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CASE REPORT

Reduced dense granules in platelet by electron microscopy in a patient with abnormal platelet aggregation with ADP and arachidonic acid: A case report of delta storage pool disorder

Nurasyikin YUSOF[1,2], Rabeya YOUSUF[2], Nur Izyan OTHMAN[2], Suria ABDUL AZIZ[1,2], Lailatul Hadziyah MOHD PAUZY[2], Dayang Anita ABDUL AZIZ[3]

[1]Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Kuala Lumpur, Malaysia; [2]Department of Diagnostic Laboratory Services, Hospital Canselor Tuanku Muhriz, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, Kuala Lumpur, Malaysia, [3]Department of Surgery, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Kuala Lumpur, Malaysia.

Abstract

Introduction: Delta storage pool disease (δ-SPD) is a platelet function disorder due to the decreased number and contents of dense granules causing bleeding symptoms. Diagnosis of δ-SPD is a complex procedure due to the variability of test results in platelet aggregometry and also it requires specialised tests. Electron microscopy (EM) is a promising tool to help in the diagnosis of this disorder. We report here a rare case of δ-SPD confirmed by EM. *Case report:* A 42-year-old lady presented with prolonged bleeding history from a leech bite for 3 days. She also has a history of bleeding of variable severity for more than 20 years. On presentation, blood was oozing from the bite mark on her right wrist and there were multiple small bruises over her lower limbs. Full blood count, peripheral blood smear, coagulation profile, factor VIII assay, factor IX assay, von Willebrand Factor antigen and activity, bleeding time, and clot retraction test were normal. Platelet aggregation tests showed poor aggregation with ADP with a lag phase >60 seconds with arachidonic acid. There was poor ATP release reaction with ADP and arachidonic acid suggesting a storage defect. Subsequently, the EM of the platelets was performed and showed reduced dense granules indicating delta storage pool deficiency (δ-SPD). She was counselled about her diet and medication which seems to control her symptoms. *Conclusions:* This case report highlights rare δ-SPD confirmed by EM. Diagnosis of this disorder is crucial in managing the patient. Highly specialised tests including platelet aggregometry, EM and molecular analysis are helpful in diagnosing this rare SPD.

Keywords: Platelet function defect, electron microscopy, storage pool deficiency, platelet aggregometry, dense granules

INTRODUCTION

Platelets are small anucleated blood cells, crucial for maintaining normal haemostasis and to stop bleeding in vascular injury.[1] During the primary haemostatic plug formation, platelets undergo four steps; adhesion, aggregation, contraction and secretion. Upon damage to the vascular wall with exposure to collagen, platelets adhere to these substances, through the von Willebrand factor. Following that, aggregation is mediated by the binding of fibrinogen, or other adhesive proteins, to GP IIb-IIIa complexes of platelets. Subsequently, platelets contract and change their shape and leading to the secretion of their granular contents which further potentiates aggregation.[2] The defect at any stage of these biological functions of platelets can give rise to platelet function disorder. The inherited platelet function disorders are rare diseases causing bleeding syndromes of autosomal

**Address for correspondence*: Associate Professor Dr Nurasyikin Yusof, Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaakob Latiff, Bandar Tun Razak, Cheras, 56000 Kuala Lumpur. Tel: 603-91455373 Email: *drsyikin@hctm.ukm.edu.my*

dominant, recessive or X-linked inheritance in patients. It ranges from low circulating platelets to changes in platelet morphology, function, or a combination of both with altered megakaryopoiesis and a defective platelet response resulting in blood platelets failing to fulfil their haemostatic function.[3]

One of the diseases that causes platelet dysfunction is storage pool disease (SPD) which involves a range of disorders due to decreased number and contents of alpha granules (α-granules), dense granules (δ-granules) or both.[4] Alpha-SPD is characterised by severe reduction or absence of α-granules in the platelet and associated with some disorders such as Gray Platelet Syndrome, Paris–Trousseau or Jacobsen syndrome, Quebec platelet syndrome or arthrogryposis-renal dysfunction cholestasis (ARC). Delta-SPD (δ-SPD) is a rare autosomal dominant disease characterised by a reduction in dense granules and their specific constituents of calcium, ADP, ATP, pyrophosphate and serotonin within platelets which are required for platelet aggregation.[5] The combined deficiency of α-granules and δ-granules is known as αδ-SPD (e.g. X-linked thrombocytopenia, Wiskott-Aldrich syndrome).[4,5]

