Analysis of Preplatelets and Their Barbell Platelet Derivatives by Imaging Flow Cytometry

Tracking no: ADV-2021-006073R2

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Abstract:
Circulating large "preplatelets" undergo fission via barbell platelet intermediates into two smaller, mature platelets. In this study, we determine whether preplatelets and/or barbells are equivalent to reticulated/immature platelets by using ImageStream flow cytometry (ISFC) and super-resolution microscopy. Immature platelets, preplatelets and barbells were quantified in healthy and thrombocytopenic mice, healthy human volunteers, and patients with immune thrombocytopenia (ITP) or undergoing chemotherapy. Preplatelets and barbells were 1.9%±0.18/1.7%±0.48 (n=6) and 3.3%±1.6/0.5%±0.27 (n=12) of total platelet counts in murine and human whole blood, respectively. Both preplatelets and barbells exhibited high expression of HLA-I with high thiazole orange and mitotracker fluorescence. Tracking dye experiments confirmed that preplatelets transform into barbells and undergo fission ex vivo to increase platelet counts, with dependence upon the cytoskeleton and normal mitochondrial respiration. Samples from antibody-induced thrombocytopenia in mice and patients with ITP had increased levels of both preplatelets and barbells correlating with immature platelet levels. Furthermore, barbells were absent post-chemotherapy in patients. In mice, in vivo biotinylation confirmed that barbells, but not all large platelets, were immature. This study demonstrates that a subpopulation of large platelets are immature preplatelets that can transform into barbells and undergo fission during maturation.

Conflict of interest: No COI declared

COI notes:

Preprint server: Yes; BioRxiv https://doi.org/10.1101/2021.07.30.454421

Author contributions and disclosures: SK generated the data and wrote the paper. AD generated advanced microscopy images. PH conceived, funded the project and wrote the paper. GL and PN provided clinical input and clinical samples for the study and edited the paper. ST and SPW provided advice and edited the paper. YS provided mice edited the paper and contributed to funding.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: emails to corresponding author

Clinical trial registration information (if any):
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Running title: Imaging Flow Cytometry of Platelet Precursors

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Abstract

Circulating large “preplatelets” undergo fission via barbell platelet intermediates into two smaller, mature platelets. In this study, we determine whether preplatelets and/or barbells are equivalent to reticulated/immature platelets by using ImageStream flow cytometry (ISFC) and super-resolution microscopy. Immature platelets, preplatelets and barbells were quantified in healthy and thrombocytopenic mice, healthy human volunteers, and patients with immune thrombocytopenia (ITP) or undergoing chemotherapy. Preplatelets and barbells were 1.9%±0.18/1.7%±0.48 (n=6) and 3.3%±1.6/0.5%±0.27 (n=12) of total platelet counts in murine and human whole blood, respectively. Both preplatelets and barbells exhibited high expression of HLA-I with high thiazole orange and mitotracker fluorescence. Tracking dye experiments confirmed that preplatelets transform into barbells and undergo fission ex vivo to increase platelet counts, with dependence upon the cytoskeleton and normal mitochondrial respiration. Samples from antibody-induced thrombocytopenia in mice and patients with ITP had increased levels of both preplatelets and barbells correlating with immature platelet levels. Furthermore, barbells were absent post-chemotherapy in patients. In mice, in vivo biotinylation confirmed that barbells, but not all large platelets, were immature. This study demonstrates that a subpopulation of large platelets are immature preplatelets that can transform into barbells and undergo fission during maturation.

Key points

Preplatelets and barbell platelets are equivalent to reticulated/immature platelets
Preplatelets are a subpopulation of large platelets that can transform into barbells and undergo fission during maturation
Introduction

Platelets are anucleated discoid blood cells that originate from bone marrow megakaryocytes (MK).\(^1\) As human platelets exhibit a short lifespan of \(\sim 10\) days, over 100 billion new platelets are required per day to sustain the platelet count (150–400 x 10\(^9\) / L).\(^2\) The youngest platelets are termed reticulated platelets (RP),\(^3\) and were first described in acute blood loss.\(^4\) RP have been traditionally measured by flow cytometry using nucleic acid dyes (e.g. thiazole orange).\(^5\) Biotinylation studies performed in mice have also confirmed that they are immature platelets.\(^3\) RP levels can distinguish between thrombocytopenic conditions due to enhanced peripheral destruction or lack of production.\(^6\)-\(^8\) Commercial methods for quantification include the immature platelet fraction (IPF),\(^9,10\) which although standardised tends to overestimate numbers in macrothrombocytopenia and bone marrow suppression/failure due to non-specific labelling.\(^11,12\) Recent efforts have tried to overcome such limitations identifying HLA-I (MHC-I) as a potential new marker of platelet immaturity.\(^13\)

During maturation, MKs form long proplatelet extensions, which can subsequently release platelets.\(^14-16\) These proplatelets are released into the bone marrow sinusoids, with platelet maturation continuing within the bloodstream\(^16-19\) and the lungs.\(^20,21\) Furthermore, proplatelet-like barbell structures (consisting of a continuous, barbell-shape marginal band) undergo fission \textit{in vitro} to form progeny, thus increasing the count.\(^22\) This led to the discovery of a new terminal stage of maturation which takes place in the bloodstream through a platelet intermediate the “preplatelet”.\(^18\) Balduini has recently highlighted that similar structures and platelet division were originally described by Perroncito back in 1921.\(^23\) Circulating preplatelets are large platelet precursors (3-10 \(\mu\)m),\(^24\) which divide by converting into barbells before undergoing fission into mature platelets (<3 \(\mu\)m).\(^18,22\) These
structures have been identified and quantified in healthy human platelet-rich plasma (PRP) using laser scanning cytometry (3.6% and 0.05% respectively). However, barbells have been rarely reported probably because both IPF and blood film analysis use EDTA anticoagulated blood, causing swelling and irreversible conversion of barbells back into preplatelets.

