



ORIGINAL ARTICLE

Confocal microscopy of the paediatric haemostatic system

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Introduction

As the current population continues to rise, so will the aging population where age-related diseases such as cardiovascular disease and stroke are the major causes of morbidity and mortality worldwide. Cardiovascular disease (CVD) is the leading cause of death in the world with cardiovascular disease causing approximately 54 million deaths globally. One of the main mechanisms of cardiovascular complications occurs from an imbalance in the blood clotting system (haemostatic system), resulting in thrombosis or an occlusion of a blood vessel. Blood vessel occlusion by thrombosis is the typical final mechanism of almost all related diseases such as ischemic heart disease and stroke.

Scientific research holds the key to addressing the social, clinical and financial problems of the ageing population.

A concept known as developmental haemostasis has shown that there are differences in the way the haemostatic system coagulates blood in children when compared to adults. The aim of this study is to further characterise the differences between children and adults where these differences will reinforce the concept of developmental haemostasis and further explain the thromboprotective elements that children

possess against cardiovascular disease and stroke when compared to adults.

Haemostasis is the basic physiological response that prevents significant blood loss following an injury to the vascular system [1]. Haemostasis relies on a series of synchronized complex events that involve platelets and the activation of specific blood proteins to induce coagulation (blood clotting). The haemostatic system has two main functions, which are to maintain blood in a fluid state and to induce a rapid, localized response to damaged vessels. When injury occurs, the haemostatic system responds via the establishment of a haemostatic plug (blood clot) that includes platelets, sub endothelium and plasma coagulation proteins [2].

The process of haemostasis consists of 3 distinct phases:

1. The initial phase, primary haemostasis, induces the formation of a platelet plug.
2. The second phase, secondary haemostasis, results in the production of thrombin, a key haemostatic protein responsible for the conversion of fibrinogen into fibrin. Fibrin is one of the main proteins that is responsible for blood clot structure and the formation of a fibrin clot represent the final step in blood coagulation [3].
3. The final phase, known as tertiary haemostasis or fibrinolysis (clot lysis)



represents the removal of the blood clot once the need for it is no longer required. Fibrin itself plays a role during fibrinolysis, functioning as both a cofactor and substrate for the enzyme plasmin, which is the main protein that causes fibrinolysis.

Within the healthy population, rigorous regulation of the haemostatic system ensures that clotting only ensues at the sites of vascular injury, without disturbing systemic blood flow [4]. Unfortunately, the haemostatic system can become dysfunctional (i.e. lack of clotting factors) which can lead to pathological thrombosis and subsequent morbidity and mortality [5]. These imbalances lead to diseases that include: cardioembolic stroke, pulmonary embolism (PE), atherothrombosis (thrombosis triggered by plaque rupture) and venous thromboembolism (VTE). Therefore, understanding the haemostatic system is crucial when examining disease states linked with thrombosis.

Developmental haemostasis

A term first used by Andrew et al. in papers published in *Blood* from 1987-1992 [5-6], 'developmental haemostasis' describes the physiological differences in the haemostatic system between neonates, children and adults, particularly in relation to coagulation proteins. The haemostatic system begins developing in utero, continues throughout adolescence and all the way throughout adulthood. All the vital constituents of the haemostatic system exist at birth however age-related differences in

structure and function of coagulation proteins occur during the development from neonates to children to adults. The haemostatic system changes rapidly, as developmental changes occur over a series of months, weeks or even days [5, 6, 8, 9]. However, the understanding of the haemostatic system in children and neonates is limited when compared to the level of understanding in adults.

Developmental haemostasis supports the idea that neonates and children appear to have a protective advantage over the adult haemostatic system [5, 10, 11]. To further support this concept, long-term outcomes of thrombotic complications in neonates and children are very different when compared to adults [5].

The use of confocal microscopy to image and identify each element of the haemostatic system in paediatrics provides an excellent scope into clot formation. This, coupled with further research can provide a basis for understanding diseases of burden such as cardiovascular disease and stroke.

Patient description

Blood was taken from a 5-year-old male paediatric patient, undergoing elective surgery, with no history of any cardiovascular or haematological disorders.

Materials and methods

Whole blood clot fluorescent dye and antibody staining. Whole blood clots were formed from



using an adult sample using a modification of a previously published method [13]. After the formation of Whole blood clot, the clots were washed three times In Phosphate Buffered Saline (PBS) for 3-5 minutes per wash. Fibrin fibres and red blood cells (RBC) were labelled separately on a formed clot, platelets were labelled separately on a blood clot and white blood cells (leukocytes, WBC) were labelled on a separate blood clot.

