Thrombopoietin mutation in congenital amegakaryocytic thrombocytopenia treatable with romiplostim

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Editorial Decision:	20 July 2017
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editors: Roberto Buccione and Céline Carret

1st Editorial Decision 20 July 2017

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three Reviewers whom we asked to evaluate your manuscript.

As you will see the reviewers are very positive and suggest a number of improvements to increase the impact and quality of presentation.

I will not delve into any detail, as their evaluations are quite straightforward and clear. I would just mention that reviewer 3 would like you to provide further data in support of your contention that there is an impairment of THPO secretion. Also, reviewers 2 and 3 would both like more context and discussion on the use of romiplostim and also suggest you incorporate a discussion of the Kim et al, 2017 paper (PMID: 28283061).

In conclusion, while publication of the manuscript cannot be considered at this stage, we would be pleased to consider a revised submission, with the understanding that the Reviewers' points must be addressed including further experimentation as necessary. Eventual acceptance of the manuscript will entail a second round of review.

I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

This is a well written and interesting manuscript. It is only the second family in whom a recessive defect in thrombopoietin is described. The long-term follow-up of 6 years of treatment are impressive, the report is clinically highly relevant for these very rare cases, and the laboratory experiments convincingly show the functional consequences of the mutation. My comments are only minor. Due to the rarity of such mutations, the manuscript will be very frequently cited in the literature on thrombopoiesis.
The medical impact is high, because this is only the 2nd family described with the genetic mutation leading to THPO deficiency. It is important to diagnose those patients, as the standard therapy for a plastic anemia/thrombocytopenia would be bone marrow transplantation which is inefficient in these patients
The model is appropriate, as the transfected cell lines show impaired release of THPO, which is fully consistent with the low levels of the protein measured in patient blood

Referee #1 (Remarks):

Pecci et al. describe the second family with inherited deficiency of thrombopoietin (THPO) causing congenital amegakaryocytic thrombocytopenia. The recessive disorder in the family is caused by a homozygous mutation in p. R119C. This leads to moderately reduced function of THPO, normal THPO production in transfected cell lines, but impaired release of the protein into the circulation. The clinical consequences are the same as in other forms of CAMT, with the major difference that THPO levels are either reduced or normal but not excessively increased like in patients with a mutation in the receptor for THPO. Treatment with the thrombopoietin receptor agonist romiplostim resulted in improvement of hematopoietic cell counts. The manuscript is interesting and very relevant for the differential diagnoses of causes of CAMT and the choice of treatment.

Minor comments:
page 2, abstract
line 9: the terminus "production" seems not to be correct in the view of the experiments with HEK cells, in which the production was normal but the release was impaired.
Line 11: the abstract states that infections had been reduced in the affected children, while this information is missing in the results section. Please add information on the infection frequencies in the description of patients in the section reporting on treatment response.
Page 5, para 3, line 5 and 7: the cell culture was performed in 96 well culture plates. Did you really incubate the cell culture with 0.5 µL only? Was the mutant media diluted in any culture buffer?
Page 7, para 3: please give information on infection frequencies
Page 8, para 1, line 2: The abbreviation gr/dl is unusual, g/dL is mostly used, please double check with the journal style.
Page 8, para 2, line 3: reports = report
page 9, para 3, line 2: again the question of the 0.5 or 1.0 µL volume
page 13, last para: the para is a copy of the first para
figures:
figure 3: legend Y axis Proliferation = proliferation;
figure 5 A and B: surnatant = supernatant; 0,5 = 0.5 (also accounts for the legend). Please use larger font size for molecular weight standard
supplementary figure 1: please consider to use multiple symbols to show presence of thrombocytopenia, infection, anemia and neutropenia, e.g. by showing 4 little black or white squares within the large square symbol

Referee #2 (Remarks):

In this paper, the authors report in Egyptian family with three children affected by thrombocytopenia, associated variably with anemia and pancytopenia, that have homozygous mutations altering the amino acids sequence of the cytokine thrombopoietin (THPO). The specific mutation is in a conserved domain of the protein, which is found in low amounts in the supernatant

of cells engineered to express the mutant THPO. In addition, the serum levels of THPO, while at normal levels, are functionally low considering the degree of thrombocytopenia. It is not known whether this mutation leads to any abnormalities in the binding of the mutant THPO to mpl. Most importantly, a clinical response was evident with the use of the THPO-mimetic romiplostim. While this response is highly clinically relevant, the intermittent nature of the medical follow-up appears to be responsible for the highly variable blood counts shown in Figure 1. However, taken together, the data support the conclusions and the discussion points raised by the authors.

