# Significance of borderline hemoglobin A2 values in an Italian population with a high prevalence of β-thalassemia

Antonino Giambona,[1] Cristina Passarello,[1] Margherita Vinciguerra,[1] Rita Li Muli,[1] Pietro Teresi,[2] Maurizio Anzà,[2] Gaetano Ruggeri,[2] Disma Renda,[1] and Aurelio Maggio[1]

[1]U.O. Ematologia II con Talassemia, Ospedale Vincenzo Cervello, Palermo and [2]Centro di riferimento Aziendale per la Prevenzione delle Talassemie, ASL 6 Palermo, Bagheria, Italy

## ABSTRACT

We report a retrospective analysis carried out on 23,485 subjects submitted to a screening program from 2000 to 2006. Of these subjects, 3,934 had borderline $HbA_2$ values from 3.1 to 3.9%; 410 samples, analyzed previously using PCR methods and sequencing because all of these were partners of a carrier of classical β-thalassemia, were selected for statistical analysis. Of 410 subjects, 94 (22.9%) were positive for a molecular defect in the β-, δ- or α-globin genes. The most prevalent molecular defects were β IVS1 nt 6 (HBB c.92+6T C), co-inheritance of severe β thalassemia and δ mutations, β-promoter mutations and triplication of α genes were detected; α-thalassemia and Hb-variants were also evident. Borderline $HbA_2$ is not a rare event in a population with a high prevalence of β-thalassemia carriers. These data support the necessity to investigate these cases at a molecular level, particularly if the partner is a carrier of β-thalassemia.

Key words: $HbA_2$ borderline, β thalassemia, carrier screening.

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## Introduction

In a country with a high prevalence of β-thalassemia, the detection of borderline $HbA_2$ subjects should be addressed to avoid missing *at risk couples*. In Sicily it has been reported that there is a 6% prevalence of β-thalassemia carriers with an expected 1/270 probability of at risk couples.[1] In fact, while the presence of a single β-thalassemia allele is usually associated with hypocromic microcytic red cells and an increase in $HbA_2$ levels, in some cases, the effect of the β-thalassemia genotype or the interaction of this genotype with other molecular defects (α-, δ-thalassemia) may render this allele completely silent with normal or borderline hematologic and $HbA_2$ levels.

Due to these factors, some individuals with β-globin gene silent mutations[2,3] or with triplication of α-globin genotypes[4] may be missed during screening programs for β-thalassemia, and may only be detected following the birth of an affected offspring. These genotypes must be considered *at risk* for having children with β-thalassemia if their partner is a carrier of the classical β-thalassemia mutation.[5] In this paper, we report the results of the molecular analysis of 410 subjects with normal or β-thalassemia carrier-like phenotypes, without iron deficiency, and with $HbA_2$ levels between 3.1 and 3.9%. The main objective of the study was to evaluate the prevalence and the significance of borderline $HbA_2$ values for subjects in our population. The secondary aim was to perform a genotype-phenotype correlation within the sub-group of subjects with β or α molecular defects.

## Design and Methods

A previous study on borderline $HbA_2$ phenotype occurrence in areas with high prevalence of β-thalassemia,[6] using HPLC procedures for $HbA_2$ determination, defined values of $HbA_2$ between 3.3 and 3.7% as borderline. However, because of the high heterogeneity of molecular defects[7,8] evident in our population, with also a prevalence of defects causing $\beta^+$- or $\beta^{++}$-thalassemia, we defined $HbA_2$ levels as borderline if they were between 3.1 and 3.9%.

A retrospective analysis was carried out on 23,485 samples obtained during a program for β-thalassemia carrier screening in the Sicilian population from 2000 to 2006. We identified

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*Correspondence: Aurelio Maggio, U.O.C. Ematologia II con Talassemia, Azienda Ospedaliera Vincenzo Cervello, via Trabucco 180, Palermo, Italy.*
*E-mail: aureliomaggio@virgilio.it*

3,934/23,485 (16.75%) samples showing borderline $HbA_2$ levels; of these, 726 were partners of carriers of β-thalassemia and all were submitted to molecular analysis; from these we selected only 410 samples, without iron deficiency anemia or hemoglobin variants, for statistical analysis. Figure 1 shows the profile of study performed in this work.

The samples showing iron deficiency anemia or hemoglobin variants were excluded from statistical analysis to avoid study bias. All samples are routinely subjected to hematologic and molecular analysis as previously described.[8] All statistical analyses were performed with STATA 9 (StataCorp, 4905 Lakeway Drive, College Station, Texas 77845 USA). Means are reported with standard deviation (SD); proportions and differences are reported with 95% confidence intervals (CI). A Receiver Operating Characteristic (ROC) analysis was performed to determine sensitivity and specificity of $HbA_2$ determination in detecting an *at risk* genotype within the 3.1 to 3.9% interval.

