

Interlaboratory exercise on the use of immunofluorescence microscopy on the blood smear for recognizing inherited platelet disorders: Communication from the ISTH SSC Subcommittee on Platelets in Health and Disease

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Abstract

Immunofluorescence-based platelet phenotyping using peripheral blood smears has recently emerged as a promising method for characterizing a subgroup of inherited platelet disorders (IPD). A single-center study demonstrated its potential for accurate diagnosis of 9 disorders with characteristic platelet structural changes. The aim of this

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study was to evaluate the reproducibility of this approach through an interlaboratory validation study. Native, air-dried blood smears from healthy controls and patients with confirmed IPD were shipped to 7 participating laboratories, blinded for the sample origin. Samples were fixed and stained using a shared panel of 13 commercially available primary antibodies and 2 fluorescence-labelled secondary antibodies. Laboratories formulated diagnostic predictions based solely on immunofluorescence findings. The pre-workshop method establishment involved other samples and feedback with the coordinating laboratory to address technical issues before blinded sample validation. All 7 laboratories (Brisbane, Greifswald, Murcia, Paris, Pavia, Perugia, and Tübingen) correctly diagnosed *MYH9*-related disease, Bernard-Soulier syndrome, Glanzmann thrombasthenia, and *GFI1B*-related thrombocytopenia. Six of 7 laboratories accurately identified *TUBB1*-related disorder and quantitative δ -storage pool disorder, while 5 of 7 correctly diagnosed *GATA1*-related thrombocytopenia. Immunofluorescence-based platelet phenotyping on peripheral blood smears demonstrated high sensitivity for diagnosing *MYH9*-related disease, Bernard-Soulier syndrome, Glanzmann thrombasthenia, and *GFI1B*-related thrombocytopenia, and reasonable sensitivity for *TUBB1*-related disorder, quantitative δ -storage pool disorder, and *GATA1*-related thrombocytopenia. Immunofluorescence analysis of blood smears may be of help in the diagnostic work-up of IPD.

KEYWORDS

Bernard-Soulier syndrome, blood, blood platelet disorders, blood platelets, fluorescence, microscopy, platelet storage pool deficiency, thrombasthenia, thrombocytopenia

1 | INTRODUCTION

Inherited platelet disorders (IPD) are a heterogeneous group of rare conditions characterized by thrombocytopenia, platelet dysfunction, or both. These disorders result from mutations in over 70 genes impacting platelet biology, structure, or function, leading to a wide spectrum of bleeding phenotypes [1,2]. Accurate diagnosis of IPD is crucial for appropriate patient management, genetic counseling, and potential targeted therapies [3–7]. However, diagnosis can be challenging, requiring a combination of detailed clinical evaluation and laboratory testing [8]. Traditional approaches rely on platelet function assays, flow cytometry, morphologic examination of blood smears, and increasingly, genetic sequencing [9–11]. Although valuable, these methods have limitations, are technically demanding, and exhibit significant inter-laboratory variability. Furthermore, molecular testing may not always identify causative mutations, particularly for novel or rare variants [12].

Immunofluorescence microscopy offers a promising complementary approach for characterizing the platelet phenotype [13]. This technique visualizes specific platelet structures using fluorescence-labeled antibodies for assessing platelet morphology and identifying potential defects. Key advantages include the requirement for small blood volumes (< 100 μ L), making it possible to assess neonates, suitability for long-distance sample shipping, and relatively low equipment costs [14].

A recent study demonstrated the feasibility of using immunofluorescence microscopy on peripheral blood smears to identify specific phenotypic changes associated with 9 forms of IPD (*MYH9*-related disease, classical- and monoallelic Bernard-Soulier syndrome, Glanzmann thrombasthenia (GT), *TUBB1*-related disorder, Wiskott-Aldrich syndrome, quantitative δ storage-pool disorder, *FLNA*-related thrombocytopenia, and *GFI1B*-related thrombocytopenia), achieving promising sensitivity and specificity in a single-center setting [15]. However, the reproducibility of this technique across laboratories and the robustness of its diagnostic potential require rigorous validation.

Therefore, this first multicenter study was designed to assess the interlaboratory reproducibility of immunofluorescence microscopy for recognizing IPD.

