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SILENT THALASSEMIAS: GENOTYPES AND PHENOTYPES

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ABSTRACT

Background and Objective. Current application of molecular biology techniques to the study of the DNA of globin genes has confirmed the existence of silent α and β thalassemias, which had already been reported on the basis of red blood cell parameters and family studies. The present work was aimed at analyzing all the aspects of the phenotype of the most common varieties of silent thalassemia.

Materials and Methods. Groups of heterozygous carriers of these varieties were examined using established techniques that determined all hematologic, hemoglobin (electrophoresis and measurement of Hb A_2 and Hb F levels), and globin synthesis (evaluation of the α/β ratio) parameters. Furthermore, all subjects underwent a complete molecular study of the α and β globin genes by means of the ARMS, SSCP, DGGE, PCR and Southern blotting techniques.

Results. 1) The –101 C→T mutation of the promoter of the β globin gene shows a normal hematological picture with the Hb A_2 level often slightly raised and the α/β globin synthesis ratio slightly greater than 1; 2) β^+ thalassemia resulting from the IVS II 844 C→G mutation has a phenotype that is even closer to normal; 3) $-\alpha^{3.7}$ deletion type I usually has a totally silent phenotype; 4) the α^{NcoI} mutation almost always gives rise to a sub-silent phenotype if it is located on gene α_2 and to a silent phenotype if it is found on gene α_1; 5) α^+ thalassemia due to the α_2^{HphI} mutation displays a sub-silent phenotype in some cases and a silent one in others; 6) triplication of the α genes gives rise to a phenotype that is quite similar to that of the –101 C→T mutation of the promoter of the β globin gene, namely one that is very often silent.

Interpretation and Conclusions. Many of these silent varieties (β^+ thalassemia due to the –101 C→T mutation; α^+ thalassemia from a deletion or point mutation of an α gene; ααα triplication) are quite frequent in the overall group of thalassemias. It is therefore important for the operators in the field of thalassemia diagnosis to possess exact knowledge of them, especially in order to prevent thalassemia major.

Key words: silent thalassemias

β thalassemia heterozygosity habitually manifests a typical hematologic, hemoglobin and globin synthesis picture, namely an increased RBC count, a reduced hemoglobin level, reduced MCV and MCH, altered erythrocyte morphology, increased Hb A_2 level, imbalance in globin synthesis with the α/β ratio greater than 1.

Immediately following the identification of this anomaly[1] through the hematological alterations observed, it became evident that there were cases (at that time these could only be detected in the parents of patients with Cooley's disease) in which these characteristics were normal.[2,3] In 1969 a silent thalassemia was reported[4] through examination not only of the hematological picture but also of the hemoglobin status and the α/β globin synthesis ratio; the carrier was undoubtedly the father of two thalassemia intermedia patients and he showed a normal hematologic picture and a normal level of Hb A_2, but the α/β globin synthesis ratio was considerably greater than 1.

In subsequent years many similar observations[5-9] confirmed the existence of varieties of silent thalassemia that could only be searched for among the apparently normal parents of thalassemia intermedia patients and could only be diagnosed through *in vitro* study of globin chain synthesis.

As the knowledge of this phenomenon progressed an analogous condition was also observed for the α thalassemia,[10] that is α° or α thal 1, easily recognizable for its thalassemic hematological picture and for the α/β globin synthesis ratio well below 1, a silent or sub-silent variety (α^+ or α thal 2) in which all these characteristics as well as the globin chain synthesis ratio were normal or very near normal. Likewise, this variety could only be

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identified in obligate carriers – the apparently normal parent of a hemoglobinosis H patient – or very rarely in non obligate carriers, through careful evaluation of slight alterations in some hematologic parameters (MCV below the normal mean, mild changes in erythrocyte morphology) or due to the presence of an α/β globin synthesis ratio a little under 1.

Finally, in the same years, different types of α globin gene triplication[11,12] and, more rarely, quadruplication[13] were described which have a hematological picture devoid of the customary thalassemic characteristics, but which interact with β thalassemic defects and are expressed as true β thalassemias.[11]

Except for the sporadic above mentioned observations, real progress in knowledge of all these silent types of thalassemia was made only when it became possible to carry out large-scale studies on globin genes thanks to the modern techniques of molecular biology.

The aim of the present work is to describe in detail the phenotype of the silent varieties of β and α thalassemia and of the triplicated α globin gene most common in Italy. All the subjects examined come from the population of individuals who daily attain to our center in order to be tested for thalassemia. They include: heterozygous carriers of the –101 C→T mutation of the β globin gene promoter; heterozygous carriers of the IVS II 844 C→G mutation of the β globin gene; heterozygous carriers of α^+ thalassemia due to deletion of one of the two α genes of a cluster ($-\alpha^{3.7I}$, $-\alpha^{3.7II}$, $-\alpha^{4.2}$); heterozygous carriers of non deletion α^+ thalassemia (α_2^{NcoI}, α_1^{NcoI}, α_2^{HphI}); heterozygous carriers of α globin gene triplication. Carriers of β+α thalassemia were excluded because their phenotype is not silent[14,15] and subjects with an isolated increase of Hb A_2 were also omitted since no alterations of the β cluster has yet been identified in many of them, and thus it is not certain that they belong to the group of β thalassemias.[15]

Subjects

Twenty-two subjects were carriers of the β –101 C→T mutation (Table 1), 13 males and 9 females who belong to a group of 60 subjects identified in our medical center over the last five years and already described in part in a previous work.[16] The ones chosen for the present study are those who were also evaluated through *in vitro* globin synthesis study and shown by DNA analysis to be carriers of a normal α genotype. The mean values of the individual parameters of this subgroup are identical to those of the overall group.

The carriers of this mutation were most often identified among the parents of mild thalassemia intermedia patients or among members of couples at risk, however, some of them were also chosen directly through detection of slight alterations in some hematologic parameter or because of a mildly elevated Hb A_2 level or even through the simple erythrocyte morphology test we carry out in the preliminary phase of school screening.

Four subjects were carriers of the IVS II 844 C→G mutation (Table 2). They belong to 3 different family lines and came to our attention either because they were relatives of thalassemia intermedia patients who showed this mutation in their genotype, or because the carrier displayed an isolated increase in Hb A_2.

The subjects with deletion α^+ thalassemia were often identified among the apparently healthy parents of hemoglobinosis H patients or among the apparently normal parents of subjects with a clear α thalassemia picture. Others were identified among subjects examined in our laboratories or in school screenings because they were carriers of hematological anomalies mild but constant over time.

Table 1. Phenotype of heterozygous carriers of β^+ thalassemia due to the –101 C→T mutation.

N.	case	age yrs.	Hb g/dL	RBC x10¹²/L	MCV fL	MCH pg	EOF 0.36%	EMA	HbA_2 %	HbF %	α/β ratio
males											
1	TA	54	16.0	5.6	84	28.6	N	–	3.7	1.8	1.15
2	TM	31	14.4	4.9	92	29.6	N	–	3.2	1.0	0.97
3	BE	27	16.5	5.5	85	29.7	N	–	3.4	1.5	0.90
4	FD	14	13.8	5.5	78	25.0	N	+	3.3	1.0	0.92
5	VM	29	17.6	5.6	94	31.3	N	–	3.6	1.0	1.03
6	BG	35	14.9	4.9	92	30.2	N	–	3.2	0.8	1.19
7	SW	42	13.7	5.1	86	26.7	N	±	3.6	2.3	1.26
8	DSC	40	15.0	5.1	88	29.2	N	±	3.3	1.0	1.03
9	NB	41	15.7	5.3	90	29.6	N	–	3.4	1.0	1.46
10	OM	28	16.4	5.8	84	28.3	N	±	3.6	2.0	1.00
11	ML	60	14.9	5.4	85	27.5	N	–	3.5	1.8	1.11
12	LGF	51	17.0	5.9	83	28.6	N	–	4.0	2.3	0.99
13	GM	14	14.2	5.2	81	27.2	N	+	3.9	2.5	1.13
females											
14	CT	12	14.5	5.0	84	28.9	N	±	3.3	0.8	1.09
15	MER	37	13.7	5.2	81	26.2	N	±	3.2	0.8	1.23
16	FCM	25	11.7	3.9	91	29.9	N	±	3.6	1.0	1.07
17	CCA	36	13.9	4.8	87	29.0	N	–	3.4	1.0	1.10
18	SFF	43	14.5	4.5	97	32.2	N	–	3.4	0.8	1.01
19	DCLR	32	11.3	4.4	81	25.3	N	±	3.3	1.0	1.30
20	DGAP	32	12.4	4.6	84	27.1	N	–	3.3	0.7	1.24
21	AL	13	13.1	5.1	81	25.6	N	±	3.1	0.7	0.99
22	GS	14	11.3	4.7	77	24.0	N	+	3.0	1.0	1.00
mean*			14.4	5.1	86	28.2			3.4		1.10
SD			±1.7	±0.5	±5.1	±2.1			±0.23		±0.14
SE			±0.4	±0.1	±1.1	±0.1			±0.05		±0.03

