Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. MD Scatter plot of $\log_2$-fold change (D, x-axis), against absolute difference in transcript number (M, y-axis). Differentially expressed genes at $q = 0.8$ are highlighted in red.

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Severe macrothrombocytopenia with platelet CD9 deficiency responsive to romiplostim

The spectrum of inherited platelet disorders with variable platelet size continues to expand.[1,2] Among them, macrothrombocytopenia cases constitute a subgroup with different genetic basis, and mild-to-moderate thrombocytopenia without major bleeding symptoms. Here we describe a child with symptomatic severe macrothrombocytopenia without a genetic aetiology and low platelet CD9 expression raising the possibility of a new form of inherited macrothrombocytopenia.

A 6-month-old female presented with diffuse ecchymotic skin lesions, severe thrombocytopenia ($<10 \times 10^9$/l) and giant platelets Fig 1A–D. She has normal growth and development without any dysmorphic features. She was unresponsive to intravenous immunoglobulin and steroid treatments. Bone marrow was normocellular with normal megakaryocyte density and morphology, and cytogenetic evaluation. Family history revealed that the parents were first-degree cousins, originally from Yemen; however, two sisters and the parents had no history of bleeding with normal platelet counts.

Genetic studies for bone marrow failure and macrothrombocytopenia including glycoprotein IBα (*GP1BA*), glycoprotein IBβ(*GP1BB*), glycoprotein IX (*GP9*) by Sanger sequencing revealed no variants. The patient was given weekly platelet transfusions leading to transient count recovery and resolution of the bleeding symptoms Fig 1E. Platelet flow cytometry analysis and ristocetin-induced aggregation studies did not show any abnormalities, as the tested platelets were the transfused cells.

With no underlying cause for macrothrombocytopenia and continued dependence on platelet transfusions, the patient was started on thrombopoietin (TPO) receptor agonist (RA), eltrombopag after reviewing the potential side-effects with parents. Eltrombopag formulation production was discontinued by the manufacturer, therefore she began treatment with another TPO-RA, romiplostim, which is administered subcutaneously on a weekly schedule providing platelet counts of $>100 \times 10^9$/l with resolution of bleeding symptoms Fig 2A. The patient has been maintained on romiplostim injections for 9 months with continued giant platelets on the periphery.

Whole exome analysis by next generation sequencing did not reveal a known variant, but heterozygous mutation in ankyrin repeat domain 26 (*ANKRD26*) and homozygous mutation in RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*) with unknown

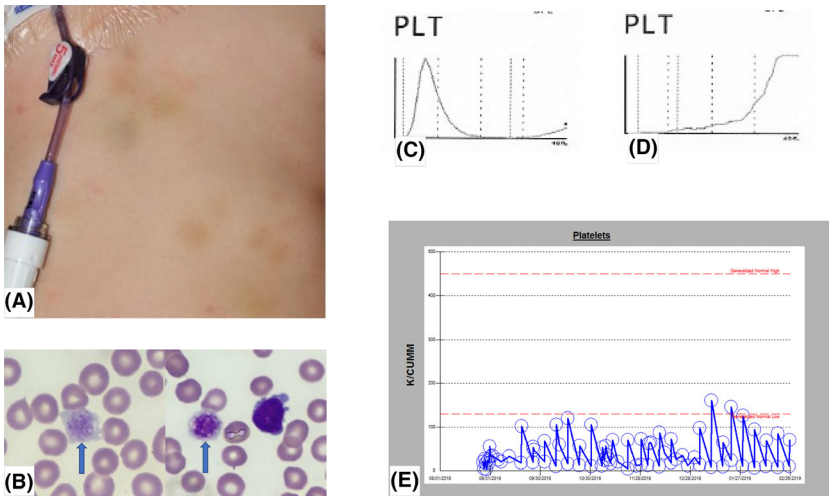

Fig 1. Clinical and laboratory findings prior to treatment with romiplostim. (A) Prominent ecchymotic skin lesions without appreciable petechiae on the trunk. (B) Very large platelets on Giemsa-Wright stained peripheral blood smears (×100). (C) Platelet size distribution on Haematology analyser in a control sample. (D) Platelet size distribution on Haematology analyser in the patient sample. (E) Response to single-donor platelet transfusions before institution of romiplostim treatment.

