Advances in the Diagnosis of Heritable Platelet Disorders

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1. Abstract

The last decade has seen large increases in the number of patients registered with heritable platelet disorders in national databases of bleeding disorders. Although individually rare, collectively they are a relatively common cause of heritable bleeding. This revolution has come about through the application of high-throughput sequencing strategies and efforts to standardize diagnostic testing. There is renewed interest in established parameters such as platelet volume and utilising simple tools such as blood smears. The diagnostic yield from peripheral blood smears can be improved with new microscopy techniques that could potentially assist in determining which patients need to be referred to tertiary centres for specialist testing. A better understanding of the other clinical features that can accompany abnormalities of platelet number or function, can lead to better clinical management and prevention of serious complications. There are challenges for clinicians who need to be aware of these developments, understand the limitations of new diagnostic techniques and keep abreast of strategies for incorporation into clinical practice. This review discusses some of these approaches, the limitations that clinicians need to be aware of and techniques that may enter clinical use in the future.

2. Introduction

Congenital abnormalities of platelets have previously been divided into platelet function disorders and heritable thrombocytopenias. While a few specific disorders, such as Glanzmann thrombasthenia (GT) and Bernard Soulier Syndrome (BSS), have characteristic diagnostic criteria that easily allow distinction from similar conditions, others have overlapping clinical and laboratory features. This makes separation into distinct disorders difficult and potentially academic for clinical management as bleeding phenotypes and therapeutic options tend to be similar. With a lack of universally agreed diagnostic criteria, terms such as 'weak agonist response defect' or 'storage pool disorder' may not necessarily define the same disease in different laboratories. They may not even do so within a laboratory as tests evolve over time. Clinical evaluation and the initial diagnostic tests used tend to be the same for all these conditions. A more useful strategy going forwards is to consider these disorders in a single overarching category with similar overlapping criteria, while maintaining sub-classification where appropriate. Recent guidelines from the International Society on Thrombosis and Haemostasis (ISTH) and The British Society for Haematology (BSH) have adopted this approach [1,2]. Heritable platelet dysfunctions and heritable thrombocytopenia are considered together under a single category of 'Heritable Platelet Disorder' (HPD). The same term is now used in the National Haemophilia Database in the UK and by other reviewers [3,4].

It has long been recognised that management of patients with rare diseases is hampered by a lack of awareness of clinical features amongst healthcare professionals and the general public, poor access to diagnostic tests and limited availability of specific treatments. National and international strategies have been brought in to improve the care of patients with rare disease [5–7]. In the last decade patients with HPD have benefited from improved diagnosis as a result of the advances implemented by these policies. The number of patients registered with a HPD in the UK database has risen from 1,195 in 2007 to 3,170 in 2019 [8]; an increase of 165%. To put this into context: during the same period registrations of von Willebrand disease increased by 8% and haemophilia 11%. HPDs collectively are now the fourth most common category in the database after von Willebrand disease, haemophilia A and factor XI deficiency. The purpose of this review is to describe the advances that have led to this increase and the effect this might have on clinical management going forwards.

3. Clinical Assessment

For any bleeding disorder systematic recording of the history of bleeding symptoms is essential. As with other mild, mucocutaneous bleeding disorders the history generally correlates better with clinical severity than laboratory abnormalities. Many HPDs are part of a syndrome that has clinical effects outside haemostasis that are not directly caused by abnormalities of platelet function or number.

Evaluating these non-haemostatic effects is another key component of the clinical assessment. Although this review does not cover acquired platelet abnormalities due to drugs or other medical conditions, these do need to be considered in the history and differential diagnosis.