Although the current dogma is that preplatelets and barbells represent intermediate structures that undergo fission, it is still unknown whether this exclusively occurs within immature platelets. Therefore, we applied ISFC to detect and quantify both preplatelets and barbells directly in whole blood. ISFC allows full preplatelet and barbell characterisation as it combines the speed, sensitivity and phenotyping abilities of conventional flow cytometry with single cell morphological and content resolution. By designing a new ISFC method to accurately discriminate preplatelets and barbells, for the first time we have quantified and characterised these structures in health and thrombocytopenic human and mouse blood and demonstrated their equivalence to immature platelets. We also confirmed that these structures can undergo terminal maturation via fission and propose morphometric quantification of preplatelet-derived barbells could be an additional tool to platelet counts and IPF for potentially diagnosing and managing thrombocytopenia.
Materials and Methods

Patient Recruitment

This study was approved by the NHS Research and Ethics Committee (NHS REC; 15/WM/0465) and by the University Hospitals Birmingham NHS Foundation Trust (RRK 5677). The study was performed in accordance with the Declaration of Helsinki. Healthy controls, patients with Immune Thrombocytopenia (ITP), and those with haematological malignancies undergoing chemotherapy-induced bone marrow ablation were recruited. Blood samples were anticoagulated in EDTA, trisodium citrate and hirudin BD Vacutainers® (Fisher Scientific and Roche respectively). See Supplementary Methods and Supplementary Table 1 for details.

Mice

Wildtype (WT) C57BL/6 mice were of mixed sex between 8-12 weeks old. All animal procedures were undertaken with UK Home Office approval under a project licence (P46252127).

Antibodies and Probes

Whole blood or washed platelets were labelled with anti-human BV421 CD62p (P-selectin; 1/100, AK4, BioLegend, 304926), BV421 CD42b (1/300, HIP1, BioLegend, 303930), FITC CD61 (β3; 1/25, RUU-PL7F12, BD, 348093), Thiazole Orange (200ng/mL; Sigma, 390062), PE CD42b (GPIbα 1/50, HIP1, BD, 555437), PE CD62p (1/100, AK4, BioLegend, 304906) AF599 mitotracker Ros CMX (mitochondrial dye 5μM, Cell Signalling, 9082), APC CD62p (1/100, AK4, BioLegend, 304910) and/or APC HLA I (1/100, W6/32, BioLegend, 311410); or anti-mouse BV421-CD62p (1/100, VI P-44, BioLegend, 304926), FITC-CD41a (1/100, MWReg30, BioLegend, 133904), FITC-conjugated streptavidin (1/10, BioLegend, 405201), BD Retic-
count (BD, 349204) and/or APC-CD41(1/100, MWR303, ThermoFisher, 17-0411-82). A polyclonal rat anti-mouse GPIb antibody (Emfret, R300) was used to induce thrombocytopenia in mice. Isotypes BV421 mouse IgG1(1/100, MOPC-21, BioLegend, 400157), BV421 rat IgG1(1/100, A110-1, BD; 562604), PE or APC mouse IgG1(1/100, MOPC-21, BioLegend, 400112 and 400120 respectively) were used to quantify CD62p exposure. Fluorescence minus one was used for all other markers. AF674 SiR tubulin probe (4μM SpiroChrome, CY-SC002) was used for ISFC tubulin labelling.

**Haematology Analysers**

Human and mouse samples were analysed on the XN1000 (Sysmex UK) and ABX Pentra 60 (Horiba) haematology analysers respectively. Platelet counts, IPF and mean platelet volume (MPV) were recorded.

**Blood Smears**

Human whole blood was stained with modified Giemsa stain (see manufacturer’s guidelines; Gentaur, 102164).

**Washing Platelets**

Platelets were washed as previously described. Following washing steps, platelets were resuspended and diluted in serum-free M199 media to 100 x 10⁶/mL.

