Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

Article

Cut-off Values of Hematologic Parameters to Predict the Number of Alpha Genes Deleted in Subjects with Deletional Alpha Thalassemia

Diego Velasco-Rodríguez [1,*], Carlos Blas [1], Juan-Manuel Alonso-Domínguez [1], Gala Vega [1], Carlos Soto [1], Aránzazu García-Raso [1] and Pilar Llamas-Sillero [2]

[1] Servicio de Hematología. Hospital Universitario. Fundación Jiménez Díaz, IIS-FJD. Universidad Autónoma de Madrid. Madrid. Spain
[2] Servicio de Hematología. Hospitales Quirón Públicos. IIS-FJD. Universidad Autónoma de Madrid. Madrid. Spain
* Correspondence: diegovelascorodriguez@gmail.com; Tel.:

Abstract: Most of α-thalassemia cases are caused by deletions of the structural α-globin genes. The degree of microcytosis and hypochromia has been correlated with the number of affected α-globin genes, suggesting a promising role of hematologic parameters as predictive diagnostic tools. However, cut-off points for these parameters to discriminate between the different subtypes of α-thalassemia remain to be clearly defined. Six hematologic parameters (RBC, Hb, MCV, MCH, MCHC and RDW) were evaluated in 174 cases of deletional α-thalassemia (92 heterozygous α+ thalassemia, 40 homozygous α+ thalassemia, 34 heterozygous α0 thalassemia and 9 cases of Hb H disease). A good correlation between the number of deleted alpha genes and MCV ($r = -0.672$, $p<0.001$), MCH ($r = -0.788$, $p<0.001$) and RDW ($r = 0.633$, $p < 0.001$) was observed. The deletion of at least two alpha genes in adult individuals with microcytosis without iron deficiency and normal values of Hb A2 and Hb F should be discarded with MCH <23.15 pg. Furthermore, MCH < 21.90 pg and/or MCV <70.80 fL are strongly suggestive of the presence of one α0 allele. Finally, an accurate presumptive diagnosis of Hb H disease can be made if both RDW ≥19.6% and MCH <18.45 pg are seen.

Keywords: alpha; thalassemia; deletional; cut-off; number of genes; microcytic anemia; differential diagnosis

1. Introduction

Alpha-thalassemia is the most prevalent isolated genetic disorder worldwide [1]. Its geographic distribution is highly variable, and the highest prevalence is seen in several regions of China and Southeast Asia [2-7], and also in the Mediterranean and Middle Eastern regions [8]. However, migration flows in the last decades have significantly increased its prevalence in the rest of the world and, consequently, the amount of people needing diagnosis and management of this condition is increasing, especially in developed countries [5, 8, 9].

The α-globin gene cluster is located at the short arm of chromosome 16 (16p13.3) and contains 2 functional α genes, a ζ gene, 3 pseudogenes (Ψα1, Ψα2, Ψζ1) and a θ1 gene of undetermined function [10, 11]. Normal subjects have 4 α genes (αα/αα), 2 on each chromosome.

The majority of α-thalassemia cases are caused by deletions of the structural α-globin genes, whereas single point mutations or nucleotide insertions (nondeletional α-thalassemia) are much less frequent [1].

There are four types of deletional α-thalassemia, and their severity is correlated with the number of affected α-globin genes [1, 11-14]. Carriers of a deletion of only one α gene (α+ thalassemia) have slightly decreased values of mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), being sometimes overlapped with normal values [1, 10-12]. Both homozygosis for α+

© 2017 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

thalassemia (–α/–α) and the heterozygous form of α^0 thalassemia (—/αα) cause mild microcytic and hypochromic anemia [1, 10-12]. These milder forms of α-thalassemia can ameliorate the severity of β-thalassemia major and sickle cell disease when they are co-inherited [15-17].

Hemoglobin (Hb) H disease is produced by the loss of three α genes, resulting in a marked decrease in the production of alpha globin. These individuals characteristically have microcytic hypochromic chronic hemolytic anemia with acute episodes of hemolysis in response to oxidant drugs and infections, splenomegaly and mild jaundice [6, 10, 12, 18]. When the four α-globin genes are deleted, a severe condition called Hb Bart hydrops fetalis syndrome is developed. The clinical presentation of these subjects consists in fetal onset of generalized edema, severe hypochromic anemia, marked hepatosplenomegaly, extramedullary erythropoiesis, and death in the neonatal period [6, 12, 18].

Since α-thalassemia carriers show normal levels of Hb A_2 and Hb F, molecular analysis of the α-globin cluster is required for the diagnosis [1]. The polymerase chain reaction (PCR) is the most common method to diagnose the different forms of deletional α-thalassemia, although other techniques such as Multiplex Ligation dependent Probe Amplification (MLPA) are widely used [19].

Differences in laboratory parameters of red blood cells (RBC) provided by hematologic counters between the different forms of deletional α-thalassemia have been evaluated by some authors, and a promising role as predictive diagnostic tools have been suggested [13, 14, 20]. However, no cut-off points for those parameters have been defined so far.

The aims of this work were to describe the phenotype of RBC based on laboratory parameters of 174 α-thalassemic individuals diagnosed by molecular techniques, and to evaluate whether the number of deleted alpha genes can be predicted by precise cut-off points of the hematologic parameters of the subject.

2. Materials and Methods

Over a 5-year period (April 2012–May 2017), all the deletional alpha-thalassemia cases diagnosed in the Fundación Jiménez Díaz University Hospital by molecular analysis were included in this retrospective and observational study (n= 174). All patient data were de-identified and anonymized prior to analysis. None of the subjects included had received a blood transfusion in the previous 3 months. All samples were collected in K3-EDTA anticoagulant (Vacutainer™; Becton-Dickinson, New Jersey, USA), and a complete blood count (CBC), an iron panel [serum iron, ferritin, transferrin, and transferrin saturation index (TSI)], high-performance liquid chromatography (HPLC) and molecular analysis were performed in all samples.

