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

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Article Summary

- A high-throughput platelet spreading assay was developed to assess patients with a suspected platelet function disorder and bleeding
- We identified spreading defects in numerous patients with normal lumiaggregometry, highlighting a role for this assay in patient phenotyping

Bleeding disorders are an extremely heterogeneous group of conditions presenting a unique diagnostic challenge. While the gold-standard platelet function assay is light transmission aggregometry (LTA), platelet defects are only detected in approximately 50% of patients with a clinical history of bleeding consistent with a platelet disorder. We tested a cohort of patients recruited to the UK-GAPP (Genotyping and Phenotyping of Platelets) study with a suspected platelet function disorder (PFD) using a high-throughput platelet spreading assay. We detected a platelet spreading defect in 32 out of 55 patients tested (58%), and of these, 16 presented with normal lumiaggregometry results despite a significant Bleeding Assessment Tool (BAT) score. Furthermore, a family identified through this approach was subsequently identified as carrying a rare genetic variant of TUBB1, a gene linked to macrothrombocytopenia. This work suggests that morphological defects detected through a high-content platelet spreading approach can identify platelet dysfunctions not detected by lumiaggregometry.

Platelet function disorders are a heterogeneous group of conditions whose clinical and laboratory diagnosis is complicated by the range of reported bleeding symptoms, as well as functional redundancy within platelet signalling pathways. To address this, patients with suspected inherited bleeding are often recruited for platelet function tests (PFTs) following initial assessment.[1] The main consideration when designing a panel of PFTs is the best use of limited sample quantity, yet despite the multitude of PFTs available, a platelet defect is not always detected in patients with a significant bleeding history. Indeed, in patients with patterns of bleeding consistent with platelet disorders, our work, and others', only finds a platelet defect in approximately 50% of patients. To address the limitations of current approaches, the GAPP study performed a range of additional PFTs including impedance aggregometry (Multiplate®), 96 well platelet aggregation, aggregation on collagen at arterial shear rates (1000 s¯1) and clot retraction assays on a subset of patients.[2, 3] Unfortunately this use of multiple PFTs has not increased the number of patients identified with a PFD. Here we investigate a high-throughput analysis of platelets spread on fibrinogen to interrogate morphological and platelet defects.

Full methods are given in supplementary information. Briefly, patients with bleeding of unknown cause were recruited to the GAPP study.[4] Peripheral blood collected from patients and healthy volunteers was used to generate Platelet Rich Plasma (PRP) for lumiaggregometry and washed platelets at $2x10^7$/ml for spreading on fibrinogen-coated coverslips. Spreading samples were incubated for 45 mins before fixation, immunostaining and fluorescence microscopy. Captured images were subjected to a semi-automated image analysis pipeline using the open source software platforms KNIME and ilastik. This produced platelet segmentations and corresponding measurements for area and circularity.[5, 6]

Patients were recruited with a variety of bleeding symptoms consistent with a platelet defect (n=55). The ISTH-BAT was used to provide a quantitative evaluation of the patients' bleeding history; scores were available for 40 individuals with the exception of paediatric patients. The overall mean BAT score was 9.825 (range 2-23), where scores of ≥4 are considered to be indicative of excessive bleeding.[7]

Lumiaggregometry was carried out as previously described.[8, 9] On the basis of this, the platelet defect in the cohort was split into six categories; cyclo-oxygenase (COX) deficiency (n=4), Gi receptor signalling defect (n=4), secretion defect (n=8), thrombocytopenia (n=8), multiple defects (n=5) and no defects (n=26) (Table 1). Upon recruitment, platelet samples from patients were also subjected to spreading on fibrinogen. Samples were fixed and stained with phalloidin to simply and accurately delineate cell morphology with the high signal/noise ratio required for robust high throughput imaging and analysis. A large scale data set was generated by taking 6 fields of view from a representative area of each slide. To establish a control data set, platelets from 5 healthy volunteers were treated identically. Images generated from these assays were subjected to a semi-automated segmentation workflow to measure area and circularity for each platelet, which were plotted with the median of the data set (Figure 1). Area and circularity measurements from 5 representative healthy controls were used to define a normal range, patient data which lay outside of this was defined as potentially defective.

