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HAEMATOLOGY

An integrated approach to inherited platelet disorders: results from a research collaborative, the Sydney Platelet Group

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Summary

Inherited disorders of platelet function (IPFD) and/or number (IPND) are heterogeneous conditions that result in variable mucocutaneous bleeding symptoms as a result of deranged primary haemostasis caused by platelet dysfunction or thrombocytopenia. Diagnosis is important to guide post-operative bleeding prophylactic strategies, to avoid treatment with inappropriate medications, and inform prognosis. Achieving an accurate diagnosis has traditionally been hampered by the requirement of multiple, often complex, laboratory tests that are not always available at single centres. To improve the diagnosis of these disorders a research collaborative was established, the Sydney Platelet Group, that explored an integrated approach combining traditional and contemporary platelet phenotypic and genetic diagnostic platforms available at four Sydney tertiary hospitals. Herein we report the outcomes of the first 50 patients evaluated using this approach. The cohort included 22 individuals with suspected IPFD and 28 with thrombocytopenia. Bleeding scores were higher in individuals with IPFD (mean 5.75; SD 4.83) than those with IPNDs (mean 2.14; SD 2.45). In cases with suspected IPFD, diagnosis to the level of the defective pathway was achieved in 71% and four individuals were found not to have a definitive platelet function defect. Dense granule secretion disorders were the most common platelet pathway abnormality detected (n=5). Mean bleeding scores in these individuals were not significantly different to individuals with defects in other commonly detected platelet pathways (dense granules, signal transduction and 'undetermined'). A molecular diagnosis was achieved in 52% of individuals with IPNDs and 5% with IPFD. Likely pathogenic and pathogenic variants detected included variants associated with extra-haematological complications (*DIAPH1, MYH9*) and potential for malignancy (*ANKRD26* and *RUNX1*). The level of platelet investigation undertaken by this initiative is currently not available elsewhere in Australia and initial results confirm the utility of this integrated phenotypic-genetic approach.

Key words: Platelet dysfunction; thrombocytopenia; diagnosis; next generation sequencing.

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INTRODUCTION

Inherited platelet disorders (IPD) include disorders of platelet function (inherited platelet function disorders; IPFD), as well as quantitative platelet disorders characterised by thrombocytopenia (inherited platelet number disorders; IPND), a proportion of which may also demonstrate qualitative functional abnormalities.[1] Bleeding symptoms associated with IPFDs range from frequent, severe episodes of spontaneous mucocutaneous bleeding and bruising (in the case of Glanzmann's thrombaesthenia) to asymptomatic conditions that may only be associated with an increased risk of bleeding following trauma or surgery. Importantly, the frequency of post-operative bleeding is higher in patients with IPFDs and varies according to the type of disorder.[2] Therefore, confirmation and characterisation of a platelet functional defect is of utmost importance to allow appropriate planning of perioperative strategies. In IPNDs, accurate diagnosis is important to reduce misdiagnosis for conditions such as immune thrombocytopenic purpura (ITP) that may predispose individuals to potentially harmful treatments including

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unnecessary exposure to corticosteroids, immunosuppressants and even splenectomy.[3,4] Moreover, with the advent of thrombopoietin agonists, diagnosis may inform decisions regarding potential for individualised therapeutic approaches[4–6] and in the case of disorders associated with pathogenic variants of *MYH9, RUNX1, ANKRD26* and *ETV6*, provides opportunity for appropriate screening for extra-haematological manifestations and cancer.[7]

In attempts to improve the diagnosis of IPD, international societal guidelines were published encouraging an algorithmic step-wise approach for disorders of platelet function;[8] likewise, with the emergence of genetic testing and the demonstration of its utility for the molecular diagnosis of IPNDs, numerous guidelines and algorithms[9–12] have been proposed incorporating this modality into diagnostic pipelines. Despite these efforts to standardise the approach to diagnosis of IPDs, currently recommended tests remain technically difficult and many lack reproducibility.[13] Hampering endeavours further is the limited number of centres at which the required diagnostic tests are available, many of which, despite being recognised as tertiary level referral centres, only offer single assays, such as light transmission aggregometry with a standard panel of agonists for the evaluation of platelet function.

In order to address the limitations of the current diagnostic capacity available at single centres reliant solely on limited phenotypic testing, a research collaborative was established, the Sydney Platelet Group (SPG), consisting of haematologists and scientists from four tertiary referral centres in Sydney. Through combining new and conventional diagnostic platforms available at each centre, this initiative aimed to provide a level of platelet investigation that is currently not available elsewhere in Australia. Herein we report the results from the first 50 patients referred to the SPG and confirm the utility of an integrated phenotypic-genetic approach.

METHODS

Establishment of an integrated pathway of assessment

A pathway for investigation of platelet disorders that integrated platelet phenotypic and genetic testing was designed by members of the SPG. This included a secure online portal through which patient referrals were received and triaged (https://bit.ly/SydneyPlateletGroup) and the establishment of a specific research clinic, held every fortnight at a single site that served as the entry point into the SPG for the patient. Finally, the team devised a strategy for same day sample collection and transport between the centres required to complete all functional and genetic investigations for these disorders (Fig. 1).

Participant selection

Patients were referred to the SPG by their treating haematologists because of diagnostic uncertainty and/or possible inherited nature of their platelet disorder. Patients were invited to participate in the investigation of inherited platelet function and number disorders study (RESP/16/19) according to the following criteria:

1. The individual had a suspected inherited platelet disorder suggested by abnormal platelet function testing but without a known diagnosis and not attributable to other causes of disordered platelet function such as medication, liver disease or renal disease, OR
2. The individual had thrombocytopenia and a family history of thrombocytopenia, OR
3. The individual had thrombocytopenia and a family bleeding history.

All research was approved by the Northern Sydney Human Research Ethics Committee (HREC/16/HAWKE/28) and all participants gave written informed consent in accordance with the Declaration of Helsinki.

Assessment of bleeding symptoms

Patient demographics (age and gender) and clinical information regarding family history of thrombocytopenia/platelet bleeding disorder, personal bleeding symptoms, previous diagnoses and treatment (including consumption of common medications/known compounds with anti-platelet affects), as well as extra-haematological manifestations (renal impairment, sensorineural deafness and cataracts) were recorded.

The International Society of Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT)[14] was used by study doctors who regularly take bleeding histories from study participants in an attempt to improve the

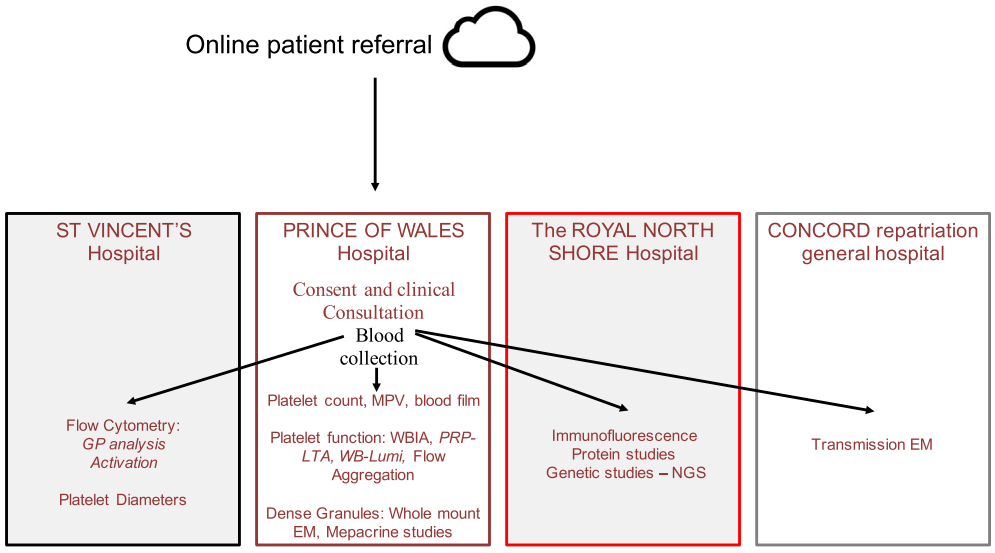

Fig. 1 A multi-site integrated approach to the investigation of inherited platelet disorders. Patient referrals were received via a secure online portal. Patients were then invited to participate in the study and were seen in a designated clinic at a single site. Here patients received informed consent and clinical consultation. Blood was then collected at this site and used for phenotypic testing and genetic studies as indicated. EM, electron microscopy; GP, glycoprotein; LTA, light transmission aggregometry; Lumi, lumi-aggregometry; NGS, next generation sequencing; PRP, platelet rich plasma; WB, whole blood; WBIA, whole blood impedance aggregometry.