2 | METHODS

2.1 | Aim of the study, participating centers, and subject enrollment

This study aimed to validate a standardized protocol for immunofluorescence staining of peripheral blood smears and to evaluate its ability to accurately identify healthy controls and patients with IPD in a blinded, multicenter setting.

Eight institutes internationally participated in the exercise: Institut für Transfusionsmedizin, Universitätsmedizin Greifswald, Germany (coordinating center); Institute for Clinical and Experimental Transfusion Medicine, University Hospital of Tübingen, Germany; Servicio de Hematología, Hospital Universitario Morales Meseguer-Centro Regional de Hemodonación Murcia, Spain; Department of Medicine and Surgery, Section of Internal and Cardiovascular Medicine, University of Perugia, Italy; Department of Internal Medicine, University of Pavia, Italy; Laboratoire d'Hématologie, Assistance Publique Hôpitaux de Paris, Hôpital Bichat-Claude-Bernard, Paris Cité University, Paris, France; Service d'Hématologie Biologique, Hôpital Lariboisière, Paris Cité University, Paris; and Department of Haematology, Pathology Queensland, Centre for Children's Health and Research (CCHR), South Brisbane, Australia. The 2 French institutes collaborated as a single center, performing the analysis jointly with 2 investigators, resulting in a total of 7 participating laboratories.

Eight patients with 8 genetically confirmed IPD were enrolled in the study. They were recruited from the following institutions: University Medicine Greifswald, Germany; University of Murcia, Spain (as part of the Spanish multicenter project on IPD of the Grupo Español de Alteraciones Plaquetarias Congénitas, GEAPC); Medical University of Vienna, Austria; and University Clinic Bonn, Germany. Healthy controls were enrolled from blood donors at the University Medicine Greifswald upon confirming normal platelet counts and general health. Written informed consent was obtained from all patients or their legal guardians, and the study was conducted in accordance with the Declaration of Helsinki.

2.2 | Standardization of procedures and materials

The previously reported methodological protocol [14] was adopted by all participating centers. Participants received a standard operating procedure, including photographic documentation for each methodological step (Supplementary Appendix). All laboratories used the same panel of 13 mouse or rabbit anti-human primary antibodies and 2 fluorescence-labeled goat anti-rabbit or -mouse secondary antibodies (Table 1). The antibodies were procured by each center independently. Although microscopic equipment varied between laboratories, all possessed objective and ocular lenses capable of sufficient magnification for platelet assessment, with filters adjusted to capture emission light at the characteristic wavelengths of the used secondary antibodies (Supplementary Table 1). Individualized training was provided to address participant-specific questions regarding procedural details (eg, antibody staining region definition, fixation, coverslipping) and morphological interpretation.

2.3 | Study design

The study comprised 2 phases. Phase 1 aimed to assess the reproducibility of procedures for i) blood smear fixation; ii) blood smear

labeling with primary and secondary antibodies; and iii) achieving comparable immunofluorescence images. To this end, unstained and unfixed native air-dried blood smears from healthy controls, prepared in the Greifswald laboratory, were shipped within 24 hours to each participating center. The shipment was carried out via regular mail at room temperature. Careful wrapping of the slide containers was used to avoid possible hemolysis-related artifacts caused by moisture condensation, which can occur during airfreight when a cool glass slide encounters warm, humid air. Samples were fixed and stored at -20°C as reported [14]. Subsequently, slides were stained and assessed using immunofluorescence microscopy. Images were taken, self-evaluated by each participant using reference images provided by the coordinating center, and shipped to the coordinating center for centralized review. Discrepancies were reported back to participants for independent refinement of their assessment.

Phase 2 aimed to assess the method's reproducibility in i) distinguishing healthy controls from patients with IPD and ii) assigning a specific IPD diagnosis based on the sole microscopic findings. Blood smears from healthy controls and patients with genetically confirmed IPD were shipped to each laboratory following the same procedure as phase 1. The blood smears from patients and controls were prepared at the site of subject enrollment. Blood slides prepared at institutions other than the coordinating center were centralized there before shipping. A total of 7 shipments were conducted over 19 months, with each round consisting of 1 to 3 sets of 20 slides. Each slide set, corresponding to a patient or a healthy control, was sent out only once to each center. Blood smears were anonymized using an alphanumeric code. Investigators were unaware of both subject identity (patient or healthy control) and of IPD diagnoses, ensuring full blinding, including with respect to differential diagnoses. However, they did know that each sample set of 20 blood smears originated either from a patient with IPD or from a healthy control. No clinical information, platelet counts, or inheritance patterns were provided. To maintain blinded conditions, smear preparation and dispatch at the centers of Greifswald and Murcia were performed by personnel separate from those conducting microscopic analyses.

