Mean Corpuscular Volume of Heterozygotes for \( \beta \)-Thalassemia Correlates With the Severity of Mutations

By Deborah Rund, Dvora Filon, Nurith Strauss, Eliezer A. Rachmilewitz, and Ariela Oppenheim

The relationship between the degree of microcytosis and the type of mutation carried by \( \beta \)-thalassemia heterozygotes was investigated. In 113 individuals, 18 different mutations were identified, correlated with mean corpuscular volume (MCV) values, and analyzed statistically. Overall, there was a wide range of MCV (56.3-87.3 fL). In almost all cases, carriers of \( \beta' \) mutations had an MCV below 67 fL, whereas all but a few \( \beta' \) heterozygotes had MCVs above this cutpoint. Mean MCV of \( \beta' \) carriers was statistically significantly lower than those of \( \beta' \) heterozygotes. The various \( \beta' \) mutations were associated with significant differences in mean MCV values. In contrast, all the \( \beta' \) (null) mutations had virtually identical ranges of MCV. The results indicate that degree of reduction in MCV is directly related to the severity of the mutation. Deviations, in four cases, were associated with concurrent \( \alpha \) gene rearrangements, whereas in three other cases, the MCV was not significantly affected by concurrent \( \alpha \) rearrangements. The MCV of \( \beta' \)-thalassemia heterozygotes is a valuable parameter in planning strategies for rapid identification of mutations in populations with great mutational diversity. Its use can be particularly advantageous in the setting of prenatal diagnosis. The pattern of mean corpuscular hemoglobin (MCH) values was similar to the MCV pattern. However, our results suggest that MCH may be preferred for carrier detection in population screening.

\[ \text{MCV} \]
GENOTYPE AND MCV OF β-THALASSEmia CARRIERS

(SPSS, Inc, Chicago, IL) software was used to perform the analysis, at the Hadassah Computer Center.

RESULTS

Individuals with β' mutations have a lower MCV than those with β'. A family study (Fig 1), which antedated the technique of PCR, illustrates the observations that led us to the present studies. A Kurdish Jewish couple (both carriers of β-thalassemia) sought prenatal diagnosis for their sixth pregnancy. They had one unaffected child and three children who were heterozygotes. A previous pregnancy had been terminated after prenatal diagnosis by fetal blood sampling. Routine hematologic tests and haplotype analysis were performed in an attempt to identify a nonthalassemic (A/A) sibling, and to facilitate DNA-based prenatal diagnosis by restriction fragment length polymorphism (RFLP). The analysis suggested that the parents carried two different mutations, with a high MCV phenotype (mother) and a lower MCV phenotype (father), which segregated in the children (Fig 1).

We therefore commenced studies of all thalassemia carriers who were available. Thus far, 113 β-thalassemia heterozygotes, who were found to carry any of 18 different point mutations, were examined. Nine of these were mutations leading to a β' phenotype and nine to a β' phenotype. Sixty-three individuals were carriers of β' mutations (55.8%) and 50 of β' mutations (44.2%). These individuals represent a number of different ethnic groups: Arabs (54.9%), Kurdish Jews (30.1%), Druze (10.6%), and others, which include Samaritans and Jews of other Mediterranean countries (4.4%). Carrierrship of β' and β' mutations was approximately equal for individuals from the major ethnic groups (Arab and Kurdish Jewish).

Figure 2 is a scattergram of the MCV of the subjects, grouped according to the mutations that they carry. The mean MCV value for β' mutations was 63.1 (SD = 3.4), whereas for β' mutations it was 69.3 (SD = 5.6). The difference between the mean values of MCV of the two groups was statistically significant ($P < .0001$).

Could MCV have a predictive value for a β' mutation versus β'? This question was answered positively by discriminant analysis, which yielded a significant discriminant function for β' versus β' that includes MCV ($P < .0001$). This means that MCV can be used to predict whether a heterozygote carries a β' or a β' mutation. Estimating Fisher's linear discriminant functions, we arrived at the classification rule that if the MCV is greater than 66.96, the mutation is β', and if the MCV is lower, it is β' (arrow, Fig 2). Using this cutpoint, we obtained the results presented in Table 1, with 81.4% of individuals correctly classified as β' or β'. The major source of erroneous predictions was the MCV of individuals with the β' mutation IVS1 nt 110, half (11 of 21) of whom had MCV values below the β' boundary (Fig 2). Another source of error was the low MCV of two individuals with the IVS2 nt 745 mutation.
There was a wide range of MCV values their distribution (Fig 66.96 fL. Correctly classified cases: 92/113 = 81.4%.