Fibrin fibres were then labelled with 100 μ L (working concentration) of antibody (Alexa Fluor 488 human fibrinogen conjugate F-13191, Molecular Probes 5 mg, Invitrogen Life Technologies), which was prepared using sodium bicarbonate buffer (pH 8.3). Following the labelling of the fibrin, the RBC's were labelled with 100 μ L of the Merocyanine-labelled RBC-emission wavelength 520 nm (Molecular Probe USA). After the Merocyanine staining, the clot was incubated at room temperature for 45 min in dark room. After the time period elapsed, the dye was washed again using PBS 3 times for 3-5 minutes each wash.

Platelets were immunolabeled with Primary Antibody (Anti-CD41 antibody M148, AB11024, ABCAM) followed by Secondary Antibody (Donkey Anti-Mouse IgL H&L (Alexa Fluor 568), AB175472, ABCAM) using manufacturer's instructions. The mixture was then placed for 45 minutes at room temperature in a dark place where the stain was then removed and the clot is washed again 3 times using PBS, at 3-5 minutes each wash.

WBC's were labelled with 5 μ L Antibody (Brilliant Violet 421 CD45 AntiMouse Human, Becton Dickinson PTY Limited, AUS). The mixture is then placed in a dark room for 45 minutes at room temperature where it was finally washed with PBS, 3 times at 3-5 minutes each wash.

Confocal Microscopy Results

Figure 1 represents a whole blood clot that has been labelled with antibodies and fluorescent dyes, where it has been observed under confocal microscopy. The fibrin fibres were labelled with a green dye, where the red blood cells were labelled with a red dye.

In Figure 2 we can see the result of the positive staining and fluorescence of a lymphocyte using the Brilliant Violet 421 CD45 Anti-Mouse Human antibody. The lymphocyte (granulocyte) was identified by its morphological features and is consistent with the antibodies used.

In Figure 3 we can observe a series of fluorescently-labelled platelets in a whole blood clot. The platelets were identified by their cellular morphology and are consistent with the antibody used.

Images

Figures 1-3 are shown on the following pages of the article.