The δ-SPD are classified into (1) congenital diseases due to genetic origin, or syndromic which are associated with albinism, ophthalmologic impairment, and immune deficiency such as Hermansky–Pudlak, Chediak–Higashi, and Griscelli syndromes; (2) acquired forms, most often associated with haematological malignancies such as myeloproliferative syndrome, acute leukaemia, or myelodysplastic syndromes or with auto-immune disease such as systemic lupus erythematosus, Sjogren's syndrome etc.[1] The δ–SPD is characterised by a bleeding diathesis of variable degree ranging from subcutaneous and mucosal bleeding to menorrhagia, gastrointestinal, post-operative or post-trauma bleeding. It depends on the relative degree of residual platelet function.[5]

Accurate diagnosis is necessary in order to provide the proper clinical management of the patient of bleeding disorder, to have an estimate of epidemiology of the disease and to obtain a deeper insight of the platelet function.[6] Diagnostic evaluation of platelet dysfunction is a challenge as it requires a number of diagnostic test and moreover there is lack of consensus on a standardisation of the diagnostic approaches and diagnostic criteria for inherited platelet function disorder.[6] Accurate bleeding history including family history of bleeding diathesis is a better way to screen patient for bleeding disorder.[7] Besides, full physical examination also must be done to assess the severity of bleeding with attention to any recognised syndrome or other diseases that are associated with platelet function disorder.[2] Initial workup consists of full blood count (FBC), peripheral blood film (PBF), and coagulation profiles i.e prothrombin time (PT) and activated partial thromboplastin time (aPTT). The other screening tests includes vWF antigen and activity to exclude vWD, fibrinogen level to exclude the hypofibrinogenemia or afibrinogenemia and thrombin time.[7] Platelet aggregation with agonist ADP, collagen, arachidonic acid, thrombin, epinephrine and ristocetin by using light transmission aggregometry (LTA) is the most valuable functional assays for assessing platelet function. This is the first step of screening of bleeding disorder.[7]

The diagnosis of δ-SPD is a complex procedure due to variability in the results from aggregation study and the requirement for specialised techniques such as flow cytometry, lumiaggregometry, sophisticated techniques of electron microscopy and molecular genetic analyses. Here, we report a rare case of δ-SPD presented with recurrent bruises and prolonged bleeding which showed variable results with aggregometry and later diagnosed as δ-SPD by electron microscopic examination. The case is a good example to illustrate the use of electron microscope (EM) in demonstrating δ-SPD.

CASE REPORT

A 42-year-old Malay lady presented with continuous blood oozing for two days from a leech bite. She noticed a huge leech was feasting on the ulnar-side of her right wrist when she went for a walk in a forest. She removed the leech with force and compressed the small bite mark immediately for ten minutes. However, the oozing of blood continued and she put a circumferential dressing around the affected area, but that did not help either. The dressing was not soaked but it needed to be changed repeatedly. On presentation at the emergency department on the 3rd day of the leech bite, her physical examination revealed normal vital signs. She had a small circular bite mark

on the ulnar-side of her right wrist measuring about 1cm × 1cm and still oozing a small amount of blood. There were also a few bruises over her legs, overlying the calves. However, she could not recall any history of trauma to the area.

On further questioning, a-long-standing history of bleeding tendencies since the age of 26 was revealed. She had episodes of menorrhagia and epistaxis for unknown causes. She had a history of recurrent unexplained haemarthrosis of the right knee requiring needle aspiration, recurrent subcutaneous haematoma of the lower limb and also a history of recurrent gross haematuria. She also had spontaneous bruising over the eyelid and haematoma outside the right carotid sheath which was confirmed by ultrasound and MRI. She noted that most of the major events were preceded by fever with chills. She had no family history of any bleeding disorder. Her significant bleeding episodes were treated with an antifibrinolytic agent. Despite previous years of abnormal bleeding history, she has not sought any medical help for the investigation of the cause of her bleeding disorder.