collection and reproducibility of the bleeding history. This tool consists of a questionnaire that explores 14 different types of bleeding symptoms. A score of 0–4 is assigned for each symptom based on bleeding events reported by the individual and a combined bleeding score is tallied. A total ISTH-BAT score of ≥3 for children, ≥4 for adult males and ≥6 for adult females is considered abnormal.[15] These cut-offs were used to define abnormal bleeding symptoms in this study.

Platelet morphology

Blood was collected from participants from the antecubital fossa, by clean venipuncture using minimal tourniquet pressure. Blood in potassium-ethylenediaminetetra-acid (EDTA) tubes was used for automated full blood count analysis and peripheral blood film preparation (Sysmex XE-5000; Sysmex, Australia). Digital slides to measure mean platelet diameters (MPDs) were prepared from blood films in cases of thrombocytopenia. Films were fixed and stained (haemotoxylin and eosin), and each slide then scanned using a 40× to 100× objective with a Zeiss Axio Scan.Z1 Slide Scanner (Carl Zeiss Pty Ltd, Australia) or a Leica DM 6000 Power Mosaic (Leica Microsystems Pty Ltd, Australia). Platelet diameters were then calculated using ImageJ software (https://imagej.nih.gov/ij) by measuring the diameter of 200 platelets. Platelet diameters were interpreted using previously published recommendations.[16,17]

Whole mount electron electron microscopy (WMEM) was performed to quantitate platelet dense granules (DG) as previously published.[18] Platelet-rich plasma (PRP) was prepared from blood anti-coagulated with buffered 3.12% tri-sodium citrate within 4 h of sample collection. PRP (~10 μL) was spotted onto Formvar-coated, 200 mesh copper-palladium grids, rapidly blotted, fixed with 0.1% glutaraldehyde in White's saline for 3–5 s, rinsed with distilled water, and air dried. Unstained grids were visualised by electron microscopy (EM) using a JEOL 1400 transmission electron microscope 40-120KeV (JEOL, Japan). The mean DG content of 50 platelets was determined in duplicate and reported as normal or reduced according to local reference range derived on 50 healthy controls.

Platelet pellets prepared from fresh blood collected in citrated tubes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The platelet pellets were then sectioned for transmission electron microscopy (TEM) and viewed under the FEI Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI, USA).

Platelet function

All participants received instructions by mail on avoidance of possible interfering substances (foods, medications, etc., consistent with recommendations from previously published guidelines),[19] and were asked to document current medications that were checked by investigators prior to testing.

Whole blood in 3.12% tri-sodium citrate tubes was collected for assessment of platelet function by whole blood impedance aggregometry (WBIA) using a Multiplate analyser (Roche Diagnostics, Switzerland) that recorded aggregation in response to thrombin receptor activating peptide (TRAP) 32 μM (Sigma-Aldrich, Australia), arachidonic acid (AA) 0.5 mM, ADP 6.0 μM (all from Diapharma Group, USA) and collagen 1 μg/mL (Helena Biosciences, Australia).

Light transmission aggregometry (LTA) was employed using a standardised protocol derived from previously published societal guidelines.[19,20] Citrated PRP was prepared from participants and healthy controls and subjected to a panel of agonists including ADP (1.25, 2.5, 5, 10 μM) (Sigma Aldrich); Horm collagen (0.5, 1, 2, 4 μg/mL) (Takeda, Germany); AA (0.8, 1.6 mM) (Helena Biosciences); epinephrine (2.5, 10 μM) (Sigma-Aldrich) and ristocetin (0.5, 1.2 mg/mL) (Helena Biosciences). Aggregation was measured using the Helena AggRAM (Helena Biosciences) four channel aggregometer. Lumi-aggregometry measured impedence and adenosine triphosphate (ATP) secretion using the luciferin-luciferase reagent (Chronolume; Chrono-Log Corporation, USA) and an ATP standard solution in the Chrono-Log Model 700 2 channel lumi-aggregometer. Citrated whole blood collected from participants and healthy controls was subjected to a panel of agonists including ADP (2.5 and 5 μM, Sigma-Aldrich), Horm collagen (1 and 4 μg/mL, Takeda), AA (0.5 and 1.2 mM, Helena Biosciences), TRAP (2.5 and 10 μM, Sigma-Aldrich) and thrombin (1 U/mL, Sigma-Aldrich). In all cases, results obtained for each participant across each platform (WBIA, LTA and lumi-aggregometry) was compared to a control on the day of testing. Reporting of WBIA was according to manufacturer's range. Reporting of LTA and lumi-aggregation was by experienced staff (DD, MK, TB) and incorporated visual inspection of aggregation curves and reference to previously established laboratory reference range.

In participants with significant thrombocytopenia (platelet count $<80\times10^9$/L), platelet aggregation was assessed by flow cytometry.[21] Here, aggregation was measured in patient whole blood in 3.12% citrate in response to agonists ADP (5 μM) (Sigma-Aldrich); collagen (1 μg/mL), AA (1.6 μM) (both Helena Biosciences) and TRAP (10 μM) (Sigma-Aldrich). For each agonist, 250 μL of citrated whole blood was incubated at 37°C with a stir bar and a baseline was set by sub-sampling 10 μL of whole blood into 30 μL of fixative (fixative as published by Fox *et al.*)[21] before the addition of agonist. Agonists were then added to the sample of whole blood from which subsamples were mixed into fixative at 10 s, 30 s and 60 s (and an additional 120 s for collagen only). Platelet aggregation was measured by the % fall in single phycoerythrin (PE)-labelled anti-CD42a platelets at 10 s, 30 s, 60 s and 5 min using the BD FACS Canto II analyser (BD Biosciences, Australia). Results were reported by experienced staff (DD, MK, TB) in comparison to healthy control.