This study was approved by the Ethical Committee of A.O. "V .Cervello" Hospital, and informed consent was obtained from all subjects.

## Results and Discussion

Among 410 subjects with borderline $HbA_2$ values, 94 (22.9%) were positive for a molecular defect in the β-, δ- or α-globin genes. No molecular defects were found in the remaining 316 individuals (Figure 1).

Among the positive samples, 55 had MCV < 80 fl (group A) and 39 had MCV ≥ 80 fl (group B). Table 1 shows the hematologic findings of the positive samples.

*Group A*. Genotypes and hematologic findings of this group of subjects are shown in Table 1A. The IVSI nt6 (HBB c.92+6T→C)[9] genotype, the presence, in cis or in trans, of severe β and δ-thalassemia mutations and the α-thalassemia genotype accounted for 81.8% of these molecular globin gene defects (Figure 1, Table 1A). In the samples with the β and δ mutations, family analysis showed that 7/11 subjects had mutations in trans. No difference was found in $HbA_2$ levels if mutated δ-globin gene was in cis or in trans with mutated β-allele but more data are necessary for a correct statistical analysis. Among those with α thalassemia, 8 showed the $-\alpha^{3.7}$ deletion (Z84721.1:g.34164_37964del3801),[10] one had the mutation in the initial codon of the $\alpha_2$ gene AUG→ACG (HbA2 c.2T→C)[11] and the last had a deletion of a pentanucleotide in the IVS1 donor site (HbA2 c.95+2_95+6delTGAGG)[12] located in the 5' region of the $\alpha_2$ globin gene.

Six samples (10.90%) were heterozygotes for -101(HBB c.-151C→T),[2] and -92 (HBB c.-142 C→T)[3] β-globin gene promoter mutations (Figure 1, Table 1A). Furthermore, 2 of these cases were also carriers of the $-\alpha^{3.7}$ deletion (Z84721.1:g.34164_37964del3801)[10] and showed lower $HbA_2$ levels (3.4% and 3.5%).

Three (7.69%) subjects were carriers of hemoglobin variants, both α and β (Figure 1, Table 1A). The hemoglobin variants detected were Hb Acharnes β-Cod 53 (HBB c.160G→A),[13] Hb Bernalda $\alpha_1$-Cod 119 (HbA1 c.358C→T)[14] and Hb Ernz β-Cod 123 (HBB c.371C→A),[15] respectively. Moreover, the carrier of Hb Ernz (HBB c.371C→A) also showed the $-\alpha^{3.7}$ deletion (Z84721.1:g.34164_37964del3801) with a greater reduction of MCV (69.7 fL) and MCH (23.4 pg) and a higher $HbA_2$ level (3.6%), without polycythemia. One sample was positive for the α-globin gene triplication ($\alpha\alpha\alpha^{anti3.7}$)[4] (Figure 1, Table 1A).

*Group B*. Two genotypes showed the α-globin gene triplication ($\alpha\alpha\alpha^{anti3.7}$)[4] and the β-globin gene promoter mutation, -101 (HBB c.-151C→T)[2] and -92 (HBB c.-142 C→T),[3] accounted for the 71.8% of these molecular glo-