Minor suggestions:
1. I would suggest moving the data from supplemental figure 3 into figure 3 of the paper. It will be useful to show the normal protein levels of the mutant THPO proteins in contrast to the levels in the supernatants of the HEK293 cells.

2. It would be useful to discuss the recent very elegant paper (Kim, et al. Cell 2017) showing that a mutation in erythropoietin is responsible for altered binding to its receptor and to altered signalling downstream of that receptor in erythroid cells. In addition, this paper describes the tragic death of the proband following unnecessary bone marrow transplantation prior to the discovery of the underlying mutation.

Referee #3 (Remarks):

In this interesting manuscript from Pecci and colleagues, a novel mutation in THPO causing congenital amegakaryocytic thrombocytopenia (CAMT) in multiple members of a single family is described. This mutation reduces the expression of THPO and thus appears to cause disease. Importantly, the authors show that romiplostim therapy can treat this rare disorder. While this manuscript is interesting and important, several issues must be addressed prior to publication:

1. The presentation of the manuscript is confusing. For example, there are experiments involving a p.R38C mutant THPO that come before the reference describing this mutation. The introduction should be revised to summarize known mutations more clearly. It is also unclear what prompted the authors to initiate therapy with romiplostim. This needs to more clearly be explained. Why not escalate romiplostim dosing?

2. The authors suggest that there is an impairment of protein secretion. Can the authors be sure that there is not a problem with protein stability and not secretion? Can further analysis of this lesion be performed? This would help the published findings tremendously.

3. The authors should reference a recent paper discussing similar mutations in other CAMT patients (Seo et al., Blood, 2017).

4. The authors should also compare and contrast this mutation with other mutations in hematopoietic cytokines. For example, a recent paper described an EPO mutation with altered downstream signaling properties in patients with congenital hypoplastic anemia (Kim et al., Cell, 2017). These other similar mutations should be referenced and discussed more fully to put these findings in context. The EPO case is particularly interesting, given the response to recombinant EPO, similar to what is observed here.

1st Revision - authors' response 15 September 2017

Referee #1 (Comments on Novelty/Model System):
This is a well written and interesting manuscript. It is only the second family in whom a recessive defect in thrombopoietin is described. The long-term follow-up of 6 years of treatment are impressive, the report is clinically highly relevant for these very rare cases, and the laboratory experiments convincingly show the functional consequences of the mutation. My comments are only minor. Due to the rarity of such mutations, the manuscript will be very frequently cited in the literature on thrombopoiesis.
The medical impact is high, because this is only the 2nd family described with the genetic mutation leading to THPO deficiency. It is important to diagnose those patients, as the standard therapy for a

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Pecci et al. describe the second family with inherited deficiency of thrombopoietin (THPO) causing congenital amegakaryocytic thrombocytopenia. The recessive disorder in the family is caused by a homozygous mutation in p. R119C. This leads to moderately reduced function of THPO, normal THPO production in transfected cell lines, but impaired release of the protein into the circulation. The clinical consequences are the same as in other forms of CAMT, with the major difference that THPO levels are either reduced or normal but not excessively increased like in patients with a mutation in the receptor for THPO. Treatment with the thrombopoietin receptor agonist romiplostim resulted in improvement of hematopoietic cell counts. The manuscript is interesting and very relevant for the differential diagnoses of causes of CAMT and the choice of treatment.

R: We thank the reviewer for the encouraging and detailed comments on our work.

Minor comments:
- page 2, abstract
line 9: the terminus "production" seems not to be correct in the view of the experiments with HEK cells, in which the production was normal but the release was impaired.