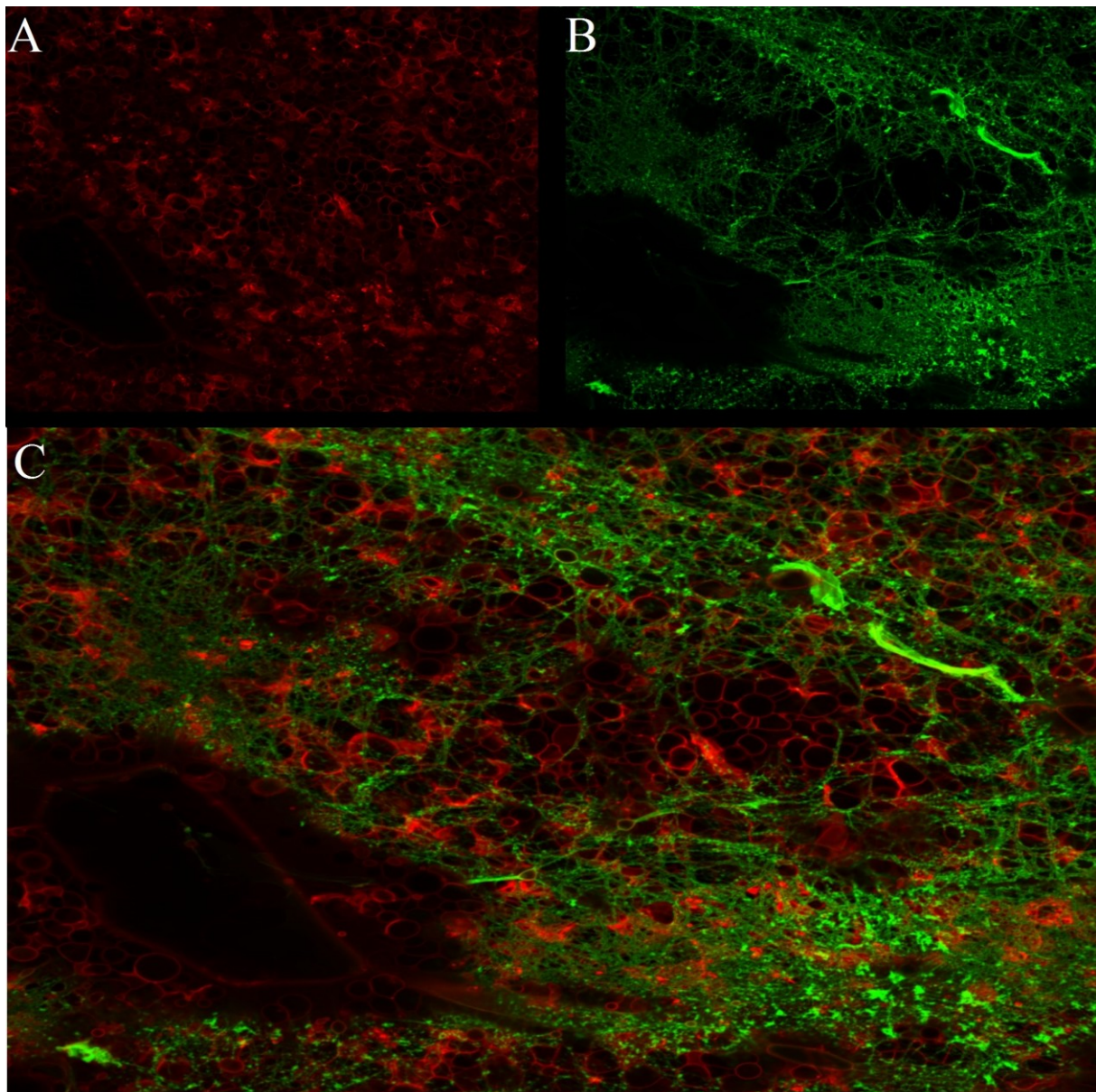


Figure 1. Whole blood clot through confocal microscopy at 40 \times magnification objective:
a) Merocyanine-labelled red blood cells;
b) Alexa Fluor 488 human fibrinogen conjugate-labelled fibrin;
c) both red blood cells and fibrin illuminated together.

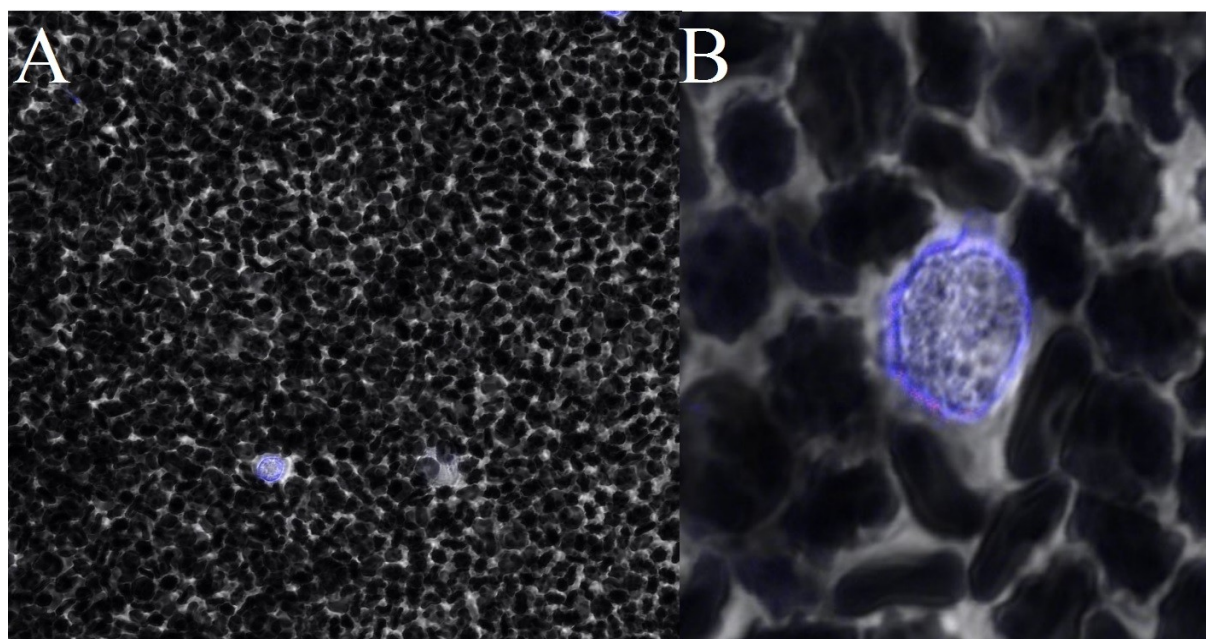


Figure 2. Confocal microscopy of fluorescently-labelled leukocyte – lymphocyte:
a) lymphocyte at 40× magnification;
b) lymphocyte at 100× magnification.

Conclusion

The near future will further bring a global ageing population throughout the world and in Australia. As a result of this, the burden of age-related diseases such CVD and stroke is promised to increase. For the first time, the present study has established identified specifics in whole blood clot structure of healthy paediatric patients using confocal microscopy.