Overall abnormal spreading morphologies were found in 32 of 55 patients tested (58%). This consisted of patients showing defects in platelet area only (n=6), platelet circularity only (n=13), both area and circularity (n=13) or no defect (n=23) (Figure 1). Abnormal area and circularity are consistent with round, unspread platelets, and such double positive hits were considered robustly indicative of morphological spreading defects. Once identified as displaying a potential platelet spreading defect, samples were correlated to existing PFTs and clinical data to establish whether spreading abnormalities predicted platelet dysfunction.

In total, spreading defects were found in 9 patients who also had an aggregation defect (Gi=3, secretion=5, COX=1). Interestingly 16 of the 32 patients (50%) who were positive for spreading defects did not demonstrate a functional abnormality through lumiaggregometry, suggesting additional platelet defects have been identified through this spreading assay (Figure 2). Furthermore, these 16 patients had a significant BAT score (median BAT score = 11). Representative immunofluorescence and segmentation images for either area (patients 2 and 4), circularity (patients 23 and 24.1) or both defects (Patients 41.1 and 41.2) are shown in Figure 2C. Of the 10 double positive platelet spreading hits with an available ISTH-BAT score, 8 patients (80%) demonstrated a significant bleeding score (median BAT= 11). Together, this data confirms that high-throughput spreading analysis can supplement data generated by lumiaggregometry, by both confirming aggregation defects and highlighting morphological abnormalities not detected by the gold-standard approach in patients with excessive bleeding.

The GAPP study aims to identify the phenotype and genetic causes of bleeding. A key question is whether platelet spreading can identify patients with a genetic defect in cases where aggregometry is normal. In this patient cohort whole exome sequencing analysis was applied using an established bioinformatic pipeline.[10] In one such patient a heterozygous TUBB1 missense variant (p.Arg359Trp) was identified (patient 41.2) but not in their sibling 41.1. This genetic defect is also in keeping with the reduced platelet count only observed in patient 41.2 (107 x10^9/L) and not their sibling (221 x10^9/L). TUBB1 defects are known to cause macrothrombocytopenia[11, 12, 13, 14] and the identification of this variant through platelet spreading analysis supports the efficacy of this tool in the study of suspected PFDs. Interestingly, this finding is consistent with previous reports showing a minimal effect of cytoskeletal inhibition on platelet aggregation. This suggests that the absence of a defect in aggregregometry in TUBB1-defective patients is likely due to a minor effect which overlaps with controls. In this instance, platelet spreading effectively detects a defect classically masked in aggregation experiments.[15, 16, 17]

This study systematically correlated high-throughput platelet spreading analysis with clinical and lumiaggregometry data to determine its utility in the investigation of PFDs. We find that patients positive for both circularity and area abnormalities, consistent with the morphology of abnormally small and round platelets, are both positive and negative for aggregation defects. 80% of patients with a spreading defect, but negative for an aggregometry defect report a significant, bleeding score. Furthermore, within this cohort we identified TUBB1 as the causative genetic variant in an individual with thrombocytopenia.

Of particular interest is that a large number of patients positive for platelet spreading defects and with a history of excessive bleeding are negative for an aggregation defect, suggesting that applying platelet spreading as part of the platelet testing panel can reveal morphological defects which would otherwise be missed by lumiaggregometry alone. Furthermore this platelet spreading assay is not dependent upon platelet count, as is the case with aggregation-based PFTs[8, 9] and could therefore be a useful tool to test for platelet function defects in patients with thrombocytopenia. Our approach validates the use of high-throughput platelet spreading analysis as an analytical tool to improve and augment existing PFTs. As high-content imaging approaches become increasingly accessible and commonplace, applying high-throughput platelet spreading strategies is likely to be an invaluable addition to the current arsenal of PFTs.

