DR. MARJILLA SEDDIQ (Orcid ID : 0000-0003-1436-2136)

DR. SUREYYA SAVASAN (Orcid ID : 0000-0001-7138-3027)

Article type : Letters

Severe Macrothrombocytopenia with Platelet CD9 Deficiency Responsive to Romiplostim

Marjilla Seddiq, MD[1], Manisha Gadgeel, MD[2], Yogindra Persaud, MD[1], Jennifer Laferty[1], RN, and Süreyya Savaşan, MD[1-3]

[1]Division of Hematology/Oncology, Children's Hospital of Michigan

[2]Hematology/Oncology Flow Cytometry Laboratory, Children's Hospital of Michigan

[3]Pediatric Bone Marrow Transplant Program, Children's Hospital of Michigan, Barbara Ann Karmanos Cancer Center, Wayne State University School of Medicine

Correspondence: Süreyya Savaşan, MD

Children's Hospital of Michigan

Division of Hematology/Oncology
Pediatric Blood and Marrow Transplantation Program
Hematology/Oncology Flow Cytometry Laboratory
Barbara Ann Karmanos Cancer Center
Wayne State University School of Medicine
3901 Beaubien Blvd.,
Detroit, MI 48201

313-745-5515 (Phone)
313-745-5237 (fax)
ssavasan@med.wayne.edu

Short title: Macrothrombocytopenia with Platelet CD9 Deficiency

Figures & Tables: 2 figures

Key words: Macrothrombocytopenia, severe, platelet CD9 deficiency, bleeding, romiplostim

Conflict of interest: None

Dear Sir/Madam:

The spectrum of inherited platelet disorders with variable platelet size continues to expand (Lambert, 2019; Noris *et al*, 2014). 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 etiology and low platelet CD9 expression raising the possibility of a new form of inherited macrothrombocytopenia.

A six-month-old female presented with diffuse ecchymotic skin lesions, severe thrombocytopenia ($<10x10^9$/L) and giant platelets (Fig1a-1c). 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 parents are first degree cousins, originally from Yemen; however, two sisters and parents had no history of bleeding with normal platelet counts.

Genetic studies for bone marrow failure and macrothrombocytopenia including *GP1BA/GP1BB/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 (Fig1d). Platelet flow cytometry analysis and ristocetin-induced aggregation studies did not show any abnormalities, since 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 that is administered subcutaneously on a weekly schedule providing platelet counts over $100x10^9$/L with resolution of bleeding symptoms (Fig2a). 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 *ANKRD26* and homozygous mutation in *RMRP* with unknown significances, which are not considered to be associated with functional abnormalities reportedly. Variants of PMRP have been associated with skeletal dysplasia conditions. Telomere length was within normal limits in leukocytes. Electron microscopy revealed normal platelet granule size, density and distribution. No antibodies against gpIb/gpIX, gpIa/IIa, gpIIb/IIIa, gpIV, and 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 above $100x10^9$/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 (Fig2b).

Platelet immunophenotyping was done using platelet rich plasma (PRP) samples by staining with several monoclonal antibodies (Beckman Coulter, Brea, CA) on Beckman Coulter Gallios flow cytometer (Beckman Coulter, Brea, CA). Platelet CD9 staining was not performed prior to 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, Canada) was used for CFU-MK colony 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 Technology) and incubated at 37 ^{0}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 analyzed on 10-color 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 (Fig2c). However, platelet CD9 expression was significantly decreased (Fig2d). 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 (Fig2e) and sequential flow cytometric characterization 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 characterized by impaired ristocetin-induced aggregation with variable platelet counts and bleeding symptoms (Boeckelmann *et al*, 2017). Decreased platelet CD9 expression was first reported in a series of BSS patients without genetic studies (Beltrame *et al*, 2009). Later, a genetically confirmed BSS patient due to *GP1BB* variant was reported to have decreased CD42a, CD42b and CD9 expression (Qiao *et al*, 2015). 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 (Rodeghiero *et al*, 2018; Zaninetti *et al*, 2020). 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 ruling out 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 tetraspanin family expressed on different tissues with unique roles in platelets (Charrin *et al*, 2014; JENNINGS *et al*, 1994). Crosslinking CD9 and CD42 can stimulate platelets signal independent of gpIIb/gpIIIa (Slupsky *et al*, 1997). 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.