Flow cytometry

Surface expression of platelet glycoproteins CD9, CD31, CD34, CD41a, CD42a, CD42b, CD61, CD110 and GPVI were measured. All antibodies (BD Biosciences) were conjugated to PE and were performed in separate tubes. Citrate anti-coagulated whole blood (5 μL) was added to flow cytometry tubes containing 2.5–5.0 μL of antibody, with the final volume made up to 50 μL using phosphate buffered saline (PBS). Quantibrite tubes (BD Biosciences) were used to quantify platelet surface expression according to the manufacturer's instructions. Platelet activation was measured by CD42b-PE, CD62p-APC (P-selectin), CD63-PE-Cy7 (LAMP) and PAC-1-FITC (GPIIb/IIIa) on agonist stimulated [TRAP, collagen-related peptide (CRP) and ADP] platelets. Citrate anti-coagulated whole blood (5 μL) was added to flow cytometry tubes containing 2.5–5.0 μL of antibody (or respective isotype controls), 10 μL of agonist or PBS, with the final volume made up to 50 μL using PBS. All samples were incubated for 20 min before the addition of 500 μL of 0.1% paraformaldehyde (ProSciTech, Australia) in 0.9% sodium chloride (Baxter Healthcare, Australia). Flow cytometry was performed using a BD LSRFortessa X-20 flow cytometer (BD Biosciences). Platelets were identified either using forward scatter vs side scatter, or forward scatter vs platelet marker. A minimum of 10,000 platelet events were collected, except in a few cases of thrombocytopenia.

Western blotting

Platelets from PRP were washed three times with Tris-EDTA buffer (10 mM Tris, 150 mM NaCl, 3 mM EDTA, pH 7.4) before lysis in SDS buffer (Tris-EDTA Buffer, 30 mM NEM, 1 mM PMSF, 2% SDS) supplemented with protease inhibitors (Protease Inhibitor Cocktail III; Merck, Australia). Platelet lysates were stored at −20°C until further testing. For western blotting, 50 μg proteins were separated by SDS-PAGE on a 6% gel, and subsequently transferred onto a nitrocellulose membrane. Membranes were blocked with 5% albumin in TBS-T (10 mM Tris, 150 mM NaCl pH 7.4 containing 0.1% Tween-20) and labelled overnight with a mouse monoclonal anti-MYH10 antibody (Santa Cruz Biotechnologies, USA). Secondary antibody labelling was performed with horseradish peroxidise (HRP)-conjugated goat anti-mouse (GAM) (Jackson ImmunoResearch Laboratories, USA) and specific binding was detected using chemiluminescence as previously described.[22]

Non-muscle myosin heavy chain IIA (NMMHCIIA) immunofluorescence staining

Immunofluorescence (IF) staining was performed as previously described.[23] Briefly, peripheral blood films were prepared and air-dried. After permeabilisation in cold acetone for 3 min, cells were blocked with 10% normal fetal bovine serum for 60 min at room temperature. Slides were washed with PBS and incubated overnight at 4°C with a mouse monoclonal antibody against NMMHC-IIA (AB55456; AbCam, Australia) or mice immunoglobulins (negative control). Following incubation, slides were washed and incubated with a secondary AF-488 goat antimouse antibody (Life Technologies, Australia) for 2 h at room temperature. ProLong Diamond Antifade Mountant with DAPI (Life Technologies) was used before examination using a

fluorescence microscope (Nikon Eclipse E800 and DSRi1 camera; Nikon Instruments, USA).

Defining subgroups of major platelet function disorders

Depending on platelet responses to activation and secretion, and following structural analysis by flow cytometry, WMEM, and when relevant, transmission electron microscopy, classification of disorders of platelet function was adapted using previously recommended subgroups/functional characteristics,[19,24,25] and are listed below:

1. Disorders of adhesion: this group included defects of GPVI, defects associated with Bernard–Soulier syndrome (BSS) including abnormalities of GP1BA, GP1BB, GPIX, as well as platelet type von Willebrand disease caused by defects of GP1BA.
2. Disorders of aggregation: abnormalities of integrin αIIbβ3.
3. Disorders of signal transduction: this heterogeneous group included defects of platelet receptors [ADP, thromboxane (TxA2), epinephrine], defects of G-protein activation, as well as other signalling pathways (phosphatidylinositol metabolism, calcium mobilisation and protein phosphorylation).
4. Disorders of arachidonic acid pathways.
5. Dense granule deficiency (storage pool deficiency).
6. Disorders of platelet secretion/release defects.
7. Disorders of alpha granules.
8. Other/undetermined type.

Genomic DNA isolation and next generation sequencing (NGS)

Genomic DNA was isolated from peripheral blood leucocytes. NGS for the first 22 patients was performed employing a custom amplicon of 47 candidate genes that was designed using the TruSeq Custom Amplicon Kit and Illumina Design Studio (Illumina, Australia). NGS libraries were prepared using the Truseq custom amplicon library preparation kit and resequencing was achieved by the MiSeq Illumina sequencer platform. Sanger sequencing was performed across *GP1BA, GP1BB* and *GP9* due to poor coverage over these targets.

An updated capture panel specific for the target regions of 343 genes was designed using NimbleDesign software (Roche Sequencing Solution) for the second 28 patients. Here, NGS capture libraries were prepared using Roche KAPA HyperPlus kit and SeqCap EZ Developer Library that targeted the 343 platelet genes following which sequencing was performed using the Illumina MiSeq next generation sequencer (Fig. 2).

Sequence variations were described following the American College of Medical Genetics and Genomics (ACMG) recommendations for standards for interpretation and reporting of sequence variations.[26,27]

RESULTS

Bleeding phenotype of individuals with IPFD/IPND

Results of the first 50 participants evaluated by the SPG are described here (Tables 1 and 2). This cohort comprised 24 male participants (median age 51 years, range 5–76) and 26 female participants (median age 37 years, range 6–71), and included 28 individuals referred for evaluation of a suspected IPND, as well as 22 with a suspected IPFD that included one individual known to be an unaffected relative.

Individuals with a possible IPFD had significantly higher (p=0.01) mean bleeding scores [n=16; mean 5.76, standard deviation (SD) 4.68] than those with thrombocytopenia (n=28; mean 2.14, SD 2.45). One individual (family 27, Table 2) identified with a subsequent PFD (platelet defect 'undetermined') had von Willebrand factor (VWF) levels consistent with 'low VWF'.[28] The presence of this potential 'compound' abnormality did not have a significant effect on bleeding symptoms reported by this individual (ISTH-BAT score 3). In individuals with thrombocytopenia, the highest bleeding score was detected in an individual (individual I, family 3, Table 1) with mosaic monosomy 21 and a complex platelet phenotype. Overall bleeding scores did not correlate with platelet count. The bleeding scores of those with mild thrombocytopenia ($100–150\times10^9$/L), moderate thrombocytopenia ($50–100\times10^9$/L) and severe thrombocytopenia ($<50\times10^9$/L) were 2.17 (n=6, SD 1.34), 2.18 (n=17, SD 2.98) and 2.20 (n=5, SD 0.98), respectively. Four individuals did not demonstrate a platelet function abnormality following phenotypic evaluation. Two of these individuals, were identified with possible abnormalities of VWF, the first with 'low VWF' levels (family 36, Table 2) and the second (family 41, Table 2) with possible