The α genotype and the serum iron levels were normal in all these subjects and were not reported in the Table (see text).
**See Table 7 for a comparison with normal individuals and carriers of other silent thalassemias; EOF = erythrocyte osmotic fragility; EMA = erythrocyte morphology alterations; N = normal.*

Table 2. Phenotype of heterozygous carriers of β^+ thalassemia due to the IVS II C→G mutation.

N.	case	age yrs.	Hb g/dL	RBC x10¹²/L	MCV fL	MCH pg	EOF 0.36%	EMA	HbA_2 %	HbF %	α/β ratio
males											
1	CL	40	15.9	5.4	90	29.5	N	–	3.0	0.8	1.28
2	AA	30	14.5	5.2	86	28.0	N	–	3.5	0.8	0.99
females											
3	CP*	50	11.3	4.7	72	23.8	N	+	3.1	0.9	1.14
4	DLG	49	12.0	4.1	89	29.6	N	–	3.2	1.5	1.19

The α genotype was normal in all these subjects (see text).
**This woman has a permanently low serum iron level (~30γ%); EOF = erythrocyte osmotic fragility; EMA = erythrocyte morphology alterations; N = normal.*

Table 3. Phenotype of heterozygous carriers of single α globin gene deletion.

N.	case	age yrs.	Hb g/dL	RBC x10¹²/L	MCV fL	MCH pg	EOF 0.36%	EMA	HbA_2 %	α/β ratio	α genotype
A - Silent phenotype											
males											
1	PS	38	15.9	6.2	80	25.8	N	±	2.3	0.82	$-\alpha^{3.7I}/\alpha\alpha$
2	AA	30	14.5	5.2	86	28.0	N	–	3.5	0.99	$-\alpha^{3.7I}/\alpha\alpha$
3	CS	34	15.8	5.6	80	28.0	N	+	3.3	0.86	$-\alpha^{3.7I}/\alpha\alpha$
4	PD	14	14.2	5.5	79	25.6	N	±	3.3	0.84	$-\alpha^{3.7I}/\alpha\alpha$
5	SA	12	13.0	5.1	80	25.4	N	+	2.6	0.82	$-\alpha^{3.7I}/\alpha\alpha$
6	UM	24	16.3	6.8	74	24.8	N	±	2.5	0.87	$-\alpha^{3.7I}/\alpha\alpha$
7	SM	31	15.0	5.5	83	27.0	N	–	2.2	0.80	$-\alpha^{3.7I}/\alpha\alpha$
8	CS	28	14.0	4.6	91	30.5	N	–	3.1	0.97	$-\alpha^{3.7I}/\alpha\alpha$
9	SA	58	15.1	5.0	92	29.9	N	–	2.5	0.98	$-\alpha^{3.7I}/\alpha\alpha$
10	TC	34	15.2	6.0	78	25.3	N	+	2.0		$-\alpha^{3.7I}/\alpha\alpha$
11	SS	73	15.3	5.6	84	27.3	N	–	2.7		$-\alpha^{3.7I}/\alpha\alpha$
12	SG	18	14.9	5.9	81	25.3	N	+	2.0		$-\alpha^{3.7I}/\alpha\alpha$
13	MA	18	16.0	6.1	81	26.2	N	±	2.7		$-\alpha^{3.7I}/\alpha\alpha$
14	BD	27	14.1	5.3	84	26.4	N	–	2.6		$-\alpha^{3.7I}/\alpha\alpha$
15	CC	30	14.3	5.6	79	25.3	N	±	2.8		$-\alpha^{3.7I}/\alpha\alpha$
16	CM	20	15.6	5.8	82	26.7	N	–	2.8		$-\alpha^{3.7I}/\alpha\alpha$
17	DA	16	15.1	5.8	78	26.0	N	–	2.5		$-\alpha^{3.7I}/\alpha\alpha$
18	FR	44	15.6	5.9	81	26.4	N	+	2.5		$-\alpha^{3.7I}/\alpha\alpha$
19	IGL	20	14.7	5.7	81	25.7	N	+	2.4		$-\alpha^{3.7I}/\alpha\alpha$
20	FN	79	16.2	5.4	92	30.2	N	–	2.8		$-\alpha^{3.7I}/\alpha\alpha$
females											
21	FD	32	13.0	5.1	75	25.7	N	±	2.7	1.02	$-\alpha^{3.7I}/\alpha\alpha$
22	CM	24	12.5	4.8	82	25.9	N	±	2.3	0.85	$-\alpha^{3.7I}/\alpha\alpha$
23	ZM	19	13.4	5.1	84	26.4	N	–	2.3	0.80	$-\alpha^{3.7I}/\alpha\alpha$
24	FA	26	10.9	4.1	77	26.3	N	±	1.5	0.90	$-\alpha^{3.7I}/\alpha\alpha$
25	DPA	35	12.2	4.4	81	27.6	N	–	2.7	0.97	$-\alpha^{3.7I}/\alpha\alpha$
26	NM	69	13.4	4.7	84	28.3	N	–	2.8	0.94	$-\alpha^{3.7I}/\alpha\alpha$
27	CSI	23	13.2	5.1	78	25.9	N	±	2.4	0.89	$-\alpha^{3.7I}/\alpha\alpha$
28	MCS	42	12.5	4.4	84	28.6	N	±	1.8		$-\alpha^{3.7I}/\alpha\alpha$
29	TR	12	13.4	5.1	79	26.0	N	±	2.4		$-\alpha^{3.7I}/\alpha\alpha$
30	TL	34	13.6	5.2	81	25.9	N	+	2.6		$-\alpha^{3.7I}/\alpha\alpha$
31	PS	17	13.1	5.3	78	24.8	N	±	2.4		$-\alpha^{3.7I}/\alpha\alpha$
32	PP	22	12.6	5.0	78	25.0	N	+	2.8		$-\alpha^{3.7I}/\alpha\alpha$
33	AG	39	12.5	4.8	80	26.0	N	–	2.1		$-\alpha^{3.7I}/\alpha\alpha$
34	AD	31	13.0	5.1	77	25.6	N	±	2.7		$-\alpha^{3.7I}/\alpha\alpha$
35	BR	15	13.0	5.1	79	25.6	N	+	2.4		$-\alpha^{3.7I}/\alpha\alpha$
36	BP	17	12.0	4.9	78	24.6	N	±	1.6		$-\alpha^{3.7I}/\alpha\alpha$
37	CM	44	12.6	4.2	90	30.2	N	–	2.7		$-\alpha^{3.7I}/\alpha\alpha$
38	CC	51	13.5	5.0	83	27.1	N	–	2.6		$-\alpha^{3.7I}/\alpha\alpha$
39	DMC	7	13.7	5.5	79	24.8	N	±	2.6		$-\alpha^{3.7I}/\alpha\alpha$
40	DPM	46	12.9	4.5	88	28.8	N	–	2.8		$-\alpha^{3.7I}/\alpha\alpha$
41	DCA	23	13.7	5.1	81	27.0	N	–	1.7		$-\alpha^{3.7I}/\alpha\alpha$
42	GF	32	14.9	5.3	87	27.8	N	–	2.5		$-\alpha^{3.7I}/\alpha\alpha$
43	LP	29	12.8	5.1	79	25.2	N	±	2.5		$-\alpha^{3.7I}/\alpha\alpha$
44	LM	47	13.9	5.1	85	27.0	N	±	2.5		$-\alpha^{3.7I}/\alpha\alpha$
45	D'AM	52	12.8	5.2	74	24.4	N	+±	2.7		$-\alpha^{3.7I}/\alpha\alpha$
46	CF	14	12.9	5.1	79	25.3	N	+±	3.0		$-\alpha^{3.7I}/\alpha\alpha$
47	SA	23	12.3	5.0	79	24.7	N	±	3.0		$-\alpha^{3.7I}/\alpha\alpha$
mean*			13.8	5.2	81	26.5			2.5	0.90	
DS			±1.3	±0.5	±4.3	±1.6			±0.40	±0.07	
ES			±0.2	±0.1	±0.6	±0.2			±0.05	±0.02	
B - Sub-silent phenotype											
males											
1	GA	14	14.1	5.6	74	24.9	N	++	2.7	0.84	$-\alpha^{3.7I}/\alpha\alpha$
2	AG	32	14.5	5.8	74	25.1	N	++	2.5	0.70	$-\alpha^{3.7II}/\alpha\alpha$
3	LA	43	14.3	5.5	82	25.7	SD	++	2.8	0.77	$-\alpha^{3.7II}/\alpha\alpha$
4	FA	62	12.0	4.5	85	26.5	N	±	2.6	0.90	$-\alpha^{4.2}/\alpha\alpha$
females											
5	MO	38	10.7	4.5	74	24.0	D	++	2.0	0.91	$-\alpha^{3.7I}/\alpha\alpha$
6	CFM	28	12.7	5.2	75	24.6	N	++	2.3	0.70	$-\alpha^{3.7I}/\alpha\alpha$
7	PF	29	12.2	5.2	73	23.5	N	++	2.4	0.86	$-\alpha^{4.2}/\alpha\alpha$

