A** = smooth membrane of aggregate, **Label B** = pseudopodia. (Scale = 1µm)

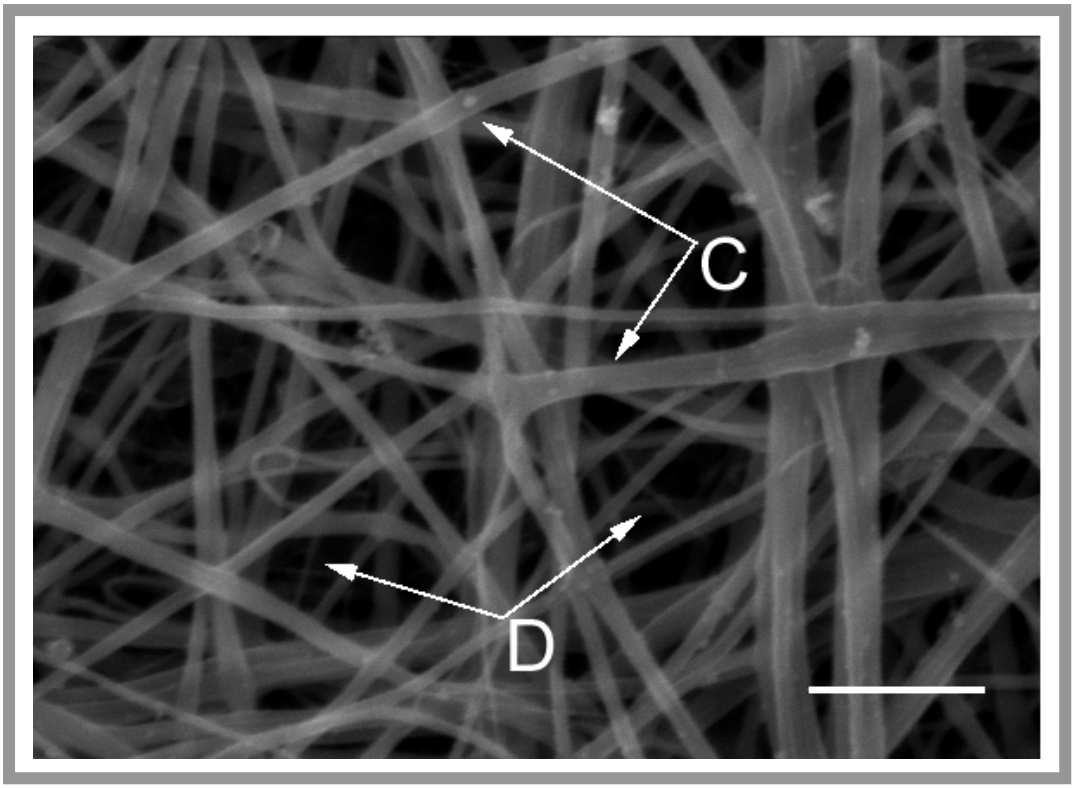

Figure 5.1b: Control fibrin network with thick, major fibers as well as thin, minor fibers. **Label C** = thick, major fibers; **Label D** = thin, minor fibers. (Scale = 1µm)