Hb A_2 levels were lower than 3.5% and Hb F levels were lower than 3.4% in all cases, ruling out heterozygous β-thalassemia and heterozygous δβ-thalassemia. Carriers of α-thalassemia and additional hemoglobinopathy were not included in the study.

All the laboratory and demographic data were extracted from the local laboratory information system.

No signed consent was obtained from the patients since all the tests had been performed as part of their clinical work-up. The study followed the ethical principles of the Helsinki Declaration and was previously approved by the ethical committee of our institution.

2.1. Analytical Methods

A GAP-PCR assay of the most frequent deletions that cause α-thalassemia ($—^{SEA}$, $—^{FIL}$, $—^{MED}$, $-\alpha^{20.5}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$) was carried out in all 174 patients as previously described [21], with minor modifications. Genomic DNA was extracted from leukocytes using QIAsymphony system (Qiagen GmbH, Hilden, Germany). Extracted genomic DNA was tested for its quality and quantity using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Thermo Fisher Scientific Inc., Wilmington, DC, USA).

The sequences of the PCR primers are listed in Table 1. Since each of the 6 deletions either partially or completely removes the α2 gene [21], its positive amplification was used to indicate heterozygosis when a deletion allele was also present. Each deletion was tested in a different reaction

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

tube, including positive and negative (H_2O without DNA) controls. The combinations of primers to detect each deletion are summarized in Table 2. Each 50 μL reaction contained 1x PCR buffer containing Tris-Cl, KCl, $(NH_4)_2SO_4$, pH 8.7; 1.5 mmol/L $MgCl_2$, 1 mol/L betaine (SIGMA, St. Louis, MO), 25 pmol of each primer, 0.2 mmol/L of each dNTP, 2 U of HotStar Taq DNA polymerase (Qiagen GmbH, Hilden, Germany) and 250 ng of genomic DNA. Reactions were carried out on a thermal cycler (SimpliAmp™ ThermalCycler, LifeTechnologies, Singapore), with an initial 15-minute denaturation at 95°C, 35 cycles of 95°C for 45 seconds, 60°C for 1 minute, 72°C for 2 minutes 30 seconds, and a final extension at 72°C for 5 minutes. Following amplification, 10 μL of product was electrophoresed through a 1% agarose, 1x TBE gel at 80V for 1 hour, stained in ethidium bromide, and visualized on an ultraviolet transilluminator.

Table 1. Primers for PCR analysis of common α-thalassemia deletions.

Name	Sequence (5'-3')	Nucleotides (GenBank ID NT_010393)
FIL-F	TGCAAATATGTTTCTCTCATTCTGTG	140821-140846
FIL-R	ATAACCTTTATCTGCCACATGTAGC	172662-172638
20.5-F	GCCCAACATCCGGAGTACATG	147041-147061
3.7/20.5-R	AAAGCACTCTAGGGTCCAGCG	167719-167699
MED-F	TACCCTTTGCAAGCACACGTAC	152260-152281
MED-R	TCAATCTCCGACAGCTCCGAC	170340-170320
SEA-F	CGATCTGGGCTCTGTGTTCTC	155257-155277
SEA-R	AGCCCACGTTGTGTTCATGGC	175909-175889
4.2-F	GGTTTACCCATGTGGTGCCTC	159269-159288
4.2-R	CCCGTTGGATCTTCTCATTTCCC	165142-165120
α2/3.7-F	CCCCTCGCCAAGTCCACCC	161883-161901
α2-R	AGACCAGGAAGGGCCGGTG	163685-163667

Table 2. Combinations of primers to detect each deletion.

Fragment	Primers	Size (bp)
FIL deletion	FIL-F + FIL-R	1166
SEA deletion	SEA-F + SEA-R	1349
20.5 deletion	20.5F + 3.7/20.5-R	1007
3.7 deletion	α2-3.7-F + 3.7/20.5-R	2022-2029
4.2 deletion	4.2F + 4.2-R	1628
MED deletion	MED-F + MED-R	807
α2 gene	α2/3.7-F + α2-R	1800

The CBC was performed with the Advia 2120 analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY). The following parameters of the CBC were assessed in all subjects: absolute RBC count, Hb, MCV, MCH, mean corpuscular hemoglobin concentration (MCHC) and RBC distribution width (RDW). Ferritin, transferrin, and TSI were measured by chemiluminescence immunoassay in the Advia Centaur (Siemens Medical Solutions Diagnostics). Subjects with ferritin levels <20 ng/mL and TSI <18% were considered to have iron deficiency, and were not included in the statistical analysis. Hb A_2 and Hb F levels were determined by HPLC on the Tosoh G7 analyzer (Horiba, Tokyo, Japan).

2.2. Statistical Analysis

The sample was divided into 4 different groups according to gender and age for the statistical analysis: 1) male and female children ≤ 2 years; 2) male and female children from 3 to 16 years; 3) females ≥ 16 years; 4) males ≥ 16 years. Cases were also classified according to the number of deleted alpha genes: 1, 2 or 3.