The β genotype was normal in all these subjects (see text). The Hb F levels and serum iron levels were normal in all these subjects and were not reported in this table.
**See Table 7 for a comparison with normal individuals and carriers of other silent thalassemias; EOF = erythrocyte osmotic fragility; EMA = erythrocyte morphology alterations; N = normal; SD = slightly decreased; D = decreased.*

Table 4. Phenotype of heterozygous carriers of α^+ thalassemia due to the $\alpha^{Nco\,I}$ mutation.

N.	case	age yrs.	Hb g/dL	RBC x10¹²/L	MCV fL	MCH pg	EOF 0.36%	EMA	HbA_2 %	HbF %	α/β ratio
Carriers of the $\alpha_2^{Nco\,I}$ mutation (genotype: $\alpha^{Nco\,I}\alpha/\alpha\alpha$)											
males											
1	CM	39	13.7	5.4	73	25.2	N	++	2.3		0.80
2	DL	12	12.6	5.2	74	24.1	N	+±	2.1		1.01
3	RR	13	12.4	5.5	69	22.6	N	++	2.5		0.79
4	RU	35	14.1	6.0	75	23.5	N	++	2.2		0.80
5	PM	6	11.7	5.1	71	22.9	D	++	2.2		0.99
6	TM	27	15.4	6.0	74	25.4	N	++	2.2		0.85
7	PM	25	14.8	5.8	74	25.4	N	++	2.4		0.86
8	SA	15	13.4	5.4	75	24.9	N	++	2.7		
9	PG	41	13.9	5.5	77	25.1	N	++	2.5		
10	SP	24	16.0	6.6	77	24.2	N	±	2.2		0.90
11	CAA	23	13.8	5.2	77	26.3	N	–	1.7		
females											
12	PM	13	12.3	5.4	72	22.9	D	++	2.1		0.75
13	VA	17	12.9	5.2	75	24.8	N	+±	2.4		0.91
14	CL	30	12.5	5.2	71	23.1	D	++	2.2		0.81
15	PD	23	12.3	5.1	76	24.2	D	++	2.8		0.82
16	CS	37	14.0	5.5	73	25.4	N	++	2.6		0.70
17	SG	68	15.0	6.1	78	24.7	SD	++	2.0		
18	CS	14	13.1	5.2	78	25.5	N	++	2.3		
19	FP	32	12.5	5.0	77	25.1	N	–	2.5		
20	DSMG	42	13.2	5.2	80	25.3	N	–	2.5		
mean*			13.5	5.5	75	24.5			2.3		0.85
SD			±1.1	±0.4	±2.7	±1.0			±0.22		±0.08
SE			±0.2	±0.1	±0.6	±0.2			±0.05		±0.02
Carriers of the $\alpha_1^{Nco\,I}$ mutation (genotype: $\alpha\alpha^{Nco\,I}/\alpha\alpha$)											
females											
21	BS	32	12.6	5.1	77	24.6	N	+±	2.6		
22	SP	44	12.7	4.6	82	27.7	N	–	2.7		1.05
23	ZA	68	14.5	5.7	82	25.5	N	–	2.7		1.00

The β genotype was normal in all these subjects (see text). The Hb F levels and serum iron levels were normal in all these subjects and were not reported in this table.
**See Table 7 for a comparison with normal individuals and carriers of other silent thalassemias; EOF = erythrocyte osmotic fragility; EMA = erythrocyte morphology alterations; N = normal; SD = slightly decreased; D = decreased.*

Table 3 lists 54 subjects (24 males and 30 females whose ages range from 7 to 79 years), of whom 50 are carriers of $\alpha^{3.7I}$, 2 are carriers of $\alpha^{3.7II}$ and 2 of $\alpha^{4.2}$. Table 4 reports 23 subjects (11 males and 12 females from 6 to 68 years of age) who are heterozygous carriers of a mutation in the initial codon of the α_2 gene (AUG→ACG) or of the α_1 gene (AUG→GUG), both of which are recognized by the restriction enzyme NcoI. Table 5 presents 46 subjects between the ages of 8 and 52 (25 males and 21 females) who are heterozygous carriers of the deletion of the TGAGG pentanucleotide located in the 5' region of the I intron of the α_2 globin gene that removes an Hph I restriction site. Carriers of the α^{Ncol} and α_2^{HphI} mutations were also identified either through hematological anomalies detected at preliminary testing or because they were the parents of α thalassemia carrier with an evident phenotype.

Table 6 includes 21 subjects (12 males and 9 females between 6 and 75 years old) who are heterozygous carriers of a triplicated α gene locus. These individuals were also the parents or children of patients with evident β thalassemia intermedia, or of subjects who upon initial testing seemed to be very obvious heterozygous carriers of a β thalassemic defect, but at the globin DNA test proved to be double heterozygotes for β thalassemia and the triplicated α gene complex. In other subjects in

Table 5. Phenotype of heterozygous carriers of α^+ thalassemia due to the $\alpha_2^{Hph\,I}$ mutation.