Initial workup revealed no abnormalities in the FBC and PBF. Her haemoglobin was 13.1 g/dL, normochromic normocytic RBCs, and total WBC count was 6.6×10^9/L with mild thrombocytopenia of 130×10^9/L. The prothrombin time (PT) was 13.1 seconds and activated partial thromboplastin time (aPTT) was 34.9 seconds. Factor VIII level was 144%, factor IX was 162%, von Willebrand factor (vWF) antigen was 115.6% and Collagen Binding activity was 106.5%, all within normal limits. Bleeding time (BT) and clot retraction test was also normal. In order to rule out autoimmune diseases, she was also fully investigated for complement C3, C4, antinuclear antibody (ANA), rheumatoid factor (RF) and the results were all negative.

She was subjected to a platelet aggregation test which was repeated four times since her presentation at our centre within four years. The agonists used were ADP, arachidonic acid, ristocetin, collagen and thrombin. Initial two platelet aggregation tests using impedance method showed reduced platelet aggregation with ADP of different strengths (5uM, 10uM, and 20uM) while it was normal with other agonists i,e arachidonic acid, ristocetin, collagen and thrombin. The 3rd and 4th aggregation tests were done using light transmission aggregometry (LTA) revealed no aggregation with ADP 10 uM. A lag phase >60 seconds with arachidonic acid 0.5 µM and poor ATP release reaction with ADP and arachidonic acid was noted in comparison with control (Figure-1). There was normal aggregation with ristocetin 1mg/mL (Figure-2) and normal aggregation with Collagen 5µg/mL and ATP release reaction with Thrombin 1 unit (Figure 3). The MYH9 immunofluorescent test

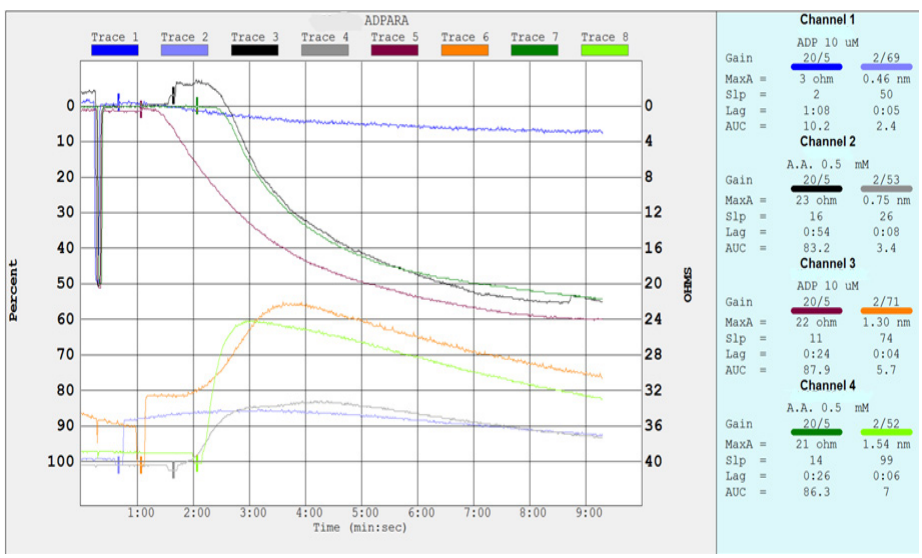

FIG 1: Platelet aggregation test showing no aggregation with ADP 10 µM (dark blue line) vs control (red line) and a lag phase >60 s with arachidonic acid 0.5 µM (black line) vs control (dark green line). There was poor ATP release reaction with both ADP 10 µM (light blue line) and Arachidonic acid 0.5mM (grey line) suggesting a storage or secretion defect.