Each laboratory conducted the investigation autonomously, reporting results using a defined evaluation grid and providing representative photo-documentation (Supplementary Figures 1–13). Each sample was evaluated by each participating center only once. Participants provided i) a qualitative assessment of each platelet marker (normal or altered relative to reference control images initially distributed by the coordinating center) and ii) a final interpretation indicating, if possible, a suspected specific IPD diagnosis. Reports and documenting pictures were shipped to the coordinating center for review.

2.4 | Statistical analysis

Categorical data were reported as counts and percentages. To evaluate the diagnostic accuracy of immunofluorescence, sensitivity, specificity, negative predictive value, and positive predictive value

TABLE 1 Primary and secondary antibodies were used in the interlaboratory exercise.

Marker protein	Antibody name	Company, catalog code	Host species	Predilution ^a	Final dilution ^b	Storage temperature
Primary antibodies						
MYH9	Antimyosin IIA, nonmuscle antibody	Sigma Aldrich, M8064	Rabbit	1:100	1:10 000	−20 °C
β1-tubulin	Anti-β-tubulin antibody	Sigma Aldrich, T4026	Mouse	1:25	1:200	−20 °C
α-tubulin	Anti-α-tubulin antibody	Abcam, ab190471	Rabbit	undiluted	1:200	−20 °C
α-tubulin	Anti-TUBA4A (TUBA1) antibody	Sigma Aldrich, T9026	Mouse	1:100	1:2000	−20 °C
Filamin A	Anti-Filamin A antibody	Santa Cruz Biotechnology, sc58764	Mouse	1:500	1:4000	4 °C
Thrombospondin-1	Anti-Thrombospondin 1 antibody	Abcam ab85762	Rabbit	1:100	1:400	−20 °C
P-Selectin	Purified mouse anti-human CD62P antibody	BD Biosciences, BD 555522	Mouse	1:100	1:400	4 °C
von Willebrand factor	Anti-human von Willebrand factor antibody	DAKO, A0082	Rabbit	1:100	1:2000	4 °C
LAMP-1	LAMP1 antibody (H5G11)	Santa Cruz Biotechnology, sc18821	Mouse	undiluted	1:20	4 °C
LAMP-2	LAMP-2 antibody (H4B4)	Santa Cruz Biotechnology, sc18822	Mouse	undiluted	1:20	4 °C
CD63	Purified mouse anti-human CD63 antibody	BD Biosciences, BD 556019	Mouse	1:100	1:5000	4 °C
GP1b/IX	Mouse anti-human CD42a antibody	Bio-Rad (AbD Serotec), MCA594	Mouse	1:100	1:500	−20 °C
GP1Ib/IIIa	CD41 unlabelled antibody	Beckman Coulter, PN IM0145	Mouse	1:100	1:2000	−20 °C
CD34	CD34 monoclonal antibody	ImmunoLogic, ILM1343-C05	Mouse	undiluted	1:100	4 °C
Secondary antibodies						
Rabbit IgG	Alexa Fluor 568	Invitrogen, A11011	Goat	undiluted	1:400	4 °C
Mouse IgG	Alexa Fluor 488	Invitrogen, A11001	Goat	undiluted	1:400	4 °C

GP, glycoprotein; Ig, immunoglobulin; LAMP, lysosome-associated membrane protein.

^a stock dilution.

^b working dilution.

were calculated. Method performance was assessed by aggregating data across the 8 IPD forms and the total investigations from each of the 7 participating laboratories (7 × 8 IPD cases + 7 × 5 healthy controls), with diagnostic metrics computed for each individual case assessment. Means and 95% confidence intervals (CIs) were computed for all parameters. Likelihood ratios were calculated to quantify the evidential strength of immunofluorescence results for or against IPD. A positive likelihood ratio (PLR) > 1 indicates that a positive test increases the odds of disease (higher values provide stronger evidence), while a negative likelihood ratio (NLR) < 1 indicates that a negative test decreases the odds of disease (lower values provide stronger evidence) [16].