ABCC4, ABCG5, ABCG8, ACTN1, ADAMTS13, ADCY7, ADCYAP1, ADRA2A, AK3, AKAP10, AKT1, AKT2, AKT3, ANKDD1A, ANKMY1, ANKRD18A, ANKRD26, ANKS1B, ANO6, AP2B1, AP3B1, AP3D1, APBB1IP, ARHGAP35, ARHGEF2, ARHGEF3, ARRB2, ARVCF, ASGR2, ATP2A1, ATP2A2, ATP2A3, ATP2B1, ATP2B4, ATXN2, AXL, BAD, BAK1, BAX, BAZ2A, BCL2L1, BET1L, BLOC1S3, BLOC1S6, BRD3, BRF1, C6orf25, C8orf22, C8orf86, CABLES1, CACNB2, CBFA2T3, CBL, CBX5, CCNDBP1, CCNE1, CD226, CD36, CD63, CDC42, CDH10, CDKN2A, CFL1, CHST14, CLEC1B, COPZ1, CPQ, CSF1, CTSZ, CYCS, DAB2, DIAPH1, DLK1, DNM3, DOCK8, DTNBP1, EGOT, EHD3, ELANE, ENTPD1, EPHA4, ERG, ETS1, ETV6, EXOC3L2, EXOC3L4, F2R, F2RL3, FAM171A2, FAR2, FBLIM1, FCER1G, FCGR2A, FEN1, FERMT2, FERMT3, FGFR1, FGR, FHOD1, FLI1, FLNA, FOS, FTSJ1, FYB, FYN, GABPA, GAS6, GATA1, GATA2, GCKR, GCSAML, GCSAML-AS1, GFI1, GFI1B, GIT1, GLIS3, GMDS, GNA12, GNA13, GNA15 , GNAI1, GNAI2, GNAO1, GNAQ, GNAS, GNE, GP1BA, GP1BB, GP5, GP6, GP9, GRTP1, GSK3a, GSK3b, GUCY1A3, GUCY1B3, HBS1L, HLA-B, HLA-DOA, HOXA11, HPS1, HPS3, HPS4, HPS5, HPS6, HSD17B13, HSPB7, ILK, IPCEF1, IRF1, ITGA2, ITGA2B, ITGA5, ITGA6, ITGB3, ITPK1, ITPR1, JAK2, JMJD1C, KALRN, KCNIP4, KCNJ3, KIAA0232, KIF1B, KXD1, LAPTM4A, LAT, LDHAL6A, LIMK1, LINC00523, LPAR1, LRRC16A, LYN, LYST, MAGI1, MAGI2, MAP2K2, MAPK1, MAPK14, MAPK3, MECOM, MEF2C, MEIS1, MFN2, MIR100HG, MKL1, MKL2, MLPH, MME, MPL, MRVI1, MYB, MYH10, MYH9, MYL12B, MYL9, MYO5A, NBEAL2, NF2, NFE2, NLRP6, NUDC, ORAI1, P2RX1, P2RY12, PAK1, PDIA5, PDK1, PEAR1, PFKP, PFN1, PHACTR1, PHLDA3, PIK3C3, PIK3CA, PIK3CB, PIK3CG, PIK3R1, PLA1A, PLA2G4A, PLAU, PLCB2, PLCB3, PLCG2, PLEC, PLEK, PLSCR1, PRKACG, PRKCA, PRKCE, PRKG1, PRNP, PSMD13, PTEN, PTGES3, PTGIR, PTGS1, PTK2, PTK2B, PTPN1, PTPN11, PTPN6, PTPRJ, RAB11A, RAB14, RAB27A, RAB31, RAB32, RAC1, RAD51B, RAF1, RANBP10, RAP1A, RAP1B RASA3, RASGRP2, RBM15, RBM8A, RCL1, RCOR1, RGS18, RGS2, RHOA, RHOB, RHOG, RNF145, RNF181, ROCK1, RPH3A, RPP25, RUNX1, S1PR1, SALL4, SATB1, SCAMP5, SCIMP, SCIN, SCUBE1, SDC1, SELP, SH2B3, SHH, SIRPA, SLC35D3, SLC39A12, SLC6A4, SLC8A3, SLFN14, SLMO2, SNORD7, SNX10, SPI1, SRC, SRF, ST3GAL4, STIM1, STX11, STXBP2, STXBP3 SYK, SYN2, TAL1, TAOK1, TBXA2R, TBXAS1, TERC , TERT, THADA, THPO, TIMP4, TLN1, TLR3? , TMCC2, TPI1, TPM1, TPM4, TRIM58, TRPC6, TRPM7, TSPYL5, TUBA8, TUBB1, TUBB4A, UBASH3A, UMPS, UNC13D, UNC5C, VASP, VAV1, VAV2, VAV3, VIPAS39, VPS33B, VPS45, VWF, WAS, WASL, WDR1, WDR66, WIPF1, YWHAZ, ZFPM1, ZFPM2

*****FGA, FGB, FGG, GATA3, SERPINE1*

Fig. 2 List of genes included in candidate gene arrays. The 343 genes included in the expanded next generation sequencing panel are shown, as well as the 47 genes used in the first gene panel (red). *Genes in the 47 gene panel that were not included in the 343 expanded panel. These included genes associated with dysfibrinogenaemia (*FGA, FGB, FGG*), fibrinolysis (*SERPINE1*) and *GATA3* encoding the transcription factor GATA3.

Table 1 Clinical and laboratory data of individuals referred for evaluation of an inherited platelet number disorder

Family (patient)	Age	ISTH-BAT score	Plt count $\times 10^9$/L	MPV (fl)	MPD (μm)	Morphology on blood film	Platelet and laboratory phenotype (other historical/clinical features)	Molecular diagnosis[a]
1 (I)	42	4	101	NR	3.46	Macrothrombocytopenia Some giant platelets Normogranular platelets	Negative IF for abnormal clusters of NMMHC-IIA	–
2 (I)	68	2	55	NR	3.80	Macrothrombocytopenia Numerous giant platelets Some hypogranular platelets	No red cell abnormalities Some hypogranular neutrophils Negative IF for abnormal clusters of NMMHC-IIA Decreased expression of platelet activation markers by flow cytometry to all agonists	–
3 (I)	6	12	69	12.9	2.75	Numerous macrothrombocytes Numerous hypogranular platelets	Red cell macrocytosis Granulocytes with dysplastic changes Reduced platelet dense granules (WMEM) Reduced mepacrine release Decreased aggregation/activation to all agonists by flow aggregation and flow cytometry	Chromosome 21 deletion encompassing *RUNX1* gene
4 (I)	31	1	107	NR	3.33	Macrothrombocytopenia Normogranular	Nil else specific (AD pattern of thrombocytopenia)	–
5 (I)	33	2	120	13.4	2.63	Macrothrombocytopenia Normogranular	Nil else specific	–
6 (I)	22	4	47	NR	3.95	Macrothrombocytopenia with numerous giant platelets Normogranular	Absent response to ristocetin (LTA) Decreased surface expression of CD42a and CD42b	*GP9*, homozygous, c.A182G:p.N61S, exon 3 Pathogenic[b]
7 (I)	42	3	107	10.8	2.41	Normal size	Increased platelet MYH10 by WB	–
(II)	68	0	117	10.4	2.34	Normogranular		
8 (I)	76	2	11	NR	3.26	Macrothrombocytopenia Normogranular with some hypogranular platelets	Mild red cell anisocytosis with some teardrop poikilocytes Polychromasia No obvious dysplastic changes seen in granulocytes Decreased activation to ADP, CRP and TRAP by flow cytometry (splenomegaly with myelofibrosis on bone marrow examination)	–
9 (I)	54	0	73	NR	3.78	Macrothrombocytopenia with numerous giant platelets	Negative IF for abnormal clusters of NMMHC-IIA	*DIAPH1*, heterozygous, c.C3637T, p.R1213X, exon 27 Pathogenic (detected in both individuals)
(II)	22	0	86	NR	3.93	Normogranular	TEM: platelets heterogeneously enlarged, some rounded with prominent peripheral microtubules; alpha-granules appeared increased and some were abnormally large and elongated (severe/profound sensorineural deafness in both individuals, second decade, asymptomatic mild neutropenia)	
10 (I)	35	3	120	NR	2.97	Macrothrombocytopenia Occasional giant platelets Normogranular	No red cell or white cell changes noted	–
11 (I)	71	2	42	NR	3.12	Macrothrombocytopenia with giant platelets Normogranlar	Abnormal clusters of NMMHC-IIA present by IF (type I)	*MYH9,* heterozygous, c.G5521A p.E1841K, exon 39 Pathogenic
12 (I)	36	2	78	10.1	2.25	Normal size Smaller platelets as well as occasional macrothrombocytes present Normogranular	Reduced aggregation to ADP, collagen, AA and epinephrine Decreased ATP release in response to multiple agonists, TRAP, ADP, collagen and AA Decreased platelet activation (CD62, PAC-1, CD63) to TRAP and CRP, but not ADP Increased platelet MYH10 and decreased MPL by WB Normal dense granules by WMEM (no personal or family history of haematological malignancy)	*RUNX1*, heterozygous, c.G514A p.G172R, exon 3 Likely pathogenic
13 (I)	50	2	80	9.5	2.43	Normal size	Thrombocytopenia (moderate)	*ANKRD26*, heterozygous, 116C>T Pathogenic (detected in individuals I-IV)
(II)	68	6	55	10.4	2.47	Most appear normogranular	WMEM with mildly reduced dense granules in individual III (no personal or family history of haematological malignancy)	
(III)	76	4	63	9.9	2.51	Occasional less granular forms		
(IV)	23	1	70	9.3	2.43			
14 (I)	20	1	99	10.5	2.53	Normal size Normogranular	Nil specific	*CYCS*, heterozygous, c.G124A, p.G42S, exon 2 Pathogenic
15 (I)	69	1	92	12.9	2.59	Macrothrombocytopenia with some giant platelets Normogranular	Negative IF for abnormal clusters of NMMHC-IIA Reduced aggregation to low concentrations of ADP and collagen Reduced aggregation to AA; normal agglutination to ristocetin	–