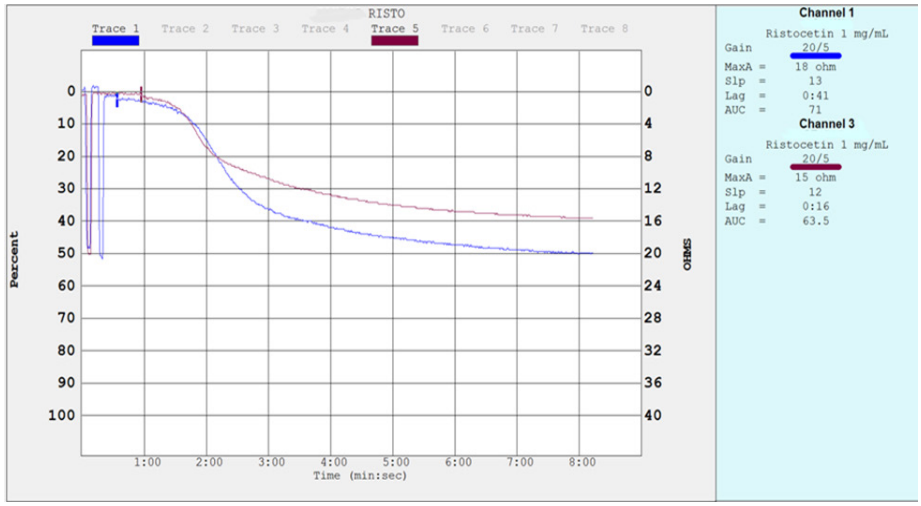

FIG 2. Platelet aggregation test showing normal aggregation with Ristocetin 1 mg/mL (patient-dark blue line, control-red line).

was done to look at the abnormal non-muscle myosin heavy chain-IIA (NMMHC-IIA) which was negative. This test was done as a screening tool to detect MYH9-related platelet disorder by looking at abnormal inclusions that are present in the neutrophils.

Subsequently, an EM examination was performed with both the whole mount and ultra-sectioning methods using transmission electron microscopy (TEM) and revealed a reduced number of dense bodies in comparison to the control (Figure 4). The mean dense granule content of 20 platelets was determined in duplicate and reported as either normal or reduced according to the method and reference range derived from 50 healthy controls based on previous study.[8,9] Therefore, she was confirmed to have δ-SPD.

After the diagnosis was made, she was counselled to take care of her diet as a number of potential food and drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), dark chocolate, caffeine, large numbers of garlic, ginseng and ginger can interfere with platelet function. She was on oral contraceptive pills (OCP) for her menorrhagia as suggested by the gynaecologist. She had strategised herself to take anti-pyretic

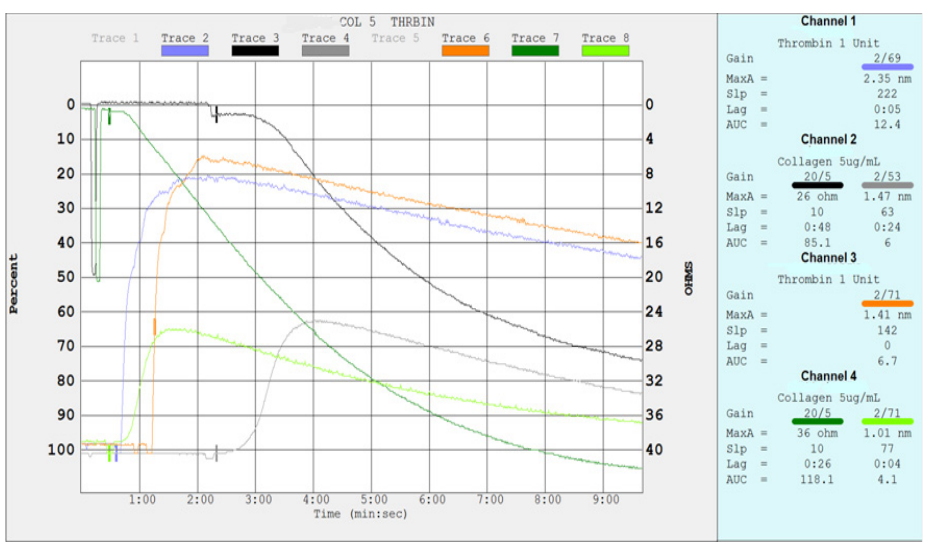

FIG 3. Platelet aggregation test showing normal aggregation with Collagen 5 μg/mL and ATP release reaction with Thrombin 1 unit.