Figure Legends

Figure1. 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 (100x). C. Platelet size distribution on flow cytometry in a control sample. D. Platelet size distribution on Hematology analyzer in the patient sample. E. Response to single-donor platelet transfusions prior to institution of romiplostim treatment.

Figure2. 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 plasma 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 (10x). F. Similar CD9 expression patterns at day 14 of the in vitro culture on culture-grown bone marrow megakaryocyte precursors from the patient prior to and while on romiplostim therapy and a healthy bone marrow donor.

References

Beltrame, M.P., Malvezzi, M., Zanis, J. & Pasquini, R. (2009) Flow cytometry as a tool in the diagnosis of bernard-Soulier syndrome in brazilian patients. *Platelets*, **20,** 229–234.

Boeckelmann, D., Hengartner, H., Greinacher, A., Nowak-Göttl, U., Sachs, U.J., Peter, K., Sandrock-Lang, K. & Zieger, B. (2017) Patients with Bernard-Soulier syndrome and different severity of the bleeding phenotype. *Blood Cells, Molecules, and Diseases*, **67,** 69–74 Available at: http://dx.doi.org/10.1016/j.bcmd.2017.01.010.

Charrin, S., Jouannet, S., Boucheix, C. & Rubinstein, E. (2014) Tetraspanins at a glance. *Journal of Cell Science*, **127,** 3641–3648.

JENNINGS, L.K., CROSSNO, J.T., FOX, C.F., WHITE, M.M. & GREEN, C.A. (1994) Platelet p24/CD9, a Member of the Tetraspanin Family of Proteins. *Annals of the New York Academy of Sciences*, **714,** 175–184.

Lambert, M.P. (2019) Inherited Platelet Disorders: A Modern Approach to Evaluation and Treatment. *Hematology/Oncology Clinics of North America*, **33,** 471–487 Available at: https://doi.org/10.1016/j.hoc.2019.01.008.

Noris, P., Biino, G., Pecci, A., Civaschi, E., Savoia, A., Seri, M., Melazzini, F., Loffredo, G., Russo, G., Bozzi, V., Notarangelo, L.D., Gresele, P., Heller, P.G., Pujol-Moix, N., Kunishima, S., Cattaneo, M., Bussel, J., De Candia, E., Cagioni, C., Ramenghi, U., et al (2014) Platelet diameters in inherited thrombocytopenias: Analysis of 376 patients with all known disorders. *Blood*, **124,** 4–11.

Qiao, J., Davis, A.K., Morel-Kopp, M.C., Ward, C.M., Gardiner, E.E. & Andrews, R.K. (2015) Low levels of CD9 coincidental with a novel nonsense mutation in glycoprotein Ibβ in a patient with Bernard-Soulier syndrome. *Annals of Hematology*, **94,** 2069–2071.

Rodeghiero, F., Pecci, A. & Balduini, C.L. (2018) Thrombopoietin receptor agonists in hereditary thrombocytopenias. *Journal of Thrombosis and Haemostasis*, **16,** 1700–1710.

Slupsky, J.R., Kamiguti, A.S., Rhodes, N.P., Cawley, J.C., Shaw, A.R.E. & Zuzel, M. (1997) The platelet antigens CD9, CD42 and integrin α(IIb)β(IIIa) can be topographically associated and transduce functionally similar signals. *European Journal of Biochemistry*, **244,** 168–175.

Zaninetti, C., Gresele, P., Bertomoro, A., Klersy, C., de Candia, E., Veneri, D., Barozzi, S., Fierro, T., Alberelli, M.A., Musella, V., Noris, P., Fabris, F., Balduini, C.L. & Pecci, A. (2020) Eltrombopag for the treatment of inherited thrombocytopenias: A phase II clinical trial. *Haematologica*, **105,** 820–828.