**Platelet Incubations**

For all incubations, platelets were incubated in M199 media (ThermoFisher, 31150022) and maintained in suspension using a microplate mixer (300rpm; StarLab, N2400-8040). Incubations were either conducted on the bench (21°C) or within a cell incubator (37°C). For whole blood experiments, 1mL of blood was incubated in a 5mL polypropylene tube for 0, 0.25, 1.5 or 3h. For washed platelets, 100 x 10⁶/mL platelets were incubated for 0, 1.5, 3, 6 or 24h.
To inhibit platelet aggregation, whole blood was incubated with 10μM eptifibatide (Sigma, SML1042) and incubated for 3h at 21°C. For disruption of cytoskeletal signalling washed platelets were incubated with nocodazole (10μM; Sigma, M1404) or cytochalasin D (1 or 0.1μM; Sigma, C6762). To inhibit mitochondrial respiration washed platelets were treated with rotenone (3μg/mL; Sigma, R8875). For dual cytosolic labelling washed platelets were incubated with CellTrace™ CFSE (green) or red (0.2μg/mL and 1μg/mL; ThermoFisher, C34570 and C34572, respectively) for 30 minutes and centrifuged at 1000g for 10 minutes with 1μM prostacyclin (PGI₂; Cayman, 61849-14-7). Green and red labelled platelets were resuspended in serum free M199 media at 100 x 10⁶/mL, mixed and incubated for 3h. Similar experimental conditions and methodology were used to track preplatelet maturation using labelling with CellTrace™ yellow (2μg/mL; ThermoFisher, C34573).

For TRAP-induced degranulation, Washed platelets were incubated with CellTrace™ yellow (2μg/mL) for 30 minutes at room temperature. Labelled platelets were centrifuged at 1000g for 10 minutes with 1μM PGI₂ to remove dye precipitates and resuspended in Tyrode’s buffer (100 x 10⁶/mL). Following this, CellTrace™ yellow labelled platelets were exposed to increasing concentrations of TRAP (1, 10, 100 or 1000 μM) for 30 minutes at room temperature to induce degranulation. Platelets were then fixed in 2% formalin for 10 minutes. Platelets were identified by ISFC using anti-human FITC CD61 (1/25) and granule release was determined by anti-human BV421 CD62p (1/100). For negative controls, washed platelets were incubated for 30 minutes without TRAP and a BV421 mouse IgG1 k isotype control (1/100, Clone: MOPC-21, BioLegend, 400158) was used to set a positive gate at 1% of the negative peak.

**Thrombocytopenia in mice**
To induce thrombocytopenia, C57BL/6 mice received an intraperitoneal injection of polyclonal rat anti-mouse GPIbα antibody (final concentration of 1.5 μg/g). Platelet counts were measured from tail bleeds on day 0 (prior to challenge), 1, 5 and 8. For preplatelet and barbell ISFC analysis, mice were culled by terminal bleed (in 1/10 volume 4% trisodium citrate) on day 5.

**In vivo platelet biotinylation**

Wild type C57BL/6 mice received two intravenous (IV) injections of NHS-biotin (4 mg/mL (Sigma, H1759), in saline) 30 minutes apart to ensure the circulating and splenic pools of platelets were labelled. A tail bleed was performed at 1h and 24h post second IV injection to measure percentage biotin positive platelets. At 24h mice were also culled via terminal bleed and whole blood was anticoagulated in 1/10 volume of 4% trisodium citrate.

**Flow cytometry**

Reticulated and biotin positive platelets were measured using the Accuri™C6 (see supplementary methods).

**Imaging Flow cytometry**

ISFC was performed using the ImageStream™ MKII single camera system with brightfield, side scattered light and lasers 405(60mW), 488(80mW) and 642nM(100mW). Images were captured using an x60 objective lens (pixel area of 0.1 μm², maximum field of view of 128 μm). Acquisitions consisted of 10,000 platelet images (anti-human FITC CD61, Figure 2A & 3-5) or BV421 CD42b (Figure 2B) or anti-mouse FITC or APC CD41 fluorescence. For human preplatelet and barbell measurements (Figure 2A & 3-5), antibody panels consisted of BV421 CD62p, Channel 1(CH01); FITC CD61, CH02; AF599 mitotracker Ros CMX, CH04 and brightfield, CH04 or CH06; For tubulin analyses, BV421 CD62p in, CH01; FITC CD61, CH02 and AF674 SiR-Tubulin, CH05. For immature platelet markers, BV421 CD42b, CH01; TO,
CH02; Mitotracker Ros CMX, CH03; PE CD62p CH04; APC HLA-1, CH05 and SSC, CH06. For mice, preplatelet and barbell analyses BV421 CD62p, CH01; FITC CD41, CH02 and brightfield, CH04 (Figure 6A-D); or for biotinylation BV421 CD62p, CH01; FITC streptavidin, CH02; brightfield, CH04 and APC CD41, CH05 (Figure 6E-F).

Captured images were analysed and optimised (fluorescent RMS gradient >20) on IDEAS (Image Data Exploration and Analysis Software). To determine barbell gating, truth populations, single circular and barbell platelets were manually selected and individually combined with pixel masks morphology (all pixels from within the outermost contour), skeleton thin (a 1-pixel wide skeletal line within an object) and erode (removes the outermost pixels from the default mask) to generate relative difference (RD) statistical feature tables. Features were chosen based on RD scores and visualised to determine optimal feature parameters to discriminate barbells from all other platelets. Preplatelets and barbells were determined by features: intensity (total pixel intensities within a mask), aspect ratio (ratio of the minor axis divided by the major axis; 1=circular), area (size of the mask in $\mu m^2$), diameter (diameter of a circle which is the same area as the object), compactness (density of pixel intensities within the object), symmetry2 (tendency of an object to have two lobes with a single axis of elongation), height (based on a bounding rectangle where the longer side is the height) or minor and major axis intensity (narrowest or widest region of an object).