(*continued on next page*)

Table 1 (*continued*)

Family (patient)	Age	ISTH-BAT score	Plt count $\times 10^9$/L	MPV (fl)	MPD (μm)	Morphology on blood film	Platelet and laboratory phenotype (other historical/clinical features)	Molecular diagnosis[a]
							Discordant reduction of platelet surface expression of GPIbα (56% of controls) and GPIX (88% of controls)	
16 (I)	23	2	49	11.6	2.78	Macrothrombocytopenia Normogranular	Decreased aggregation (flow) Aggregation in response to AA is slow and incomplete Normal aggregation to TRAP, collagen and ADP Decreased activation to TRAP, CRP and ADP (PAC-1 and CD63 only) by flow cytometry (AD pattern of thrombocytopenia)	*ANKRD26*[c] Heterozygous c.116C>T Pathogenic
17 (I)	42	4	51	NR	3.47	Macrothrombocytopenia Normogranular	Frequent stomatocytes Negative IF for abnormal clusters of NMMHC-IIA Reduced platelet aggregation (flow cytometry) to collagen and ADP Normal responses to AA and TRAP	–
18 (I)	39	1	80	11.6	2.70	Giant platelets Normogranular	Döhle-like bodies seen in neutrophils (no known extrahaematological manifestations, hearing impairment, cataracts or renal impairment)	*MYH9*, heterozygous, c.5770_5779del, p.G1924fs, exon41 Pathogenic
19 (I)	19	0	91	NR	3.72	Macrothrombocytopenia with some large and giant platelets	Platelets reduced in number	–
(II)	53	0	56	NR	5.00		Mildly reduced expression of GPIbα by flow cytometry	–
20 (I)	31	1	14	NR	5.99	Macrothrombocytopenia with large numbers of giant platelets Normogranular	Decreased expression of GPIbIX by flow cytometry	*GNE*, homozygous, c.G1624A, p.G578S, exon10 Likely pathogenic
21 (I)	66	0	64	NR	3.79	Macrothrombocytopenia Large numbers of agranular platelets	No red cell abnormalities Platelet surface expression of CD34 not detectable by flow cytometry Decreased platelet activation to ADP, CRP and TRAP with reduced expression of CD62, PAC-1 and CD63 Platelets lack alpha granules and have prominent OCS by TEM	[d]
22 (I)	43	0	56	NR	4.20	Macrothrombocytopenia with numerous giant platelets Normogranular	No Döhle-like bodies seen Relatively reduced CD42a or CD42b expression by flow cytometry	*GP1BB*, homozygous, c.G127T, p.G43W, exon2 Pathogenic

AD, autosomal dominant; IF, immunofluorescence; LTA, light transmission aggregometry; NMMHC-IIA, non-muscle myosin heavy chain type IIA; NR, not recordable; OCS, open canalicular system; TEM, transmission electron microscopy; WB, western blotting; WMEM, whole mount electron microscopy.

[a] Only likely pathogenic or pathogenic variants have been reported in this table and described in the text. In many cases, one or more variants of uncertain significance were detected.

[b] Detected by Sanger sequencing.

[c] *ANKRD26* variant detected by whole genome sequencing in this individual and confirmed by Sanger sequencing.

[d] A homozygous variant of uncertain significance in the *NBEAL2* gene was detected (*NBEAL2*, homozygous, c.T6239A, p.M2080K). Additional laboratory and family studies are required before definitively attributing the patient's phenotype to this variant.

Table 2 Clinical and laboratory data of individuals referred for evaluation of an inherited platelet function disorder

Family	Patient (age, years)	ISTH-BAT score	Platelet count ($\times 10^9$/L)	Platelet phenotype	Pathway defect	Molecular diagnosis[a]
23	I (22)	3	300	Normal WBIA Decreased platelet aggregation to ADP, epinephrine and low dose collagen Reduced ristocetin MA (LTA) Decreased dense granules by WMEM	Dense granule deficiency	–
24	I (35)	1	276	Normal WBIA Decreased platelet aggregation to ADP, epinephrine and low dose collagen Reduced ristocetin MA (LTA) Normal dense granules by WMEM	Undetermined type[b]	–
25	I (71)	17	280	Normal WBIA Mildly decreased aggregation to ADP, epinephrine and collagen low doses (LTA) Normal dense granules by WMEM Mildly decreased CD63 release by flow cytometry	Undetermined type[b]	–
26	I (45)	9	295	Mildly reduced response to AA by WBIA Reduced aggregation response to low dose collagen; mild reduction to low dose ADP; normal aggregation response to AA (LTA) Normal dense granules by WMEM	Undetermined type[b]	–
27	I (47)	3	253	Normal WBIA Increased lag time to collagen with reduced aggregation to collagen, epinephrine and low dose ADP (LTA) Normal dense granules by WMEM	Undetermined type[b]	–
28	I (52)	1	266	Mildly reduced aggregation to collagen by WBIA Reduced aggregation to ADP, collagen, and absent to epinephrine (LTA) Normal dense granules by WMEM	Undetermined type[b]	–
29	I (23)	5	272	No functional defect identified	No functional defect identified	–
30	I (63)	15	269	Normal WBIA Decreased aggregation to low doses ADP and collagen Normal aggregation to epinephrine and AA (LTA) Reduced ATP release to multiple agonists (AA, ADP, TRAP and collagen) Normal dense granules by WMEM	Signal transduction abnormality (ADP receptor)	*P2RY12*, heterozygous, c.C772A p.P258T, exon 3 Likely pathogenic
31	I (35)	10	380	Normal WBIA Mildly reduced aggregation to low doses of ADP and collagen with reduced aggregation to epinephrine (LTA) Decreased ATP release to ADP Mildly decreased expression of CD62p and CD63 in TRAP stimulated platelets, and CD63 in CRP stimulated platelets by flow cytometry Reduced dense granules by WMEM	Dense granule deficiency	–
	II (5)	4	375	Phenotype as for individual I in this family	Dense granule deficiency	–
32	I (64)	6	235	No functional defect identified	No functional defect identified	–
33	I (58)	3	253	Normal WBIA Reduced aggregation to all concentrations of ADP, epinephrine and low dose collagen (LTA) Normal dense granules by WMEM Decreased ATP release to ADP and TRAP	Signal transduction abnormality (Gi defect)	–
34	I (25)	6	256	Normal WBIA	Dense granule secretion defect	–