All measurements were expressed as the median ± standard deviation (SD). The Shapiro–Wilk test was used to assess the normality of our dataset in case of less than 30 samples per group in the comparation. An independent sample t-test was used to compare classical hematologic parameters

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

among the different subtypes of α-thalassemia. In case of less than 30 samples per group and non-normal distribution, non-parametric tests were used (Mann-Whitney). Additionally, non-parametric tests were used when any of the subgroups compared had less than 30 members. *P* values less than 0.05 were considered statistically significant. Receiver operating characteristic (ROC) curves were plotted in all the parameters that showed significant differences and their area under the curve (AUC) used to evaluate their diagnostic performance. An arbitrary value of AUC ≥ 0.8 was used as the cut-off for considering a variable to be efficient enough to discriminate between the different subgroups. For those variables, a cut-off was selected based on its sensitivity and specificity.

One-way ANOVA test was used to compare the median values of each parameter in three groups of subjects according to the number of deleted alpha genes. Kruskal-Wallis test was performed in the case of parameters of samples whose variances were not equal and groups were very different in size.

The Pearson coefficient was estimated to assess the correlation between each of the hematological parameters and the number of alpha genes deleted. An arbitrary value of R ≥ 0.6 was considered a good correlation.

SPSS version 19.0 for Windows (SPSS, Chicago, IL) was used for statistical analysis of the data.

3. Results

The reliability of the results of the complete blood count, Hb A_2 and Hb F is guaranteed with daily internal quality control (provided by the manufacturer) and external quality assessment every month (Hemqual program, Sociedad Española Hematología y Hemoterapia). Internal quality controls are performed to guarantee that results of the molecular analysis are also reliable.

Of the 174 cases included in the study, 82 (47.12%) were males and 92 (52.87%) females and the median age was 33 years (1-81).

According to the number of deleted alpha genes, each individual was allocated to one of the following groups:

1. Loss of one α gene or heterozygous α^+ thalassemia (–α/αα) (n= 92). The $\alpha^{3.7}$ haplotype was observed in 91 cases (24 men, 32 women and 35 children), whereas the $\alpha^{4.2}$ haplotype was found only in 1 woman.

2. Loss of two α genes from different chromosomes (–α/–α) (n= 40): 37 subjects with $-\alpha^{3.7}/-\alpha^{3.7}$ deletions (11 men, 20 women and 5 children), 1 man with $-\alpha^{4.2}/-\alpha^{4.2}$ deletions and 2 men were compound heterozygotes for the $-\alpha^{3.7}/-\alpha^{4.2}$ mutations.

3. Loss of two α genes from the same chromosome or heterozygous α° thalassemia (—/αα) (n= 34): This group was comprised of 20 $—^{SEA}/\alpha\alpha$ individuals (5 men, 9 women and 6 children) and 14 $–^{FIL}/\alpha\alpha$ (2 men, 9 women and 3 children). Neither $—^{MED}/\alpha\alpha$ nor $-\alpha^{20.5}$ individuals were identified. The nationalities of our ($—^{SEA}/\alpha\alpha$) cases were: 12 Filipino, 7 Chinese and 1 Bolivian. All of ($—^{FIL}/\alpha\alpha$) cases from our study were Filipino except one Spanish girl whose mother was Filipino.

4. Loss of three α genes or Hb H disease (—/–α) (n= 9): 4 subjects presented the $—^{SEA}/-\alpha^{3.7}$ genotype whereas the $—^{FIL}/-\alpha^{3.7}$ genotype was found in 4 cases. There was a man with the $—^{FIL}/-\alpha^{X}$ genotype, which means he carried, not only the $—^{FIL}$ deletion in one allele, but also a non-identified deletion in the other allele. In this case, inclusion bodies were identified with brilliant cresyl blue stain. All of them were born in the Philippines, except one Spanish girl whose grandfather was Filipino.

Hematological parameters of the 4 groups are summarized in Table 3.

No Hb Bart hydrops fetalis syndrome was diagnosed in the mentioned period of time. According to our criteria, co-existing iron deficiency was found in 30 individuals (17.14%).

Table 3. Hematologic parameters of the different subtypes of deletional α-thalassemia. Data represent mean ± SD (standard deviation).

	Gender and age	-α/αα	-α/-α	--/αα	--/-α
RBC (x10^{12}/L)	Male	6.0 ± 0.42	5.9 ± 0.64	6.6 ± 0.35	6

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

	Female	5.1 ± 0.49	5.4 ± 0.59	5.6 ± 0.46	5.8 ± 0.43
	Children 3-16 years	5.4 ± 0.33	5.8 ± 0.14	6.1 ± 0.51	6.2 ± 1.41
	Children ≤2 years	5.3 ± 0.45		5.9 ± 0.70	
Hb (g/dL)	Male	15.3 ± 0.94	14.1 ± 1.07	14.3 ± 1.03	9.6
	Female	12.9 ± 0.98	12.3 ± 0.94	11.8 ± 0.73	9.4 ± 0.61
	Children 3-16 years	13.0 ± 0.92	11.5 ± 0.59	12.3 ± 1.12	9.6 ± 0.56
	Children ≤2 years	12.3 ± 0.92		11.3 ± 0.84	
MCV (fL)	Male	79.4 ± 2.75	74.5 ± 3.33	68.1 ± 2.00	66.3
	Female	78.3 ± 3.60	73.2 ± 3.32	68.6 ± 3.52	64.6 ± 5.90
	Children 3-16 years	75.6 ± 3.82	64.4 ± 3.58	64.0 ± 3.87	61.1 ± 9.33
	Children ≤2 years	70.9 ± 3.57		60.5 ± 4.95	
MCH (pg)	Male	25.8 ± 1.68	23.1 ± 1.09	21.5 ± 1.58	16
	Female	25.1 ± 1.74	22.9 ± 1.13	21.0 ± 0.99	17.2 ± 1.18
	Children 3-16 years	24.2 ± 1.07	20.4 ± 0.86	20.1 ± 0.68	15.8 ± 2.61
	Children ≤2 years	23.4 ± 1.03		19.3 ± 0.92	
MCHC (g/L)	Male	32.3 ± 1.60	31.0 ± 0.98	32.2 ± 2.46	24.2
	Female	32.4 ± 1.45	31.2 ± 1.16	30.7 ± 1.19	25.9 ± 0.95
	Children 3-16 years	31.7 ± 1.38	31.2 ± 0.73	31.5 ± 1.00	25.9 ± 0.35
	Children ≤2 years	31.6 ± 1.48		31.9 ± 1.06	
RDW (%)	Male	13.5 ± 0.80	14.97 ± 1.45	15.08± 1.54	23.3
	Female	13.95 ± 1.28	14.60 ± 1.01	15.81 ± 2.14	21.6 ± 1.40
	Children 3-16 years	14.15 ± 0.96	14.76 ± 0.50	14.61 ± 0.79	21.65 ± 0.92
	Children ≤2 years	14.0 ± 0.81		17.15 ± 0.64	