For preplatelet and barbell gating, all analyses were performed on CD62p negative platelets to exclude microaggregates. For preplatelet quantification a CD61, CD41 or CD42b morphology pixel mask was designed to generate features aspect ratio and area of platelet images. Preplatelets were determined by an aspect ratio score of 0.8–1. From this an erode+4-pixel mask was designed to determine preplatelets with a diameter $\geq 3\mu m$. For
barbell quantification resting elongated platelets were discriminated from circular/discoid platelets using compactness and height*symmetry² feature measurements of the CD61 morphology mask. From this, a skeleton thin mask was designed to depict a single pixel thick cytoskeletal line of the barbell structure and barbells were determined by a low minor axis intensity of CD61 morphology and high area*minor axis intensity of the CD61 skeleton thin (the barbell gate). See supplementary Figure 3 for visualisation and description.

As no barbell platelets were present at 21°C, characterisation of barbell-shaped microaggregates was completed in 21°C citrate blood (using EDTA blood as a negative control) using a similar ISFC methodology to the barbell analysis where microaggregates were discriminated from elongated platelets by a high symmetry² and fluorescence intensity of a CD61 morphology mask.

**Immunofluorescence microscopy**

See supplementary methods

**Statistical Analysis**

All data analysis was performed using GraphPad Prism v8.4.3(GraphPad Software, California, USA). Statistical analyses are described within the figure legends.
Results

*Preplatelets and barbells are immature reticulated platelets in human blood*

In this study, a novel ISFC strategy was developed to accurately detect and quantify preplatelets and barbells in whole blood, as described in Supplementary Figures 1-3 (details on pre-analytical variables: labelling, detection and gating). Preplatelets are detected as rounded structures which can be distinguished and resolved from platelets by their size (>3μm in diameter), while barbells have a unique appearance which distinguishes them from platelet doublets/microaggregates that are CD62p positive and composed of 2 individual platelets (Supplementary figure 1-2). Figure 1A(i-iii) shows several examples of labelling of the marginal tubulin band and CD61 (integrin subunit β3) in platelets, preplatelets and barbells.

To characterise these structures in greater detail, α-tubulin-labelled PRP was imaged using super-resolution structured illumination microscopy (SIM). Compared to platelets (green arrows), barbell platelets consisted of a marginal band with microtubules extended through the narrow cytoplasmic bridge or shaft which looped back at the distal ends to form two teardrop-shaped structures (Figure 1B, blue arrows). Large preplatelets(yellow arrows) and a new population of intermediate elongated preplatelets, characterised by an oval-shaped marginal band (Figure 1C, purple arrows), were resolved which appear to be in the process of forming barbells. Using ISFC, preplatelets and barbells can be further distinguished from
platelets by their greater granularity (side scatter (SSC)), labelling with the immature platelet markers thiazole orange (TO), HLA-1, and mitotracker which detects platelets containing greater numbers of mitochondria (Figure 2A). Using the ISFC protocol (Supplementary Figure 3), the mean number of preplatelets and barbells in whole blood was 3.3±1.6% and 0.5±0.27% (n=12) of the platelet population, respectively, correlating with the %IPF (Figure 2B). These data suggest that preplatelets and barbells are immature.

**Preplatelets and barbells in acquired thrombocytopenia**

Preplatelets and barbells were quantified by ISFC within ITP whole blood (n = 7) relative to controls (n=12). ITP is characterised by low platelet counts (mean 46.5±36.9 x 10^9/L versus 250.9±52.5 x 10^9/L), a higher MPV (mean 12.3±0.8 fl versus 9.7±0.8 fl) and higher percentage of immature platelets (IPF mean 20.5±12.7% versus 4.1±1.7%; Supplementary Figure 4). The percentage of preplatelets (10.5±4.7%) and barbells (1.8±1.6%) in ITP was significantly (p<0.05) greater than controls (3.2±1.5% and 0.46±0.21%, respectively), and correlated with the increase in the %IPF (Figure 3A). While preplatelets and barbells in ITP show similar labelling for tubulin (Figure 3B) they were significantly (p< 0.05) larger than in controls (~10% increase, respectively; Figure 3B and C). Sex had no effect on the above measurements but an increase sample size is needed to definitively show this (Supplementary Table 1).

Chemotherapy (in the context of autologous stem cell transplantation) mediates bone marrow ablation and terminates platelet production and can therefore be used to verify whether preplatelets and barbells are part of the newly formed IPF. Preplatelets and barbells were quantified within whole blood from patients (n=6) with lymphoma or myeloma prior to and post chemotherapy. The patients’ platelet count and %IPF prior to
bone marrow ablation were $231 \pm 27 \times 10^3 / \mu L$ and $3.2 \pm 0.74\%$, respectively. Preplatelets and barbell levels were $3.8 \pm 1.3\%$, and $0.45 \pm 0.04\%$, respectively (Figure 3D). Post-chemotherapy, the platelet counts were significantly reduced ($35.0\pm35.5 \times 10^3 / \mu L$), but surprisingly the proportion of IPF and preplatelets was not significantly different ($2.0\pm1.1\%$ and $3.3\pm1.3\%$, respectively). In contrast, barbells were rarely detected ($0.08\% \pm 0.06$; Figure 3D) suggesting they are formed from immature platelets which will be absent after bone marrow ablation. Large platelets (>3μm in diameter) must also therefore represent mature platelets and are a heterogeneous population of different ages including immature preplatelets.