N.	case	age yrs.	Hb g/dL	RBC x10¹²/L	MCV fL	MCH pg	EOF 0.36%	EMA	HbA_2 %	α/β ratio
Genotype $\alpha^{Hph\,I}\alpha/\alpha\alpha$										
males										
1	SP	38	15.2	5.8	79	26.2	N	±	2.3	0.82
2	UM	28	13.8	5.2	79	26.5	N	±	2.6	0.92
3	MA	47	16.2	5.6	86	28.8	N	–	2.4	1.05
4	MR	14	13.8	5.5	75	24.8	N	+	2.6	0.87
5	NG	43	14.8	5.7	77	25.8	N	+	2.1	
6	ML	26	14.9	5.9	79	25.4	N	+	2.5	
7	AG	32	14.4	5.6	79	25.8	N	+	2.2	
8	CGL	27	13.9	5.5	77	25.2	N	+±	2.4	
9	SA	12	14.7	5.6	77	26.2	N	+±	2.5	
10	MG	45	14.2	4.9	87	28.7	N	–	2.0	
11	CL	17	14.5	5.8	76	25.0	N	++	2.3	0.88
12	QA	29	14.8	5.8	79	25.5	D	++	2.6	0.87
13	SM	21	14.8	5.7	79	25.8	N	++	2.1	0.87
14	CA	33	14.8	6.2	73	23.7	N	++	2.2	0.68
15	DLG	37	15.1	6.1	77	24.9	N	++	2.9	0.70
16	MG	23	15.2	6.1	75	25.0	N	++	2.3	0.90
17	BM	18	15.0	6.0	76	34.6	N	++	2.5	
18	PR	13	13.0	5.5	74	23.6	N	++	2.2	
19	LE	13	12.3	5.7	67*	21.4	SD	++	2.3	
20	MS	31	14.5	5.7	83	25.3	N	++	2.2	
21	TL	14	12.4	4.9	75	25.1	N	++	2.9	1.03
22	PA	45	14.0	5.3	81	26.1	N	–	2.7	
23	BG	37	16.4	6.1	80	27.0	N	±	2.1	
24	BP	32	14.8	5.6	79	26.6	N	–	2.1	
25	NG	43	14.8	5.7	77	25.8	N	+	2.1	
females										
26	CNP	38	12.0	4.7	77	25.4	N	+±	2.6	
27	RP	36	13.4	5.0	81	26.7	N	±	2.2	0.94
28	TE	17	12.8	4.8	81	26.8	N	–	2.6	0.98
29	BA	21	13.1	5.3	79	24.4	N	±	2.1	0.89
30	PC	16	12.5	5.1	82	24.4	N	+	1.9	
31	SSR	25	14.1	5.6	79	25.2	N	+	1.7	
32	CA	27	12.0	5.2	77	23.2	N	+	2.6	
33	BR	23	11.6	5.0	73	23.2	N	+±	2.4	
34	GB	22	12.4	5.0	79	24.6	SD	+±	2.7	
35	CA	26	12.3	4.9	76	25.2	N	+±	2.2	
36	TM	19	11.9	4.8	76	24.7	N	+	2.2	
37	IM	18	12.9	5.1	75	25.3	N	+±	2.5	
38	SGM	52	14.1	5.6	75	25.3	N	+±	2.2	
39	DLE	33	12.8	5.3	72	23.9	N	++	2.7	1.04
40	FA	31	13.5	5.6	73	24.2	N	++	2.4	0.91
41	TM	14	12.5	6.5	62*	19.3	D	++	2.9	0.68
42	AMC	33	13.3	5.6	76	23.8	SD	++	2.7	
43	RV	8	11.7	5.0	73	23.3	N	++	2.3	
44	MM	12	13.4	5.4	77	24.8	N	++	1.9	
45	MF	13	12.2	5.2	72	23.5	SD	++	2.4	
46	RA	31	13.4	5.1	83	26.1	N	±	2.6	
mean°			13.7	5.5	78	25.3			2.4	0.88
SD			±1.2	±0.4	±3.4	±2.1			±0.27	±0.12
SE			±0.2	±0.1	±0.5	±0.3			±0.04	±0.03

The β genotype was normal in all these subjects (see text). The Hb F levels and serum iron levels were normal in all these subjects and were not reported in this table.
**This value was not computed in the mean (see Figure 1). °See Table 7 for a comparison with normal individuals and carriers of other silent thalassemias; EOF = erythrocyte osmotic fragility; EMA = erythrocyte morphology alterations; N = normal; SD = slightly decreased; D = decreased.*

this group the diagnosis was made by searching directly for the triplicated α gene on the basis of the phenotype individual characteristics.

In addition to the above mentioned subjects, another group of individuals was investigated (Table 7) at the same time for comparison: 113 normal subjects, 70 heterozygous carriers of mild β^+ thalassemia, and 33 homozygotes or compound heterozygotes for α^+ thalassemia.

Table 6. Phenotype of heterozygous carriers of a triplicated α gene locus.

N.	case	age yrs.	Hb g/dL	RBC x10¹²/L	MCV fL	MCH pg	EOF 0.36%	EMA	HbA_2 %	α/β ratio
Genotype $\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$										
males										
1	MVC	9	13.9	5.4	78	25.7	N	+±	2.6	1.64°
2	MN	29	14.5	5.0	86	28.7	N	–	3.4	1.28
3	ED	14	12.9	4.4	87	29.4	N	–	3.2	1.24
4	TS	19	13.3	5.3	78	24.9	SD	++	2.3	1.40
5	TR	8	11.6	4.9	76	23.7	N	++	2.4	1.10
6	TD	20	13.8	5.0	81	27.4	N	+	2.7	1.41
7	BL	26	15.2	4.7	95	32.4	N	–	3.0	1.15
8	CS	25	16.3	5.4	86	30.3	N	–	3.1	1.20
9	GB	50	17.5	5.5	87	31.7	N	–	2.9	
10	CF	6	12.0	4.8	76	24.7	N	+	2.9	1.26
11	ZG*	61	16.1	5.4	87	29.8	N	–	2.4	1.38
12	MM	57	15.4	5.1	89	30.5	N	–	3.0	1.42
females										
13	GA	30	13.4	5.6	84	28.3	N	–	3.0	1.00
14	EBE	44	11.7	3.7	96	31.6	N	–	2.7	
15	LDND	44	13.1	4.4	86	29.5	N	–	2.3	1.10
16	LE	14	13.5	5.1	78	26.3	N	–	3.0	1.05
17	GR	26	14.6	5.1	79	28.4	N	–	3.0	1.33
18	RZMC	23	13.0	4.3	83	30.3	N	±	3.2	1.02
19	RMA	59	12.5	4.3	82	29.3	N	–	2.7	1.36
20	BD	46	13.0	5.0	76	26.1	N	±	2.7	1.30
21	BSA	75	12.3	4.6	79	26.5	N	–	1.3°	0.98
mean#			13.8	4.9	83	28.3			2.8	1.22
SD			±1.5	±0.5	±5.7	±2.4			±0.31	±0.14
SE			±0.3	±0.1	±1.2	±0.5			±0.07	±0.03

The β genotype was normal in all these subjects (see text). The Hb F levels and serum iron levels were normal in all these subjects and were not reported in this table.
**This subject carries an $\alpha\alpha\alpha^{anti\,4.2}$ genotype. °This value was not computed in the mean (see Figure 1). #See Table 7 for a comparison with normal individuals and carriers of other silent thalassemias. EOF = erythrocyte osmotic fragility; EMA = erythrocyte morphology alterations; N = normal; SD = slightly decreased; D = decreased.*

Methods

Besides the basic hematologic tests (determination of blood parameters, hemoglobin electrophoresis, measurement of Hb A_2 and Hb F levels, serum iron level), *in vitro* globin synthesis studies and molecular analysis of the β and α globin genes, and in some cases of the δ gene as well, were performed.

Hematological tests: red blood cell (RBC) indices were determined with a Technicon H1 automatic cell counter; erythrocyte osmotic fragility (EOF) was measured using the single saline solution at a concentration of 0.36%; RBC morphology was examined on thin, uncolored blood smears 3 (EMA); reticulocyte count and the search for RBC inclusion bodies were carried out on fresh blood preparations colored with brilliant cresyl blue. Serum iron levels were also determined by means of traditional methods in all subjects.

Hemoglobin study and the measurement of the various hemoglobin fractions were performed with the semiautomatic methodology standardized in our laboratories, which, in our experience, is still the most suitable technique today both for detecting minimal alterations in the Hb A_2 level and for studying large numbers of individuals.

According to this method the hemolysate is deposited in

Table 7. Means and distribution of some hematological parameters in subjects with silent β or α thalassemia.