When analyzed according to their gender and age, differences in the hematologic parameters were not significant in any thalassemic group, except for a low level of Hb and MCV and MCH in children aged ≤ 2 years. Therefore, children aged ≤ 2 years were not included in the subsequent analysis. Additionally, Hb levels were lower in females than in males in all the subgroups.

Statistically significant differences ($p<0.05$) were observed in all the hematological parameters between α^+-thalassemia carriers and those subjects with at least 2 alpha genes deleted [(–α/–α), (—/$\alpha\alpha$) and (—/–α)] (Table 4). Individuals with two or more alpha genes deleted presented significantly higher RBC count (5.79 $\times 10^{12}$/L vs 5.53 $\times 10^{12}$/L, $p=0.016$), lower Hb (12.29 g/dL vs 13.71 g/dL, $p<0.001$), lower MCV (69.56 fL vs 77.33 fL, $p<0.001$), lower MCH (21.30 pg vs 24.83 pg, $p<0.001$), lower MCHC (30.61 g/dL vs 32.11 g/dL, $p<0.001$) and higher RDW (15.82% vs 14.07%, $p<0.001$). Only MCV and MCH demonstrated to be efficient enough to discriminate between these two groups. MCV showed an AUC of 0.889 and the cut-off point of 74.05 provided a sensitivity of 85.7% and a specificity of 80.8%. MCH showed an AUC of 0.810 and the cut-off point of 23.15 provided a sensitivity of 85.7% and a specificity of 80.8%.

Table 4. Comparison of hematologic parameters in subjects with loss of 1 alpha gene and those with at least two genes affected. Data represent mean ± SD (standard deviation). *P* values less than 0.05 were considered statistically significant.

	Loss of 1 gene (-α/$\alpha\alpha$)	**Loss of 2 or 3 genes [(–α/–α), (—/$\alpha\alpha$) and (—/–α)]**	***P* value**
RBC (x10^{12}/L)	5.53 ± 0.53	5.79 ± 0.63	0.016
Hb (g/dL)	13.7 ± 1.41	12.3 ± 1.61	<0.001

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

MCV (fL)	77.33 ± 3.75	69.56 ± 5.37	<0.001
MCH (pg)	24.83 ± 1.68	21.30 ± 2.25	<0.001
MCHC (g/dL)	32.11 ± 1.47	30.61 ± 2.12	<0.001
RDW (%)	14.07 ± 1.07	15.82 ± 2.69	<0.001

Comparison of (–α/–α) and (—/αα) revealed significantly lower MCV (67.34 fL vs 72.51 fL, p<0.001) and lower MCH (21.02 pg vs 22.59 pg, p<0.001) in (—/αα) subjects (Table 5). No significant differences were found in RBC, Hb, MCHC and RDW. MCV showed an AUC of 0.815 and the cut-off point of 70.20 provided a sensitivity of 82.8% and a specificity of 77.8%. MCH showed an AUC of 0.810 and the cut-off point of 21.90 provided a sensitivity of 82.8% and a specificity of 64.9%.

Table 5. Comparison of hematologic parameters of homozygous α^+ thalassemia (–α/–α) and heterozygous α° thalassemia (—/αα). Data represent mean ± SD (standard deviation). *P* values less than 0.05 were considered statistically significant.

	(–α/–α)	**(—/αα)**	***P* value**
RBC (x10^{12}/L)	5.56 ± 0.61	5.95 ± 0.61	0.064
Hb (g/dL)	12.7 ± 1.33	12.5 ± 1.38	0.420
MCV (fL)	72.51 ± 4.56	67.34 ± 3.88	<0.001
MCH (pg)	22.59 ± 1.44	21.02 ± 1.12	<0.001
MCHC (g/dL)	31.17 ± 1.07	31.25 ± 1.55	0.794
RDW (%)	14.74 ± 1.04	15.41 ± 1.92	0.079

Comparison of [(–α/αα), (–α/–α)] vs [(—/αα), (—/–α)] was also performed in order to identify parameters that could discriminate subjects at risk of having children with Hb H disease or Hb Bart hydrops fetalis syndrome (Table 6). The [(—/αα), (—/–α)] group presented significantly higher RBC count (5.92 ×10^{12}/L vs 5.58 ×10^{12}/L, p=0.004), lower Hb (11.84 g/dL vs 13.33 g/dL, p<0.001), lower MCV (66.70 fL vs 75.45 fL, p<0.001), lower MCH (20.05 pg vs 23.96 pg, p<0.001), lower MCHC (30.07 g/dL vs 31.74 g/dL, p=0.001) and higher RDW (16.87% vs 14.33%, p<0.001). An AUC ≥0.8 was found only in MCV and MCH. MCV showed an AUC of 0.909 and the cut-off point of 70.80 provided a sensitivity of 85.9% and a specificity of 86.5%. MCH showed an AUC of 0.920 and the cut-off point of 21.9 provided a sensitivity of 84.8% and a specificity of 86.5%.