**Preplatelets convert into barbells that can undergo fission**

Previous studies report that preplatelet and barbell transformation is promoted when incubating washed platelets under cell culture conditions within serum free media at 37°C. To study barbell formation *in vitro* by ISFC, human control citrate blood and washed platelets were incubated at 37°C in serum-free M199 media for up to 24h. Barbell formation significantly increased approximately 4x in washed platelets compared to whole blood up to 3h and perimeter measurements and tubulin labelling confirm they originate from platelets >3μm in diameter and share similar morphology with human barbells (Figure 4A and Supplementary Figure 1-3). Figure 4B demonstrates barbells can be detected 1.5-6h but are completely absent by 24h. There was an inverse relationship between decreasing numbers of preplatelets with increasing barbells over time (Figure 4B). Dual labelling of washed platelets with cytosolic CellTrace™ green or red cytosolic dyes confirm that barbells originate from single large platelets (Figure 4C).

To further track the fate of preplatelets and barbells, washed platelets were labelled with CellTrace™ and incubated as above. Baseline measurements confirm that 100% of platelets
were positive for CellTrace<sup>TM</sup>. After 24h, ~10% of platelets appeared with significantly reduced CellTrace<sup>TM</sup> labelling (Figure 5A), a phenomenon unaffected by TRAP-induced granulation (Supplementary Figure 5). Interrogation of CellTrace<sup>TM</sup> MFI suggested preplatelets may have undergone at least two rounds of fission to increase the platelet count. Newly formed platelets not only exhibited higher Mitotracker labelling (Figure 5B) but the mitochondrial electron transport chain inhibitor rotenone (3µg/mL) inhibited barbell formation, suggesting mitochondrial function and energy is important. Furthermore, barbell formation was also inhibited by nocodazole and cytochalasin D (Figure 5C). Preplatelets are therefore capable of undergoing fission, which is dependent on cytoskeletal remodelling and mitochondrial respiration.

**Preplatelets and barbells in murine blood**

To confirm that preplatelets and barbells are not exclusive to human blood, we performed measurements in mouse blood and anti-platelet antibody-treated blood as a model of ITP. We used in vivo biotinylation labelling to conclusively confirm whether preplatelets and barbells represent newly formed immature platelets. Using a similar ISFC protocol (Supplementary Figure 6A), preplatelets and barbells (with similar tubulin cytoskeletal morphology to human) were also detected in murine blood under the same conditions (Supplementary Figure 6B-D) such as temperature and anticoagulant as humans (1.9%±0.18 and 1.7%±0.48, respectively) and correlate with immature RP levels (7%±2.1; Figures 6A and 6B). WT mice were treated with anti-GPIbα to induce severe thrombocytopenia and the time course of recovery of platelets was monitored, to investigate the temporal relationship between preplatelets, barbells and platelets. Figure 6C shows the kinetics of the platelet count with a nadir at day 1, increasing to 288 x 10<sup>3</sup>/µL at day 5 and near normal by day 7. At
day 5, the percentage of RP and MPV increased by 76.5% and 32.5%, respectively, confirming the platelet population consisted predominantly of immature platelets (Figure 6C). Preplatelets and barbells were not only larger than baseline (Supplementary Figure 7) but also significantly increased in number and correlated with the fraction of RP at days 0 and 5 (Figure 6D). To conclusively confirm whether preplatelets and barbells are immature newly formed platelets, mice were intravenously injected twice with NHS biotin to ensure that 100% of circulating platelets were labelled (Figure 6E). At 24h 11.2% ± 5.1 of circulating platelets were shown to be biotin negative and thus represent newly formed immature platelets (Figure 6E). The barbell structures were all biotin negative confirming that they originate from immature cells. In contrast, preplatelet (>3 μm in diameter) and other circulating platelets (<3 μm in diameter) were positive for biotin (Figure 6E-F). Moreover, the biotin-negative platelets were not exclusively large, confirming their heterogeneity (Figure 6F).
Discussion

Using ISFC, we have designed and optimised a high throughput image-based approach to not only accurately quantify preplatelets and barbells in whole blood but to further study the dynamics and regulation of platelet fission.\textsuperscript{18,24} Using this method we have demonstrated for the first time that preplatelets/barbells are analogous to RP (Figure 2A). Furthermore, quantification of preplatelets/barbells were shown to be equivalent to RP/IPF measurements (Figure 2B) and the rate of platelet production in thrombocytopenia (ITP) associated with enhanced peripheral destruction and turnover of platelets (Figure 3A) but virtually absent in patients at the nadir of the platelet count following chemotherapy-induced BM ablation (Figure 3D). Also, preplatelet maturation occurred in vitro and increased the platelet count (Figures 4B & 5A) thus supporting why more barbells were observed in ITP blood compared to healthy and chemotherapy patients. As platelet size in ITP is dependent on MK ploidy,\textsuperscript{28} and is therefore a good indicator of platelet turnover in hyper-destructive disorders,\textsuperscript{29} it was unsurprising preplatelets also positively correlated with the rate of thrombopoiesis in ITP. However, both the IPF and percentage preplatelets were unchanged at the nadir of platelet counts following chemotherapy (Figure 3D). As IPF and
MPV have been shown to be a poor discriminator of BM failure patients,\textsuperscript{11,30} this supports the consensus that under some conditions platelet size and age are independent.\textsuperscript{31-33}