Cohen's κ (unweighted) was used to assess the agreement of individual laboratories versus true diagnosis across all cases, and the interlaboratory agreement via $n = 21$ pairwise comparisons [17,18]. The overall average κ value integrated both components. κ values

were interpreted as follows: $\kappa < 0$ = poor agreement; 0.01 to 0.20 = slight; 0.21 to 0.40 = fair; 0.41 to 0.60 = moderate; 0.61 to 0.80 = substantial; 0.81 to 1.00 = almost ($\kappa = 1.00$ = perfect agreement) [19,20]. All statistical analyses were performed using JASP software, version 0.95.4 [21].

3 | RESULTS

3.1 | Patient enrollment, and morphologic features of the investigated IPD

Eight unrelated subjects affected by 8 different forms of IPD with previously reported alterations in platelet structure were enrolled (Table 2 [22–31]). In addition to the well-established morphologic changes [13,14], some enrolled diseases showed additional

TABLE 2 Enrolled patients with inherited platelet disorders (IPDs) confirmed at the molecular level.

Subject number	Sex, age (y)	IPD diagnosis	Genetic variant	Variant interpretation		
				ACMG ^a	ClinVar ^b	References
1	F, 31	Delta storage-pool disorder due to Hermansky-Pudlak syndrome	<i>HPS4</i> : c.2054delC; p.Pro685LeufsTer17 (homozygous)	P	P/LP	[22]
2	F, 39	<i>MYH9</i> -related disease	<i>MYH9</i> : c.5521G>A; p.Glu1841Lys (heterozygous)	P	P/LP	[23]
3	F, 26	<i>DIAPH1</i> -related thrombocytopenia	<i>DIAPH1</i> : c.3637C>T; p.Arg1213Ter (heterozygous)	P	P	[24]
4	F, 36	<i>TUBB1</i> -related disorder	<i>TUBB1</i> : c.1075C>T; p.Arg359Trp (heterozygous) <i>GFI1B</i> : c.580T>C; p.Cys194Arg (heterozygous)	VUS VUS	VUS/LB/ B -	[25]
5	F, 23	Bernard-Soulier Syndrome	<i>GP1BB</i> : c.80C>T; p.Pro27Leu (homozygous)	LP	LP	[26]
6	F, 38	<i>GATA1</i> -related thrombocytopenia	<i>GATA1</i> : c.652G>A; p.Asp218Asn (heterozygous)	LP	LP/VUS	[27]
7	F, 43	<i>GFI1B</i> -related thrombocytopenia	<i>GFI1B</i> : c.551insG; p.Ser185LeufsTer3 (homozygous)	P	LP	[28]
8	M, 50	Glanzmann Thrombasthenia	<i>ITGA2B</i> : c.1882C>T; p.Arg628Ter (homozygous)	P	P	[29,30]

B, benign; LB, likely benign; LP, likely pathogenic; P, pathogenic; VUS, variant of uncertain significance.

^a Variant classification following the guideline from the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology [31].

^b Variant classification according to ClinVar submission (<https://www.ncbi.nlm.nih.gov/clinvar/>; accessed on January 5th, 2026).

phenotypic characteristics (Table 3). In particular, abnormalities of platelet cytoskeleton and faint platelet expression of CD34 were reported in the blood smears of individuals with *DIAPH1*-related thrombocytopenia (*DIAPH1*-RT) [16], and, alongside platelet changes, aggregates in the erythrocytes consisting of nonmuscular myosin IIA (NMMIIA) were observed in *GATA1*-related thrombocytopenia (*GATA1*-RT) [32].

3.2 | Shipment and delivery of blood smears

Blood smears were delivered within 24 to 48 hours to the participating centers residing in Europe. To the Australian laboratory, the blood smears were delivered within 48 to 72 hours in all but 1 case, in which the parcel arrived after 5 days. Even in that case, no significant alterations of the quality of the blood smears and/or the staining outcome were reported.

3.3 | Method implementation (phase 1)

Each group was able to apply the protocol without major technical shortcomings. Minor issues were signal weakness when staining against the cytoskeletal protein α -tubulin and excessive background signal when staining against the dense granule marker LAMP-2. Regarding the

α -tubulin staining, a better outcome was obtained with an alternative antibody by 2 groups, which has then been included in the antibody panel (Table 1). Some participants repeated 1 or more immunostainings due to preanalytical issues or to confirm pathologic findings. No participant requested additional slides beyond the 20 provided per subject—which theoretically allowed at least 1 repetition per marker—indicating that 20 slides from a subject under investigation represent an appropriate number for routine shipment to a reference laboratory for diagnostic purposes.