Acknowledgements

This study was partially supported by funds from Children's Hospital of Michigan Foundation.

Authors do not have any conflict of interest.

Author contributions:

MS: Drafted the manuscript

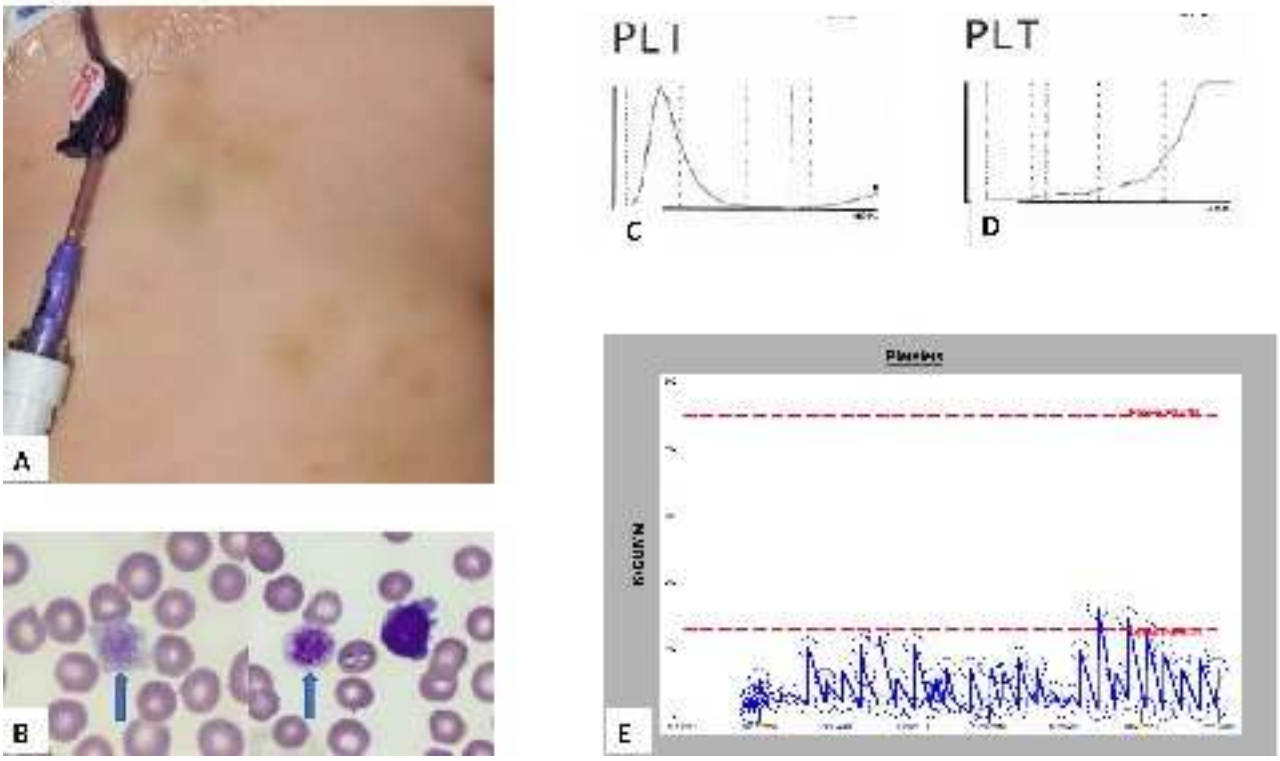
MG: Conducted laboratory experiments, prepared the materials and methods, provided some of the illustrations and edited the manuscript