Table 6. Comparison of hematologic parameters of subjects with and without an α° allele. Data represent mean ± SD (standard deviation). *P* values less than 0.05 were considered statistically significant.

	(–α/αα), (–α/–α)	**(—/αα), (—/–α)**	***P* value**
RBC (x10^{12}/L)	5.58 ± 0.56	5.92 ± 0.62	0.004
Hb (g/dL)	13.33 ± 1.45	11.84 ± 1.74	<0.001
MCV (fL)	75.45 ± 4.70	66.70 ± 4.51	<0.001
MCH (pg)	23.96 ± 1.93	20.05 ± 2.20	<0.001
MCHC (g/dL)	31.74 ± 1.40	30.07 ± 2.70	<0.001
RDW (%)	14.33 ± 1.11	16.87 ± 3.33	<0.001

Comparisons of the hematological parameters of the two most frequent forms of α° thalassemia in our sample are summarized in Table 7.

Table 7. Hematologic parameters of (—^{SEA}/αα) and (—^{FIL}/αα). Data represent mean ± SD (standard deviation). *P* values less than 0.05 were considered statistically significant.

	(—^{SEA}/αα)	**(—^{FIL}/αα)**	***P* value**

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

RBC (x10¹²/L)	5.96 ± 0.58	5.93 ± 0.67	0.918
Hb (g/dL)	12.4 ± 1.33	12.5 ± 1.39	0.694
MCV (fL)	67.06 ± 4.56	67.64 ± 3.14	0.694
MCH (pg)	20.95 ± 1.34	21.09 ± 0.88	0.745
MCHC (g/dL)	31.29 ± 1.97	31.21 ± 0.99	0.894
RDW (%)	15.29 ± 1.75	15.81 ± 2.01	0.097

When compared to subjects with two alpha genes deleted [(—/αα), (–α/–α)], the Hb H disease (—/–α) group presented a significantly higher degree of anemia (9.47 g/dL vs 12.50 g/dL, p<0.001), more microcytosis (63.72 fL vs 67.72 fL, p= 0.012) and hypochromia (16.16 pg vs 22 pg, p<0.001), and a marked anisocytosis (22.61% vs 15.28%, p<0.001) (Table 8). No significant differences were found in RBC. Hb showed an AUC of 1 and the cut-off point of 10.55 provided both sensitivity and specificity of 100%. MCH showed an AUC of 1 and the cut-off point of 18.45 provided both sensitivity and specificity of 100%. MCHC showed an AUC of 1 and the cut-off point of 27.80 provided both sensitivity and specificity of 100%. RDW showed an AUC of 0.987 and the cut-off point of 19.6 provided a sensitivity of 100% and a specificity of 96.9%.

Table 8. Comparison of hematologic parameters of subjects with 2 versus 3 alpha genes deleted. Data represent mean ± SD (standard deviation). *P* values less than 0.05 were considered statistically significant.

	(–α/–α), (—/αα)	**(—/–α)**	***P* value**
RBC (x10¹²/L)	5.79 ± 0.62	6 ± 0.73	0.818
Hb (g/dL)	12.50 ± 2.25	9.47 ± 0.57	<0.001
MCV (fL)	67.72 ± 7.27	63.72 ± 5.97	0.012
MCH (pg)	22.00 ± 1.26	16.16 ± 1.77	<0.001
MCHC (g/dL)	31.12 ± 1.26	25.39 ± 1.47	<0.001
RDW (%)	15.28 ± 1.60	22.61 ± 2.02	<0.001

Differences in Hb, MCV, MCH, MCHC and RDW values in carriers of the deletion of 1, 2 and 3 genes were statistically significant (p<0.001). Pearson coefficient showed good correlation between the number of deleted alpha genes and each of the following parameters: MCV (r = -0.672, p<0.001), MCH (r = -0.788, p<0.001) and RDW (r = 0.633, p<0.001).

4. Discussion

To date, very few studies have compared hematologic parameters of the distinct subtypes of deletional α-thalassemia [13, 14, 20]. Despite describing differences in several corpuscular indices, no precise cut-off points have been defined for each parameter in these studies.

According to our results, MCV, Hb and, to a lesser extent, MCH are influenced by age in carriers of any deletion in alpha genes. It is well known that values of both Hb and MCV are lower in children than in adults and lower in women than in men [22, 23, 24]. Since MCV rises with age, it seems reasonable not to screen for thalassemia in asymptomatic children with low MCV. Both MCH and MCHC are known to remain stable throughout childhood, and no differences between sexes are found [23]. Our findings are in agreement with previous studies [13, 14, 20].