3.4 | Blinded evaluation of patients with IPD and healthy controls (phase 2)

The final reports of each laboratory (randomly assigned number from 1 to 7) are summarized in Tables 4 and 5. Documenting microscopy photos are given in the Supplementary Figures 1–13. These pictures also provide an overview of the overall high reproducibility of platelet features among the different laboratories.

3.4.1 | Patients with confirmed IPD

Seven out of 7 participants (100%) correctly identified *MYH9*-related disease (*MYH9*-RD), Glanzmann thrombasthenia (GT), Bernard-

TABLE 3 Previously reported, morphologic features of the investigated inherited platelet disorders.

Disorder	Morphologic pattern
Quantitative δ -storage-pool disorder due to Hermansky-Pudlak syndrome	Reduced number of granule structures or altered expression (diffused or prevalent on the outer membrane vs intracellular expression) upon staining against dense granule markers LAMP-1, LAMP-2, and CD63.
MYH9-related disease	Platelet macrocytosis; inclusion bodies consisting of the cytoskeletal protein NMMIIA in the neutrophils' cytoplasm; diffused vs ring-shaped NMMIIA expression in platelets.
TUBB1-related disorder	Platelet macrocytosis; misdistribution (diffused, tangled, or wool ball-like vs ring-shaped expression) of the cytoskeletal protein β 1-tubulin in platelets.
Glanzmann Thrombasthenia	Reduced-to-absent expression of the platelet surface GP IIb/IIIa.
Bernard-Soulier syndrome	Platelet macrocytosis; reduced-to-absent expression of the platelet surface GP Ib/IX.
GATA1-related thrombocytopenia	Platelet macrocytosis; reduced expression of platelet alpha granule markers vWF, TS, and PS; aggregates consisting of NMMIIA in the erythrocytes.
GFI1B-related thrombocytopenia	Platelet macrocytosis; reduced expression of platelet alpha granule markers vWF, TS, and PS; expression of the stem-cell antigen CD34 on platelet surface; aggregates consisting of NMMIIA in the erythrocytes.
DIAPH1-related thrombocytopenia	Platelet macrocytosis; misdistribution of cytoskeletal makers (diffuse or tangled vs ring-shaped expression of β 1- and/or α -tubulin) in platelets; faint expression of CD34 on platelet surface.

GP, glycoprotein; LAMP, lysosome-associated membrane protein; NMMIIA, nonmuscular myosin IIA; PS, P-selectin; TS, thrombospondin-1; vWF, von Willebrand factor.

Soulier syndrome (BSS), and *GFI1B*-related thrombocytopenia (*GFI1B*-RT). Six out of 7 participants (86%) correctly identified *TUBB1*-related disorder (*TUBB1*-RD) and quantitative δ -storage-pool disorder due to Hermansky-Pudlak syndrome. Five out of 7

participants (71%) correctly predicted *GATA1*-RT. Despite not making the exact diagnosis because of the nonspecific alterations of platelet structures, 6 out of 7 participants (86%) reported the blood films of the *DIAPH1*-RT patient as pathologic and recognized altered expression of cytoskeletal markers (NMMIIA, α - and β -tubulin) and, in 2 cases, faint expression of CD34 on platelet surfaces.

One sample each of quantitative δ -storage-pool disorder, *GATA1*-RT, and *DIAPH1*-RT was reported as normal. In one case of *GATA1*-RT and 1 of *TUBB1*-RD, the formulated suspicion was *ITGA2*-related thrombocytopenia and *MYH9*-RD, respectively.

Note: The genetic analysis of subject 4 identified an additional heterozygous mutation in *GFI1B* beyond the *TUBB1* variant (Table 2). As the *GFI1B* variant was present in a family member without thrombocytopenia, a diagnosis of *TUBB1*-RD was made. Immunofluorescence revealed morphologic features indicative of pathogenicity for both variants (disrupted β -tubulin ring and faint CD34 expression), suggesting a joint pathogenic effect. For this interlaboratory exercise, in this sample, either *TUBB1*-RD or *GFI1B*-RT was accepted as a correct diagnostic prediction.

