Formulas for the Detection β-Thalassemia Carriers Are Affected by Changes in Red Cell Parameters

We read with interest the article by Roth et al.,[1] introducing a newly developed formula for the detection of β-thalassemia carriers—a complex mathematical calculation using erythrocyte parameters—which the authors report can detect carriers with a sensitivity of 98% and negative predictive value (NPV) of 99.77%, superior results to all currently existing formulas. Indeed, β-thalassemia major is a devastating disease with an economic burden, particularly in Mediterranean populations. Detecting carriers of this recessively inherited disease and its eventual eradication are critical. Achieving this goal at minimum cost and with maximum accuracy is of utmost importance. Scientists have been endeavoring for years to develop formulas using erythrocyte parameters on complete blood count (CBC), and Roth et al. are congratulated for their contribution toward this goal. However, it *cannot be overlooked* that formulas developed to detect β-thalassemia carriers inherently rely on the changes in erythrocyte parameters. Therefore, these formulas are useful only if the β-thalassemia mutation effects changes in these parameters. Otherwise, regardless of their complexity, the formulas fail. Their ineffectiveness may also be attributed to the fact that erythrocyte changes are not as expected or due to the presence of confounding factors in erythrocyte parameters. In these situations, carriers may be missed. I herein provide a number of such examples illustrating how the formulas do not function in certain circumstances.

Currently, the "expected" red cell parameters in β-thalassemia carriers are as follows: decreased/normal hemoglobin (Hb), decreased mean corpuscular volume (MCV), decreased mean corpuscular hemoglobin (MCH), increased red blood cell (RBC) count, and normal red cell distribution width (RDW).[2] To detect the carriers, formulas run mathematical methods incorporating these expected changes. However, there are β-thalassemia mutations that do not lead to changes in red cells. Mutation of the distal and proximal CACCC boxes within the β-globin gene promoter is characterized by substitution at nucleotide position and leads to silent β-thalassemia.[3] In the carriers of this mutation, all erythrocyte parameters, even MCV, 'the key diagnostic indicator', are normal, and hence, none of the formulas may be effective in detecting these carriers. Mutations in the human *KLF1* gene cause silent β-thalassemia minor.[4] These mutations occur in the CACCC box and affect the binding and responsiveness to erythroid Krüppel-like factor (KLF1), which is essential for expression of the β-globin gene. Since these mutations may still retain some binding ability and affinity to the promoter, they result in a mild phenotype. In these carriers, all erythrocyte parameters, especially MCV and MCH—the basic parameters of the new formula—are within normal range. None of the formulas developed thus far would detect these carriers. Mutations involving the catabolite activator protein (CAP) sites or the 5' or 3' untranslated regions are extremely mild β-thalassemia alleles and are also unidentifiable in heterozygotes.[5]

Formulas basically have been developed to differentiate β-thalassemia minor from iron

deficiency (ID), and the most effective parameters for discrimination between the two are RBC and RDW. While the other parameters are similar in the two conditions, increased RBC is expected in β-thalassemia minor and increased RDW in ID. Roth et al. agree with this expectation by their statement "*...but the red blood cell (RBC) count and red cell distribution width (RDW) can differentiate between the two*".[1] Although this expectation is generally true, RBC is not increased in all carriers (Aslan D, unpublished observation). In fact, it can sometimes be increased even in pure ID.[6] Again, conflicting with general expectation, RDW could be increased in pure β-thalassemia minor (Aslan D, unpublished observation). In those situations in which the expected erythrocyte changes are not present, formulas cannot function properly and cannot identify carriers. In brief, depending on the β-thalassemia mutation, the accuracy of formulas may be altered.

These atypical mutations are not rare or clinically silent. Mutations in the promoter region of the β-globin gene and *KLF1* gene are common in Mediterranean populations.[3,4] While they do not cause erythrocyte changes alone, when accompanied by a classical mutation of β-thalassemia, they result in β-thalassemia intermedia.[7] Of note, those patients usually have severe transfusion-dependent β-thalassemia, a human resource- and cost-intensive disorder as seen in β-thalassemia major. It is similar for the mutations with unexpected changes.

When ID, as a confounding factor in parameters, accompanies β-thalassemia minor, RDW increases, and formulas may misinterpret the carriers as ID. δβ-thalassemia minor, a subgroup of β-thalassemia minor characterized by increased RDW,[8] may likewise be missed by formulas.

The authors reported a sensitivity of 98% and a NPV of 99.77%. Sensitivity of a formula shows the percentage of affected people who are correctly identified as having the condition (here, β-thalassemia minor). Where the β-thalassemia mutation does not effect red cell changes, no identification is available. The sensitivity of a formula that is not working is, naturally, irrelevant. NPV reflects the probability that a person who is a test-negative is a true-negative (here, for β-thalassemia minor). Atypical carriers are not true-negative. They have a mutation but with no influence on erythrocyte parameters;

therefore, the situation appears, and is interpreted, as if these individuals do not have a mutation. In this case, NPV is false high and inaccurate. In the case of the formula presented by Roth et al., if the reliability of the sensitivity and negative predictivity are in question, the other features of the study (e.g., the size and the homogeneity of the population, or the integration of the formula to the automated systems) lose their relevance. As is for accuracy, the sensitivities and negative predictivities for the formulas are related to the underlying mutations, as was also previously reported.[9]

HbA2 elevation, as the next step in detecting β-thalassemia carriers, is reported as the gold standard. However, as previously reported, HbA2 level may be normal in β-thalassemia minor (namely, "Normal A2 β-thalassemia minor").[5] HbA2 level may be normal, or even low, due to accompanying ID and also in δβ-thalassemia minor.[8] For these reasons, absence of HbA2 elevation does not exclude β-thalassemia minor.