The (–α/αα) group accounted for 52.29% of all our α-thalassemic cases, whereas the percentage of (–α/–α), (—/αα) and (—/–α) were 22.40%, 19.54% and 5.17% respectively. As reported in several studies [1, 13], $-\alpha^{-3.7}$ deletion is the commonest α-thalassemia determinant in our geographic area. We believe the proportion of α^{+} thalassemia and α^{0} thalassemia found in our cohort is not representative of people living in Spain, based on previous reports [25]. The real prevalence of α^{+} thalassemia is probably underestimated, since this condition produces minor hematologic changes that are clinically silent and carriers usually go unnoticed.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

When subjects with two or more alpha genes deleted were compared to those with heterozygous α^+ thalassemia, all the parameters showed significant differences (Table 4). The ($-\alpha/\alpha\alpha$) subjects had no anemia and showed only mild microcytosis and hypochromia. A known α^+ thalassemia carrier by definition will transmit at least one *HBA* gene to his/her offspring, thus there is no risk of having children with Hb Bart hydrops fetalis syndrome. However, the importance of identifying a carrier of α^+ thalassemia relies on warning him/her that, if his/her couple shows microcytosis, a molecular study of α-thalassemia should be performed prior to having children. Our results in ($-\alpha/\alpha\alpha$) subjects are in accordance with those published by many authors [13, 14, 20]. Two parameters stood out as the most efficient to identify the deletion of at least two alpha genes: MCH (AUC= 0.916) and MCV (AUC= 0.889). Cut-off points of 23.15 pg and 74.05 fL for MCH and MCV respectively represented the best combination between sensitivity and specificity for both parameters and can be used to predict the deletion of at least two alpha genes.

Despite the detection of two deleted alpha genes has no considerable clinical impact in the carrier, since these individuals show only mild anemia, it allows an adequate genetic counseling to at-risk couples. Loss of two alpha genes can be due to heterozygosis of α^0 thalassemia ($--/\alpha\alpha$) or homozygosis for α^+ thalassemia ($-\alpha/-\alpha$). Although overlapping Hb, RBC, MCHC and RDW values were found in both conditions, the ($--/\alpha\alpha$) group presented a higher degree of microcytosis (67.34 fL vs 72.51 fL, $p<0.001$) and hypochromia (21.30 pg vs 24.83 pg, $p<0.001$). Although both MCV and MCH showed an AUC ≥0.8, their optimal cut-offs were neither sensitive nor specific enough to accurately discriminate between both conditions.

Identification of individuals with α^0 thalassemia is of great importance, since they may have children with Hb H disease or Hb Bart´s disease if their couples have α^+ thalassemia or α^0 thalassemia respectively. Since an adequate genetic counseling is essential for these subjects, it would be of great interest if subjects with one α^0 allele [($--/\alpha\alpha$) or ($--/-\alpha$)] could be easily identified. For this purpose, the discriminant efficiency of the hematologic parameters in this setting was assessed. MCH had the best AUC (0.920), followed by MCV (0.909). Therefore, according to our results, the presence of one α^0 allele should be suspected if the MCH <21.90 pg and/or the MCV <70.80 fL.

Among ($--/\alpha\alpha$) cases, no significant differences were observed in any parameter between the deletions $--^{SEA}$ and $--^{FIL}$. All of the corpuscular indices were almost identical in these two subgroups (Table 7). The $--^{SEA}$ deletion removes nearly 20 kb DNA and extends from the 3′ end of the *HBZps* gene through the *HbA1* gene [1]. It has been observed at high frequencies in several Southeast Asian populations [1]. The $--^{FIL}$ deletion extends for approximately 30-34 kb and removes the entire ζ-α-globin gene cluster [25]. It has been described mostly in Filipino population or individuals with Filipino ancestry, but also in people from other countries of the southeast of Asia [27, 28]. Although the $--^{FIL}$ deletion involves a larger fragment compared to the $--^{SEA}$ deletion [25, 28, 29], this fact has no consequences in the erythrocytic phenotype of these subjects. However, there is a subtle clinical difference. Whereas homozygosis for the $--^{FIL}$ deletion results in early intrauterine death since neither embryonic ($\zeta_2\gamma_2$) nor fetal ($\alpha_2\gamma_2$) Hb can be produced due to loss of the entire ζ-α-globin gene cluster, homozygotes for the $--^{SEA}$ deletion usually survive until birth since the sparing of the embryonic gene allows enough functional embryonic Hb [1, 4].

We found interesting the absence of individuals with the $--^{MED}$ in our study, since it has been previously described in Spain [25, 30].

There has been a remarkable increase in the prevalence of several forms of α-thalassemia over the last decades due to the immigration flows in non-endemic countries for these conditions, thus they have become a global problem. As an example to illustrate this phenomenon, all the subjects from our study who had at least one α^0 allele were Southeast Asian (81.39% Filipino, 16.27% Chinese) or had ancestries from this geographic area. In Spanish people, only sporadic cases of α^0 thalassemia are found, observed in isolated families [29]. Although there have been described several forms of α^0 thalassemia of local ethnicity ($--^{MA}$, $--^{CANT}$, $--^{SPAN}$) in Spain [13], most of α^0 thalassemia cases in our country are $--^{SEA}$ or $--^{FIL}$ deletion described in Asian people. Understanding the genotype/phenotype relationship of the various mutations of α-globin genes can lead to identify carriers of these defects.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

Compound heterozygosis for these α^0 deletions and deletions removing a single α gene results in what is called Hb H disease (—/–α). As previously described by several authors [6, 12, 13, 14, 18, 20], a more severe degree of anemia in this subset of cases was found in our study, with Hb levels 9-10 g/dL. Decreased hemoglobinization (lower values of MCH and MCHC) and impaired α-globin chain synthesis lead to a higher number of divisions in erythroid precursors and therefore to microcytosis (lower values of MCV) in these patients. Our phenotypic data related to the size and chromia of erythrocytes are in agreement with previous reports of Hb H disease patients. Finally, a RDW higher than 20% was found in all the 9 cases of Hb H disease included in our study. There are no specific data of the differences in RDW in the different types of deletional α-thalassemia in the work of Villegas et al, although a marked increase in RDW of subjects with Hb H disease in comparison to other subgroups is mentioned [13]. Akhavan-Niaki et al did not evaluate RDW in α-thalassemia subjects [14]. However, Ahmad et al described a marked anisocytosis in Hb H disease, with mean RDW values of 26.2% ± 6.7 [20]. The imbalance in the α/β-globin chain ratio produced in the Hb H disease leads to ineffective erythropoiesis, since the unstable free β-globin chain tetramers precipitate in erythroid precursors [12]. Another possible reason is the elevated reticulocyte count of these subjects in comparison to other forms of α-thalassemia [13, 20]. Since reticulocytes have a larger size than RBC, a higher degree of anisocytosis can consequently be expected.