R: The referee is right. The abstract has been modified according to the reviewer's suggestion and to the author guidelines (not more than 175 words). The word "production" does not appear any longer.

- Line 11: the abstract states that infections had been reduced in the affected children, while this information is missing in the results section. Please add information on the infection frequencies in the description of patients in the section reporting on treatment response.
- Page 7, para 3: please give information on infection frequencies

R: We thank the reviewer for his/her suggestion. Before romiplostim administration, the patient had episodes of febrile neutropenia, or fever and otitis media, requiring hospitalization every 1-2 months. Conversely, no further infectious episodes were recorded during romiplostim administration. The missing information was included in the revised version of the manuscript (Results: page 4, second paragraph, lines 2-3; page 5, first two lines).

- Page 5, para 3, line 5 and 7: the cell culture was performed in 96 well culture plates. Did you really incubate the cell culture with 0.5 µL only? Was the mutant media diluted in any culture buffer?
- Page 9, para 3, line 2: again the question of the 0.5 or 1.0 µL volume

R: The cells were incubated in 96-well plates with 250 µL of culture buffer containing 0.5 or 1.0 µL of THPO-conditioned supernatants from HEK293T cells cultures (wild-type, mutant, or empty vector). The culture buffer was MEM Alpha medium. The methods of the experiments were the same as those previously reported in the study by Dasouki et al. on the effects of the p.R38C mutation (Dasouki *et al*, 2013). We used the same protocol to be able to reproduce their results and compare the effects of the p.R119C variant with those of the p.R38C.
We understand that the previous version of the paper might have been a bit confusing on this point. We have thus modified the manuscript with more accurate explanations (Results: page 6, second paragraph of "The p.R119C affects both secretion of THPO and THPO/MPL signalling", lines 2-4; Patients and Methods: page 13, in "MTT cell proliferation assay", lines 3-7; page 14, in "Analysis of signaling kinases downstream MPL", lines 6-8; legend of Figure 3).

- Page 8, para 1, line 2: The abbreviation gr/dl is unusual, g/dL is mostly used, please double check with the journal style.
Page 8, para 2, line 3: reports = report
page 13, last para: the para is a copy of the first para

R: We apologize for the typo and thank the reviewer for the helpful indications. The manuscript was modified accordingly.

figures:

figure 3: legend Y axis Proliferation = proliferation; figure 5 A and B: surnatant = supernatant; 0,5 = 0.5 (also accounts for the legend). Please use larger font size for molecular weight standard supplementary figure 1: please consider to use multiple symbols to show presence of thrombocytopenia, infection, anemia and neutropenia, e.g. by showing 4 little black or white squares within the large square symbol

R: The figures were modified according to the reviewer's suggestions.

Referee #2 (Remarks):

In this paper, the authors report in Egyptian family with three children affected by thrombocytopenia, associated variably with anemia and pancytopenia, that have homozygous mutations altering the amino acids sequence of the cytokine thrombopoietin (THPO). The specific mutation is in a conserved domain of the protein, which is found in low amounts in the supernatant of cells engineered to express the mutant THPO. In addition, the serum levels of THPO, while at normal levels, are functionally low considering the degree of thrombocytopenia. It is not known whether this mutation leads to any abnormalities in the binding of the mutant THPO to mpl. Most importantly, a clinical response was evident with the use of the THPO-mimetic romiplastim. While this response is highly clinically relevant, the intermittent nature of the medical follow-up appears to be responsible for the highly variable blood counts shown in Figure 1. However, taken together, the data support the conclusions and the discussion points raised by the authors.

R: We thank the reviewer for the favorable and specific comments.

Minor suggestions:

1. I would suggest moving the data from supplemental figure 3 into figure 3 of the paper. It will be useful to show the normal protein levels of the mutant THPO proteins in contrast to the levels in the supernatants of the HEK293 cells.