3.1. Bleeding Assessment Tools

Modern bleeding assessment tools (BAT) were initially developed to quantify the extent of bleeding in a systematic fashion during studies of type 1 von Willebrand disease [9]. As this disorder has a similar bleeding phenotype to many HPDs, the ISTH has reviewed and evaluated their BAT for the diagnosis of HPD [2]. Higher BAT scores were associated with a greater risk of subsequent haemorrhage. While this might seem axiomatic, it reinforces the importance of early diagnosis for preventing subsequent bleeding. Clearly this is a lesson that still needs to be learnt judging by the length of time patients wait (several years in some cases) between onset of bleeding symptoms and diagnosis [10]. While BAT scores are useful for comparing the severity of clinical phenotype between individuals they do have some obvious shortcomings. There is a dependence on haemostatic challenges limiting utility in young men or boys and girls prior to menarche. The hope that these tools could be used in primary care and non-specialist clinics to determine which patients should be referred for specialist evaluation, has not yet been realised in practice. This may be because studies have generally used trained investigators to apply the BAT leading to a perception that they are less useful in non-expert hands and too time-consuming. However, there is evidence that completion by the patient or a parent prior to a clinic visit can be done quickly and with a reasonable sensitivity [11]. At the moment it seems unlikely that a BAT may be used to identify patients referred for investigation of a bleeding tendency that can be discharged without recourse to expensive and laborious platelet function analysis.

3.2. Non-haemostatic features of Heritable Platelet Disorders

A clinician managing patients with HPD is perfectly placed to identify related abnormalities outside haemostasis. Indeed it may be incumbent upon us to do so as preventative measures may then be applied. For example sitosterolemia due to variants in the *ABCG5* or *ABCG8* (ATP Binding Cassette subfamily G member 5 or 8) genes is associated with premature cardiovascular disease due to accumulation of plant sterols [12]. Presentation may be with isolated thrombocytopenia or in association with other haematological abnormalities such as mild haemolysis. This occurs well before any signs of heart disease. Recognition of the syndrome allows introduction of lipid-lowering medication that can prevent life-threatening arterial disease. Similarly *MYH9* (Myosin Heavy Chain 9)-associated thrombocytopenia, when previously referred to as May-Hegglin anomaly, was considered a relatively benign disorder. It is now recognised that variants in this gene are also the cause of the

much more serious conditions: Epstein, Fechtner and Sebastian syndromes, which are associated with renal impairment, deafness, liver enzyme abnormalities and presenile cataracts[13]. These features can be monitored for and preventative measures taken, such as the avoidance of nephrotoxic medication and ophthalmic screening. Simply joining these eponymous syndromes together under the umbrella of *MYH9*-associated thrombocytopenia is useful for preventing incorrect assumptions about clinical severity that occur when using the old terminology. However, a lack of correlation between genotype and phenotypic consequences previously hampered management [14]. This might result in some patients being advised to take preventative measures unnecessarily. International collaborative efforts are improving our understanding of specific variant effects and indicate, for example, that variants in the globular domain of this particular gene are more likely to be associated with renal impairment [15]. Further work on the genotype – phenotype correlation is needed before screening can be restricted to specific variants for this particular gene.

For other conditions genomic testing has been very useful for identifying patient groups with specific phenotypes. Through next generation sequencing studies we now have at least 11 sub-types of Hermansky-Pudlak syndrome (HPS). This has allowed identification of which subtypes (HPS 1, 2 and 4) are most likely to develop pulmonary fibrosis and might therefore benefit from screening and interventions such as smoking cessation [16]. Other subtypes do not require these interventions as they are not associated with pulmonary fibrosis. As they have the same presenting features of oculocutaneous albinism and platelet storage pool disease, subtype distinction and appropriate clinical management requires identification of the causative genetic variant.

Perhaps it is the heritable thrombocytopenias that are associated with an increased risk of haematological malignancies where intervention can be of greatest benefit. Through the efforts of international registries we already have a better understanding of the relative risk associated with variation in different genes. Leukaemia occurs in about 40% of cases with pathogenic variants in *RUNX1* (which codes for Runt-Related Transcription Factor 1)[17,18]. There is a lower risk associated with variants in *ETV6* (which codes for E26 transformation-specific Variant Transcription factor 6) of about 30%, while it is about 5% with variants in *ANKRD26* (which codes for Ankyrin Repeat Domain 26). It may be that the risk can be further defined to specific types of genetic alteration or variants affecting particular functional protein domains. Ultimately the expectation is that understanding of the underlying pathophysiology will lead to targeted and specific molecular therapies, as exemplified by the development of specific tyrosine kinase inhibitors after elucidation of the *BCR-ABL* (Breakpoint Cluster Region - Abelson Tyrosine-Protein Kinase 1) rearrangement in chronic myeloid leukaemia[19].