In vivo biotinylation of platelets, has definitively demonstrated that RP are newly formed platelets.\textsuperscript{3,34,35} Following in vivo biotinylation experiments in WT mice in our study, interrogation of biotin negative and positive platelet images (by ISFC) at 24h showed immature and mature platelets consisted of both platelets <3\( \mu \text{m} \) in diameter and large preplatelets >3\( \mu \text{m} \) (Figure 6E & F) thus, confirming that not all large platelets are therefore immature. In contrast, it was quite striking that all barbell platelets were exclusively biotin negative at 24h, confirming they originate from newly formed immature preplatelets. However, these findings were limited by the 24h biotinylation time point. As thousands of platelets are formed per second (in mice),\textsuperscript{36} measurements at earlier time points (< 24h) would have clarified the exact timing of their appearance and kinetics within the circulation.

Based on our findings (Figure 4 & 5) and others,\textsuperscript{18,22} preplatelet maturation may occur to regulate physiological platelet counts. Furthermore, taking into consideration our barbell estimates in human and mouse whole blood (0.5% and 1.7% respectively; Figure 2B & 6A), the total number of circulating platelets and blood volume, and differences in the rate of turnover across species,\textsuperscript{36,37} barbells would need to divide approximately every 2.4 or 4.1 hours respectively to maintain normal counts. Although our human and mouse data are in the same order of magnitude and seem feasible, these estimates not only assume that each preplatelet produces 2 daughter platelets, but may also not consider highly transient maturation events and those which have already occurred within highly turbulent environments e.g. lung capillary beds.\textsuperscript{20,38}
Intriguingly, platelet progeny following incubation consisted of hyperpolarised mitochondria and inhibiting complex I of the mitochondrial respiratory chain with rotenone prevented barbell formation from occurring in vitro (Figure 5B). A mitochondrial hyperfused, polarised state has been reported in other cell types during the G1/S phase transition.\textsuperscript{39} Also, platelets exhibit the capacity to translate mRNA into protein.\textsuperscript{40} In consideration of this, it is possible during preplatelet maturation, mitochondria become fused and hyperpolarised to increase ATP production to potentially support proteome replication. In addition, mitochondrial health is essential for platelet lifespan.\textsuperscript{41,42} Despite this, off target effects of rotenone could also have prevented barbell formation via inhibition of microtubule assembly.\textsuperscript{43} However, it is also possible that preplatelets employ dynein-driven microtubule sliding and not assembly when converting into barbells.\textsuperscript{44,45} Further investigation will be required to determine the importance of mitochondria in driving platelet fission/maturation.

The current clinical use of measuring immature platelets is hindered by non-specific labelling of nucleic acid dyes and disease context.\textsuperscript{5,11,46} As measuring barbell platelets is based on morphometric evaluation, it has potential to overcome such limitations and could be an invaluable, non-invasive, rapid tool to distinguish peripheral thrombocytopenia from dysfunctional platelet production,\textsuperscript{8} accurately defer unnecessary prophylactic platelet transfusions\textsuperscript{47} and/or be used as a biomarker/risk factor in antiplatelet therapy and cardiovascular disease, respectively.\textsuperscript{48,49} Previous work by Thon and colleagues shows, preplatelet/barbell conversion is dependent on cortical microtubule bundle thickness and diameter.\textsuperscript{24} Therefore, it is likely platelet fission is inhibited in giant platelet disorders (e.g.MYH9-RD) where platelets have up to a 20-fold increase in the number of peripheral microtubule coils.\textsuperscript{50} Although there are many challenges to consider when implementing specialized instruments such as the ISFC into a clinical setting, digital morphological analysis
of blood films is feasible as demonstrated with a haematology analyser (i.e. Cobas m511, Bloodhound® technology; Roche, Basel, Switzerland). However, these approaches may lack the sensitivity to accurately quantify all barbell platelets within 2D blood films. ISFC technology could also still be usable within a clinical setting by designing a machine learning approach based upon brightfield imaging. Thus a pre-designed imaged-based counting method might be possible using appropriately fixed, unlabelled blood samples. Furthermore, continuing instrument evolution with increased computing power could eventually result in a haematology analyser that performs ISFC analysis of all blood cells.