An accurate presumptive diagnosis of Hb H disease based on hematological parameters is easier in comparison to the rest of α-thalassemia groups, since its erythrocytic phenotype is much different. Three parameters demonstrated an outstanding AUC to discriminate subjects with Hb H disease from those with two alpha genes deleted: MCH (1), Hb (1), MCHC (1) and RDW (0.987).

Since the main utility of hematologic parameters in deletional α-thalassemia seems to be to predict the number of alpha genes deleted, it would be of great importance to identify a parameter not only with an excellent AUC but also with a good correlation between its values and the number of alpha genes deleted. Our results demonstrate that three parameters are strongly affected by the number of alpha genes deleted: MCV, MCH and RDW. The Pearson coefficient was estimated to assess the correlation between each of the hematological parameters and the number of alpha genes deleted. The strongest correlation was observed in MCH ($r = -0.788$, $p<0.001$), followed by MCV ($r = -0.672$, $p<0.001$) and RDW ($r = 0.633$, $p<0.001$). The more alpha genes deleted, the lower values of MCH and MCV, whereas the RDW showed an opposite trend. These three parameters had consistently shown statistically significant differences and high values of AUC in most of comparisons between subgroups throughout the statistical analysis, especially MCH. Moreover, the stability of MCH during storage of blood samples is higher compared to MCV [31, 32]. Additionally, as stated before, MCH seems to be less influenced by age. Based on these considerations, many authors recommend using MCH instead of MCV to screen for thalassemia. Our results are consistent with this recommendation.

5. Conclusions

Unequivocal diagnosis of α-thalassemia can only be made with molecular studies, but corpuscular indices provided by hematological counters can be of great utility as predictive markers of the number of alpha genes deleted. Identification of at-risk couples prior to pregnancy by hematologic parameters could prevent the most severe forms of the disease, especially if the couple belongs to a population at risk for α^0 thalassemia (—/$\alpha\alpha$).

To our knowledge, this is the first study that defines cut-off points of several corpuscular indices to discriminate between the different subtypes of deletional α-thalassemia, adding value to an initial diagnostic approach of these conditions.

There might be two possible drawbacks in our study. First of all, non-deletional α-thalassemia cases were not included, although only a minority is due to point mutations. Secondly, not all of the deletional forms of α-thalassemia were evaluated. However, the GAP-PCR used in this study detects the most frequent deletional α-thalassemia determinants. Detection of inclusion bodies with supravital stains was not systematically performed.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

In conclusion, according to our results it is mandatory to discard the deletion of at least two alpha genes in adult individuals with microcytosis without iron deficiency and normal values of Hb A_2 and Hb F when MCH levels are lower than 23.15 pg. Additionally, the presence of one α^0 allele should be suspected with MCH <21.90 pg and/or MCV <70.80 fL**.** In this setting, Hb H disease will be the most likely diagnosis if RDW ≥19.6% and/or MCH <18.45 pg and/or MCHC <27.8 g/dL are seen. Further prospective validation of these cut-off points is needed to establish their real utility in daily clinical practice.

Acknowledgments: All sources of funding of the study should be disclosed. Please clearly indicate grants that you have received in support of your research work. Clearly state if you received funds for covering the costs to publish in open access.