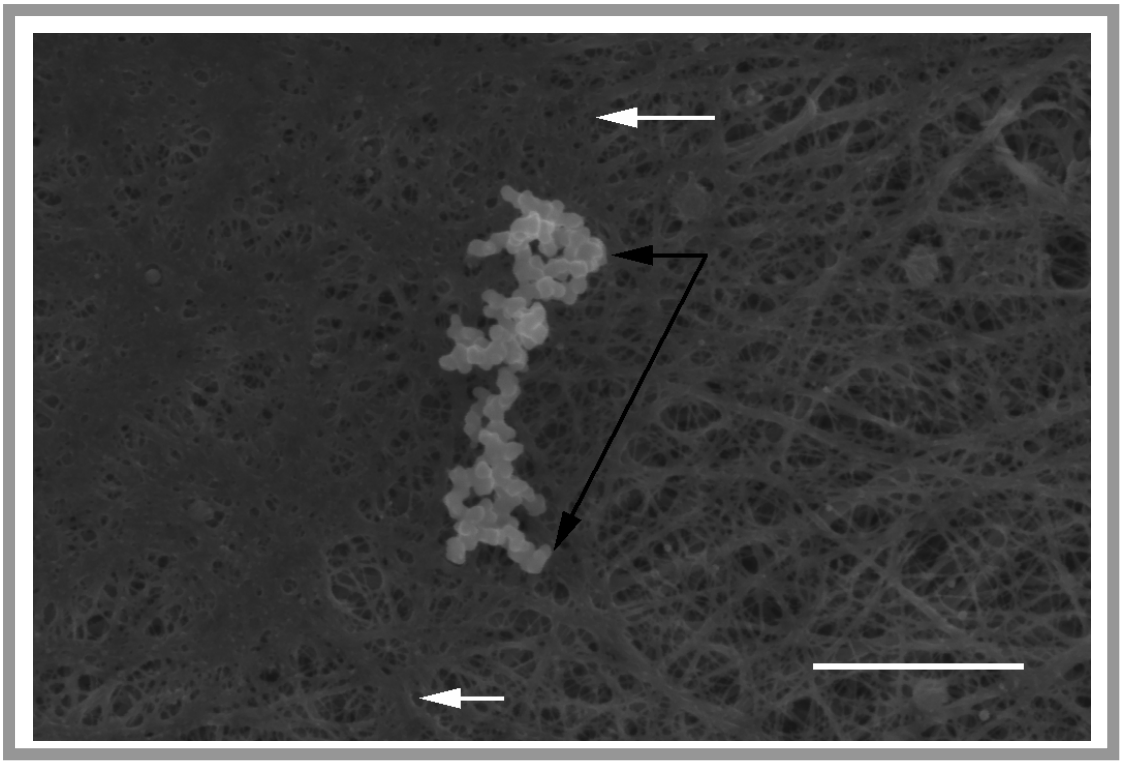

Figure 5.1c: Platelet aggregate and fibrin network from asthmatic mouse from the BALB/c asthmatic animal model. Loosely arranged platelet aggregate shown by thick black arrow; thick white arrow showing matted fibrin network. (Scale = 1µm)