R: Figure 3 shows the proliferation of the UT7-TPO cells induced by the THPO-conditioned supernatants of the HEK293T cells. The reduction observed for p.R119C and p.R38C is not only due to the low level of the mutant THPO in the supernatant but also to defective activity in stimulating the signaling downstream of MPL, as shown in Figures 5 and 6. For this reason, we think it is more appropriate to move supplementary Figure 3 into Figure 2 of the paper. The new Figure 2 recapitulates all the immunoblotting experiments to investigate the THPO expression in HEK293T cells and now shows that the expression level of the different forms of THPO is maintained for 24 and 48 hours after transfection, the time points at which the supernatants were collected for cell proliferation assays. Accordingly, in the revised version of the manuscript Figure 2 and its relative legend were modified.

2. It would be useful to discuss the recent very elegant paper (Kim, et al. Cell 2017) showing that a mutation in erythropoietin is responsible for altered binding to its receptor and to altered signaling downstream of that receptor in erythroid cells. In addition, this paper describes the tragic death of the proband following an unnecessary bone marrow transplantation prior to the discovery of the underlying mutation.

R: We thank the reviewer for his/her suggestion. We have included a paragraph commenting on the paper by Kim and colleagues in the Discussion section (page 10, third paragraph).

Referee #3 (Remarks):

In this interesting manuscript from Pecci and colleagues, a novel mutation in THPO causing congenital amegakaryocytic thrombocytopenia (CAMT) in multiple members of a single family is described. This mutation reduces the expression of THPO and thus appears to cause disease. Importantly, the authors show that romiplostim therapy can treat this rare disorder. While this manuscript is interesting and important, several issues must be addressed prior to publication:

1. The presentation of the manuscript is confusing. For example, there are experiments involving a p.R38C mutant THPO that come before the reference describing this mutation. The introduction should be revised to summarize known mutations more clearly.

R: We thank the reviewer for the constructive comments, which have undoubtedly helped us to have a significant improvement of the manuscript. The introduction was modified according to the reviewer's suggestion. Reference to the p.R38C mutation and the other two *THPO* mutations very recently identified by Seo *et al* have been now included (Introduction: page 3, third paragraph).

It is also unclear what prompted the authors to initiate therapy with romiplostim. This needs to more clearly be explained.

R: The proband was started on romiplostim due to lack of a donor for hematopoietic stem cell transplantation and of any other available therapeutic options - as an empirical attempt to increase platelet count in the child who had severe, life-threatening hemorrhages and required very frequent platelet transfusions. Of note, the proband initially received the diagnosis of idiopathic aplastic anemia and, before empirical romiplostim administration, he was treated with cyclosporine A for 4 months, without any response, and then with oxymetholone, again without any improvement. The two siblings of the proband were started on romiplostim in view of the efficacy of the treatment in the proband.

Following the reviewer's suggestion, the manuscript has been revised to clarify these aspects (Results: page 4, third paragraph, lines 1-5).

- Why not escalate romiplostim dosing?

R: Romiplostim was administered to the three children using a non-conventional schedule and low doses (4 µg/Kg/monthly) because of difficulties of the family in accessing healthcare facilities in their country (Egypt), as reported in the manuscript (page 4, third paragraph, lines 6-7). For the same reason, it was not possible to escalate the romiplostim dose. Nevertheless, all the three children maintained sustained hematological and clinical responses.

2. The authors suggest that there is an impairment of protein secretion. Can the authors be sure that there is not a problem with protein stability and not secretion? Can further analysis of this lesion be performed? This would help the published findings tremendously.

R: We thank the reviewer for this very appropriate suggestion. To investigate whether the p.R119C (and p.R38C) substitution could affect stability of the THPO protein, we studied stability of the wild type and mutant forms by the standard cycloheximide chase assay. After blocking the *de novo* protein synthesis in HEK293T cells by treatment with cycloheximide, the kinetics of degradation of the wild type and mutant THPO proteins were assessed over 48 hours. The assay showed that the two mutant proteins have similar stability to that of the wild type form. These new data have been included in the revised version of the manuscript (Results: page 7, second paragraph; Patients and Methods: page 15, "Cycloheximide chase assay"; new Figure 4 with relative legend).

3. The authors should reference a recent paper discussing similar mutations in other CAMT patients (Seo et al., Blood, 2017).