4. Laboratory Assays

There are many assays that can be used to assess platelet number, size, shape and function. This section focuses on some of the key assays and new developments.

4.1. Platelet Aggregometry

Light transmission platelet aggregometry (LTA) was described 60 years ago and remains the primary test for assessing platelet function [20]. This is despite the test being laborious, prone to interference from pre-analytical variables and restricted to specialist laboratories. In some ways it is surprising that an assay with these limitations has stood the test of time. Tests such as the platelet function analyser (PFA) were developed to overcome these limitations but have been unable to supplant LTA, or even indicate which patients need to go on to further testing, because of insufficient sensitivity for detecting mild HPD [21] Standardisation of LTA components, such as how to prepare platelet-rich plasma and which agonists and concentrations should be included, has been difficult to achieve, perhaps because no one protocol is demonstrably superior to another [22,23]. Automation has helped to make the test more reproducible when comparing results from different laboratories [24]. However, one of the intrinsic limitations of using light transmission as the indicator of platelet aggregation is that red and white cells have to be removed. Although this has the advantage of allowing platelets to be tested without interference from other blood components, it has the disadvantage that interactions with these components do have a role in physiological haemostasis. Impedance-based methods overcome this limitation and allow platelet aggregation to be assessed in whole blood. This also means that the premature platelet activation that can occur during the preparation of platelet-rich plasma shouldn't be an issue. Comparison of these two methods has shown that mild abnormalities are sometimes detected in one assay but not the other [25]. This should not be surprising as the techniques are fundamentally different. Perhaps attempts to show that one technique is superior to the other should give way to an acceptance that they provide different information about platelet aggregation. One could consider them to be complimentary in which case use of both together would be appropriate. Of course this adds to the cost of testing and, as there is overlap in results particularly for more severe HPDs, is not an approach that many clinical labs would take. Thus laboratories are choosing either LTA or impedance-based aggregometry resulting in greater heterogeneity in how aggregometry is measured.

Variability in techniques used to assess platelet aggregometry is compounded by a lack of uniformity in how results should be reported. The most basic reporting simply provides the percentage aggregation as a maximum value obtained or at a specific time point. While a numerical value is easy to enter into pathology reporting systems, it does not convey the phases, lag time or overall shape of the aggregation trace. Descriptive reports can achieve this but are difficult to standardise. External

quality assurance for platelet aggregometry is generally not feasible because of the relatively short time available between sampling and testing. Freezing of plasma is used for quality assurance of other coagulation assays but isn't suitable for platelets because they lyse. Internal quality assurance and occasional sample sharing between geographically close laboratories are the main suggested options [26]. Consensus-based guidelines for interpreting platelet aggregometry are likely to be the most effective way of standardising reporting [27].

4.2. Platelet count and volume

Platelet count and volume are inversely correlated [28]. As platelet function is dependent on the overall mass of platelets in the body, reliance on platelet count alone can be misleading in some situations. Reporting of the mean platelet volume alongside the platelet count may then provide a better association of platelet parameters with disease [29,30]. Despite this being a standard measurement in automated analysers, it is underutilised in clinical practice. Perhaps lack of standardisation is one of the reasons [31,32].

The gold standard method for counting platelets is flow cytometry using antibodies against platelet glycoprotein receptors but is seldom used in clinical laboratories because of cost [33]. The most frequent method for automated platelet counting uses electrical impedance. This gives highly accurate results with normal platelets. However, impedance changes when the platelet volume and/or shape is not normal. This occurs frequently with heritable thrombocytopenias when impedance counting can miss a significant proportion of abnormal platelets. Optical counting based on light scatter may be more accurate but is also likely to miss larger or abnormally shaped platelets [34]. Counting using fluorescent dye staining of platelet RNA may be the most accurate but further comparative studies in patients with these disorders are needed. The problem is demonstrated by the platelet size distribution plots obtained by impedance counting shown in figure 1. In both the control and immune thrombocytopenia plots the distribution has a normal bell-shaped pattern with a single peak. The plots from patients with Gray Platelet Syndrome and *MYH9*-associated thrombocytopenia show multiple peaks with giant platelets intruding into the red cell window above 40 fL. Most of these would not be automatically counted as platelets. The pattern is very easily distinguishable from normal and these plots could be a useful addition to the diagnostic toolbox. As the platelet count in heritable thrombocytopenias often does not correlate well with bleeding, these inaccuracies may not affect diagnosis. However, for invasive procedures a specific threshold for the platelet count may be required, often with little or no supporting evidence even for thrombocytopenias with morphologically normal platelets. This results in patients with heritable thrombocytopenias receiving unnecessary platelet transfusions. Unfortunately, clinicians carrying out invasive procedures seem to