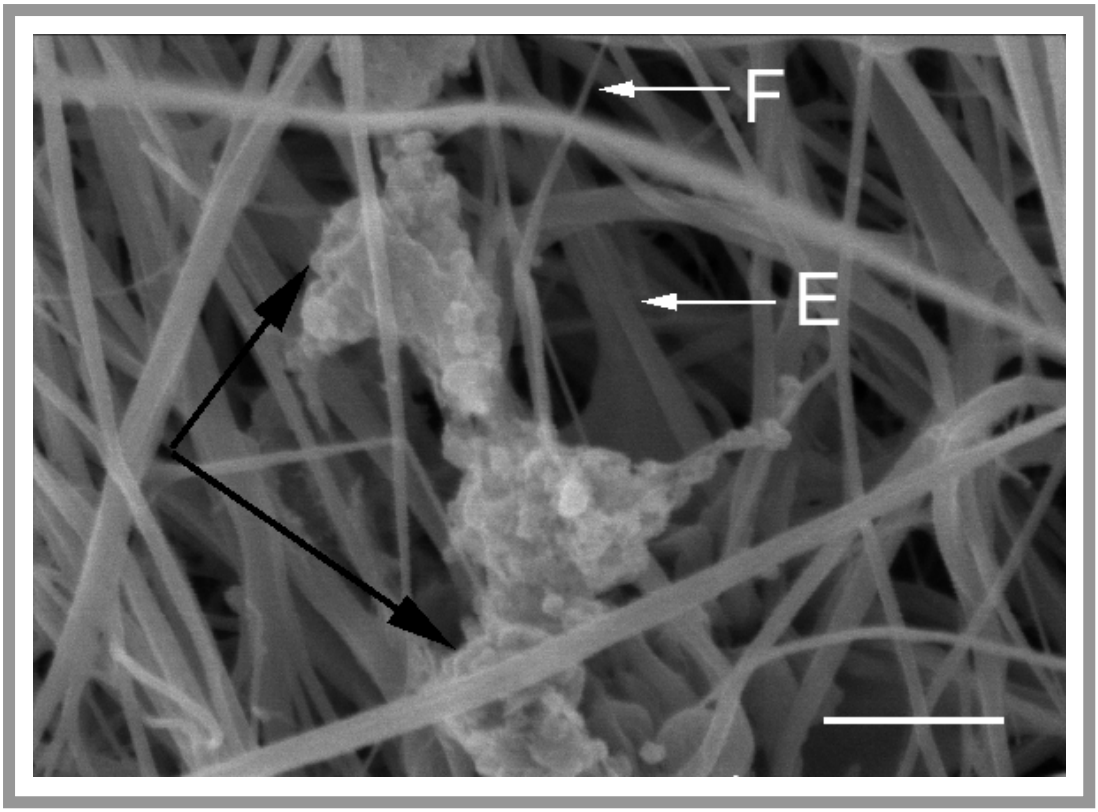

Figure 5.2: Platelet aggregate from controlled asthmatic subject, with course, granular aggregate indicated with thick black arrow. Fibrin network is seen with thick, major fibers as well as thin, minor fibers. **Label E** = thick, major fibers; **Label F** = thin, minor fibers. (Scale = 1µm)

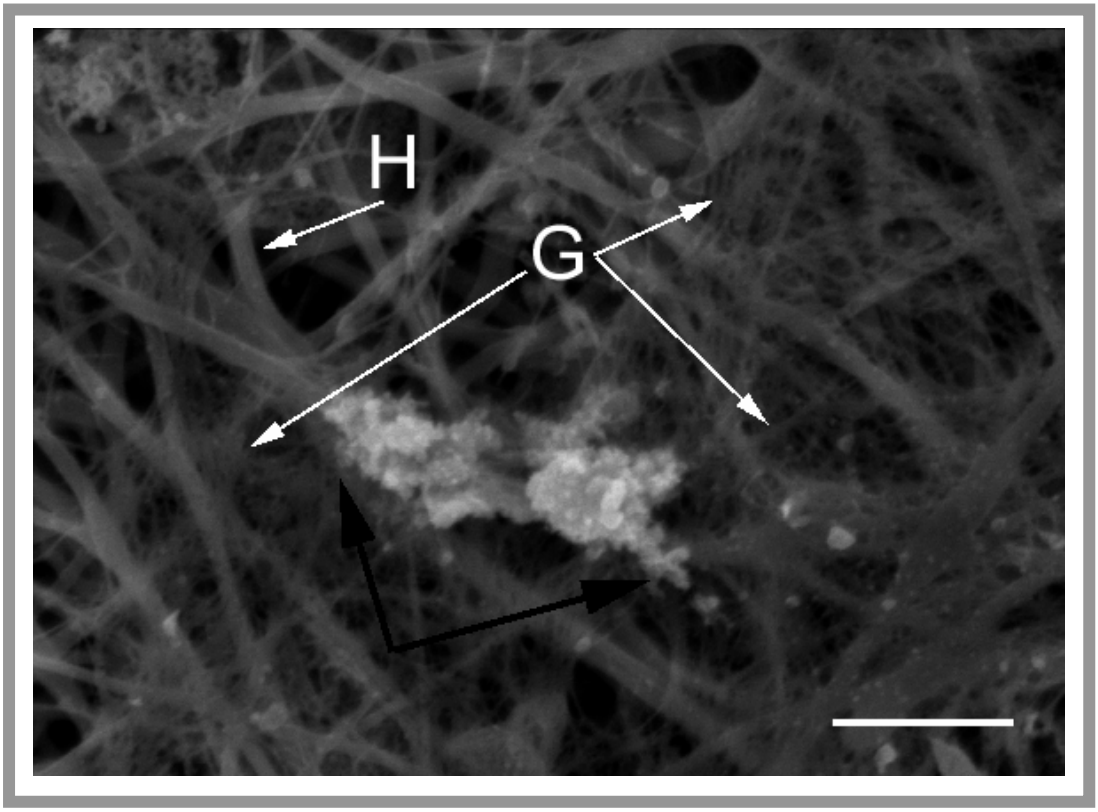

Figure 5.3: Platelet aggregate and fibrin network from uncontrolled asthmatic individual. Thick black arrow shows course, loosely arranged platelet aggregate. **Label G** = major fibers; **Label H** = minor fibers showing matted fibrin network. (Scale = 1µm)

In order to study the SEM results, each clot area (diameter 3mm) was studied by systematically viewing the whole clot using SEM. This was done to make sure the morphology seen in the given figures was indeed representative of the whole clot structure. Previous ultrastructural results using the BALB/c asthma model have shown that platelets and fibrin have a changed ultrastructure in asthma.