3.4.2 | Healthy controls

Among the 5 enrolled healthy controls, 2 were reported as normal by 6 out of 7 laboratories (86%), 2 were reported as normal by 5 out of 7 laboratories (71%), and 1 was reported as normal by 4 out of 7 laboratories (57%). When blood smears from healthy controls were interpreted as pathologic, the reported morphologic alterations concerned α - or δ -granule markers (partially diminished expression, 4 cases), surface glycoprotein (GP) IIb/IIIa (partially diminished expression, 2 cases), cytoskeletal protein β 1-tubulin or Filamin A (disarranged expression, 2 cases), and CD34 (weak platelet expression, 1 case).

3.5 | Diagnostic accuracy and interlaboratory agreement

Cohen's κ values ranged from 0.45 to 1.00 for individual laboratories versus true diagnosis, and from 0.049 to 1.00 across all 21 interlaboratory comparisons. Individual κ values with 95% CIs are provided in the [Supplementary Table 2](#). The overall mean κ value, integrating both components, was 0.532, indicating a moderate agreement [18]. These results support the reliability of immunofluorescence predictions despite minor discrepancies in specific cases.

3.6 | Estimation of diagnostic performance metrics

Although the limited sample size warrants caution in interpreting these statistical estimates, we report them to characterize the diagnostic performance of immunofluorescence microscopy in identifying phenotypic changes associated with the 8 IPD forms.

TABLE 4 Report of each participating laboratory upon immunofluorescence assessment of blood smears from patients with inherited platelet disorders (IPDs).

Participating laboratory	Reports							
	IPDs							
	Quantitative δ -SPD	MYH9-RD	DIAPH1-RT	TUBB1-RD	BSS	GATA1-RT	GFI1B-RT	GT
1	incorrect	correct	correct	correct	correct	incorrect	correct	correct
2	correct	correct	correct	correct	correct	correct	correct	correct
3	correct	correct	correct	correct	correct	correct	correct	correct
4	correct	correct	incorrect	correct	correct	correct	correct	correct
5	correct	correct	correct	correct	correct	correct	correct	correct
6	correct	correct	correct	correct	correct	correct	correct	correct
7	correct	correct	correct	correct	correct	correct	correct	correct

δ -SPD, δ storage-pool disorder; BSS, Bernard-Soulier syndrome; *DIAPH1*-RT, *DIAPH1*-related thrombocytopenia; *GATA1*-RT, *GATA1*-related thrombocytopenia; *GFI1B*-RT, *GFI1B*-related thrombocytopenia; GT, Glanzmann thrombasthenia; *MYH9*-RD, *MYH9*-related disease; *TUBB1*-RD, *TUBB1*-related disorder.

correct interpretation with prediction of the specific IPD.

correct interpretation without prediction of the specific IPD.

incorrect interpretation.

TABLE 5 Report of each participating laboratory upon immunofluorescence assessment of blood smears from healthy controls.

Participating laboratory	Reports				
	Healthy controls				
	Control 1	Control 2	Control 3	Control 4	Control 5
1	correct	incorrect	correct	correct	correct
2	correct	incorrect	incorrect	correct	incorrect
3	incorrect	correct	incorrect	correct	correct
4	correct	correct	incorrect	incorrect	correct
5	correct	correct	correct	incorrect	correct
6	correct	correct	correct	correct	correct
7	correct	correct	correct	correct	correct

correct interpretation.

incorrect interpretation.

The estimated sensitivity and specificity of the immunofluorescence assessment in recognizing IPD were 93.1% (95% CI, 86.5%-97.6%) and 73.6% (95% CI, 61.0%-85.0%), respectively. The corresponding negative and positive predictive values were 88.3% (95% CI, 77.1%-96.1%) and 83.0% (95% CI, 72.9%-91.1%), respectively. The Figure visually summarizes the distribution of true-positive, false-positive, true-negative, and false-negative results.

The NLR was 0.09 (95% CI, 0.03-0.19), and the PLR was 3.57 (95% CI, 2.35-6.24). Values of NLR < 0.1 and PLR > 5 are generally considered to provide strong evidence for ruling-out and ruling-in disease, respectively, supporting immunofluorescence as a valuable test for exclusion but not as conclusive for confirmation [16].