be fixated on platelet count when simply taking an accurate bleeding history gives a much better prediction of bleeding risk.

4.3. Platelet Morphology

The incessant drive towards laboratory automation has meant that characteristics that cannot be easily numericalised become marginalized. Morphology is one such art. In fact the humble blood smear can tell us a great deal such as when impedance-based counting is likely to be less accurate. With application of antibodies against eight key structures or proteins, immunofluorescence microscopy on peripheral smears shipped to specialists centres can produce a similar diagnostic yield to that from conventional platelet function testing [35]. This approach is most sensitive to structural abnormalities and may not detect signalling defects.

Electron microscopy provides the greatest detail of platelet morphology. However, despite first being described more than 50 years ago, it remains inaccessible in routine clinical practice. Super-resolution light microscopy can provide similar levels of detail and, in combination with image capture software, can provide automated counting of platelet structures relatively easily [36]. This technique has only been applied to dense granule defects so far but is only limited by the availability of fluorescently-labelled antibodies specific for distinct platelet structures (figure 2). Unlike platelet function testing a major advantage of peripheral blood smears is that the patient can have blood collected locally and the slide sent by ordinary post to the testing facility at a later date. Further studies evaluating these techniques in other structural platelet disorders are required before this can be rolled out for clinical use.

5. Genomic Testing

Understanding the genetic basis of heritable disease is essential for the development of new treatments targeted for specific disorders and ultimately, individualised for particular patients. The benefits of genetic testing have been realised for the haemophilias. Since the discovery of the *F8* gene in 1984 [37] and *F9* in 1985 [38], there has been a steady accumulation of genetic knowledge harnessed in freely available variant databases [39]. Without this hard graft we would not have gene therapies and recombinant molecules that have revolutionised the management of these otherwise fatal conditions. Single gene sequencing based on polymerase chain reaction (PCR) amplification, has proved adequate, if slow, for the elucidation of monogenic diseases. However, different technologies were required for the testing of HPDs where tens or hundreds of genes may harbour pathogenic variants. These technologies were developed during the Human Genome Project and have now resulted in high-throughput sequencing technology becoming available in routine clinical practice

[40]. Clinical practice has had to adapt to keep up with these rapid developments and an important consideration is appreciation of which genes have established links with haemostatic disease and which are still under research. The Genomics in Thrombosis and Haemostasis Subcommittee of the ISTH maintains a list of the genes that should be included in genomic testing of haemostatic disorders [41]. At the time of writing the list contains 103 genes of which 69 cause HPD, easily the largest category. Each year the committee considers evidence for adding new genes to the list with the result that testing laboratories need to incorporate annual updates into the panels offered for clinical use. Ensuring that panel versions and dates are printed on reports is therefore essential, particularly for negative reports and those with variants of uncertain significance. In these cases retesting may well be required when new versions of a panel with additional genes that may be causative of the phenotype under investigation. For the same reason it is important that the phenotype is accurately captured and included in the information provided to the laboratory at the time of the initial test request. Deep phenotype capture and categorisation were an essential component of genomic research studies in haemostasis but this has not transitioned into clinical practice [42,43].