(*continued on next page*)

Table 2 (*continued*)

Family	Patient (age, years)	ISTH-BAT score	Platelet count ($\times 10^9$/L)	Platelet phenotype	Pathway defect	Molecular diagnosis[a]
				Reduced aggregation to low dose ADP and epinephrine (LTA) Normal dense granules by WMEM Decreased ATP release multiple agonists (ADP, AA and TRAP)		
35	I (55)	8	290	Normal WBIA Reduced platelet aggregation to low dose ADP with delayed aggregation to epinephrine (LTA) Normal ATP release by lumi-aggregometry Reduced dense granules by WMEM	Dense granule deficiency	–
36	I (20)	6	221	No functional defect identified	No functional defect identified	–
37	I (32)	9	164	Decreased aggregation to AA by WBIA Normal aggregation to all agonists by LTA Decreased ATP release to APD, collagen, AA and TRAP Mildly decreased activation to TRAP (CD62p and PAC-1) and CRP (CD62p, PAC-1 and CD63) by flow cytometry Normal dense granules by WMEM	Dense granule secretion defect	–
38	I (41)	2	235	Normal WBIA Normal aggregation to all agonists by LTA Reduced ATP release with all agonists (ADP, AA, Collagen, TRAP) Mildly decreased activation to TRAP and decreased activation to CRP by flow cytometry Normal dense granules by WMEM	Dense granule secretion defect	–
39	I (30)	5	281	Normal WBIA Reduced platelet aggregation to ADP (all concentrations) and mildly reduced aggregation to epinephrine (LTA) Mildly reduced ATP release to low dose ADP and TRAP Normal dense granules by WMEM	Signal transduction abnormality (Gi defect)	–
40	I (17)	2	225	Normal WBIA Normal aggregation to all agonists by LTA Abnormal ATP release in response to ADP (low dose) and TRAP (all doses) Decreased expression of CD63 following activation by CRP, ADP and TRAP by flow cytometry Normal dense granules by WMEM	Dense granule release defect	–
	II (46)	0	ND	Unaffected parent	Unaffected parent	–
41	I (57)	5	170	No functional defect identified	No functional defect identified	–
42	I (38)	1	272	Normal WBIA Decreased aggregation to ADP; delayed and reduced aggregation to collagen; normal aggregation to epinephrine and AA (LTA) Decreased ATP release to all agonists (ADP, AA, collagen, TRAP and thrombin) Normal dense granules by WMEM	Dense granule release defect	–

Gi, G-protein(i); LTA, light transmission aggregometry; WBIA, whole blood impedance aggregometry; WMEM, whole mount electron microscopy.

[a] Only likely pathogenic or pathogenic variants have been reported in this table and described in the text. In many cases, one or more variants of uncertain significance were detected.

[b] Categorised as an 'undetermined type' platelet function disorder if abnormalities were detected by LTA but ATP was not assessed and dense granules were normal.[25]

(confirmatory testing required) von Willebrand disease (VWD) (type 2M). These individuals had bleeding scores of 6 and 5, respectively; these scores were equivalent to the mean scores of the other two individuals without abnormalities (family 29 and 32, scores 5 and 6, respectively), which in turn were not significantly different (p=0.84) from those with a platelet defect detected by phenotypic testing. This finding is consistent with previous reports suggesting that bleeding scores are not predictive of a platelet defect on lumi-aggregometry.[29] Moreover, there was no significant difference in bleeding scores between individuals identified with different platelet pathway defects (signal transduction, secretion/release, dense granule deficiency, undetermined) or between these individuals and those referred with abnormal bleeding symptoms but without a defect detected by laboratory testing (Fig. 3), suggesting an inability of the bleeding score alone to differentiate individuals with different platelet pathway/function abnormalities.

Outcomes of phenotypic testing

Inherited platelet function disorders

Twenty-two individuals were referred for evaluation of a possible IPFD (Table 2) and comprised 15 females (median age 35 years; range 20–71) and seven males (median age 48 years; range 5–58). One individual was a known unaffected relative and was not tested further (sample taken for NGS only), and four individuals were found not to have a demonstrable platelet functional defect following testing. Of these four individuals, two had been referred with mucocutaneous bleeding symptoms and historically normal LTA traces, and one had been referred with bleeding symptoms and historically variable WBIA results. In these cases, an absence of a detectable platelet function defect was concordant with referral information. In the last individual, reported normal findings were discordant with the referral information that had described abnormal platelet responses to ADP and collagen (traces not seen) by LTA. Of the remaining 17 individuals, 12 (71%) received a diagnosis to the level of defective platelet pathway. The most common platelet pathway defects were dense granule release defects (n=5), dense granule deficiencies (n=4) and signal transduction defects (n=3). Five cases were found with normal dense granules by WMEM and demonstrated abnormalities by LTA but did not have ATP release assessed by lumi-aggregometry and hence were referred to as platelet pathway defects 'undetermined type'.

Platelet aggregation responses were assessed by both WBIA and LTA. An abnormality in aggregation by WBIA was not detected in 13 of the 17 individuals identified with a functional platelet defect by subsequent LTA or lumi-aggregometry (3/3 identified with signal transduction abnormalities, 4/5 with dense granule secretion defects, 4/4 with dense granule deficiency and 3/5 with platelet defects 'undetermined'), whilst LTA was normal in three individuals (3/16, 18.7%) who were shown to have significant defects in ATP release by lumi-aggregometry, consistent with dense granule secretion defects. In one of these cases (individual I, family 40, Table 2) the normal LTA result was discordant with the referral LTA study that had reported abnormal aggregation in response to ADP, epinephrine and collagen. On both occasions LTA had been performed in the same laboratory (Prince of Wales Hospital, Sydney).

Dense granule content was assessed semi-quantitatively by WMEM and by mepacrine uptake by flow cytometry. In all cases where decreased dense granules were quantitated by WMEM, a corresponding decreased uptake of the fluorescent marker, mepacrine, was not seen, suggesting low sensitivity of mepacrine uptake for the detection of dense granule deficiency. Moreover, in all five cases where a dense granule secretion defect was identified by lumi-aggregometry, a corresponding abnormal mepacrine release following agonist stimulation was not seen, suggesting lack of sensitivity of this measure as a surrogate for dense granule secretion.

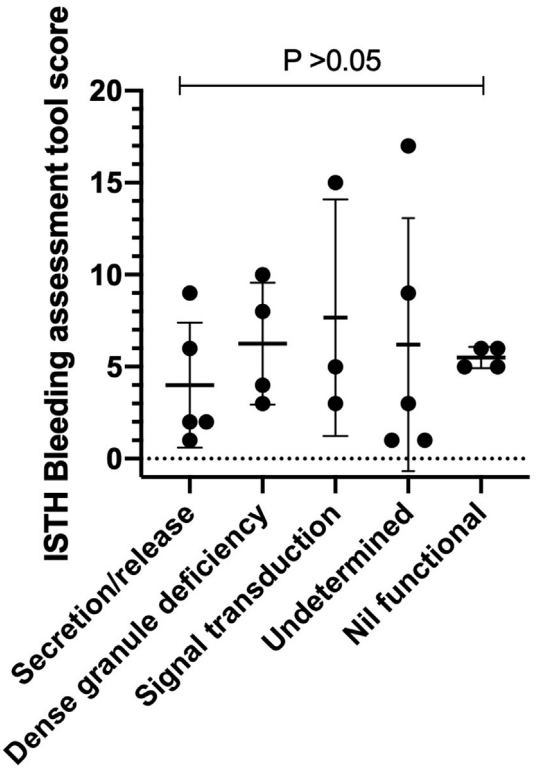

Fig. 3 Association between the ISTH bleeding assessment tool score and the type of platelet function defect. Box plots represent the 25th and 75th quartiles. Statistical analysis was performed with the non-parametric Kruskall–Wallis test, with Dunn's adjustment. NS, no significant difference.