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Author Contributions. AOK, GCL, SPW and NVM designed the research. GCL provided patient samples and clinical data. GCL and NVM undertook the research governance of the study. AOK, AM, PLRN, RAG, SGT, JAP and NVM performed the research and analyzed data. NVM and AOK wrote the paper and all authors critically reviewed and edited the paper.

Declaration of Interest. The authors report no conflicts of interest.

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Table 1. Phenotypes of all patients recruited to the GAPP study that were included in platelet spreading investigations. Families with multiple affected individuals were labelled .1 and .2 etc. F – Female, M – Male, NA – not applicable e.g. for children. ^Mean platelet counts are shown, normal reference range is 150-400 x 10^9 /L, thrombocytopenia is defined as platelet count <150 x10^9 /L; $Mean platelet volume (MPV) is shown, normal reference range is (7.83-10.5 fL);*ATP secreted in response to 100 μM PAR-1 receptor specific peptide SFLLRN, 5th centile in healthy volunteers is 0.65 nmol / 1x10^8 platelets; Lumiaggregometry was used to simultaneously test platelet aggregation and secretion and platelet defects were categorised as cyclo-oxygenase deficiency (COX), Gi receptor signalling defect (Gi), Gq receptor signalling defect (Gq) secretion defect, thrombocytopenia and no defect; ΨISTH-BAT score), 95th percentile (score of 4) calculated from healthy volunteers;[7] Code for bleeding symptom: 1 - Epistaxis, 2 - Cutaneous bleeding, 3 – Petechiae, 4 – Oral cavity bleeding, 5 – Bleeding from minor wounds, 6 – bleeding after tooth extraction, 7- Bleeding after surgery, 8 – Hematuria, 9 – GI bleeding, 10 – Ovulation bleeding, 11 – Menorrhagia, 12 – Post-partum haemorrhage, 13 – Muscle haematomas.

Individual/ Family	Age	Gender	Platelet count (x 10^9/L)^	Mean Platelet Volume (fL)$	ATP Secretion (nmol ATP/1 x 10^8 platelets)*	Platelet defect based on lumiaggregometry findings	BAT score ψ	Bleeding phenotype
1	78	M	91		0.45	Thrombocytopenia	2	2, 4
2	20	F	182	12.5	2.35	Gi	5	4, 5, 11
3	72	M	113	10.2	1.05	Thrombocytopenia	13	1, 7, 8, 9
4	80	F	204	11.6	0.74	No defect	9	1, 2, 4, 5
5	6	F	230	10.3	0.39	Secretion	NA	1, 2
6.1	7	F	287	8.8	1.01	No defect	NA	
6.2	73	F	150	10.5	0.5	Secretion		
7	6	M	104	8.9	N+	Thrombocytopenia	NA	2, 3
8.1		F	103	15.0	0.21	Thrombocytopenia/Gq	8	1, 7, 9
8.2	35	F	107	15.1	0.26	Thrombocytopenia/Gq	4	1, 9
9	77	F	417	10.1	1.72	No defect	23	1, 2, 4, 6, 8, 11, 12
10	45	F	375	10	1.22	Gi	8	4, 5, 6, 7
11	54	F	239	11.9	0.74	No defect	16	2, 7, 8, 10, 11
12	49	F	289	11.5	1.3	No defect	6	4, 5, 11
13		F	270	12	1.11	No defect	7	2, 4, 8, 9, 11
14		F	352	10.5	1.27	No defect	15	4, 5, 7, 9, 11
15	12	F	355	8.3	0.75	No defect	NA	
16	50	M	270	11.4	1.39	Gi		1
17	22	F	323	11.5	0.66	No defect	3	1, 5, 6
18	19	F	269	11.9	0.73	No defect	16	1, 2, 4, 5, 7, 9, 11
19	26	F	295	10.8	0.89	No defect	3	1, 5
20	29	F	225	12.1	0.55	No defect	10	2, 4, 5, 10, 11
21	32	F	122		1.47	Thrombocytopenia/Gi	10	2, 4, 11, 12
22	49	F	195	10.4	0.63	No defect	14	2, 4, 5, 7, 11, 12
23	38	F	101	12.1		Thrombocytopenia		1, 2
24.1	23	M	428	9.1	0.39	Secretion	10	1, 4, 5, 7, 9
24.2	20	F	345	10.2	0.59	COX	12	2, 4, 5, 6, 11
24.3	17	M	329	10.9	0.25	Secretion	3	2, 5
25	76	M	120	14.6	0.97	Thrombocytopenia + COX	3	
26	43	F	331	10	0.56	Gi	14	1, 2, 4, 5, 11, 12
27	29	F	225	12.1	0.55	No Defect	10	2, 4, 5, 11, 12
28	18	F	235	11	0.97	No defect	11	1, 2, 4, 5, 10, 11
29	53	F	298	11.1	0.72	No defect	13	2, 4, 7, 9, 11
30	32	F	292	9.7	0.61	COX		1, 2, 3, 7, 8, 12
31	40	F	232	10.2	0.77	No defect	6	2, 7, 11
32		F	371	10.5	0.86	No defect	5	4, 5, 10, 11
33		F	281	11.3	1.04	No defect	12	2, 4, 5, 6, 8, 11
34	7	F	170	10.5	0.55	Secretion	NA	
35	68	F	43		0.34	Thrombocytopenia		
36.1	30	M	59	13		Thrombocytopenia	8	1, 4, 5, 7
36.2	52	M	183	12	0.66	No defect	2	5, 7
37	6	M	140	10.1	0.53	Thrombocytopenia/ secretion	NA	2, 3
38	36	F	221	11.7	1.13	No defect	13	1, 2, 4, 11, 12