5.1. Limitations of Genomic testing

The technological advances of high-throughput sequencing have made identification of causative variants feasible for all monogenic coagulation factor deficiencies. Although discovery of new genes causing HPD is the largest growth area in haemostasis genetics, testing of HPD samples does not currently identify a pathogenic variant in many cases. With multi-gene panels a pathogenic or likely pathogenic variant is identified in 20-50% of cases of suspected heritable thrombocytopenia [44]. This figure drops to 10-20% for platelet function disorders. In contrast it is well above 90% for coagulation factor deficiencies. Variant detection is not the main problem, rather it is the relatively high proportion of Variants of Uncertain Significance (VUS) as shown in figure 3 [45]. This poor rate of return is partly explained by less discriminatory testing during research studies which may have included some acquired causes of thrombocytopenia or platelet dysfunction. More careful selection of patients with attention paid to family history, medication and the presence of multi-system clinical features, will result in these percentages increasing. Nevertheless it does demonstrate that for some common categories of HPD, such as non-syndromic storage pool disorder or platelet signalling defects, the genetic causes are not well characterised. This is partly because there are still plenty of candidate platelet genes that need investigation. The UK Genotyping and Phenotyping of Platelets study included more than 300 genes in the panel of which the vast majority are still research grade [46]. The lesson from haemophilia genetics is that systematic genotyping of all cases with collation of results in international databases is the most effective way of defining pathogenicity. With developments in

bioinformatics we can be confident that this will not take the 40 years that were required after discovery of the *F8* and *F9* genes.

An important limitation of high-throughput sequencing techniques is that sensitivity differs depending on variant type. The sensitivity for single nucleotides variants or indels up to 50 base pairs in coding regions, is greater than 99%. Copy number variants were not detected by most of the early high-throughput sequencing platforms and required specific assays such as Multiplex Ligation-dependent Probe Amplification. More recently read depth analysis has been incorporated into next generation sequencing platforms to assess this issue [47]. Sensitivity remains poor for variants in the non-coding space and complex rearrangements are not detectable by high-throughput sequencing platforms in clinical use today. This is not a problem for previously discovered rearrangements, such as the inversions causing 40% of severe haemophilia A, because tailored PCRs were developed for their detection years ago [48]. As the screening technique that was originally used to discover these inversions, Southern Blotting, falls into disuse without an obvious replacement, it is difficult to see how novel rearrangements will be discovered in clinical practice today. Awareness of these limitations of genomic testing is not widespread amongst clinical requestors.

There are new techniques in development that may overcome some of these limitations. High-throughput sequencing (second generation if Sanger sequencing is considered first generation) relies upon analysing vast short sequences in parallel, while third generation techniques read single DNA molecules at a time. The advantage is that this results in much longer read lengths of hundreds of kilo-base pairs compared with the few hundred base pairs achieved by second generation methods [49]. This may be extendable to tera-base pairs in the future. Such long reads overcome problems caused by repetitive regions, pseudogenes and rearrangements. Some of the platforms have a very small footprint, making point-of-care testing in remote, resource-poor environments a possibility. The main drawback at the moment is the relatively high error rate of 3-15% which is unacceptable for heritable diseases mostly caused by single nucleotide variation.

Guidelines for the use of genomic testing initially focused on laboratory methods rather than clinical practice. There are now national and international guidelines on how to obtain informed consent and convey the results of genomic reports to patients for frontline clinicians [50,51].

6. Future Considerations

We need new assays for assessing platelet function that overcome some of the limitations discussed above. Techniques that assess platelet biomechanics can be useful in demonstrating the effects of structural abnormalities [52,53]. The advent of whole blood aggregometry has demonstrated the

importance of considering platelet function in relation to other blood components. Going forwards we also need to take account of the interaction with blood vessels and harness techniques that consider platelets in the microfluidic parameters of normal physiological and pathological environments [54]. While advances in therapeutics are outside the remit of this review, there are already promising new treatments for HPD. Molecules that target specific platelet receptors offer one possible approach towards precision medicine [55].

Practice guidelines by their nature focus on the utility of assays for making a diagnosis. This may give the impression that following the procedures and recommendations set-out inevitably leads to a diagnosis. Unfortunately we have to acknowledge that, even with the full gamut of tests that we now have available, we are still sometimes unable to distinguish heritable from acquired platelet disorders. Even when we do make the distinction, we are often left with vague diagnoses like 'platelet dysfunction' that tell us little about underlying pathological mechanisms. We have made great strides in the diagnosis of HPD in the last decade but there is still a long way to go before we can claim mastery.