Different \( \beta^* \) mutations result in different MCV values. There was a wide range of MCV values (56 to 87 fL), and their distribution (Fig 2) is nonrandom. We asked whether the different mutations have MCV values that are distinguishable from each other in a statistically significant fashion.

We tested this hypothesis using analysis of variance. All of the groups (a set of individuals carrying a given mutation) carrying \( \beta^* \) mutations were found to have no statistically significant intergroup variation in MCV (\( P = .86 \)). That is, all carriers of \( \beta^* \) mutations behave as a single group with regard to MCV. In contrast, the different \( \beta^+ \) mutations showed statistically significant differences in mean MCV (\( P < .0001 \)) between groups.

We wished to test how useful these data would be in predicting mutations among the \( \beta^* \) individuals, as an aid in mutational screening of heterozygotes. This was performed using discriminant analysis in a similar way to the analysis of \( \beta^+ \) versus \( \beta^* \) mutations. The results showed that even though the present data are limited, ranges of expected MCV values could be delineated for the different mutations. For certain mutations there was an overlap in the MCV ranges. That is, an individual with a certain MCV value would be expected to have any of several mutations.

It is a common practice in mutational screening to use ethnic origin as a guideline. Therefore, we also tested the effect of introducing ethnic origin as an additional variable. This resulted in a better ability to discriminate among the various mutations on the basis of MCV. This is primarily because the ethnic background helped pinpoint the mutation; some of the \( \beta^+ \) mutations (three of nine) are ethnic group specific. These results, and a more expanded statistical treatment of the data, will be published elsewhere.

MCV and ethnic background. Because specific mutations are characteristic of different ethnic groups, the different MCVs could reflect the ethnic background rather than the type of mutation. However, the findings thus far also raised the interesting possibility that the reduction in MCV directly results from the severity of the mutation. We therefore wanted to determine whether MCV is influenced by ethnic background for any given mutation. The availability of mutations that are widespread across different ethnic groups enabled us to do the analysis.

Regression analysis was performed on the carriers of the \( \beta^+ \) mutations only, because \( \beta^+ \) mutations were homogeneous and thus were not expected to be informative. To test this hypothesis directly and eliminate a factor that could introduce bias, individuals with known \( \alpha \)-globin gene rearrangements were excluded. Using this analysis, the only factor of significance in determining MCV was found to be the severity of mutation (\( P < .0001 \)) and the ethnic origin had no additional significant contributory effect (\( P = .46 \)).

Confounding factors. Because concomitant presence of \( \alpha \)-thalassemia may affect the MCV of heterozygotes, \({ }^{1,2} \) analysis of \( \alpha \)-globin gene number was performed in 32 of the individuals. Because we wanted to find whether \( \alpha \)-thalassemia affected the MCV distribution, the individuals were selected in order to increase the likelihood of identifying those with concomitant \( \alpha \)-globin rearrangements. Most of the individuals selected for analysis had an MCV that was outside, or at the extremes of, the range of MCV seen in others with the same mutation. These individuals are designated (by a diagonal line) in Fig 2.

Twenty-five of the 32 individuals examined had a normal \( \alpha \)-globin gene pattern. Of the remaining seven, three had one \( \alpha \) gene deleted (-\( \alpha^+/-\alpha \)); in a fourth, two \( \alpha \) genes were deleted in trans; in the fifth and sixth, a triplicated \( \alpha \) gene was present (\( \alpha \alpha^+/-\alpha^+ \alpha \alpha \alpha \alpha \alpha \)); and in the seventh, an \( \alpha \)-globin quadruplication was found (\( \alpha \alpha \alpha \alpha /\alpha \alpha \alpha \alpha \)). The high percentage of individuals found to have \( \alpha \)-globin gene rearrangements (7 of 32, 21.8%) is most likely due to the biased sampling, as already explained, because \( \alpha \)-thalassemia is not known to be highly prevalent in Israel.\(^{13} \)

In evaluating the effect of concurrent \( \beta^+ \) and \( \alpha \)-thalassemia on the MCV of the heterozygotes tested, we found that in two of these individuals, \( \alpha \)-globin gene deletion(s) appeared to contribute to the unexpectedly high MCV (inverted triangle and diamond, Fig 2). In two other individuals, the triplicated \( \alpha \)-globin locus may have contributed to an unexpectedly low MCV (triangles, Fig 2). In three individuals the MCV stayed within the range despite \( \alpha \) rearrangement. Overall, statistical analysis for the values described (Table 2) were only slightly changed if carriers doubly heterozygous for \( \alpha^+ \) as well as \( \beta^+ \)-thalassemia were omitted from the analysis. This is remarkable considering that four of the seven data points omitted were MCV values that deviated considerably from the mean.