4 | DISCUSSION

This first multicenter exercise demonstrated the technical reproducibility of immunofluorescence staining of platelets on peripheral blood smears, even when implemented by some laboratories for the first time. The method provided reproducible results in predicting 7 forms of IPD exclusively based on microscopic findings. Notably, the correct diagnosis of 4 disorders—*MYH9*-RD, BSS, GT, and *GFI1B*-RT—was achieved unanimously by all participants. A correct diagnosis was also achieved by the vast majority of participants for 3 additional IPDs: quantitative δ -storage-pool disorder, *TUBB1*-RD, and *GATA1*-RT.

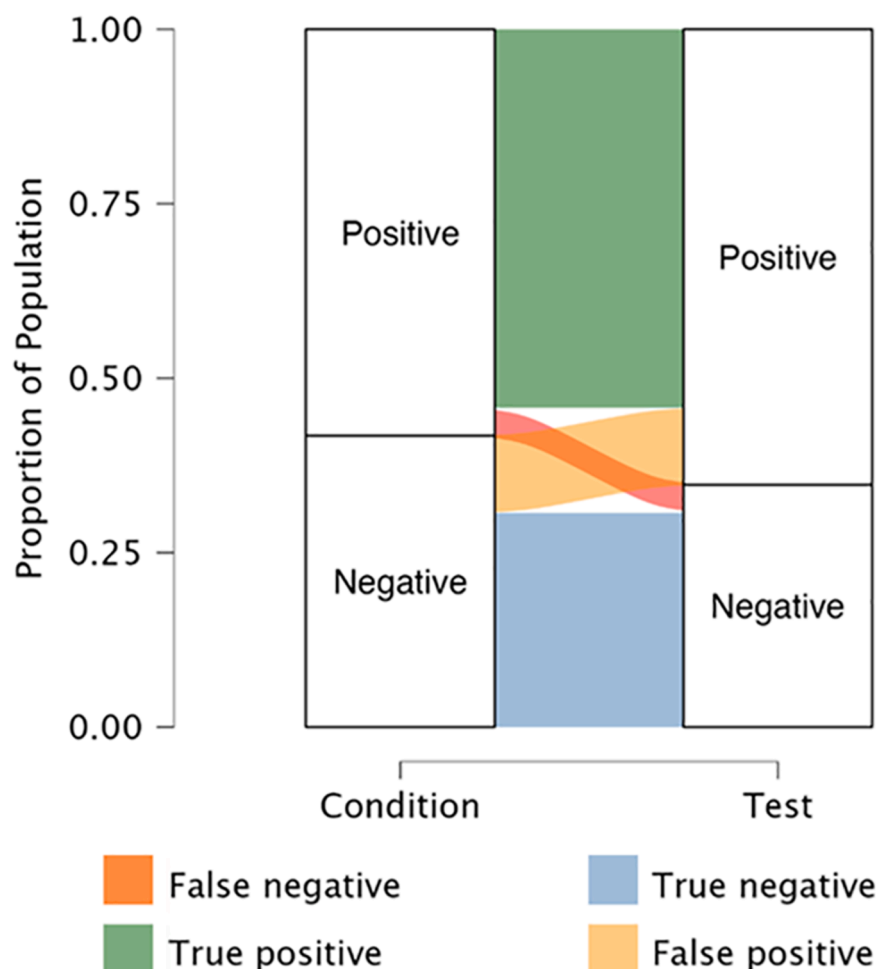


FIGURE Distribution of true/false-positive and negative results and their contribution to overall diagnostic performance. Flow diagram showing the proportion of patients with IPD and healthy controls in the study population (condition, left) and according to the diagnostic prediction (test, right). Prediction errors are highlighted as false negatives (orange) and false positives (yellow), with true positives (green) and true negatives (blue) indicating correct predictions.

These findings position immunofluorescence microscopy as a valuable tool in the diagnostic work-up of suspected IPD, offering excellent capability for ruling-out (NLR, 0.09) and supportive, albeit not definitive, evidence for ruling-in the condition (PLR, 3.57). Although positive results warrant confirmatory testing, possibly more expensive, negative results can prioritize or streamline the diagnostic work-up, especially in cases with low clinical suspicion.