7. Practice Points

- HPDs have become the fourth most common category of heritable bleeding disorders in national registries.
- Detection of HPDs involves a variety of specialist laboratory techniques that generally require the patient to attend tertiary centres. Point-of-care testing is not sensitive enough to indicate which patients require referral.
- Diagnostic yield, in terms of the detection of pathogenic variants from genomic testing, is relatively low for HPDs compared with coagulation factor deficiencies.
- Recently updated guidelines can assist with standardisation of testing methodology.
- A significant proportion of HPDs remain uncharacterizable at the molecular level.

8. Research Agenda

- Techniques that assess platelet function under conditions that mimic normal physiological and pathological interactions are under development.
- New approaches for utilising blood smears provide details of platelet morphology and structure that can be developed for clinical use.
- 'Big data' approaches that correlate genotype with phenotype in international collaborative databases will lead to improved understanding of the molecular basis of HPD and pave the way for targeted therapies.

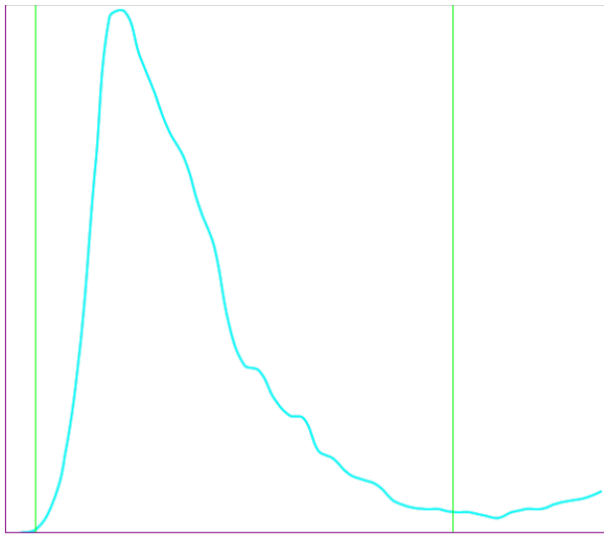
- Third generation sequencing strategies should be able to overcome some of the limitations of current genomic testing platforms but the error rate requires marked improvement.

9. Disclosure

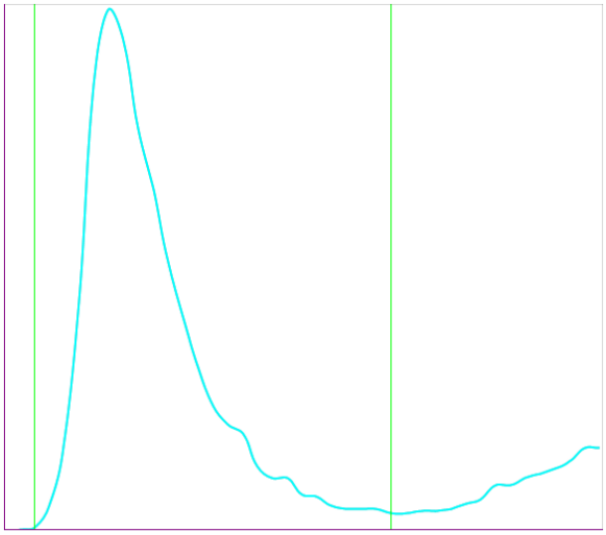
None.

Figure 1. Platelet size distribution plots generated by impedance counting in a Sysmex XN10. Platelet volume is on the X-axis and number on the Y-axis. Green vertical lines represent lower and upper discriminators of the platelet window.