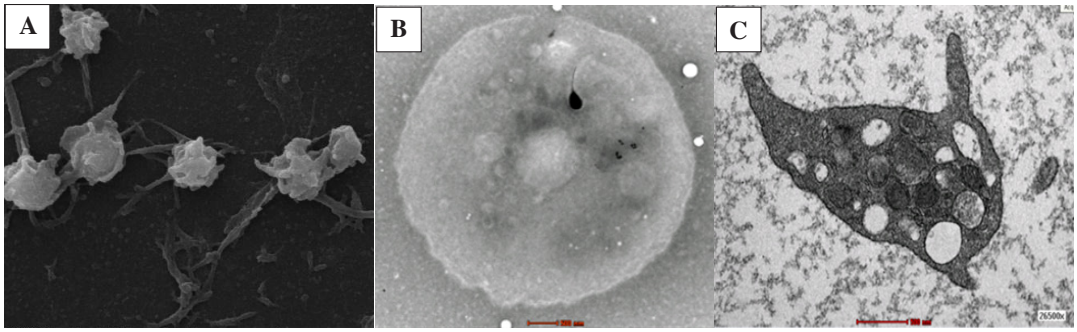

FIG 4. Electron micrograph showing the normal rugose surface of the patient (A). (B) and (C) showed the dense granule is reduced in the whole mount and ultra sectioning TEM preparation in the patient's platelet.

drug (paracetamol) early when she had a fever for unknown reason. She has been on regular follow-up for the last seven years. Currently, although she is still experiencing intermittent bruises and haematoma at various sites in her body, there have not been any major bleeding episodes. She is peri-menopausal and has not had any menorrhagia anymore. Lifestyle and diet control seem to be able to control her symptoms.

DISCUSSION

In this present case, the patient presented with a variable degree of bleeding severity from an earlier age, although she had no known medical illness, no family history of bleeding disorder and no history of intake of herbal medicine or supplements that can affect the platelet function. Though initially she was not investigated thoroughly, recurrent bleeding warranted her to seek further investigation and medical treatment and all initial screening results for bleeding disorder were revealed as normal.

It is mentioned that patients with δ-SPD often shows variable results[10] even normal aggregation was reported in responses to agonists ADP, epinephrine, and collagen by LTA.[11] Milder form of platelet defect may show normal LTA results, thus unable to exclude platelet function disorders.[12] This happens to the patient in this present case report where the platelet aggregation test results were variable with different agonists. Poor aggregation was noted with different strengths of ADP in all four-aggregation studies, however, other agonists showed normal aggregation in the first two aggregation studies. In her last two results of LTA, poor aggregation with ADP and a lag time of >60 seconds with low dose arachidonic acid together with poor ATP release reaction with ADP and arachidonic acid was noted which are suggestive of poor ADP receptor activity, could be due to SPD. Agonist ADP binds to the ADP receptor on the surface of platelets and causes the release of intracellular calcium and change the shape of platelets leading to the primary wave of aggregation. The subsequent secondary wave reflects the release of ADP from platelet storage granules. In δ–SPD, platelet aggregation studies typically show a significantly impaired second wave of aggregation when stimulated by ADP, or thrombin due to a low content of dense bodies.[7,11] However, in this present case report, although ADP showed impaired aggregation, thrombin showed normal aggregation.

Despite, LTA is considered a gold standard test in diagnosing platelet function disorder, there are some limitations regarding LTA results. It still cannot accurately mimic the whole physiological process such as platelet adhesion, activation and aggregation in the formation of primary platelet plug. This is because separated platelets in platelet-rich plasma (PRP) only form aggregates following the addition of agonists under low shear conditions of stirring. Also, LTA does not give a true picture of structural malformation of platelet.[13] Furthermore, there is also lack of standardisation because of variability in methodology. Even though, a panel of different agonists has been introduced, including arachidonic acid, epinephrine, adenosine diphosphate (ADP), collagen and ristocetin, the concentration of the agonists used for testing differ widely and lack guidelines on how laboratories should perform the platelet function test.[12]

Subsequent test that can be applied for the assessment and diagnosis of δ-SPD may include the ATP released from dense granules during platelet activation by bioluminescent

assay. Similarly, nnucleotides determination and serotonin determination by high performance liquid chromatography, serotonin uptake and release by radio-labelled method can be used. Lumi-Aggregometry is the fastest and widely used technique where simultaneously platelet aggregation by light transmission or impedance method and ATP release into the extracellular medium throughout the aggregation process are done.[1] These are highly specialised tests available in specialised laboratories, requiring technical expertise and equipment and also it needs several days for the result which causes a delay in diagnosis and appropriate management of patients with bleeding.[1,14.]