Recently we have begun to successfully unravel the final stages of platelet formation which occur in the bloodstream. Using such critical information, we demonstrate preplatelet or immature platelet maturation occurs in a subpopulation of large platelets and the rate in which this takes place signifies the rate of platelet production and BM activity in health and disease. This may develop into a new and improved approach to perhaps overcome the limitations of current immature platelet measurements (see model in Figure 7). We propose quantification of preplatelet-derived barbells could be an invaluable tool for diagnosing and managing thrombocytopenia. With the use of appropriate temperature regulated anticoagulants, fixatives and labels with incorporation of image-based analyses into future haematology analysers, this may offer a unique and more affordable automated approach than current ISFC to measure preplatelet-derived barbell structures in whole blood.
Acknowledgements

This project was funded by a British Heart Foundation 3-year PhD studentship grant (FS/17/29/32828) and supported by a BHF Accelerator Award (AA/18/2/34218) and by the CRN (Clinical Research Network West Midlands). We thank Dr. Jun Mori and Dr. Timo Voegtle for their advice and technical help with the mice studies. We also thank Dr. Charles Percy, Dr. Hayder Hussein, Dr. Will Lester, Dr. Suzanne Morton and Beth Lovell for their clinical input and help with patient recruitment. The authors would like to acknowledge the Imaging Suite at the University of Birmingham for support of imaging experiments. Imaging facilities used in this project were funded by the University of Birmingham, COMPARE and the BHF.

Authorship Contributions

SK and AD generated the data and wrote the paper. PH conceived, funded the project and wrote the paper. GL and PN provided clinical input and clinical samples for the study and
edited the paper. ST and SPW provided advice and edited the paper. YS provided mice
edited the paper and contributed to funding.

Disclosure of Conflicts of Interest

There are no relevant conflicts of interest
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Figure Legends

Figure 1: Imaging of Platelets, preplatelets and barbell platelets in normal human blood

A) Healthy control trisodium citrate blood was labelled immediately after phlebotomy with FITC anti-CD61 and AF674 SiR tubulin (4μM) for 30 minutes at 37°C and 10,000 CD61 positive platelet images were acquired using ISFC. SiR tubulin labelling clearly depicts the marginal band of (i) platelets, (ii) preplatelets and (iii) barbell platelets discriminated by ISFC (x60). Images are representative of a single experiment (n=5). Scale bar = 7μm. B) PRP was separated form citrated whole blood immediately after phlebotomy at 37°C and was labelled for α-tubulin and imaged using super-resolution SIM microscopy (x100 magnification, n=3). Circular platelets (≤3μm; green arrows) and preplatelets (≥3μm; yellow arrows) are shown along with barbell platelets (blue arrows). C) Circular platelets are shown along with Intermediate elongated preplatelets (purple arrows). Scale bars= 5μm.

Figure 2: Quantification of reticulated platelets, preplatelets and barbells in human whole blood.

Healthy control trisodium citrate whole blood was labelled immediately after phlebotomy with anti-CD42b BV421 and thiazole orange (TO), mitotracker AF599 or anti-HLA APC (n=5; scale bar 7μm) and granularity was determined by side scattered light SSC. Representative images of preplatelets and barbells with each label are shown (A). MFI was normalised to cellular perimeter. (B) Under the same conditions, citrate blood was labelled anti-CD61 FITC and CD62p BV421 for 15 min at 37°C and percentages of immature platelets (IPF) was measured by Sysmex XN1000 and, preplatelets and barbells were quantified by ISFC and correlated with IPF (n=12). (A) Platelets were then labelled IPF: immature platelet fraction. (A & B) One-way anova with Dunnett’s multiple comparisons test; (B) Pearson’s correlation coefficient. Sig. * <0.05, ** <0.01, *** <0.001, ****<0.0001. +/- 1 SD.

Figure 3: Quantifying reticulated platelets, preplatelets and barbell proplatelets in acquired thrombocytopenia

All measurements were performed in trisodium citrate anticoagulated whole blood. (A) Healthy control (HC; green dots: n=12) and immune thrombocytopenia (ITP; red dots: n=7)
blood was incubated at 37°C for 1.5h and platelet count, MPV and IPF was measured by the XN1000 haematology analyser (Sysmex) and preplatelets and barbells by ISFC with correlations between % reticulated platelets (IPF%) and preplatelets and/or barbells. (B) α-tubulin immunofluorescence imaging of control and ITP PRP displaying preplatelets (yellow arrows), barbells (blue arrows) and elongated preplatelets (purple arrows; n=3; Leica DM6000 widefield microscope, x60 lens, scale bar 5μm). (C) Mean diameter of preplatelets and area of barbell platelets measured by ISFC comparing healthy controls (n=15) with ITP (n= 8; scale bar = 7μm). (D) Blood samples were taken from patients with high grade lymphoma prior to chemotherapy (baseline; day -1), 5-7 days post stem cell autograft (nadir). Platelet counts and IPF were measured by the XN 1000 analyser and preplatelets and barbells by ISFC (n=6). (A, B & D) Unpaired t-test; (A) Pearson’s correlation coefficient. Sig. * <0.05, ** <0.01, *** <0.001; **** <0.0001. +/- 1 SD.

Figure 4: Kinetics of preplatelet maturation.