Condition	N. cases	Hb g/dL	MCV fL	EMA –	EMA +	EMA ++	EMA +++	EOF N	EOF SD	EOF D	HbA_2 %	α/β ratio°	genotype β	genotype α
Normal*	113	14.0 ±1.3 ±0.1	87 ±3.8 ±0.4	112	1			113			2.6 ±0.23 ±0.02	1.00 ±0.04 ±0.01	β^A/β^A	αα/αα
Mild β^+ thalassemia	70	13.1××× ±1.2 ±0.1	73××× ±4.4 ±0.5	4	9	57		39	21	10	4.0××× ±0.84 ±0.10	1.49××× ±0.10 ±0.03	β IVS I-6/β^A or β –87 C→G/β^A	αα/αα
β thal due to –101 C→T mutation	22	14.4 ±1.7 ±0.4	86 ±5.1 ±1.1	11	11			22			3.4××× ±0.23 ±0.05	1.10×× ±0.14 ±0.03	–101/β^A	αα/αα
Heterozygosity for $\alpha\alpha\alpha^{anti3.7}$	21	13.8 ±1.5 ±0.3	83××× ±5.7 ±1.2	14	4	3		20	1		2.8×× ±0.31 ±0.07	1.22××× ±0.14 ±0.03	β^A/β^A	$\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$
Silent α^+ thalassemia due to the $-\alpha^{3.7\,I}$ mutation	47	13.8 ±1.3 +0.2	81××× ±4.3 ±0.6	18	27	2		47			2.5× ±0.40 ±0.05	0.90××× ±0.07 ±0.02	β^A/β^A	$-\alpha^{3.7\,I}/\alpha\alpha$
Silent α^+ thalassemia due to the $-\alpha_2^{Nco\,I}$ mutation	20	13.5 ±1.1 ±0.2	75××× ±2.7 ±0.6	3	1	16		15	5		2.3××× ±0.22 ±0.05	0.85××× ±0.08 ±0.02	β^A/β^A	$\alpha^{Nco\,I}\alpha/\alpha\alpha$
Silent α^+ thalassemia due to the $\alpha_2^{Hph\,I}$ mutation	46	13.7 ±1.2 ±0.2	78××× ±3.4 ±0.5	5	15	26		40	4	2	2.4××× ±0.27 ±0.04	0.88××× ±0.12 ±0.03	β^A/β^A	$\alpha^{Hph\,I}\alpha/\alpha\alpha$
Homozygosity or compound heterozygosity for α^+ thal	33	12.5××× ±1.3 ±0.2	70××× ±3.7 ±0.6			31	2	5	5	23	2.3××× ±0.29 ±0.05	0.69××× ±0.10 ±0.02	β^A/β^A	α^+ thal/α^+ thal of various types and combinations

Mean values are followed by standard deviation (SD) and standard error (SE). ××× symbolyze statistical significance of comparisons with normal values at the level of $p \leq 0.05$, $p < 0.01$, $p < 0.001$, respectively. EOF = erythrocyte osmotic fragility; EMA = erythrocyte morphology alterations; N = normal; SD = slightly decreased; D = decreased.
**These subjects were examined in the same period and with the same methods and instrumentation as the members of the other groups.*
°The subjects examined for in vitro globin synthesis included: 21 normal individuals; 12 carriers of mild β^+ thalassemia; 22 carriers of β^+ thalassemia due to the –101 mutation; 19 carriers of the $aaa^{anti3.7}$; 16 carriers of the $-\alpha^{3.7}$ deletion; 13 carriers of the $\alpha_2^{Nco\,I}$ mutation; 17 of the $\alpha_2^{Hph\,I}$ mutation; 27 homozygotes or compound heterozygotes for α^+ thalassemia (Figure 3).

microzones on strips of Helena cellulose acetate; the electrophoretic run is conducted with 0.125 M NaOH buffer, 1.4 M glycine – pH 8.7 – at 280 volts for 24 min. The strips are colored with a 0.4% *ponceau* red solution and rendered transparent with a Helena solution. Then the optical density of the hemoglobin bands is read with a semiautomatic Preference Ciampolini densitometer and the levels of Hb A_2, Hb F and any abnormal Hb present are determined.

In vitro globin chain synthesis studies were effected using the technique of Weatherall *et al.*:[18] *in vitro* incubation of reticulocytes in a mixture of amino acids containing tritium-labelled leucine; separation of the globin chains in a Perkin Elmer HPLC apparatus and measurement of the radioactivity of the eluates corresponding to the various peaks by means of a TRI Carb Packard spectrometer; calculation of the α/β ratio.

For the study of molecular defects genomic DNA was extracted from leukocytes in circulating blood with the salting out technique.[19]

Defects of the β globin gene were identified through the ARMS (amplification refractory mutation system) technique,[20] which is based on allele-specific amplification carried out with oligonucleotide primers having at their 3' extremity a base complementary to the sequence of the mutation in question. The relative primers were utilized to search for the most common β globin gene defects found in Italy, namely:

–101 (C→T); –87 (C→G) and (C→T); –88 (C→T); –31 (A→C); –28 (A→C); frameshift cod. 6 (–A); cod. 27 Hb Knossos (G→T); cod. 30 Hb Monroe Arg→Thr (G→T); IVS I nt 1 (G→A); IVS I nt 2 (T→A); IVS I nt 5 (G→A), (G→T) and (G→C); IVS I nt 6 (T→C); IVS I nt 110 (G→A); cod. 39 (C→T) frameshift cod. 44 (–C); IVS II nt 1 (G→A); IVS II nt 705 (T→C); IVS II nt 745 (C→G); IVS II nt 844 (C→G).

The rare defects were identified through a two-step strategy: preliminary examination of β globin gene DNA with the SSCP (single strand conformation polymorphism) technique, modified in our laboratory,[21] or with the DGGE (denaturing gradient gel electrophoresis) method[21bis] – both of which signal the DNA region containing a probable defect. Successively, the direct nucleotide sequencing of the DNA region found to be alterd was carried out.

The ARMS technique was also employed to search for the most common δ gene defects.

Defects of the α globin genes were identified through a technique developed in our laboratories and already described in a previous issue of this journal.[22] This technique involves polymerase chain reaction (PCR) and specific primers that amplify sections of DNA in which the defect being looked for is located. The DNA fragments thus obtained allow recognition not only of the deletions responsible for α° thal that remove the two a genes of a cluster [in Italy, –MED and $-(\alpha)^{20.5}$], but also the majority of the molecular defects that cause α^+ thal: type I or II of the $-\alpha^{3.7}$ deletion in the heterozygous or homozygous condition; the $-\alpha^{4.2}$ deletion; the $(\alpha)\alpha^{5.3}$ deletion; the α_2^{NcoI}, α_1^{NcoI} and α_2^{HphI} point mutations in the heterozygous and the homozygous conditions (after having performed separate amplification of the α_2 and α1 genes and digestion with the appropriate restriction enzyme); the $\alpha\alpha\alpha^{anti\ 3.7}$ in the heterozygous condition. In order to identify the homozygous condition of this triplication, the heterozygous and homozygous conditions of the $\alpha\alpha\alpha^{anti4.2}$ triplication and the quadruplication of the α genes, which still cannot be detected with this technique, Southern blotting[23] was employed. This latter method also reveals previously unknown

Table 8. Distribution and frequency of some silent thalassemias in the Latium region of Italy.

Province	N. examined	Normal subjects	Carriers of a single α globin gene deletion	Carriers of α^+ thal due to the $\alpha_2^{Hph\,I}$ mutation	Carriers of β^+ thal due to the –101 C→T mutation
Rome	117	0.78 (91/117)	0.068 (8/117)	0.077 (9/117)	0.077 (9/117)
Frosinone	24	0.67 (16/24)	0.125 (3/24)	0.208 (5/24)	0
Latina	9	0.67 (6/9)	0.222 (2/9)	0.111 (1/9)	0
Viterbo	5	0.80 (4/5)	0	0	0.200 (1/5)
Rieti	16	0.56 (9/16)	0.125 (2/16)	0.063 (1/16)	0.250 (4/16)

defects. Before definitive classification all subjects underwent a complete analysis of their β and α globin genes and, when the Hb A_2 level was very low, of the δ gene as well, in order to exclude the presence of other recognizable molecular defects in addition to the one under investigation.

Results

During the course of the study the participants were all tested many times for their hematological and biochemical parameters, and the values obtained were always consistent. The tables report the results of the last tests performed.