Author Contributions: DV-R, JMA-D and CB conceived and designed the study; CB performed the molecular analysis; CS validated the hematologic parameters; DV-R, AG-R and GV collected the data; JMA-D and AG-R analyzed the data; DV-R wrote the paper; JMA-D, CB, CS, GV, AG-R and PL reviewed the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Harteveld CL, Higgs DR. Alpha-thalassaemia. Orphanet J Rare Dis. 2010 May 28; 5: 13. DOI: 10.1186/1750-1172-5-13.
2. Lau YL, Chan LC, Chan YY et al. Prevalence and genotypes of alpha- and beta-thalassemia carriers in Hong Kong -- implications for population screening. N Engl J Med. 1997 May 1; 336 (18): 1298-301. DOI: 10.1056/NEJM199705013361805
3. Li Z, Li F, Li M et al. The prevalence and spectrum of thalassemia in Shenzhen, Guangdong Province, People's Republic of China. Hemoglobin. 2006; 30 (1): 9-14. DOI: 10.1080/03630260500453818
4. Vichinsky EP. Alpha thalassemia major--new mutations, intrauterine management, and outcomes. Hematology Am Soc Hematol Educ Program. 2009: 35-41. DOI: 10.1182/asheducation-2009.1.35
5. Michlitsch J, Azimi M, Hoppe C et al. Newborn screening for hemoglobinopathies in California. Pediatr Blood Cancer. 2009 Apr; 52 (4): 486-90. DOI: 10.1002/pbc.21883
6. Lal A, Goldrich ML, Haines DA et al. Heterogeneity of hemoglobin H disease in childhood. N Engl J Med. 2011 Feb 24; 364 (8): 710-8. DOI: 10.1056/NEJMoa1010174
7. Vichinsky E. Advances in the treatment of alpha-thalassemia. Blood Rev. 2012 Apr; 26 Suppl 1: S31-4. DOI: 10.1016/S0268-960X(12)70010-3
8. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ. 2001; 79 (8): 704-12.
9. Williams TN, Weatherall DJ. World distribution, population genetics, and health burden of the hemoglobinopathies. Cold Spring Harbor Perspect Med. 2012 Sep 1; 2 (9): a011692. DOI: 10.1101/cshperspect.a011692.
10. Higgs DR, Vickers MA, Wilkie AO et al. A review of the molecular genetics of the human α-globin gene cluster. Blood 1989; 73: 1081-104.
11. Higgs DR. The molecular basis of α-thalassemia. Cold Spring Harb Perspect Med. 2013 Jan 1; 3 (1): a011718. DOI: 10.1101/cshperspect.a011718
12. Vichinsky EP. Clinical manifestations of α-thalassemia. Cold Spring Harb Perspect Med. 2013 May 1; 3 (5): a011742. DOI: 10.1101/cshperspect.a011742
13. Villegas A, Porres A, Sánchez J et al. Red blood cell phenotypes in alpha-thalassemias in the Spanish population. Haematologica. 1998 Feb; 83 (2): 99-103.
14. Akhavan-Niaki H, Kamangari RY, Banihashemi A et al. Hematologic Features of Alpha Thalassemia Carriers. Int J Mol Cell Med. 2012 Summer; 1(3): 162–167.
15. Ataulfo González F, Blázquez C, Ropero P et al. Association of hemoglobinopathy and alpha thalassemia. Study of 45 patients. Med Clin (Barc) 2005; 124: 726–9.
16. Wambua S, Mwacharo J, Uyoga S, et al. Co-inheritance of alpha+-thalassaemia and sickle trait results in specific effects on haematological parameters. Br J Haematol 2006; 133: 206–9. DOI: 10.1111/j.1365-2141.2006.06006.x

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 21 September 2017 doi:10.20944/preprints201709.0100.v1

Peer-reviewed version available at Int. J. Mol. Sci. 2017, 18, 2707; doi:10.3390/ijms18122707

17. Gringras P, Wonke B, Old J et al. Effect of alpha thalassaemia trait and enhanced gamma chain production on disease severity in beta thalassaemia major and intermedia. Arch Dis Child. 1994 Jan; 70 (1): 30-4.
18. Fucharoen S, Viprakasit V. Hb H disease: clinical course and disease modifiers. Hematology Am Soc Hematol Educ Program 2009:26-34. DOI: 10.1182/asheducation-2009.1.26
19. Harteveld CL, Voskamp A, Phylipsen M et al. Nine unknown rearrangements in 16p13.3 and 11p15.4 causing α- and β-thalassaemia characterised by high resolution multiplex ligation-dependent probe amplification. J Med Genet. 2005; 42: 922-931. DOI: 10.1136/jmg.2005.033597
20. Ahmad R, Saleem M, Aloysious NS et al. Distribution of alpha thalassaemia gene variants in diverse ethnic populations in Malaysia: data from the institute for medical research. Int J Mol Sci. 2013 Sep 10; 14 (9): 18599-614. DOI: 10.3390/ijms140918599
21. Chong SS, Boehm CD, Higgs DR et al. Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassemia. Blood. 2000 Jan 1; 95 (1): 360-2.
22. Dallman PR, Siimes MA. Percentile curves for hemoglobin and red cell volume in infancy and childhood. J Pediatr. 1979 Jan; 94 (1): 26-31.
23. Aldrimer M, Ridefelt P, Rödöö P et al. Population-based pediatric reference intervals for hematology, iron and transferrin. Scand J Clin Lab Invest. 2013 Apr; 73 (3): 253-61. DOI: 10.3109/00365513.2013.769625
24. Williams TN, Maitland K, Ganczakowski M et al. Red blood cell phenotypes in the alpha + thalassaemias from early childhood to maturity. Br J Haematol. 1996 Nov;95(2):266-72.
25. Villegas A, Ropero P, González FA et al. The thalassemia syndromes: molecular characterization in the Spanish population. Hemoglobin. 2001 Aug; 25(3): 273-83.
26. Fischel-Ghodsian N, Vickers MA, Seip M et al. Characterization of two deletions that remove the entire human globin gene complex (THAI and FIL). Br J Haematol 1988; 70: 233–8.
27. Ko TM, Hwa HL, Liu CW et al. Prevalence study and molecular characterization of alpha-thalassemia in Filipinos. Ann Hematol. 1999 Aug; 78 (8): 355-7.
28. Chong SS, Boehm CD, Cutting GR et al. Simplified multiplex-PCR diagnosis of common Southeast Asian deletional determinants of alpha-thalassemia. Clin Chem. 2000 Oct; 46 (10): 1692-5.
29. Nicholls RD, Fischel-Ghodsian N, Higgs DR. Recombination at the human-globin gene cluster: sequence features and topological constraints. Cell 1987; 49: 369–78.
30. Villegas A, Sánchez J, Sal del Río E. Alpha globin genotypes in a Spanish population. Hemoglobin 1992; 16: 427-9.
31. Ryan K, Bain BJ, Worthington D, et al; British Committee for Standards in Haematology. Significant haemoglobinopathies: guidelines for screening and diagnosis. Br J Haematol. 2010; 149: 35-49. DOI: 10.1111/j.1365-2141.2009.08054.x.
32. Brugnara C, Mohandas N. Red cell indices in classification and treatment of anemias: from M. M. Wintrobe's original 1934 classification to the third millennium. Curr Opin Hematol. 2013; 20: 222-230. DOI: 10.1097/MOH.0b013e32835f5933.