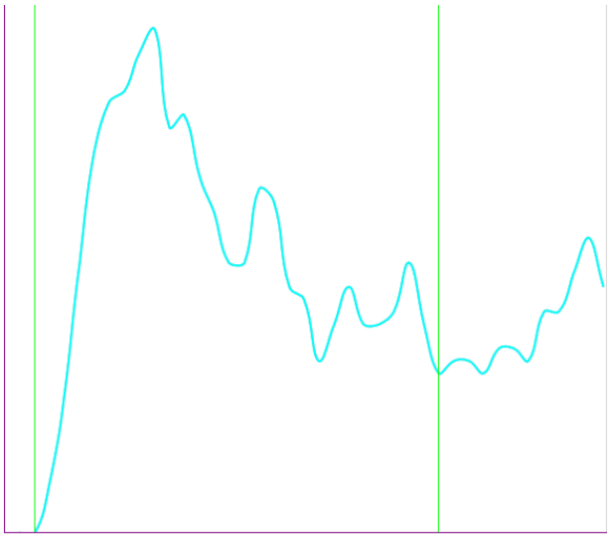
A) Control



B) Immune Thrombocytopenia



C) Gray Platelet Syndrome



D) *MYH9*-Associated thrombocytopenia

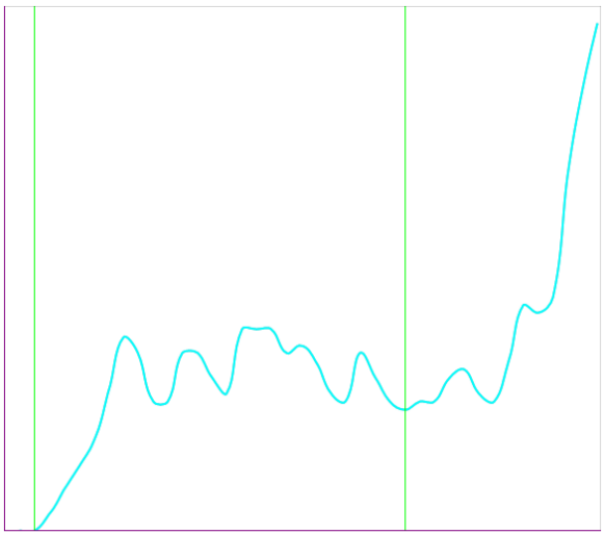


Figure 2. Imaging platelets with Structured Illumination Microscopy (SIM). The platelet perimeter is labelled with anti-tubulin (red) and dense granules are labelled with anti-CD63 (green). Scale bar = 1 µm. A) The same platelet imaged by SIM (lower right) has much higher resolution than with conventional diffraction-limited widefield microscopy (upper left). B) Control platelet with normal number of dense granules. C) Hermansky-Pudlak syndrome platelet with far fewer dense granules [36].