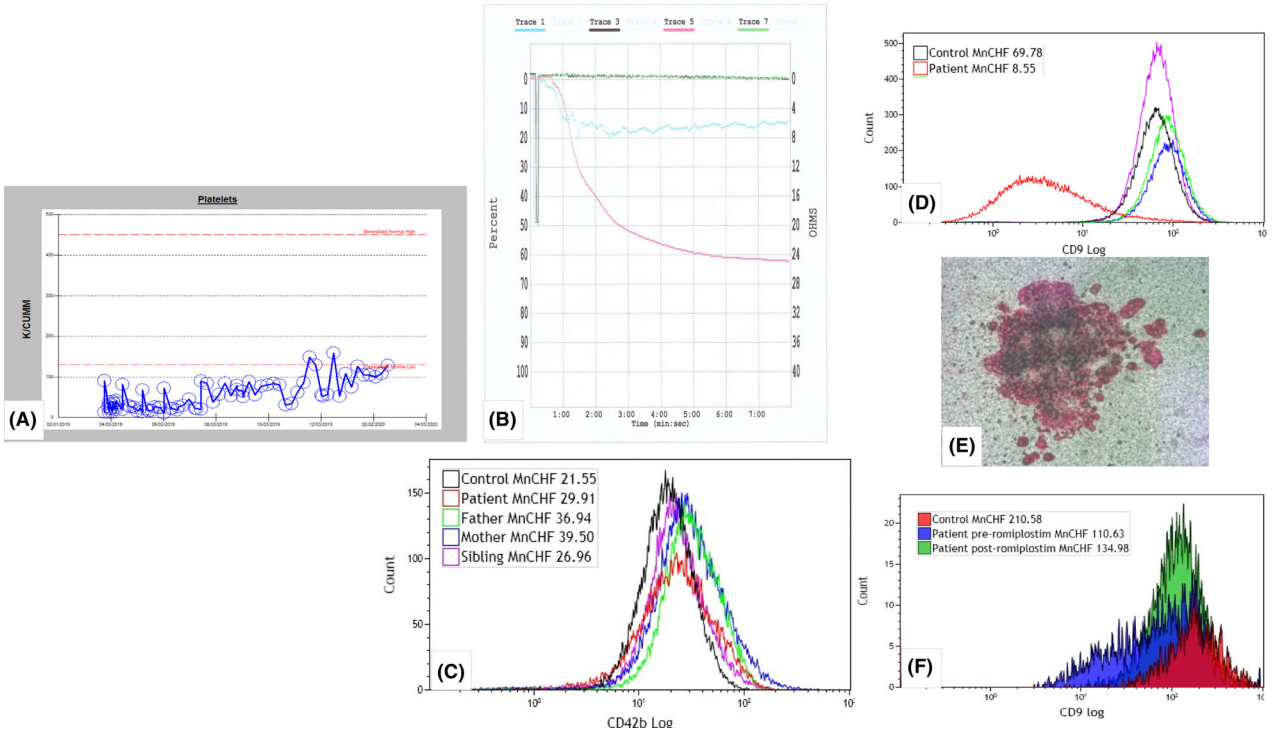

Fig 2. Laboratory observations after starting romiplostim therapy. (A) Platelet count recovery and maintenance on weekly romiplostim treatment. (B) Moderately decreased ristocetin-induced platelet aggregation in the patient platelet-rich plasm sample. Light blue line represents the patient, lavender line healthy control and black and green lines negative controls. (C) Similar CD42b expression on platelets from the patient, family members and a control. MnCHF stands for mean channel fluorescence, reflecting expression intensity of the marker. (D) Significantly decreased CD9 expression on platelets from the patient in comparison with a control sample. (E) Bone marrow megakaryocyte colony development (×10). (F) Similar CD9 expression patterns at day 14 of the *in vitro* culture on culture-grown bone marrow megakaryocyte precursors from the patient before and while on romiplostim therapy and a healthy bone marrow donor.