The reticulocyte number was always normal in all the carriers of the various silent thalassemias, RBC inclusion bodies were absent or less than 1% after 6-7 days of observation, and serum iron levels were within the normal range. Therefore the individual values of these analyses were not included in the Tables. The hematologic-hemoglobin picture in all the different silent thalassemia varieties, while manifesting different frequencies between one type and another, was sometimes completely normal and at other times marked by faint alterations of some parameters that permitted a distinction between a silent and a sub-silent phenotype. These alterations, however, were always so slight and so far from those found in mild β^+ thalassemias, which are already very modest, as to justify completely in every case the classification of all these varieties in a single group of silent thalassemias.

Since the samples we collected were not *at random*, it is possible that the reciprocal frequency of the two phenotypes is not exactly what is reported here and that the frequency of the sub-silent phenotype was overestimated with respect to that of the silent one. At this time, however, there is no other possible approach to the problem and besides it is not one of the primary concerns of this work.

The –101 (C→T) mutation of the β globin gene

The hematological parameters of the 22 subjects investigated are in the normal range (Table 1 and Figure 1), except for rare cases (MCV less than 80 fL in two subjects). Hb A_2 levels (Figure 2) range from 3.0 to 4.0%, i.e. slightly or modestly above the norm. The mean values of the hematological parameters (Hb, MCV) are identical to the normal values (Table 7), while they are significantly different from those found in carriers of mild β^+ thalassemia (for Hb: t=3.99, d.f.=90, p<0.001; for MCV: t=11.6, d.f.=90, p<<0.001). As in all silent thalassemias, in this β^+ thalassemia too it appears that MCV and MCH are strongly correlated.

The IVS II 844 C→G mutation of the β globin gene

The 4 subjects identified (Table 2 and Figure 1) present a normal hematological picture except for #3, who shows slight alterations that are, however, attributable to a marked, persistent iron deficiency. Hb A_2 levels are at the upper normal limits and in one case are clearly increased (Figure 2). The α/β globin synthesis ratio is slightly high in 3 cases (Figure 3). The mean values of the various parameters are quite similar to the normal levels and therefore very far from those of carriers of mild β^+ thalassemia.

The $-\alpha^{3.7}$ and $-\alpha^{4.2}$ deletions

The hematologic parameters in the 47 subjects with the $-\alpha^{3.7\,I}$ defect listed in Table 3A are normal except for a slight reduction of MCV in many cases (Figure 1). Hb A_2 levels are normal in all but 6 cases where they are borderline, a phenomenon that has already been reported in the literature[24] and has no explanation at this time. The α/β globin synthesis ratio is slightly under 0.90 in only half of the cases, and in our laboratory this value (0.90) represents the lower normal limit (Figure 3). With respect to normal values, the mean Hb level is identical, while those of MCV and the α/β ratio are significantly different (for MCV: t=8.73, d.f.=157, p<<0.001; for α/β: t=5.49, d.f.=35, p<<0.001). The mean values of the hematologic parameters (Hb, MCV) and of the α/β globin chain synthesis ratio (Table 7) are all significantly different from those found in homozygotes and compound heterozygotes for α^+ thalassemia (for Hb: t=4.39, d.f.=78, p<<0.001; for MCV: t=11.9, d.f.=78, p<<0.0001; for α/β: t=7.38, d.f.=41, p<<0.001).

Besides this larger group of subjects with a silent phenotype (for example, cases #2, 8, 9, 26, 40 of Table 3A), there is a smaller group (Table 3B) of carriers of the $-\alpha^{3.7\,I}$, $-\alpha^{3.7\,II}$ and $-\alpha^{4.2}$ defects who have a sub-silent phenotype and present MCV and α/β globin synthesis ratio values that are similar to the lowest levels of these parameters found in carriers of the $-\alpha^{3.7\,I}$ mutation with a silent phenotype (Figures 1 and 3).

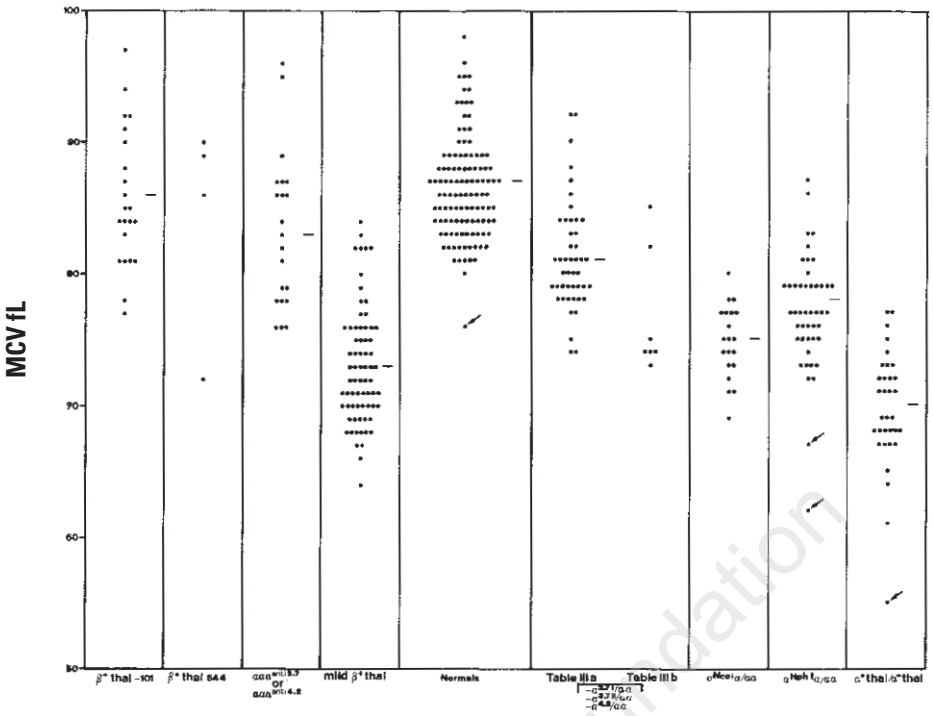

Figure 1. MCV in the various classes of heterozygous carriers of silent thalassemias: comparison with normal subjects, heterozygous carriers of mild β^+ thalassemia, homozygotes and compound heterozygotes for α^+ thalassemia.
The solid black line (—) in every column indicates the mean value (Table 7). Arrowed data were not included in the calculation of the mean value.

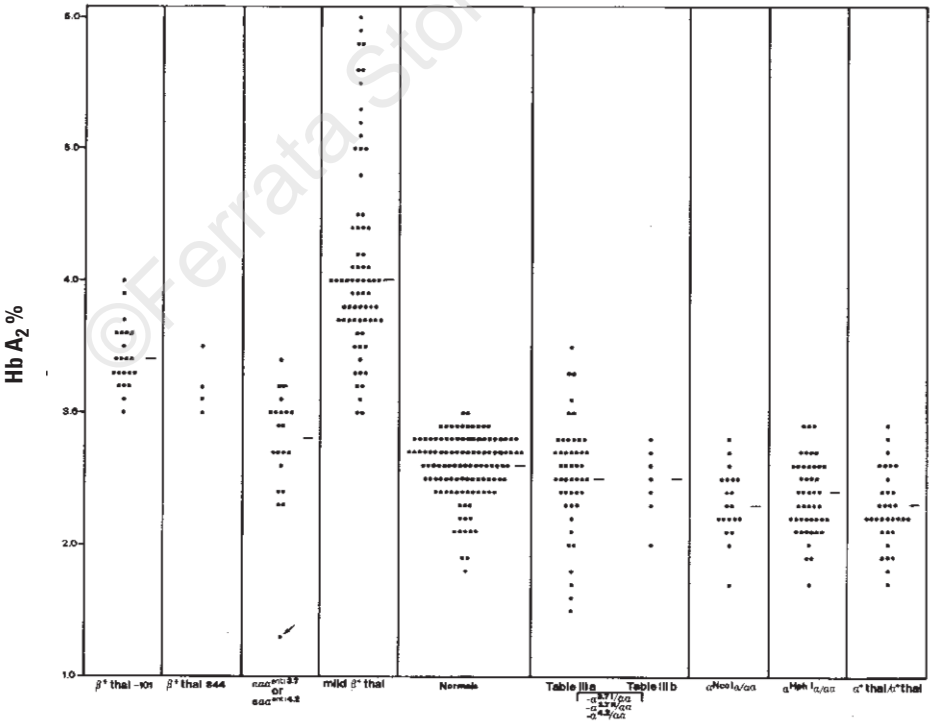

Figure 2. Hb A_2 in the various classes of heterozygous carriers of silent thalassemias: comparison with normal subjects, heterozygous carriers of mild β^+ thalassemia, homozygotes and compound heterozygotes for α^+ thalassemia.
The solid black line (—) in every column indicates the mean value (Table 7). Arrowed data were not included in the calculation of the mean value.