Future studies should focus on studying blood clot formation and structure in different clinical scenarios, like those on extracorporeal membrane oxygenation (ECMO) or in patients who suffer CVD and stroke. This further investigation would describe the unknown gaps of knowledge in the aetiology of thrombosis related diseases and their treatments.

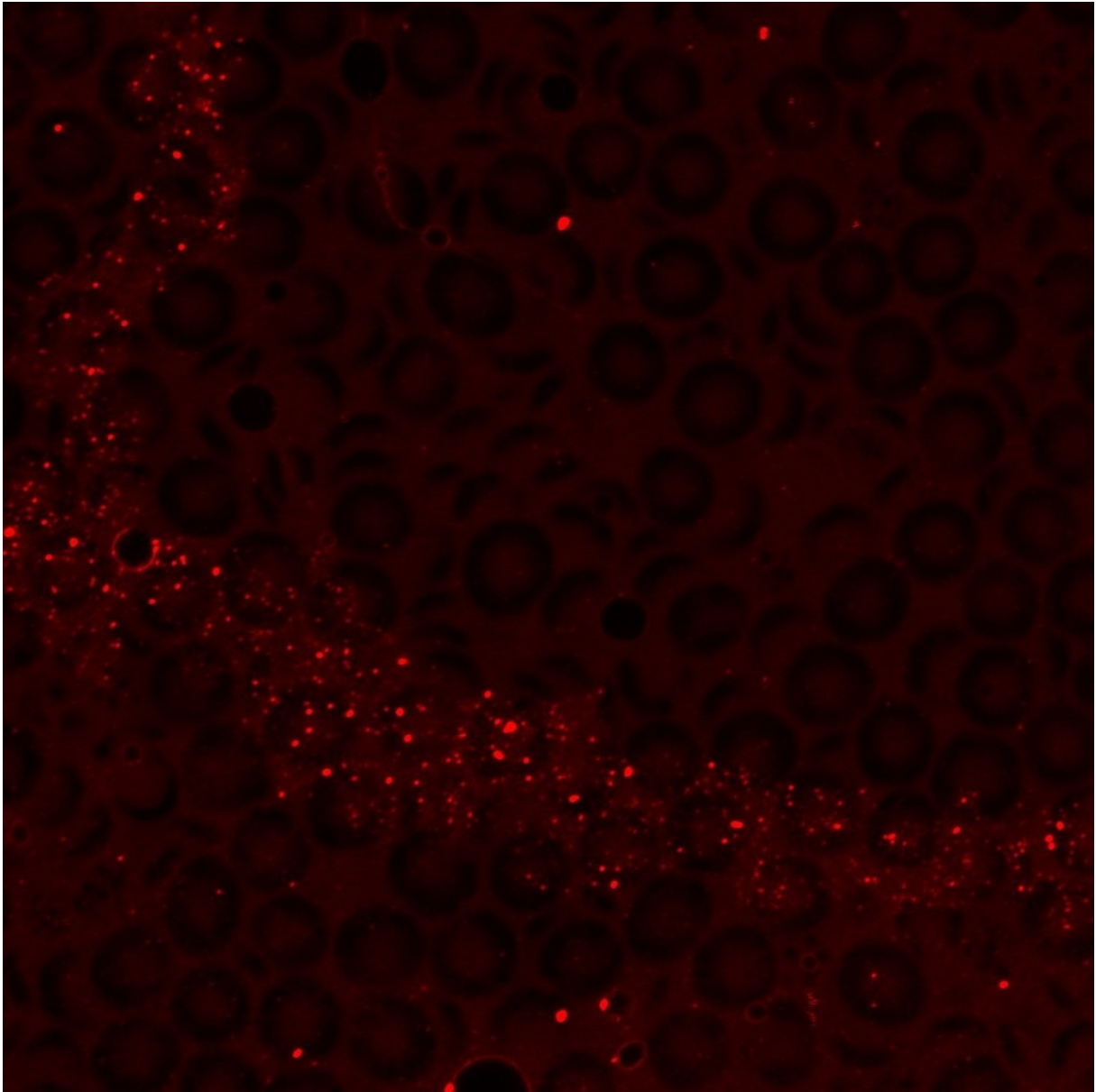
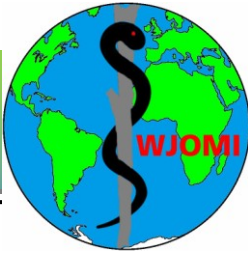


Figure 3. Confocal microscopy of fluorescently-labelled platelets in a whole blood clot.



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Conflict of interest: none declared

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