In any situation in which red cell parameters are not as expected or even when the HbA2 level is not elevated, family history remains a good guide. Presence of subtle laboratory findings in any sibling or parent strongly suggests the possibility of β-thalassemia minor.

In conclusion, formulas detect and interpret erythrocyte changes. If the β-thalassemia mutation does not effect such changes, none of the formulas, including the one presented by Roth et al., can function properly. Further, if the red cell parameters are not as expected or are affected by confounding factors, formulas can miss the identification of carriers. Awareness of these critical points is essential for achieving eradication of β-thalassemia.

Response letter from the authors of the original paper. We thank the authors of this letter for their interest in our study and the important comments.

The arguments that the authors of this letter arose lead to the final conclusion that any screening is not good enough and just molecular analysis can be the state of art for the diagnosis of thalassemia carriers.

But any screening procedure, as its name means, is just a screening. As the authors of this

letter wrote "achieving this goal (screening) at minimum cost and with maximum accuracy is of utmost importance", principally when thalassemia carriers are under diagnosed all over the world, principally in low developed countries.

Of course, "formulas developed to detect β-thalassemia carriers inherently rely on the changes in erythrocyte parameters, …. these formulas are useful only if the β-thalassemia mutation effects changes in these parameters".

In our study all of the "false negative" results belongs to individuals with MCV ≥77.6 fl and based in molecular analysis performed in some of those carriers, indeed they carried mild mutations like -101 C>T (c:-151C>T) which is a mild mutation. Unfortunately, since the data was obtained from the screening databases not all the patients / carriers were analyzed molecularly. It should be done in the future.

In the future when we perform validation of both formulas presented in our study in a cohort of adult males we can suggest incorporating one of those formulas in the electronic counters and provide an alert flag indicating that this is a blood count suspected to belong to a thalassemia carrier. Same analysis should be done in proven α thalassemia.

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References:

1. Roth IL, Lachover B, Koren G, Levin C, Zalman L, Koren A. Detection of ß-Thalassemia Carriers by Red Cell Parameters Obtained from Automatic Counters using Mathematical Formulas. Mediterr J Hematol Infect Dis. 2018;10:e2018008. https://doi.org/10.4084/mjhid.2018.008 PMid:29326805 PMCid:PMC5760065

2. Clarke GM, Higgins TN. Laboratory investigation of hemoglobinopathies and thalassemias: review and update. Clin Chem. 2000;46:1284-1290. PMid:10926923

3. Maragoudaki E, Kanavakis E, Traeger-Synodinos J, Vrettou C, Tzetis M, Metaxotou-Mavrommati A, Kattamis C. Molecular, haematological and clinical studies of the -101 C --> T substitution of the beta-globin gene promoter in 25 beta-thalassaemia intermedia patients and 45 heterozygotes. Br J Haematol. 1999;107:699-706. https://doi.org/10.1046/j.1365-2141.1999.01788.x PMid:10606872

4. Perseu L, Satta S, Moi P, Demartis FR, Manunza L, Sollaino MC, Barella S, Cao A, Galanello R. KLF1 gene mutations cause borderline HbA(2). Blood. 2011;118:4454-4458. https://doi.org/10.1182/blood-2011-04-345736 PMid:21821711

5. Weatherall DJ. The Thalassemias. In: Beutler E, Coller BS, Lichtman MA, Kipps TJ, Seligsohn U., Eds. Williams Hematology. 6th Ed., New York: McGraw-Hill Company. 2001; 547-580. https://doi.org/10.1006/rwgn.2001.1286

6. Aslan D, Altay C. Incidence of high erythrocyte count in infants and young children with iron deficiency anemia: re-evaluation of an old parameter. J Pediatr Hematol Oncol. 2003;25:303-306. https://doi.org/10.1097/00043426-200304000-00007 PMid:12679644

7. Rund D, Oron-Karni V, Filon D, Goldfarb A, Rachmilewitz E, Oppenheim A. Genetic analysis of beta-thalassemia intermedia in Israel: diversity of mechanisms and unpredictability of phenotype. Am J Hematol. 1997;54:16-22. https://doi.org/10.1002/(SICI)1096-8652(199701)54:1<16::AID-AJH3>3.0.CO;2-7

8. Velasco-Rodríguez D, Alonso-Domínguez JM, González-Fernández FA, Villarrubia J, Ropero P, Martínez-Nieto J, de la Fuente F, Guillén R, Acedo N, Serí C, Cava F. dß-Thalassemia trait: how can we discriminate it from ß-thalassemia trait and iron deficiency anemia? Am J Clin Pathol. 2014;142:567-573. https://doi.org/10.1309/AJCPPBQ8UB1WHXTS PMid:25239426

9. Bordbar E, Taghipour M, Zucconi BE. Reliability of Different RBC Indices and Formulas in Discriminating between ß-Thalassemia Minor and other Microcytic Hypochromic Cases. Mediterr J Hematol Infect Dis. 2015;7:e2015022. https://doi.org/10.4084/mjhid.2015.022 PMid:25745549 PMCid:PMC4344165