Inherited platelet number disorders

Twenty-eight individuals were referred for evaluation of a possible IPND and comprised 17 males (median age 54 years, range 22–76) and 11 females (median age 39 years, range 6–68) (Table 1). Six individuals had mild thrombocytopenia, 17 had moderate thrombocytopenia and five had severe thrombocytopenia.

An automated mean platelet volume (MPV) could not be reported in 16/28 (55%) of IPND patients due to the presence of large platelets in those cases; however, MPD was successfully calculated in all individuals with thrombocytopenia and was used for initial classification. Six cases were classified as 'disorders with giant platelets', 11 cases as 'disorders with large platelets' and 11 cases as 'disorders with normal or slightly increased sized platelets'. A significant correlation between forward scatter (FSC) by flow cytometry and MPD (p<0.0001), PDLCR (p<0.0001) and MPV (p=0.0031) was

noted, indicating the potential applicability of this parameter for initial classification of these disorders.

Nine individuals (32.1%) with thrombocytopenia had associated platelet function defects and included one individual with BSS with a characteristically absent response to ristocetin, as well as two individuals with variants or loss of *RUNX1*, and one individual with phenotypic characteristics in keeping with Gray platelet syndrome (GPS) (Table 1). The aetiology of thromobocytopenia in the remaining five individuals with associated functional defects remains uncharacterised (Table 1).

Outcomes of molecular testing

NGS was performed for 49 individuals; this included 27/28 individuals referred for evaluation of an IPND and 22 for evaluation of an IPFD. Data were not generated for one patient in the IPFD group due to poor quality/quantity of DNA obtained. DNA was not analysed for one individual (individual I, family 16) as this patient had consented to whole genome sequencing via a separate study and results were still pending at the time of participation in this project. A pathogenic variant in the 5′ untranslated region (UTR) of *ANKRD26* was subsequently detected and confirmed by Sanger sequencing. In individuals with IPNDs, 14 (52%) (14 individuals in 10 families) received a diagnosis at a molecular genetic level through the identification of disease causing variants (likely pathogenic or pathogenic) in these individuals. Of these cases, only two individuals (family 6 and family 11) had been referred with a probable clinical and laboratory phenotype (BSS and *MYH9*-RD, respectively) indicating that a diagnosis was achieved in 12/25 (48%) (12 individuals from 8 families) referred with an IPND of uncertain aetiology. Details of the variants detected are shown in Fig. 4, and included disorders associated with extra-haematological manifestations such as severe sensorineural hearing impairment (*DIAPH1*), as well as renal failure, cataracts and sensori-neural hearing loss (*MYH9*). *RUNX1* mutations are associated with thrombocytopenia and a propensity to myeloid neoplasms. A *RUNX1* deletion was confirmed in a young child with a complex chromosomal anomaly (*de novo* mosaic monosomy 21) (family 3, Table 1) and associated thrombocytopenia, and a novel likely pathogenic missense variant (*RUNX1*, c.G514A, p.G172R) was detected in a second, unrelated family (family 12, Table 1). Here, application of western blotting demonstrated increased expression of MYH10 (NMMHC IIB), supporting abnormal regulation of this protein. Another novel likely pathogenic homozygous variant (c.G1624A, p.G578S) in the *GNE* gene that encodes a key enzyme in sialic acid biosynthesis, UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase, was detected in one individual (family 20, Table 1) of middle eastern background.

NGS was performed in 22 individuals with possible IPFD and included one individual who was an unaffected relative. Analysis was not possible in one case as the DNA sample was inadequate for analysis (family 23, Table 2). A single likely pathogenic variant was detected in one individual 1/20 (5%) (Table 2). A number of variants of uncertain significance were detected in other patients and are under evaluation (Supplementary Tables 1 and 2, Appendix A). This low detection rate is in keeping with published literature[13,30] and reflects the complex regulation of platelet function in individuals with mild platelet defects characterised by abnormalities of signal transduction, dense granule formation and secretion.[30]

NGS was performed using a candidate gene panel of 47 genes for the first 22 patients. This panel was revised to include 343 genes (Fig. 2) for analysis of the second 28

47 gene panel*		343 gene panel**	
DIAPH1	NM_001314007: Heterozygous, exon 27 c.C3637T, p.R1213X **Pathogenic**	*ANKRD26*	NM_014915: Heterozygous, -116C>T **Pathogenic**
MYH9	NM_002473: Heterozygous, exon 39 c.G5521A p.E1841K **Pathogenic**	*CYCS*	NM_018947: Heterozygous, exon2 c.G124A, p.G42S **Pathogenic**
RUNX1	NM_001001890: Heterozygous, exon 3 c.G514A p.G172R **Likely pathogenic**	*GP1BB*	NM_000407: Homozygous, exon 2 c.G127T, p.G43W **Pathogenic**
RUNX1	Ch21 RUNX1 deletion	*MYH9*	NM_002473: Heterozygous, exon 41 c.5770_5779del, p.G1924fs **Pathogenic**
		GNE	NM_001128227: Homozygous, exon 10 c.G1624A, p.G578S **Likely pathogenic**

Fig. 4 Pathogenic and likely pathogenic variants detected in individuals with inherited platelet number disorders. *This panel was used to sequence the first 22 patients referred to the SPG. **This panel was used to sequence the second 27 patients referred to the SPG.

patients. Considering individuals with IPNDs, 14 were analysed by the 47 gene panel and 13 by the 343 gene panel. Molecular diagnosis was achieved in five individuals from four families using the first panel (Sanger sequencing only performed for individual I, family 6) and in eight individuals from five families using the revised panel (Fig. 4). Two families were found with mutations in genes included in the second panel that were not part of the candidate list in the first panel, *CYCS* and *GNE*, respectively (Fig. 4).

DISCUSSION

An integrated approach to the diagnosis of IPDs consisting of extensive phenotypic and genetic testing methods was accomplished by combining resources and expertise from four centres in the Sydney region. This strategy resulted in 71% of individuals referred with possible IPFDs receiving a diagnosis to the level of the defective platelet pathway. This number exceeds the 28.2% reported by an international survey conducted by the International Society of Thrombosis and Haemostasis (ISTH).[13] The ability to provide a comprehensive report and phenotypic diagnosis is beneficial to clinicians involved in devising peri-operative bleeding prophylaxis plans for these patients. Moreover, detailed analysis has implications for the patient who historically might have been required to visit multiple sites on separate occasions in order achieve an equivalent result.