This workshop also provided evidence of feasibility that laboratories lacking prior experience can implement this diagnostic protocol and achieve staining proficiency and key pattern recognition within weeks (typically 3-4 weeks of training according to participant feedback), provided they possess an appropriate microscope and the needed reagents, a comprehensive standard operating procedure, and remote support from an experienced reference center. For cases with strong clinical suspicion of specific disorders (eg, BSS, GT, MYH9-RD), using a reduced panel of antibodies (< 13) may suffice, thus lowering both antibody costs and procedure complexity. However, we strongly recommend always applying the entire panel of antibodies to obtain sufficient experience on the expression pattern of markers in different forms of IPD. In addition, as shown by subject 4, sometimes more than 1 mutation is present in an individual, which can only be recognized if the entire panel of antibodies is used. The

costs per patient under investigation are difficult to estimate, as they strongly depend on the number of samples assessed. If only a single patient is assessed, the costs for controls are the same as for assessing a batch of 10 patients. In addition, overall costs are substantially lower if the antibodies are used before expiration, compared with a scenario in which a substantial proportion of the antibodies must be discarded.

Compared with its effectiveness in identifying patients with IPD, immunofluorescence showed in this study lower accuracy in correctly recognizing healthy controls. The moderate tendency toward false-positive results can be at least partially attributed to the lack of expertise of some participants. In fact, it is a common experience that, in the initial stages of learning microscopic practices, there is a tendency to attribute pathologic significance even to minor alterations. The blinded design of the workshop, without providing any clinical information, represented a worst-case scenario for recognizing normal samples.

Due to the limited sample size (8 patients and 5 healthy controls) and the rarity of IPDs in the general population [8,33], diagnostic metrics should be interpreted with caution, as these factors erode statistical power, increase estimate uncertainty, and reduce generalizability. For these reasons, a prospective evaluation in subjects with suspected primary hemostasis disorders visiting outpatient

clinics is required before definitive diagnostic performance estimates can be made.

A strength of our study was that the diagnostic prediction was exclusively based on morphologic findings. This allows us to reasonably expect increased diagnostic efficacy when this method is used in a real diagnostic setting, with preselection of patients with suspected IPD based on clinical and family history and other laboratory tests (eg, platelet count). The use of different fluorescence microscopes further strengthens the generalizability of the findings. Possible limitations include i) the small number of disorders investigated in this workshop; ii) the use of 1 single patient for each disorder, not allowing consideration of possible interpatient variability in immunofluorescence findings; and iii) the interobserver variability inherent in manual microscopic assessment. Ongoing efforts toward immunofluorescence automatization, with the introduction of quantitative approaches and artificial intelligence-assisted strategies to limit artifacts due to epifluorescence assessment and operator subjectivity [34,35], represent promising steps forward to address these issues. Further larger cohort studies are needed to broaden the spectrum of disorders included in the differential diagnosis when evaluating suspected patients with IPD using immunofluorescence and to establish quantitative and qualitative thresholds to distinguish, objectively, between changes of likely or unlikely pathologic significance, ultimately improving the specificity of this approach.

5 | CONCLUSION

This first interlaboratory exercise confirmed the technical reproducibility of immunofluorescence microscopy on peripheral blood smears for evaluating platelet phenotype. The method showed high sensitivity for MYH9-RD, BSS, GT, and GFI1B-RD, and reasonable sensitivity for quantitative δ -storage-pool disorder, TUBB1-RD, and GATA1-RT.

Especially in children with suspected IPD, immunofluorescence analysis of blood smears offers major advantages. The method allows for sample shipment by regular mail with minimal risk of preanalytical errors and is affordable in low- and middle-income settings.

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AUTHOR CONTRIBUTIONS

C.Z. and A.G. conceived and designed the research; L.B., V.B., A.Z.C., K.A., G.U., S.B., A.N., D.F., A.S., C.F., S.S., G.L.B., T.B., A.P., P.G., T.T.,

C.Z., J.R., and A.G. performed the investigations and interpreted data. A.G., J.R., L.K., and S.O. enrolled patients. C.Z. wrote the manuscript. All authors critically reviewed and approved the final version of the manuscript.

DECLARATION OF COMPETING INTERESTS

The authors state that they have no conflicts of interest related to the work presented in this manuscript.

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SUPPLEMENTARY MATERIAL

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