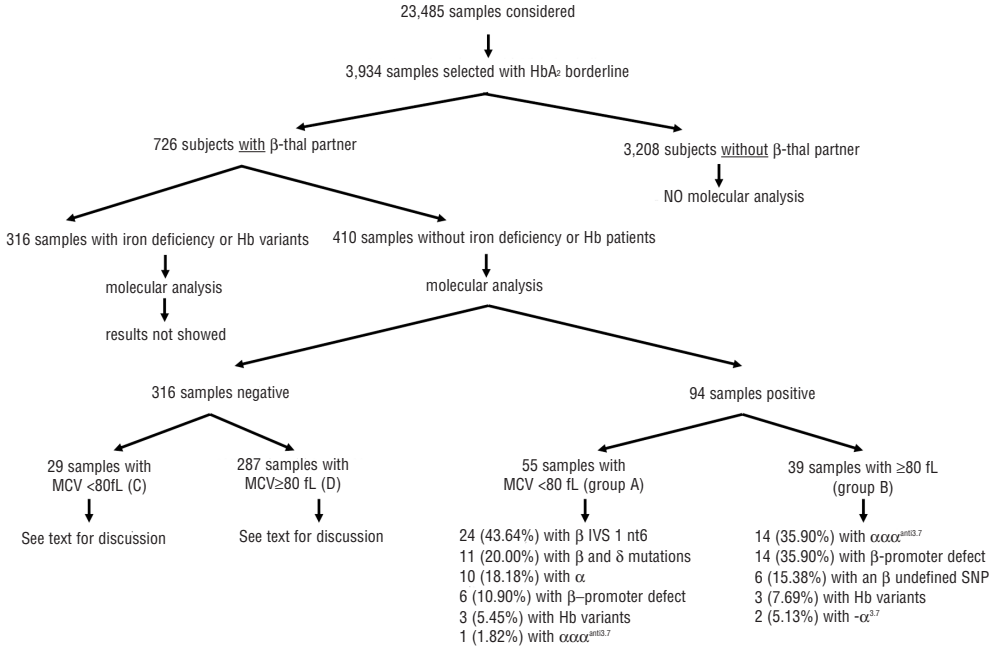


**Figure 1. Profile of study used to evaluate the genotypes β-thal or α-thal presented in samples with $HbA_2$ between 3.1 % and 3.9%.**

**Table 1.** Hematologic parameter mean values and Standard Deviation (SD). (A) parameters of the genotype group found in sample with MCV<80 FL. Among double heterozygotes for severe β- and δ-thalassemia, 5 subjects showed αβ IVSI nt110 (HBB c.93-21G→A), 3 had an association with δ $HbA_2$-Yialousa (HBD c.82G→T) and 2 with IVSII nt 897(HBD c.316-2 A→G), 2 subjects a β IVSI nt1 (HBB c.92+1 G→A) and $HbA_2$-Yialousa, 3 subjects had a β codon 39 (HBB c.118C→T) with $HbA_2$-Yialousa and the last individual showed a double heterozygosis for αβ IVSII nt745 (HBB c.316-106C→G) and $HbA_2$-Yialousa. (B) Parameters of the genotype group found in sample with MCV≥80 FL.

| A Groups | Frequence | RBC ($10^6$/$mm^3$) | Hb(g/dL) | MCV (fL) | MCH (pg) | RDW (%) | $HbA_2$ (%) | HbF (%) |
|---|---|---|---|---|---|---|---|---|
| IVS 1 nt 6 heterozygote | 24 | 5.77±0.62 | 12.94±1.26 | 69.18±2.58 | 22.47±0.85 | 14.47±0.62 | 3.69±0.14 | 0.53±1.36 |
| severe β heterozygote; δ heterozygote | 11 | 5.82±0.56 | 11.66±0.85 | 61.53±2.91 | 20.08±1.12 | 15.1±0.70 | 3.49±0.17 | 1.20±0.76 |
| β promoter heterozygote | 6 | 5.19±0.57 | 14.36±1.10 | 77.73±1.48 | 27.93±3.40 | 13.43±0.58 | 3.67±0.19 | 0.88±0.92 |
| β Variant* heterozygote | 3 | 5.08±1.02 | 12.60±1.73 | 74.03±3.80 | 25.00±1.42 | 14.83±1.62 | 3.37±0.21 | 0.63±0.21 |
| $\alpha\alpha\alpha^{anti3.7}$ heterozygote | 1 | 4.70 | 12.30 | 75.20 | 26.01 | 13.10 | 3.10 | 0.50 |
| -α heterozygote | 10 | 5.23±0.80 | 12.83±1.05 | 74.41±6.96 | 24.87±2.87 | 14.33±1.49 | 3.13±0.67 | 0.29±0.25 |

| B Groups | Frequence | RBC ( $10^6$/$mm^3$) | Hb (g/dL) | MCV (fL) | MCH (pg) | RDW (%) | $HbA_2$ (%) | HbF (%) |
|---|---|---|---|---|---|---|---|---|
| $\alpha\alpha\alpha^{anti3.7}$ heterozygote | 14 | 4.66±0.61 | 13.89±1.47 | 86.51±2.92 | 29.94±1.24 | 12.56±0.56 | 3.35±0.12 | 0.60±0.56 |
| β promoter heterozygote | 14 | 4.56±0.35 | 13.35±1.33 | 85.98±3.21 | 29.25±1.37 | 13.06±0.52 | 3.70±0.96 | 1.29±1.34 |
| bSNP heterozygote | 6 | 4.39±0.36 | 13.28±1.32 | 88.55±3.66 | 30.27±1.11 | 12.58±0.65 | 3.33±0.16 | 0.30±0.19 |
| β Variant** heterozygote | 3 | 5.14±0.41 | 15.20±1.35 | 86.70±1.08 | 29.50±0.44 | 13.23±0.93 | 3.27±0.16 | 0.67±0.45 |
| -α heterozygote | 2 | 4.40 | 12.30 | 80.30 | 27.80 | 13.50 | 3.40 | 0.00 |
| | | 5.00 | 13.40 | 80.60 | 26.80 | 13.90 | 3.10 | 0.50 |