significances, which are not considered to be associated with functional abnormalities reportedly. Variants of *RMRP* have been associated with skeletal dysplasia conditions. Telomere length was within normal limits in leucocytes. Electron microscopy revealed normal platelet granule size, density and distribution. No antibodies against gpIb/gpIX, gpIa/IIa, gpIIb/IIIa, gpIV, and human leucocyte antigen (HLA) class-I were detected in the serum. Factor VIII activity, von Willebrand factor (vWF) antigen, activity, and multimers were within normal limits. Once platelet count was $>100 \times 10^9$/l, platelet aggregation was performed using ADP, collagen, thrombin, arachidonic acid, ATP and ristocetin and showed moderate decrease in ristocetin-induced aggregation with normal responses to other reagents Fig 2B.

Platelet immunophenotyping was done using platelet-rich plasma (PRP) samples by staining with several monoclonal antibodies (Beckman Coulter, Brea, CA, USA) on Beckman Coulter Gallios flow cytometer (Beckman Coulter). Platelet CD9 staining was not performed before romiplostim therapy. Patient serum was co-incubated with the control PRP and platelet immunophenotyping was performed to test for CD9 expression inhibition. Similarly, CD9 was studied on peripheral blood monocytes.

Bone marrow samples were collected after signed consents were obtained in an Institutional Review Board-approved study to investigate bone marrow failure syndromes during planned procedures. Megakaryocyte colony growth was assessed using archived bone marrow mononuclear cells from a healthy control, and pre- and on-romiplostim patient samples. MegaCult™-C Complete Kit with Cytokines and MegaCult-C staining kit (StemCell Technologies, Inc., Vancouver, BC, Canada) was used for colony-forming unit-megakaryocyte (CFU-MK) evaluation.

Bone marrow $CD34^+$-enriched cells obtained by magnetic bead separation, were re-suspended in StemSpan SFEM (Serum free medium) supplemented with StemSpan Megakaryocyte Expansion Supplement (StemCell Technologies) and incubated at 37°C and 5% CO_2 for 14 days. Megakaryocyte precursors were harvested on days 7, 10 and 14 and stained with several monoclonal antibodies and analysed on 10-colour Gallios flow cytometer.

There were subtle changes in platelet CD41, CD61, CD42a, CD42b, CD40, CD31 and CD62P expression, if any, when compared to a healthy control or other family member samples Fig 2C. However, platelet CD9 expression was significantly decreased Fig 2D. There was no change in platelet CD9 expression, after PRP from healthy control was co-incubated with patient serum suggesting a lack of blocking anti-CD9 antibody. Monocyte CD9 expression was within normal limits. Bone marrow megakaryocyte colonies were generated Fig 2E and sequential flow cytometric characterisation of the culture-grown megakaryocyte precursors confirmed their maturation. There was very minimal CD9 expression decrease at day 14 of the culture in both patient samples compared with the control subject.

The presented case here has unique features of severe thrombocytopenia with bleeding symptoms, very large platelets, and significantly decreased platelet CD9 expression. Bernard-Soulier syndrome (BSS) is a type of macrothrombocytopenia characterised by impaired ristocetin-induced aggregation with variable platelet counts and bleeding symptoms.[3] Decreased platelet CD9 expression was first reported in a series of patients with BSS without genetic studies.[4] Later, a patient with genetically confirmed BSS due to *GP1BB* variant was reported to have decreased CD42a, CD42b and CD9 expression.[5] It could be debatable, if the presented patient is in the spectrum of BSS. However, flow cytometric analysis revealed normal platelet CD42a or CD42b expression in contrast to above-referenced BSS case, which also had *GP1BB* mutation, thus, making this case a form of BSS unlikely.