Platelets from the control BALB/c animals showed a smooth membrane surface with pseudopodia. Fibrin networks of control animals showed thick, major fibers and thin, minor fibers (Pretorius *et al*., 2007a; Pretorius *et al*., 2007b), as was also found in the current study as indicated in chapter 4. However, platelets from the asthmatic animals showed a tendency to from loosely connected, granular aggregates, while minor fibers of asthmatic mice have a netlike appearance covering the major fibers. This was also seen in the current study when looking at Figure 5.1c.

The question that arose was whether platelets and fibrin networks of humans with asthma would show a similar ultrastructure. The current research investigated 2 individuals with asthma; the one subject has controlled asthma, while the second subject has chronic, uncontrolled asthma.

Figure 5.1a and b show a platelet aggregate and fibrin network of a typical control subject, respectively. Typical control platelet aggregates (Figure 5.1a), possess smooth membranes (Label A) with pseudopodia (Label B) protruding from the body of the aggregate. Figure 1b shows fibrin networks from the control human subject. Fibrin fibers can be morphologically divided into major, thick fibers (Label C) and minor, thin fibers (Label D). The major, thick fibers branch off from each other and the fibers are typically seen as single fibers forming relative straight lines. Thin, minor fibers are much thinner than the major, thick fibers and are found sparsely distributed among the major fibers.

Figure 5.1c shows a platelet aggregate and fibrin network of a typical asthmatic mouse model. A loosely connected, granular aggregate is shown (thick black arrow) while minor fibers of asthmatic mice have a netlike appearance covering the major fibers (thick white arrow). These results were also found and discussed in Chapter 4.

Figure 5.2 shows a platelet aggregate and fibrin network of a controlled asthma subject. Platelet aggregates in this human subject from loosely connected, granular aggregates (thick black arrow), similar to that previously found by Pretorius *et al*., 2007a in the asthmatic BALB/c murine model and the same results were found in the current study (Figure 5.1c, thick black arrow). However, in this subject, fibrin networks appeared as that of the controls with major, thick fibers (Label E) and minor, thin fibers (Label F).

Figure 5.3a shows a platelet aggregate and fibrin network of the subject with uncontrolled, chronic asthma. Platelet aggregates also appeared loosely connected and granular (thick white arrow), as is seen in the asthmatic mouse model (Figure 5.1c). Also, fibrin networks were similar to that found in the asthmatic BALB/c animals (Figure 5.1c). In the human subject, the thick, major fibers (Label G) were covered with a fine net of minor, thin fibers (Label H) as seen in the asthmatic animal model (Figure 5.1c, thick white arrow).

5.4 Discussion

These results suggest that the BALB/c asthmatic murine model mimics human asthma when studying the ultrastructure of platelets and fibrin networks. Additionally, these results may also perhaps be useful in planning treatment regimes for asthma. Here it shows that a subject with controlled asthma still has changed platelet morphology (Figure 5.2) similar to asthmatic animals (Figure 5.1c), however, fibrin networks seem to be stabilized if the asthma is under control, and appear similar to

that of human control fibrin networks (Figure 5.1b). The subject with uncontrolled asthma showed both changed platelet morphology and fibrin networks (Figure 5.3). It is known that patients with asthma have extra-vascular thrombin, fibrinogen, and fibrin have been found in the sputum of patients with asthma (Banach-Wawrzenczyk *et al*., 2000; Pizzichini *et al*., 1996; Wagers *et al*., 2004). There might be a link between the presence of fibrinogen and fibrin in the sputum and the fibrin net covering the major fibers. However, this cannot be confirmed with the present study.

5.5 Conclusion

Therefore it can be concluded that human platelet and fibrin asthmatic ultrastructure is similar to that found in the BALB/c murine asthmatic model. The question that always arises when using animal models is whether the model adequately mimics the human disease; the current research therefore shows additional support for the use of this model in asthma.