**Hb Acharnes (HBB c.160 G→A); Hb Ernz (HBB c.371 C→A); Hb Bernalda (HBA1 c.358 C→T); **Hb Kokomo (HBB c.223G→A); Hb Ernz (HBB c.371 C→A).*

bin gene defects (Figure 1, Table 1B).

An undefined Single Nucleotide Polymorphism (SNP) in the β-globin gene was shown in 6 cases (15.4%) (Figure 1, Table 1A). Among these 5 unrelated samples, there was a T→C substitution at 12 nts 5' to the poly A site or +1570 from the CAP site (HBB c.*+96T>C). This final substitution was described previously as a β thalassemia mutation[16] and recently as a polymorphism.[17] A point nucleotide substitution, not previously described, was also found in one sample at position β -54 G→A (HBB c.-104G→C) (*M. Vinciguerra, personal communication, 2006*).

Three samples (7.7%) presented an Hb Variant of the β-globin gene (Figure 1, Table 1B): 2 subjects with Hb Ernz (HBB c.371C→A)[15] and one with Hb Kokomo (HBB c.223G→A) (*M. Gallivan, personal communication, 2005*). Two samples (5.1%) showed the $-\alpha^{3.7}$ deletion (Z84721.1:g.34164_37964del3801) (Figure 1, Table 1B).

The importance of detecting all β-thalassemia carriers is very relevant for a prevention screening program aimed at the identification of *at risk couples*. Moreover, although borderline $HbA_2$ values were previously described during a β-thalassemia screening program in a high risk population,[6] so far, we do not have any accurate data on their prevalence and clinical significance.

The results of this retrospective analysis, although limited by the selection of samples, suggest both that this event is not rare in our population and that it may be associated with *at risk* β-thalassemia genotypes.

The most severe β molecular gene defects were found in Group A, and included samples with borderline $HbA_2$ values and MCV <80 fL (Figure 1). In fact, if we select the four main genotypes found in the A and B groups for a cut-off MCV value of 80 fL (Figure 2), we find that in the MCV≥80 range it is possible to detect β-globin gene promoter mutations and triplication of the α globin gene, while the most severe genotypes at the IVSI nt 6 (HBB c.92+6T→C) and the co-inheritance of severe- β and δ thalassemia mutations were detected only in the MCV <80 range (Table 1, Figure 2).

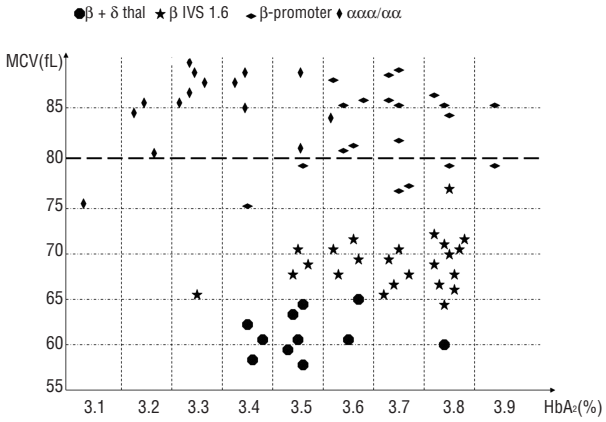


**Figure 2.** Distribution of $HbA_2$ and MCV values of the four genotypes most presented in the group A and B, among the selected borderline $HbA_2$ levels.

Furthermore, in the group with MCV≥80 fL, the β-promoter mutation carriers showed a higher $HbA_2$ level (Table 1, Figure 2) with a statistically significant difference (t=8.08; $p<0.05$) in comparison with the α globin gene triplication group (Table 1, Figure 2).

