High-throughput platelet spreading analysis: a tool for the diagnosis of platelet-based bleeding disorders

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Supplementary Information

Supplementary Methods

Patient recruitment and sample preparation. Patients were recruited to the GAPP study from multiple collaborating Haemophilia Centres across the UK and Ireland. Recruitment of patients was based on a diagnosis of bleeding or thrombocytopenia of unknown cause as defined by ISTH-BAT. The GAPP study was approved by the UK National Research Ethics Service by the Research Ethics Committee of West Midlands (06/MRE07/36) and participants gave full, written, informed consent in accordance with the Declaration of Helsinki. Peripheral blood was collected from patients and healthy volunteers into 3.2% trisodium citrate by venepuncture. Whole blood cell counts were obtained from running analysing EDTA anticoagulated blood on the Sysmex XN-1000™ Haematology analyser (Sysmex, UK). For lumi-aggregometry Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 200 g for 20 minutes at room temperature.

Patient Cohort. Our study cohort consisted of 55 patients with a strong history of bleeding and suspected of having a PFD of unknown cause (patients' clinical and platelet function results are summarised in table 1). Patients were recruited from both paediatric and adult referral centres, with an average age of 39.6 years, ranging from 6 to 78 years. The patient cohort consisted of 80% female (n=44) and 20% male (n=11) participants. The majority of patients suffered from a variety of bleeding symptoms including epistaxis, cutaneous bruising, bleeding from minor wounds, haematuria, gastrointestinal bleeding, oral cavity bleeding, and bleeding after tooth extraction, surgery, major trauma or child birth. The International Society of Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT) was used to provide an objective evaluation of the patients bleeding history and scores were available from 40 individuals apart from the paediatric patients (where the BAT is not valid). There was a mean BAT score of 9.825 overall (ranging from 2 - 23) where a score of 4 or more is considered to be elevated and indicative of excessive bleeding. Platelet counts for the patient group ranged from 43 - 428 $x10^9$/L and the average platelet count was 232 $x10^9$/L. 12 patients had a platelet count of <150 $x10^9$/L which is below the normal range and therefore labelled as having thrombocytopenia. The overall platelet counts in the control group ranged from 67 - 420 $x10^9$/L and the average platelet count in this group was 224 $x10^9$/L (n=32). The Mean Platelet Volume (MPV) in patients tested ranged between 8.3 fl and 15.1 fL (mean reference range for healthy controls ±2 SD = 7.83 - 10.5 fL).

Platelet Spreading assay. A platelet spreading assay was carried out for each patient alongside a healthy volunteer using $2x10^7$ /ml washed platelets prepared using modified Tyrodes buffer (134 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 12 mM $NaHCO_3$, 0.34 mM NA_2HPO4, 1 mM $MgCl_2$, 45 mg of glucose) at pH 7.3 and left to rest for 20-30 minutes before spreading. 300 µl of washed platelets at $2x10^7$/ml were added to coverslips pre-coated with fibrinogen (Enzyme Research Laboratories, UK) and allowed to spread for 45 minutes at 37 °C. The platelet solution was then removed and coverslips washed 1 x in PBS. Platelets were fixed in 500 µl 10% formalin for 10 minutes then washed 3 x in PBS.

The coverslips were incubated for 5 minutes with 0.1% triton and washed 3 x with PBS. Coverslips were then incubated for 20-30 minutes in blocking buffer (1% BSA [First Link UK Ltd., UK], 5% goat serum [Gibco™, USA] in PBS). -tubulin fibres were stained with primary antibody, Monoclonal Anti--Tubulin DM1A (Sigma-Aldrich®, UK), was added (1:200) to blocking buffer for 1 hour. Coverslips were washed 3 x in PBS and secondary antibodies Alexa Fluor® 647 goat anti-mouse IgG (1:250) (Thermo Fisher Scientific™, USA) and Alexa Fluor® 488- conjugated Phalloidin (1:300) (Thermo Fisher Scientific™, USA) were added to blocking buffer to stain actin fibres, and coverslips were incubated in the dark for 1 hour. Coverslips were then washed with PBS, H_2O, blotted dry and mounted on glass microscope slides with Hydromount (Scientific Laboratory Supplies Ltd., UK). Before imaging, the slides were left to dry overnight in the dark at 4°C.

Imaging. All images were taken using a DM IRE2 Leica Inverted microscope, SP2 confocal system running Leica Confocal Software Version 2.61 Build 1537. Confocal imaging of the spread platelets was performed using the 488 nm line of an Argon-Ion laser 457-514 nM (to image Alexa Fluor® 488) and the 633 nm line of an Helium-Neon-Ion laser (to image Alexa Fluor® 647) with an HCX Plan Apo lbd.BL 63x NA 1.4, Leica objective. Z-stacks were taken and the average intensity projection of 10 fields of view per control or patient were analysed.

Image Analysis. To isolate signal from background an ilastik pixel classifier was trained on a subset of the data.[5] This was run on the full dataset within KNIME to produce binary images.[6] Holes within the binary images smaller than 10 μm^2 were filled. To separate touching platelets points were manually placed at the centre of each platelet using KNIME's interactive annotator node. These points were used as seeds for a watershed transformation of the binary image which produces a labelled segmentation of individual platelets. Objects smaller than 1 μm^2 were removed.

Measurements for each platelet, specifically area covered (A), perimeter (P), circularity (C) and mean fluorescent intensity were then calculated. Circularity is defined as

$$C = \frac{4\pi A}{P^2}$$

and has a maximum value of 1 for a perfect circle. Platelet area and circularity measurements was displayed and visualised using a web app PlotsOfData.[18]

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