YP: Edited the manuscript

JL: Edited the manuscript

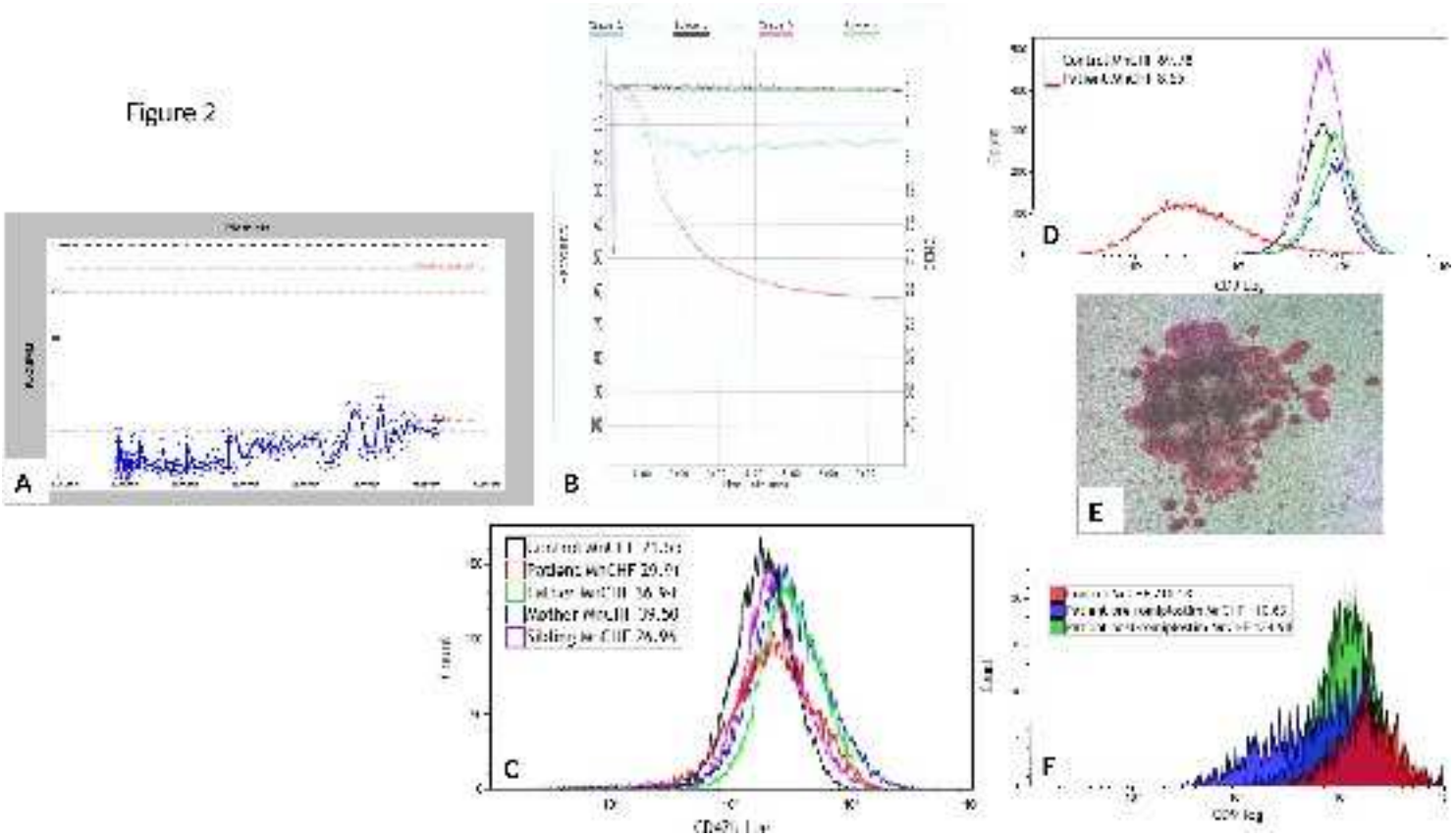
SS: Planned the report, designed laboratory studies, drafted and edited the manuscript, provided some of the illustrations

Figure 1



bjh_16812_f1.tiff

Figure 2



bjh_16812_f2.tiff