39	57	F	261	9.3	0.82	COX	12	1, 5, 6, 11
40	22	F	291	11	1.03	No defect	13	2, 4, 8, 11
41.1	41	F	221	13.9		Secretion	NA	
41.2	66	M	107	large		Thrombocytopenia	NA	
42	57	F				Thrombocytopenia	NA	
43	52	F			1.49	No defect	16	1, 4, 5, 11, 12
44	20	F				No defect		2, 5, 9
45	54	F	238	10.9	0.92	No defect	7	2, 4, 5, 11, 12
46	27	F	256	11.1	0.75	No defect	9	1, 2, 5, 6, 7, 11
47	60	F	184	12.2	0.46	COX	21	1, 2, 4, 5, 7, 9, 10, 11, 13
48	23	F	204	12.4	0.59	Secretion	8	1, 2, 5, 10, 11
49	72	F	270	10.1	0.57	Secretion	13	1, 2, 6, 11

Figures

Fig. 1. High-throughput measurement of platelet area and circularity. Area and circularity measurements were generated using a semi-automated image analysis workflow and abnormalities were identified by comparison to a control range established by spreading data from healthy volunteers (red box) (n=5). Patient samples with medians and confidence intervals outside of the control range were classified as positive hits (blue boxes) for either a defect in platelet area or circularity, while samples which fall outside both normal ranges were classified as double positives (orange boxes).

Fig. 2. Distribution of platelet spreading defects associated with lumiaggregometry defects. Panel A. A heat map showing the presence of a Spreading or Aggregation defect. Each row represents an individual patient. Each column depicts a platelet spreading abnormality (A - platelet area spreading defect, B - platelet circularity spreading defect, C - Platelet circularity and area spreading defect) or (D- Thrombocytopenia defect, or lumiaggregometry defect, E - platelet secretion defect, F - COX defect, G - Gi signalling defect or H - multiple aggregation defect). In red: double positive hits. In dark green: single hits for either spreading or aggregation defect. In black: multiple hits within a category. In light green: no hits for either test. Patients with a double positive hit and increased BAT score are highlighted in red font. **Panel B**. A pie chart indicating the distribution of 'double positive' platelet spreading defects'. **Panel C** - Representative platelet spreading immunofluorescent and segmentation images of selected patients with clear defects on circularity or area. Top panel shows a representative control healthy donor, Lower panels; Patients 2 and 4 were found to have a platelet area spreading defect only, patients 23 and 24.1 had a platelet spreading