Table 1. Use of MCV in Predicting Whether Heterozygotes Carry \( \beta^* \) or \( \beta^+ \) Mutations: Classification Results

<table>
<thead>
<tr>
<th>Type of Mutation Found</th>
<th>Type of Mutation Predicted</th>
<th>No. of Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta^* )</td>
<td>( \beta^* )</td>
<td>60 (95.2%)</td>
</tr>
<tr>
<td></td>
<td>( \beta^+ )</td>
<td>3 (4.8%)</td>
</tr>
<tr>
<td>( \beta^+ )</td>
<td>( \beta^* )</td>
<td>18 (36.0%)</td>
</tr>
<tr>
<td></td>
<td>( \beta^+ )</td>
<td>32 (64.0%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>113</td>
</tr>
</tbody>
</table>

The MCV value used to predict the type of mutation (\( \beta^* \) or \( \beta^+ \)) was 66.96 fL. Correctly classified cases: 92/113 = 81.4%.

Table 2. Effect of Concurrent \( \alpha \)-Gene Rearrangement on Mean MCV of \( \beta \)-Thalassemia Heterozygotes

<table>
<thead>
<tr>
<th>( \alpha )-Thalassemia</th>
<th>Mean MCV</th>
<th>Cutpoint for ( \beta^* ) or ( \beta^+ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals Included</td>
<td>63.1</td>
<td>66.96</td>
</tr>
<tr>
<td>Individuals Not Included</td>
<td>67.9</td>
<td>66.74</td>
</tr>
</tbody>
</table>

| No. of individuals tested | 113      | 106                                      |
the MCH is less sensitive to the difference between \( \beta^+ \) and severe \( \beta^- \) mutations. Almost all of the individuals (21 of 22) with the \( \beta^- \) mutation intervening sequence (IVS) nt 110 had MCH below the 20.94 cutpoint. Similarly, some heterozygotes for IVS1 nt 5 and IVS1 nt -1 would have been misinterpreted as \( \beta^- \) carriers if MCH had been used as a guideline for mutational screening.

**HbA\(_2\)** levels did not correlate with specific mutations. HbA\(_2\) levels are a parameter less readily available than MCV. These data were available on 77 of the individuals. There were significant differences (\( P = .0008 \)) in HbA\(_2\) levels for \( \beta^- \) (mean = 5.13, SD = .937) versus \( \beta^+ \) carriers (mean = 4.28, SD = 1.193). However, the difference was less significant than for the analogous data for MCV and MCH, with a considerable overlap in values (data not shown). We tested for differences among mutations within groups of \( \beta^- \) and \( \beta^+ \) heterozygotes separately. The analysis showed no significant differences for the \( \beta^- \) mutations as well as for the \( \beta^+ \) group (\( p = .46 \)). This means that, in contrast to MCV, HbA\(_2\) cannot be used to predict type of \( \beta^- \) mutation. Others have also found heterogeneity in HbA\(_2\) levels of different thalassemia mutations.\(^4\),\(^16\)

**DISCUSSION**

As has been noted by Weatherall and Clegg,\(^1\) it would be useful to differentiate between \( \beta^- \) and \( \beta^+ \) thalassemia in heterozygous carriers. Attempts to do this have been made in various populations (Jamaican blacks, Thais, and Greeks) before the advent of molecular techniques. In blacks, there was a significant difference in the average MCV and MCH of \( \beta^- \) versus \( \beta^+ \) carriers. However, in the other groups studied, such a distinction was not found. Weatherall and Clegg concluded that the heterozygous state for \( \beta^- \) thalassemia and the more severe form of \( \beta^- \) thalassemia were indistinguishable by the techniques that were then available.

Our results confirm and extend these findings. We found that the \( \beta^- \) mutations were homogeneous, as would be expected due to their null phenotype. This homogeneity was supported by statistical analysis. However, the use of molecular techniques and precise identification of the various \( \beta^- \) mutations showed that not only were they distinguished from \( \beta^- \) mutations, but the various \( \beta^- \) mutations were associated with different MCV values. In general, it would appear that internal IVS mutations (activation of cryptic splice sites) cause lower MCV than mutations in consensus sequences of splice signals. Transcriptional mutations and polyadenylation signal mutations are milder. Moreover, it is of note that a mutation in the TATA box lowers the MCV more than one in the CACACCC upstream element. A deletion of 5 bp in the poly(A) signal sequence is more severe than a point mutation in the signal.