R: We thank the reviewer for the suggestion that gave us the opportunity to comment on this interesting paper. Accordingly, the revised manuscript has been modified to include the main findings reported by Seo and colleagues (Introduction: page 3, third paragraph, lines 4-5; Discussion: page 8, third paragraph, lines 7-12; Discussion: page 9, first paragraph, lines 14-15; Discussion: page 10, first paragraph, lines 4-5 and second paragraph, lines 4-10).

4. The authors should also compare and contrast this mutation with other mutations in hematopoietic cytokines. For example, a recent paper described an EPO mutation with altered downstream signaling properties in patients with congenital hypoplastic anemia (Kim et al., Cell, 2017). These other similar mutations should be referenced and discussed more fully to put these findings in context. The EPO case is particularly interesting, given the response to recombinant EPO, similar to what is observed here.

R: We thank the reviewer for his/her suggestion. We have included a paragraph commenting on the paper by Kim and colleagues in the Discussion section (page 10, third paragraph).

2nd Editorial Decision 23 October 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending editorial amendments.

***** Reviewer's comments *****

Referee #2 (Remarks for Author):

The authors have responded effectively to all of the suggestions/critiques of the reviewers with the addition of data, changes to the text, including reorganization of the presentation of data and a discussion of other papers that helps to put this paper in a wider context.

Referee #3 (Comments on Novelty/Model System for Author):

This paper is much improved and I have no additional comments

Referee #3 (Remarks for Author):

Very nice revision.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Anna Savoia
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2017-08168-V2

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Not applicable. For each functional in vitro assay to investigate the effects of THPO mutants, we carried out n=3 separate and independent experiments. In particular, immunoblotting for detection of exogenous THPO levels in HEK293T cells, ELISA assay for mesurement of THPO concentration in HEK293T cells supernatants, and cycloeximide chase assay were carried out on cells collected after n=3 independent trasfection experiments. Immunoblotting for detection of exogenous THPO levels in HEK293T cells and ELISA assay for mesurement of THPO concentration in HEK293T cells supernatants were performed on the same HEK293T cells samples. For MTT assay and analysis of signaling kinases downstream MPL, we performed n=3 separate and independent experiments by stimulating UT7-TPO cells with the same HEK293T supernatants collected in the transfection experiments referenced above. Details are provided in the Patients and methods section.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable. All the performed experiments were included in the analyses.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not applicable.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Not applicable. Results of the reported experiments (e.g. optical density read by ELISA plate reader in the ELISA assay; absorbance read by the BioPhotometer in the MTT assay; densitometric analysis of immunoblotting bands) are all objective data and are not at risk of subjective bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Yes. We used standard unpaired two-sided Student's t-test to analyze the results of in vitro functional studies to investigate the effects of THPO mutants.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used standard two-sided Student's t-test to analyze the results of in vitro functional studies to investigate the effects of THPO mutants. Details are provided in the appropriate Figure Legends and relative Source Data.
Is there an estimate of variation within each group of data?	Standard deviation was used to estimate variation of the results of the in vitro functional studies.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
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http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	For each antibody, we provided the name of the clone and/or the company from which the antibody was purchased. Any details are provided in the Patients and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The Human embryonic kidney (HEK)293T cells were purchased from the National Institute for Cancer Research, Genoa, Italy. UT7-TPO human megakaryoblastic cell line is a kind gift of Dr. Hana Raslova (Institute Gustave Roussy, Villejuif, France). Both cell lines were recently authenticated and tested for mycoplasma contamination (no contamination). Details are provided in the Patients and Methods section.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The studies on patients' samples carried out to ascertain the etiology and pathogenesis of their disease (molecular screening of THPO and MPL genes, assessment of plasma THPO levels, revision of peripheral blood and bone marrow samples) were approved by the Institutional Review Board of the IRCCS Policlinico San Matteo Foundation, Pavia, Italy. Details are reported in the Patients and Methods section.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The patients' parents signed written informed consent for the studies, for treatment with romiplostim, and for reporting their clinical data in an anonymous form. All the procedures were performed in accordance with the principles set out in the WMA of the Declaration of Helsinki and the Human Services Belmont Report. Details are reported in the Patients and Methods section.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	None.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Not applicable
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Not applicable
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	None.