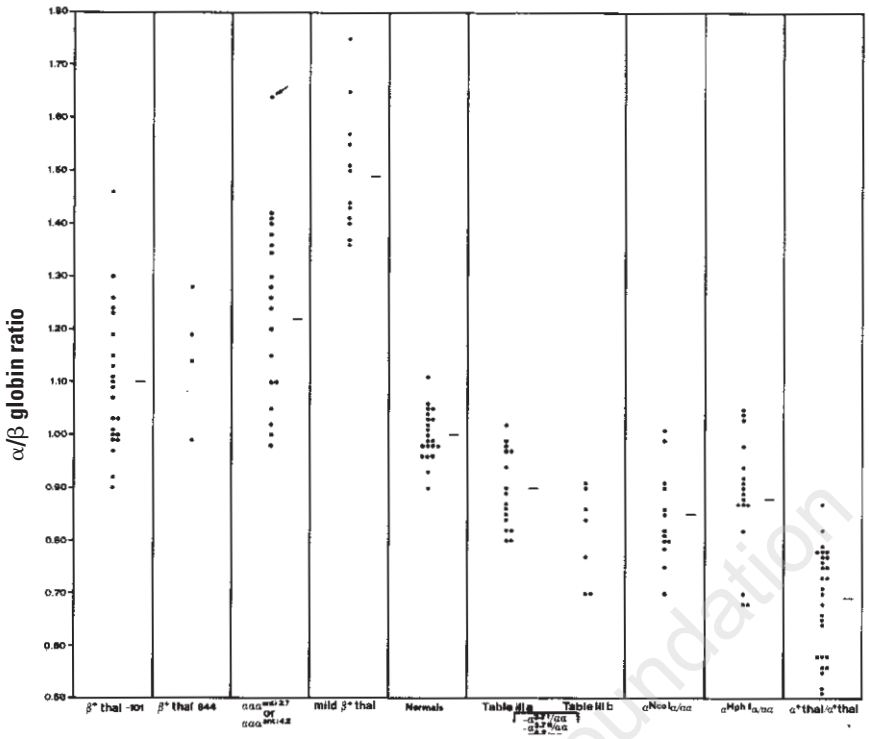

Figure 3. α/β globin chan synthesis ratio in the various classes of heterozygous carriers of silent thalassemias: comparison with normal subjects, heterozygous carriers of mild β^+ thalassemia, homozygotes and compound heterozygotes for α^+ thalassemia.
The solid black line (—) in every column indicates the mean value (Table 7). Arrowed data were not included in the calculation of the mean value.

The α_2^{NcoI} and α_1^{NcoI} mutations

In the 20 subjects with this mutation in the α_2 gene (Table 4), the phenotype is almost always sub-silent. MCV and α/β ratio values (Figures 1 and 3) are practically always below normal levels and RBC morphology is almost always altered. In this group the mean Hb value (Table 7) is identical to that of carriers of the $-\alpha^{3.7\,I}$ deletion, while the mean MCV level is significantly lower than that of heterozygous carriers of the $-\alpha^{3.7\,I}$ deletion ($t=5.76$, $d.f.=65$, $p<<0.0001$) and higher than that of homozygotes and compound heterozygotes for α^+ thalassemic defects ($t=5.23$, $d.f.=50$, $p<<0.001$). The α/β globin synthesis ratio is not significantly different between α_2^{NcoI} and $-\alpha^{3.7\,I}$ ($t=1.79$, $d.f.=27$, $p > 0.05$), but it is between heterozygotes for α_2^{NcoI} and homozygotes or compound heterozygotes for α^+ thalassemia ($t=5.03$, $d.f.=38$, $p<<0.001$).

In the 3 subjects with the α_1^{NcoI} defect the phenotype was silent (Table 4).

The α_2^{HphI} mutation

The phenotypes of the 46 carriers of this mutation show more variability than in the other varieties of silent thalassemia (Table 5). At the two ends of a long line of intermediate forms there are a completely silent phenotype (cases #3, 10, 22) and a sub-silent phenotype with deviations, sometimes even considerably marked, of some parameter (cases #11, 15, 16, 43). In the present study these two groups are numerically equal. With respect to carriers of the α_2^{NcoI} mutation, MCV values (Figure 1) are completely superimposable in most cases and only in a smaller group they are identical to normal values or to those of homozygotes or compound heterozygotes for α^+ thalassemia. This relationship is repeated for the α/β globin chain synthesis ratio (Figure 3).

The mean values of the individual parameters (Table 7), calculated cumulatively for the entire group (since they proved to be identical to those calculated separately for the silent and sub-silent carriers), are in turn completely and suggestively superimposable on those found in carriers of α^+ thalassemia due to the α_2^{NcoI} mutation for Hb ($t=0.63$, $d.f.=64$, $p>0.2$) and for α/β ratio ($t=0.77$, $d.f.=28$, $p>0.2$), and slightly but significantly different for MCV ($t=3.47$, $d.f.=62$, $p\cong0.001$).

$\alpha\alpha\alpha^{anti3.7}$ triplication

In approximately 40% of the heterozygous carriers of this defect, the hematologic parameters and RBC morphology are normal (Table 6, Figure 1). Hb A_2 (Figure 2) is around 3.0% or slightly higher in 40% of the cases and normal in the others; the α/β

globin synthesis ratio falls between 1.10 and 1.40 in the majority of the subjects and within the normal range in the remaining ones (Figure 3).

The individual MCV and α/β ratio values in the majority of cases are superimposable on those found in carriers of the –101 C→T defect, while they are identical to the values measured in carriers of mild β^+ thalassemia in only a portion of the cases (Figures 1 and 3). In the heterozygous ααα condition it is not rare to find carriers with a completely silent phenotype (for example, cases #7, 13, 15 in Table 6). The difference in the mean Hb and MCV values between $\alpha\alpha\alpha^{anti3.7}$ and β^+ thalassemia due to the –101 C→T mutation is not significant (for Hb: t=1.22, d.f.=41, p > 0.20; for MCV: t=1.82, d.f.=41, p > 0.05), but this difference is significative for Hb A_2 and the α/β ratio (for Hb A2: t=7.17, d.f.=40, p<<0.001; for α/β ratio: t=2.69, d.f.=38, p≅0.01).

Among our cases a heterozygous carrier of an α globin gene quadruplication ($\alpha\alpha\alpha\alpha^{anti3.7}$) was also identified, but this subject is not included in Table 6. His hematological phenotype was practically silent, analogously to what has been described by others,[13] but the α/β globin synthesis ratio – determined many times – was always found to be < 1 (0.70-0.80), even though the two α genes on the other chromosome were normal. This subject is still under investigation.

Discussion

The existence of a variety of silent β and α thalassemias is therefore a confirmed fact that is also of notable importance.

Among the silent β thalassemias, the one caused by the –101 C→T mutation of the promoter of the β globin gene is the best known and perhaps the most frequent in Italy as well (1.14% of all β thalassemias).[25] The initial observations made by Gonzalez Redondo *et al*.[26] and other authors[27-29] indicated a normal hematologic picture, an increased or normal Hb A_2 level and an α/β globin synthesis ratio slightly greater than 1 in this variety.