These findings suggest that, according to the evaluation of both MCV and $HbA_2$, it is possible to differenti-

ate mild mutations from more severe β-globin gene defects. Moreover, this suggests that genotypes previously described (mutations of β-promoter and α globin gene triplication) to be linked with a risk for mild or intermediate thalassemia phenotypes, if the partner is a carrier of a classical β thalassemia mutation,[18] were present only in the group with borderline $HbA_2$ values and MCV ≥80 fL. A ROC analysis was performed considering each single $HbA_2$ value from 3.1 to 3.9% as a cut-off point. At the 3.5% $HbA_2$ cut-off value, sensitivity and specificity were 77.81% and 67.90% respectively. Using the 3.5% $HbA_2$ value as a cut-off, we correctly classified the 75.85% of cases with a Likelihood Ratio + of 3.06.

The $\alpha^+$ thalassemia genotype was found among samples from Group A and B (Figure 1, Table 1). In Group A, $\alpha^+$ thalassemia showed classical hematologic findings associated with an anomalous slight increase in $HbA_2$ level (Table 1A). Similar results were reported by Galanello[19] during screening programs in Sardinia. Regulation factors may play a role in the increase in $HbA_2$ levels, although so far there is no plausible explanation.

Hemoglobin variants were also detected among samples from Group A and B (Figure 1, Table 1). Among these, three Hb variants, Hb Acharnes (HBB c.160G→A),[13] Hb Bernalda (HbA1 c.358C→T)[14] and Hb Ernz (HBB c.371C→A),[15] previously described in Italians, Greeks and in a family of Moroccan origin, were detected in Group A, while in Group B one case with Hb Kokomo (HBB c.223G>A) (*M. Gallivan, personal communication, 2005*) and 2 cases with Hb Ernz (HBB c.371C→A)[15] were found (Figure 1, Table 1). Probably, the β-globin gene variants, detected in Group A and B, caused a mild reduction in the expression of mutated alleles, allowing the carriers to present a normal hematologic profile with a slight increase in $HbA_2$ level.

The possible interaction of $\alpha^+$ thalassemia or of these Hb variants with a heterozygote for a classical β thalassemia mutation does not change the carrier phenotype[15,20] except for Hb Acharnes (HBB c.160G→A). As reported in a previous study, a subject with intermediate thalassemia resulted from the association of Hb Acharnes with β IVSI nt1 (HBB c.92+1 G→A) mutation.[13] Finally, other genotypes detected only among Group B were an undefined Single Nucleotide Polymorphism (SNP) in the β-globin gene (Figure 1, Table 1B). These last samples were difficult to evaluate and it was necessary to incorporate family history to better understand their potential influence on the phenotype. However, so far there is no evidence regarding their possible role as *at risk* β-thalassemia genotypes.

Concerning the 316 negative samples with borderline $HbA_2$ levels (Figure 1), we may only speculate as to some possible explanations. In fact, while samples with MCV < 80 fl could be due to some rare and as yet unexplored molecular defects such as $-\alpha^{5.3}$ (Z84721.1:g.28684_33930del5246),[21] or to nucleotide sequence changes in LCR or Enhancer regions of the β-globin gene,[22] those with MCV ≥80 fL could be explained by different factors: increase of δ-globin gene expression, use of drugs like zidovudine (AZT),[23] presence of hyperthyroidism[24] or *Pseudoxanthoma Elasticum* (PXE)[25] or it is also possible to speculate that these phenotypes could be due to mutations in regulatory genes involved in the synthesis of specific protein factors. However, further studies are necessary to clarify this issue. For both groups the method used for $HbA_2$ determination (electrophoresis, HPLC) and interpretation of results must be considered. Paleari[26] evaluated the inter-laboratory variation and accuracy in the $HbA_2$ assay among 48 Italian laboratories routinely measuring $HbA_2$ HPLC analyzers; during this study unacceptable results were demonstrated from 17% to 31.9% and the worse results were obtained using a borderline $HbA_2$ sample. In conclusion, borderline $HbA_2$ levels are not a rare event in a population with a high prevalence of β-thalassemia carriers, with the most severe genotypes associated with microcytosis. These data support the necessity to investigate these cases at a molecular level, particularly if the partner is a carrier of β-thalassemia.

## Authorship and Disclosures

Both AG and CP were mainly responsible for the study. They contributed to designing the study, establishing the research plan, performed molecular analysis on α and δ globin genes, analyzed the data, conducted the statistical analysis and drafted the article; MV and RL collected some of the patients for this study and performed hemoglobin and molecular analysis for β globin gene; PT, MA, GR collected part of the samples and data, and performed hemoglobin analysis; DR assisted in data analysis and interpretation; AM conceived the project, assisted in data interpretation, and checked and revised the final version of manuscript.

The authors reported no potential conflicts of interest.

Some of the data of this study were presented in part at the 41st Congress of the Italian Society of Hematology, Bologna, Italy, 2007.

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