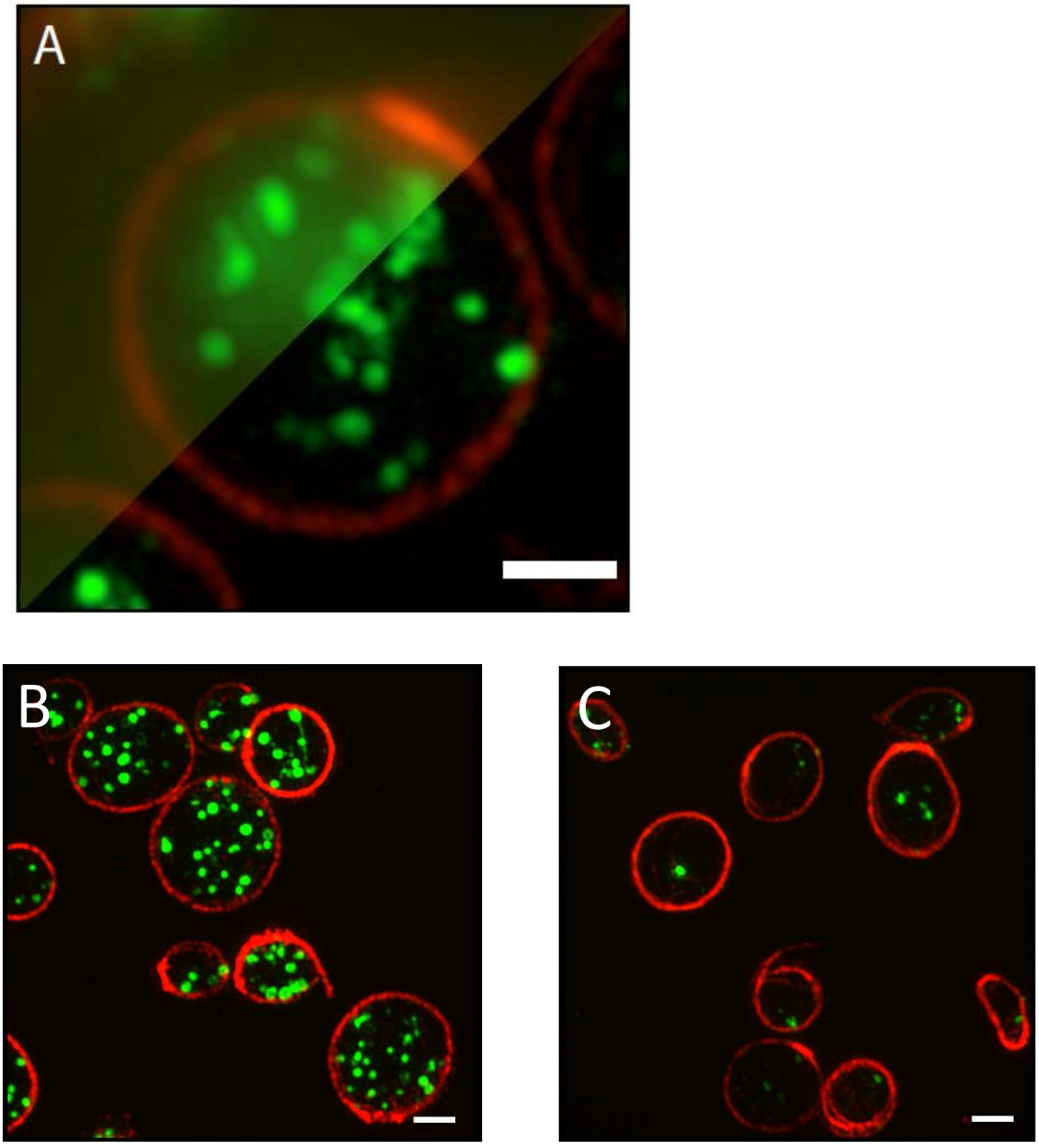
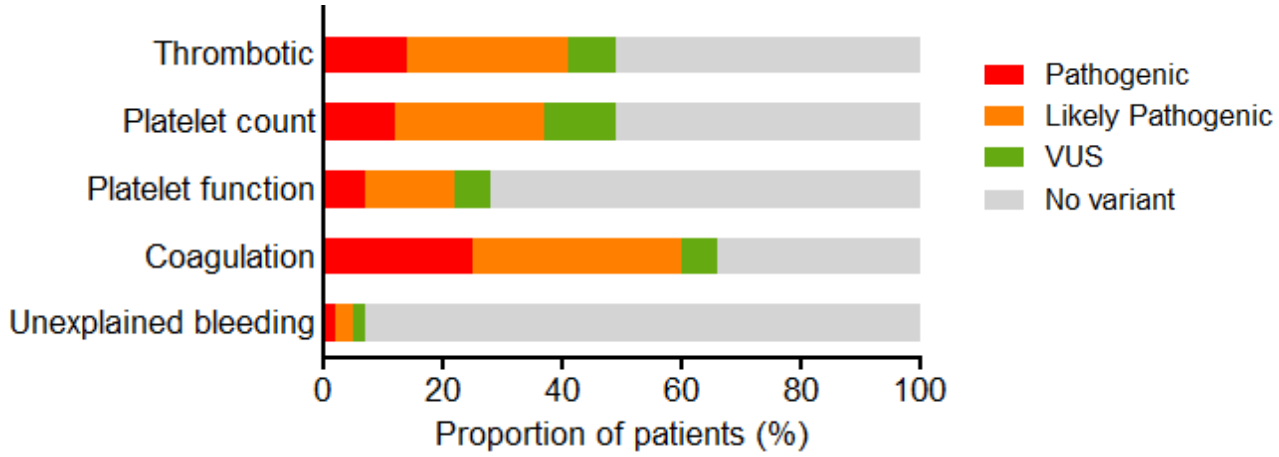


Figure 3. Diagnostic yield from 2,394 patients with bleeding, platelet and thrombotic disorders submitted to the ThromboGenomics study. Variant pathogenicity classification is shown by colour according to the legend. Fewer variants are found in patients with abnormalities of platelet count or function than those with coagulation factor deficiency and the proportion of Variants of Uncertain Significance (VUS) is higher [45].



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