Among patients investigated in our cohort, 19% did not demonstrate a platelet function defect by the phenotypic tests employed. In these cases, a lack of a detectable functional defect by the tests used in this study does not necessarily mean an absolute lack of platelet dysfunction, as defects may exist which are either not detectable by the methods used or alternatively lie below the limits of detection/sensitivity of the assays. One individual was referred with abnormalities by LTA that were not replicated by our group highlighting that in cases of discordance, repeat testing may be required as some cases of platelet dysfunction may be associated with variable and heterogeneous *in vitro* aggregation findings.[31]

Detailed phenotypic analysis was performed for every patient. This provided an opportunity to critically appraise the diagnostic efficacy of various testing platforms. Most individuals referred in our study cohort had platelet function disorders associated with milder bleeding phenotypes reflected by a relatively low mean BAT score for males and females (mean score 5.76, SD 4.68). In this setting, our results suggest that WBIA is insensitive in detecting individuals with milder platelet function disorders. This finding is consistent with previously published reports using this platform in the diagnosis of IPFDs.[32] Moreover, LTA was normal in 18.7% of cases found with a platelet function defect. Taken together, these results highlight the importance of centres not to rely solely on either WMIA or LTA (without lumi-aggregometry or a test for dense granule release) when investigating possible IPFDs. In the scenario of a finding that is discordant to a historical report, repeat testing should always be considered, especially in the setting of a suspected dense granule secretion defect where variability and heterogeneity of *in vitro* aggregation studies is well established.[31] In addition, we found that WMEM provided a better semi-quantitative measure of dense granules compared to mepacrine staining by flow cytometry and that this latter method was also inferior to lumi-aggregation as a measure of dense granule secretion.

Our study re-enforced the utility of simple measures using MPD measurements for initial classification of thrombocytopenia.[16,17] We also showed the potential use of platelet FSC by flow cytometry for the recognition of altered platelet size and demonstrated correlation of this measurement with MPD. Given the time-consuming nature of MPD measurement, platelet size analysis by flow cytometric FSC is an attractive alternative in these disorders. Except in cases where a diagnosis is confirmed by phenotypic features, such as the presence of neutrophil Döhle-like bodies detected by blood film or IF (family 11 and 18, Table 1), phenotypic testing merely raises diagnostic suspicion in most cases of IPNDs, partly because phenotypes are often diverse (e.g., FPD/AML caused by *RUNX1* variants)[33] or subtle (e.g., *CYCS*-RT),[34] but also because on the whole, detailed diagnostic criteria for these disorders are lacking. Therefore, NGS has emerged as an important tool in this set of disorders. The SPG provided a molecular diagnosis to 48% of individuals referred with possible IPNDs of uncertain aetiology. This result is comparable to results reported by Spanish and UK investigators employing large candidate gene panels[35,36] and affirms the utility of NGS gene panels in the diagnostic setting. Our NGS approach also identified two individuals from two unrelated families with novel likely pathogenic variants in *RUNX1* and *GNE*, respectively. Pathogenic variants of *RUNX1* cause thrombocytopenia associated with an increased lifetime risk of haematological malignancy.[37] RUNX1 and another important transcription factor, FLI1, silence the expression of MYH10 during terminal maturation of megakaryocytes through negative regulation of the *MYH10* gene. Increased expression of MYH10 in platelets has been suggested as a biomarker for alterations in these two transcription factors.[38] We showed by western blotting that MYH10 expression was increased by the novel RUNX1 G172R variant, supporting the potential functional significance of this variant. However, the sensitivity and specificity of increased MYH10 expression for the detection of variants in *RUNX1* and *FLI1* and/or other genes is not known. This is highlighted by the case of two related individuals (family 7, Table 1) in our cohort whose platelets demonstrated increased MYH10 expression by western blotting but were not found to have variants in either *RUNX1* or *FLI1* by NGS or additional single nucleotide polymorphism (SNP) microarray that was performed at a later stage to exclude the possibility of large a deletion that may have failed detection by conventional short read NGS.[39] The second novel variant was detected in *GNE*. Homozygous variants of *GNE* causing a change of platelet surface sialyation with subsequent increased platelet clearance have only recently been described causing thrombocytopenia and bleeding in affected individuals.[40,41] In this case, further functional studies are being performed to confirm the significance of the homozygous variant detected in our cohort.

Pathogenic and likely pathogenic variants were detected in two genes, *CYCS* and *GNE*, respectively, in the 343 gene panel. These genes were not included in the 47 gene panel, highlighting the important need of ongoing curation of gene panels to include genes not previously targeted for which literature supports their inclusion, as in the case of *CYCS*,[34] as well as those that may be newly implicated in platelet disorders, as in the case of *GNE*.[40,41] It is also important to recognise that despite the scope of our expanded gene panel of

343 genes, a clear molecular diagnosis was not established in ~50% of individuals with IPNDs. This highlights limits of variant detection by NGS gene panels that include the possibility that causative genes are not included in the panel and, like whole exome sequencing (WES) approaches, gene panels may fail to detect large deletions, duplications, copy number variations or complex structural variants.[12,42] Finally, unlike whole genome analysis sequencing, gene panels and WES do not assess non-coding regions of target genes.

A causal genetic variant was only identified in one family referred for evaluation of an IPFD. The individual in this family reported life-long mucocutaneous bleeding symptoms that included epistaxis, bruising, abnormally prolonged bleeding following minor wounds, gastro-intestinal bleeding, post-operative bleeding, as well as menorrhagia (ISTH-BAT 15). Previous testing had excluded VWD and haemophilia and had suggested a platelet function defect. Bleeding symptoms were described (but not confirmed) in other family members including the proband's father, two sisters and a nephew (who was diagnosed with a storage pool disorder) suggesting an autosomal dominant expression. NGS identified a likely pathogenic missense variant of the *P2RY12* gene (c.C772A). This single nucleotide change predicts a threonine substitution for the proline at position 258 of the $P2Y_{12}$ receptor. This amino acid is located in the extracellular loop 3-transmembrane 6 (EL3-TM6) region of the receptor that has been shown to be important in signal transduction.[43–46] The platelet functional defect (family 30, Table 2) seen in this individual was similar to those reported in other cohorts harbouring the P258T variant.[47,48] The low rate of causal variants detected in our cohort with an IPFD is not wholly unsurprising, as patients in this group were found with milder, non-syndromic disorders of signal transduction and secretion, as well as dense granule deficiency, for which a number of potential candidates have been identified,[30] but for which, on the whole, causative genes remain elusive.[49] The extension of whole genome sequencing approaches, currently used predominantly in gene discovery, into clinical diagnostic platforms, will undoubtedly increase diagnostic ability through capacity to detect variants in non-coding regions in the genome, as has been demonstrated in the case of Quebec platelet disorder (*PLAU*) and thrombocytopenia absent radius syndrome (*RBM8A*) caused by variants in the regulatory regions of these genes.[50,51]

We recognise that the level of platelet investigation performed by the SPG collaborative is limited by the requirement of multiple platforms for efficient evaluation of IPDs, which may not be available even at large single tertiary centres. This issue can be partially circumvented by the demonstrated efficacy of genetic testing in cases of thrombocytopenia and the stability of DNA, which allows transport over long distances, thereby permitting smaller metropolitan and regional centres to courier samples to referral laboratories. However, the pre-analytical requirements, the need for a fresh blood sample and the lack of a suitable screening test for platelet function disorders means that at this point in time individuals with suspected platelet function disorders should ideally be referred to centres where combined testing is possible.

In conclusion, our results confirm the utility of an integrated phenotypic-genetic approach for the diagnosis of IPD. Through combining multiple phenotypic and genetic testing strategies and performing them uniformly in all patients, we have provided a level of platelet diagnosis that is currently not available elsewhere in Australia, and we have been able to critically appraise testing methods for these conditions. Observations from this work are being incorporated into future projects aimed at streamlining investigations and optimising genetic diagnosis.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2019.10.005.

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