Other investigators have reported MCV values for heterozygotes that are consistent with our findings. For example, in U.S. blacks, heterozygotes for a different TATA box mutation, \(-29 \rightarrow \) G, had MCV 70-72.8 fl, \(^6\) almost identical to our findings for \(-28 \rightarrow \) C. The \(-101 \rightarrow \) T mutation in Turkey was associated with a normal to high MCV\(^7\) and a normal MCV in Italians,\(^8\) as we found. In addition, three other point mutations in the poly(A) signal were reported to lead to MCVs in the same range (71-78 fl),\(^9\) as found for heterozygotes for the poly(A) mutations in our region. This suggests that the nature of the mutation is a crucial determinant of the MCV even in disparate populations.

The clustering of MCV values for each mutation is striking in view of the many factors known to influence the volume of the erythrocyte. Some of the factors that lower the MCV are those that decrease Heme production, such as iron deficiency and lead poisoning. Other factors can raise the MCV, for example, ethanol abuse and folate deficiency. The latter are rare in Israel. The incidence of concurrent iron deficiency in Israeli \( \beta^- \)-thalassemia carriers was reported to be low (4%) and confined, in the adult population, to women of childbearing age.\(^10\) Because ours was primarily a retrospective analysis, iron deficiency was not tested. Young adult males, in the age range of most of the heterozygotes we studied, were not iron deficient,\(^11\) as was also reported for other Middle Eastern populations.\(^3\)

One of the confounding factors affecting MCV values is concurrent \( \alpha^- \)-thalassemia or \( \alpha^- \)-globin gene multiplicity. Because for every mutation there is a range of MCV values, it is expected that in some individuals the MCV will remain within the range in spite of coexistent \( \alpha^- \)-globin gene abnormalities. Indeed, that was found for three individuals in our present study (Fig 2). In four other individuals, \( \alpha^- \)-gene rearrangements appeared to be responsible for deviations in MCV. The reasons for MCV values that
departed from the expected range in several other individuals remains unexplained. These may be due to nondele-
tional α-thalassemia, which was not excluded in the present study (or to any of the other possible confounding factors already mentioned).

Despite the many variables affecting MCV, this parameter is extremely useful in planning strategies for the rapid characterization of mutations. We found that the MCV is a reliable indicator in planning how to prioritize which mutations are to be sought. In Israel, ethnic group identification is extremely useful in planning strategies for the rapid screening criterion. Only three of these would ameliorate the chain imbalance. Indeed, the three individuals with α-gene deletions for whom both MCV and MCH values were available show a shift in their MCH values (Fig 3), but we found the MCH to be a less reliable discriminator between the α and some of the more severe β forms of thalassemia trait. However, for the purposes of carrier detection, MCH seemed superior to MCV. Using accepted normal values (MCV 84 ± 7 fL, MCH 29 ± 3 pg/cell) six β-thalassemia heterozygotes would not have been identified as carriers using MCV as a screening criterion. Only three of these would have been missed using MCH (Figs 2 and 3). This supports the conclusion of Modell and Berdoukas, who suggested that the MCH is more useful for population screening.

Our results suggest that the MCV is not affected by the general genetic background or by the haplotype of the β-globin gene cluster. Mutations that are found in different ethnic groups, and are associated with different β-globin haplotypes (unpublished results) do not seem to result in markedly different MCV values. It will be interesting to reexamine this point as additional data accumulate from other populations. In homozygous β-thalassemia patients, disease severity may vary according to the haplotypic background of the mutation, generally in conjunction with variations in Hb F production.

There is considerable evidence that the nature of the β-thalassemia mutation directly affects the level of β-globin chain production. The direct relationship to the hemoglobin content of the erythrocyte (MCH) implies, perhaps not surprisingly, that in β-thalassemia heterozygotes the level of β-chains is a major limiting factor in hemoglobin production. However, the direct genotype-phenotype relationship between mutations and MCV is intriguing. We would like to speculate that the relative excess of α-chains plays an important role. It is known that precipitated excess α-chains have a profound effect on the structure of the erythrocyte cytoskeleton. One might predict that MCV (more than MCH) would be sensitive to α-gene deletions, because these would ameliorate the chain imbalance. Indeed, the three individuals with α-gene deletions for whom both MCV and MCH values were available show a shift in their MCV to higher values compared with other individuals carrying the same mutation. The MCH value for only one of these individuals (with two α-genes deleted, carrying IVS2 nt 1) was elevated, but to a lesser degree than the MCV (compare Figs 2 and 3). The precise mechanism by which the level of chain synthesis lowers the volume of the erythrocyte remains to be elucidated.

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