The use of flow cytometric methods for mepacrine assay and CD63 assay to detect the dense granule disorder is an excellent method which facilitates appropriate real-time management of patients with suspected dense granule disorder. Mepacrine is a fluorescent marker that is rapidly taken up and binds to adenine nucleotides and localised in dense granules which can be determined by flow cytometry. CD63 is the antibody marker of δ-granules membrane protein, the expression of which increases on activated platelet membranes and can be detected on platelet surface by flow cytometry.[7,11,14] EM is the most sophisticated method for identification of the dense granules. Genome screening which is only available in the most developed, high-income countries has increased the number of diagnosed cases of inherited platelet function disorder with identifiable genetic causes.[6]

In this present case report, we have used the electron microscope to confirm the diagnosis of δ-SPD which showed a reduced number of dense granules compared to control. The electron microscope can visualise dense granules because dense bodies are rich in calcium, which are inherently electron-opaque and thereby can be easily counted.[7,13] Additionally, EM can also give structural information in other disorders such as platelet membrane defect and platelet cytoskeletal defect. Therefore, EM is considered as a tool to study and understand the biology of platelets and to classify many platelet disorders. Earlier studies have recommended the use of EM to assess dense granule numbers as part of an evaluation for platelet function disorder if the facility is available and even if other tests exclude defects in platelet aggregation and dense granules ATP release.[15] However, this method needs specialised equipment and techniques operated by experienced persons which are not readily available in most hospitals, and it is also very time consuming.[13]

Management of patients with SPD associated with bleeding should be treated accordingly depending on the severity of the bleeding. Local bleeding such as gum bleeding and epistaxis can be stopped by local measures which is direct compression at the site of injury or use fibrin sealants.[7] Antifibrinolytic agents such as tranexamic acid can be applied topically or given orally or intravenously when the patient presents with bleeding particularly in mucocutaneous bleeding and menorrhagia but not suitable with major internal bleeding. Desmopressin also can be given to control the bleeding during emergency or surgery. Platelet transfusions are required for severe bleeding patients. However, it should be reserved for cases where tranexamic acid and/or desmopressin are ineffective.[1,11] Recombinant factor VIIa is also used in patients suffering from life threatening bleeding that cannot be stopped by conventional treatments.[11] Unnecessary platelet transfusions should be avoided as patients may develop platelet antibodies which make future transfusion less effective.[1] For women, to avoid menorrhagia, oral contraceptive pill, and hormone supplementation with either progesterone alone or progesterone with oestrogen can be used.[16] Our patient was prescribed with OCP to control her menorrhagia.

It is suggested that patients with SPD should carry a health care card describing their condition and written information about the disease, therapy, contraindications (antiplatelet drugs), as well as the telephone number of the attending physician.[7] Additionally, patients with SPD are prone to being exposed to blood products such as packed cells and platelets. Thus, it is important for the patients to get vaccinated against hepatitis A and B.[7] Affected patients should be advised to avoid certain drugs that can affect platelets such as aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), blood thinner and certain herbal medicines to prevent bleeding.[16] Certain foods that can affect platelet functions should also be avoided such as caffeine, ginkgo, garlic, green tea and ginger.[16] Such in our patient, her symptoms improved when she took care of her diet and lifestyle. They also should be informed regarding dental hygiene to avoid gum bleeding.

CONCLUSION

This case report highlights an unusual case of δ-SPD, presented with variable degrees of bleeding symptoms. The platelet aggregation study was suggestive of δ-SPD and was confirmed by EM examination. Her diet and lifestyle changes helped her improve her symptoms. It is important to diagnose SPD to manage patients optimally. It is also easier to educate them when they know what diseases they are suffering from. In order to diagnose patients with SPD, it is crucial to get a detailed bleeding history and subject patients to specialised tests including platelet function test, electron microscope, flowcytometry and molecular analysis to confirm the diagnosis.

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Authors' contributions: Conceptualization: N Yusof, DA Abdul Aziz, S Abdul Aziz. Data collection and analysis: R Yousuf, NI Othman. Writing: R Yousuf, N Yusof. Review and editing: S Abdul Aziz, LH Mohd Pauzy, DA Abdul Aziz. All authors have read and agreed to the published version of the manuscript.

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