The patient was responsive to romiplostim treatment. Successful responses to TPO-RA in inherited thrombocytopenia cases, some with macrothrombocytopenia have been reported.[6,7] We investigated, if decreased CD9 expression is related to romiplostim treatment. Megakaryocyte precursors developed *in vitro* did not show significantly decreased CD9 expression in either pre- or on-romiplostim samples excluding a possible effect of romiplostim. Lack of decreased monocyte CD9 expression stresses cell-specific nature of CD9 deficiency. We also did not find any evidence of antibody-mediated CD9 blockade. Therefore, low platelet CD9 expression maybe a secondary phenomenon that occurs in later stages of platelet development.

CD9 is a membrane protein in the tetraspanin family expressed on different tissues with unique roles in platelets.[8,9] Crosslinking CD9 and CD42 can stimulate platelets signal independent of gpIIb/gpIIIa.[10] Therefore, it can be speculated that decreased CD9 may lead to impaired platelet aggregation. In conclusion, role of CD9 in platelet development, survival and function warrants further investigation. Decreased platelet CD9 expression in this patient may represent an independent anomaly causing a yet undescribed bleeding disorder.

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Conflict of interest

None.

Author contributions

Marjilla Seddiq: Drafted the manuscript. Manisha Gadgeel: Conducted laboratory experiments, prepared the materials and methods, provided some of the illustrations and edited the manuscript. Yogindra Persaud: Edited the manuscript. Jennifer Lafferty: Edited the manuscript. Süreyya Savaşan:

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Secondary plasma cell leukaemia treated with single agent venetoclax

Patients with multiple myeloma (MM) are at risk of developing secondary Plasma Cell Leukaemia (sPCL), with associated hyperviscosity. In such patients combination therapies for MM are associated with a short median overall survival of just 1·3 months.[1] Translocation (11;14) is reported in 33% of sPCL and involves the immunoglobulin heavy chain locus on 14q32.[2] Selective inhibition of Bcl-2 with venetoclax has shown single agent activity in MM patients harbouring this translocation, however, single agent activity has not been reported in sPCL. We demonstrate a rapid and deep haematologic response with reversal of symptomatic hyperviscosity in sPCL with single agent venetoclax treatment.

Plasma cell leukaemia occurs in the setting of pre-existing multiple myeloma (sPCL) or arises as a *de novo* aggressive haematological malignancy characterized by circulating plasma cells $> 2 \times 10^9$/l in peripheral blood.[3] sPCL has a dismal prognosis with no standard or effective therapy available. In recent years the frequency of sPCL has increased,[1] likely explained by improved overall survival in MM, leading to a higher prevalence of the disease, and development of clonal evolution in patients who are treated with multiple novel agents. sPCL occurs in 1·8–4% of MM patients and is associated with immunoglobulin heavy chain (IgH) translocations on locus 14q32 in 80% of cases, the most common being t(11;14), seen in 25–65% of patients.[1] Bcl-2 family proteins are critical regulators of apoptosis with anti-apoptotic (Mcl-1, Bcl-2, Bcl-x_L), multidomain pro-apoptotic (Bax, Bak) and BH3-only members (Bim, Noxa, Bad).[4] MM cells that harbour t(11;14) express high levels of Bcl-2 relative to Bcl-x_L and Mcl-1.[5] Venetoclax, a first in class oral BH3 mimetic, which selectively inhibits Bcl-2 but not Bcl-x_L or Mcl-1, has shown single agent activity in MM.[5] BH3 profiling has previously correctly identified Bcl-2-dependent leukaemias; in this case we apply this method to sPCL.[6,7] Single agent activity of venetoclax has not been reported for primary or secondary plasma cell leukaemia, although, given the rate of t(11;14) translocations in this disease it follows that clinical responses may be achieved and successful combination therapy with venetoclax in primary PCL has been reported.[8]