(A) Whole blood and washed platelets anticoagulated with trisodium citrate was incubated for 3h at 37°C to determine change in barbell formation quantified by ISFC at 0, 1.5 & 3h and labelled with FITC anti-CD61 and AF674 SiR tubulin (4μM; n=6). Washed platelet barbell perimeter measured by ISFC (n=6). SiR tubulin live cell labelling of washed platelet barbells (n=3, scale bar 7μm). (B & C) Experiments were conducted with washed platelets from human control citrate blood incubated in M199 media at 37°C for a maximum of 24h. (B) Barbell platelet formation at 0, 1.5, 3, 6 and 24h time points (visualised using the image flow cytometry barbell gate described in Supplementary Figure 3). Quantification of preplatelets and barbells at 0, 1.5, 3, 6 and 24h time points (n=10). (C) Prior to incubating, washed platelets were labelled with either CellTrace™ green (0.2μg/mL) or red (1μg/mL) cytosolic dyes, mixed and incubated for 3h to demonstrate barbells originate from single platelets (n=3, imaged by ISFC, x60 lens, scale bar = 7μm). A*Maj-Ax-Int: Area*Major Axis Intensity; Min-Ax-Int: Minor Axis Intensity. (A -B) Two-way Anova with Bonferroni multiple comparisons test. Sig *<0.05, **<0.01, ***<0.001. +/- 1 SD.
**Figure 5: Mapping preplatelet maturation in vitro**

Washed platelets were labelled with 2μg/mL of a CellTrace™ yellow cytosolic dye and incubated in serum-free M199 media for 24h. (A) Scatter plots demonstrate the appearance of a discrete population of platelets termed “Progeny” which display a decrease in CellTrace™ yellow mean fluorescence intensity (MFI; images depicted by CD61 and CellTrace™ fluorescence using ISFC, x60 lens, scale bars = 7μm). Percentage increase in platelet count, CellTrace™ fluorescence profile at 0 and 24h is also demonstrated. (B) AF599 Mitotracker Ros CMX MFI of platelets and platelet progeny with representative images (scale bar 7μm) and the effect of rotenone (3μM) on barbell formation when incubating washed platelets for 6h. (C) Washed platelets were incubated for 6h with nocodazole (10μM) or cytochalasin D (1 or 0.1μM) and barbells were quantified by ISFC. To show the effect of either cytoskeletal drug, marginal band morphology was depicted using AF674 SiR tubulin live cell labelling (n=3). (A) paired t-test; (B) Mann-Whitney U test and Wilcoxon test; (C) two-way anova. Sig. ***=<0.001; **<0.01 & *<0.05. +/- 1 SD.

**Figure 6: Preplatelets are newly formed immature reticulated platelets**

(A) Trisodium citrate whole blood from wild type mice was incubated for 1.5h at 37°C and percentages of reticulated platelets (RP) by flow cytometry, preplatelets and barbells by ISFC using anti-CD61-FITC were quantified. (B) Preplatelets and/or barbells correlated with RP (n=6). (C) Mice (n=5) were treated with a GPIbα polyclonal antibody (1.5μg/mL) and platelet counts and MPV were measured by ABX Pentra 60 (Horiba) haematology analyser and RP by flow cytometry using thiazole orange labelling on day 0, 1, 5 or 7. Also, preplatelets and barbells were quantified by ISFC using FITC CD41 at day 0 (prior to platelet depletion) and day 5 (platelet engraftment) and (D) correlated with reticulated platelets (RP). (Mice (n=4) were injected twice intravenously with NHS biotin (4mg/mL) to label all circulating blood cells with biotin, bled 0 and 24h later and labelled with APC anti-CD41 and FITC conjugated streptavidin. (E) 100% of circulating platelets were verified as biotin positive at baseline and newly formed platelets at 24h post biotinylation were determined as biotin negative. Representative ISFC images show biotin positive and negative platelet morphology (x60 lens, scale bars = 7μm). Biotin mean fluorescence intensity of platelets, preplatelets and barbells was also measured by ISFC at 24h. (F) ISFC to determine the size distribution of...
biotin negative (immature) and positive (mature) platelets 24h post injection (representative images are depicted with brightfield ISFC imaging, x60 lens, scale bars = 7µm). (A & E) one-way anova with Tukey test; (B & D) Pearson’s correlation coefficient; (C) unpaired t-test. Sig. ** <0.01; ***<0.001; ****<0.0001. +/- 1 SD.

Figure 7: Model of Platelet Maturation in the Bloodstream

In (A) circulating platelets are heterogeneous in size and age. Immature platelets >3 µm in diameter are termed preplatelets (determined by a green cytoplasm). These platelet progenitor cells mature by continuously transforming into barbell platelets and undergoing fission into two smaller platelets until reaching a size threshold of <3 µm in diameter. Under steady state production, not all large platelets are immature. Unlike preplatelets, these lack the capacity to undergo maturation. Therefore, mature platelets consist of small and large platelets (determined by a grey cytoplasm). (B) Barbell platelets originate from immature preplatelets consisting of a greater nucleic acid content, granule content, number of mitochondria and HLA I expression compared to mature small platelets. In contrast, mature large platelets contain similar numbers of granules and mitochondria which are non-specifically labelled with dyes used for measuring IPF and RP. Large mature platelet express slightly less HLA I than immature preplatelets which could potential separate preplatelets from mature large platelets.