In our experience this mutation is completely silent in almost half of these cases and in the rest of them it can be suspected on the basis of a slight reduction in MCV, or minimal changes in RBC morphology, or the Hb A_2 level, which in our cases is always increased or borderline. These slight signs can also raise the suspicion that this mutation is present in non obligate carriers. While maintaining the necessary caution required when dealing with *non at random* case collection, we have observed that the α/β globin chain synthesis ratio is slightly higher than 1 in only half of these subjects, thus demonstrating that this mutation determines only a modest reduction in the expression of the β globin gene and therefore allows the carrier to exhibit a normal hematologic picture. That this is a very mild thalassemic defect is also confirmed by many observations of other authors[26-29] and also our,[16] which indicate that thalassemia intermedia patients, who carry this mutation in their genotype, present a very mild clinical and hematologic picture which sometimes is almost identical to that of simple heterozygosity for β thalassemia with very marked characteristics.[30]

Another type of β thalassemia that was already classified as silent[31] and which may not be rare in Puglia, if one considers that 2 of the 3 families we identified were originally from Puglia, is that caused by the β IVS II 844 C→G mutation. In our 4 cases, with one exception that presents minimal alterations probably produced by a persistent iron deficiency, the hematological picture is completely normal, the Hb A_2 levels are at the upper normal limits and the α/β globin synthesis ratio is barely over 1. This variety is surely among the most difficult to identify, but it is also among the forms that are the most important to recognize because in this case if a couple at risk is formed, the consequences for the children are very serious: one of our two patients has a clinical picture very similar to that of transfusion-dependent thalassemia major and the other presents a picture of very marked thalassemia intermedia.

The literature contains descriptions of other silent forms of β thalassemia, for example: β thalassemia due to mutations in the cleavage-polyadenylation site of pre-mRNA β, which in all the cases where the hematologic phenotype was studied[32,33] displayed practically normal characteristics with borderline Hb A_2 levels; some thalassemias caused by β globin gene mutations (+44 G→C, IVS II 478 C→A) that also have a sub-silent phenotype and therefore cannot be recognized in heterozygous carriers;[31,34] β thalassemia due to a –92 C→T transcription defect, which was defined as silent from the very first case in which it was described[35] and which in subsequent observations always presented[36,37] a normal hematologic picture with Hb A_2 slightly increased and an α/β globin chain synthesis ratio a little over 1.

The fact that the silent –92 C→T mutation, like the silent –101 C→T defect, is located in the farthest region of the β globin promoter suggests that on the average the farther a mutation of 5' flanking region of the β globin gene is from the coding sequence, the milder are its phenotype consequences.

We believe it is justified to include the α globin gene triplication and quadruplication in the group of the silent β thalassemias, not only because thalassemic characteristics are present in many of these cases, albeit in a very mild form, but above all because of the clearly thalassemic nature these conditions manifest when they interact with a β tha-

lassemic defect. Many observations already reported in the literature[38-51] and a large number of our own personal observations (which are currently the object of another work in preparation[30]) demonstrate that subjects with this association (β thalassemia defect and α globin gene triplication) can present an extremely vast array of clinical pictures ranging from a classical, very marked heterozygous β thalassemia to, more frequently, a more or less serious β thalassemia intermedia.

As far as heterozygous carriers of α globin gene triplication are concerned, previous observations[11-13,39,44,45,50,51,54,55] have indicated a silent hematological and hemoglobin picture in these subjects, with a normal or mild β thalassemic type of α/β globin chain synthesis ratio. Our cases demonstrate (Table 6 and Figures 1-3) that about half of the subjects present a completely normal phenotype and the other half show a phenotype with some minimal β thalassemic alterations of one or more parameters, and that the α/β globin synthesis ratio is, as in –101 β^+ thalassemia, only slightly and not always greater than 1, with a distribution of values that largely coincides in the two groups (Figure 3).

Therefore the phenotypic manifestations of an α gene triplication are very similar to those of –101 C→T β^+ thalassemia and, in particular, common to both is a Hb A_2 level that is often slightly raised or at least borderline (Table 6). This fact, which is also found in other silent β^+ thalassemias (e.g. those caused by the IVS II 844 C→G mutation, by the –92 C→T mutation, and by mutations in the cleavage-polyadenylation site), indicates that the carriers of all these silent varieties of β^+ thalassemia belong to that heterogeneous group of subjects, already described in the literature,[24] with a normal hematologic picture but a slightly raised (or borderline) Hb A_2 level. This leads to the supposition that other varieties as well of silent β^+ thalassemia that present this particular characteristic could still be included in the above group, which thus deserves further investigation.

This problem is by no means of secondary importance if we consider the decisive contribution that the identification of the genotype *β thalassemia + ααα* has made to the recognition of many mild forms of thalassemia intermedia which had not been diagnosed until then. The frequency of the unequal crossing-over that gives rise to the triple α gene is surely high. The fact that more than 20 such cases were identified in little more than a year in our laboratories is certainly significant, even though it was accomplished through targeted investigation of carriers of borderline Hb A_2 levels. The frequency of the ααα haplotype has already been evaluated statistically in other countries: in Greece, for example, it was found to be similar to that of defects of one α gene only (0.05 vs 0.07).[12] This knowledge led to the formulation of the hypothesis that the elevated frequency of the ααα haplotype, at least in some countries, might be the result of an undefined selective advantage.[12,46] This explanation appears to be possible if one also considers the fact that the frequency with which the two haplotypes (the one with only one α gene and the other with three α genes) are produced, is the same.

In the area of the α thalassemias, the varieties determined by the absence of one of the 4 α genes of the normal individual, defined as α^+ thalassemias or α thal 2, represent another important group of silent or sub-silent thalassemias. The most common type in Italy (9.3% of all the α thalassemias in the population of Latium)[52] is that caused by the $-\alpha^{3.7\,I}$ deletion, which in our experience and in agreement with data published in the literature[53] presents a silent phenotype (Table 3 and Figures 1 and 2). Rare cases with this mutation and all those we observed with the $-\alpha^{3.7\,II}$ deletion or the $-\alpha^{4.2}$ deletion seem to have a sub-silent phenotype (Table 3).

In the α^+ thalassemias due to deletions as well as in those due to point mutations (see below), the α/β globin chain synthesis ratio is just slightly less than 1 or about the normal value, except for rare cases in which it coincides with the levels of homozygotes or compound heterozygotes for α^+ thalassemic defects (Figure 3).

The completely normal picture found in the $-\alpha^{3.7\,I}$ thalassemia could be explained by the observation made by Liebhaber[54] that normally α_2 mRNA production is 2.6 times that of α_1 mRNA, and in the case of the $-\alpha^{3.7\,I}$ the expression of the hybrid α_2-α_1 gene is 1.8 times that of the normal α_1 gene, the end result being that overall synthesis of the α chains is only slightly less than normal. On the contrary, in the $-\alpha^{4.2}$ thalassemia it is likely that the α_1 gene (the only one remaining after deletion) cannot compensate sufficiently for the α globin chain deficiency, and this gives rise to a sub-silent phenotype.

Regarding the few cases of α^+ thalassemia due to type I or type II $-\alpha^{3.7}$ deletion which in the present study population and in constrast to the above mentioned interpretation are sub-silent, it is still possible that these exceptions are due to an undetected α^+ mutation.

In keeping with the predominant synthesizing activity of the α_2 gene with respect to the α_1 gene, it was also ascertained (Table 4) that in the α thalassemia recognized by the NcoI restriction enzyme the phenotype as a rule is sub-silent when the defect is on the α_2 gene, and silent (at least in the cases we have observed so far) when it is located on the α_1 gene.

On the other hand, the α^+ thalassemia recognized by the Hph I restriction enzyme does not fit this interpretation. This form of thalassemia can be detected in carriers with variable phenotypes,

among which there are two quite distinct and approximately numerically equal types: a silent phenotype and a sub-silent one. In the latter the alterations sometimes reach the same intensity found in homozygous or compound heterozygous carriers of α^+ thalassemic defects (Table 5, Figures 1 and 3).

It is true that in one case that was originally classified as sub-silent, another α^+ thalassemic defect was later found, and in another case a new defect was detected. But all the other silent and sub-silent cases show an $\alpha^{Hph\,I}\alpha/\alpha\alpha$ genotype without α gene triplication that could give rise to silent cases, and without any other detectable α^+ thalassemic defects that could cause sub-silent phenotypes. Therefore it seems that in all probability the variability of expression is a phenomenon specific to this mutation and for which, at the moment, there is no explanation.

Regarding the distribution of the various silent thalassemias in the regions of Italy, the observations collected so far are insufficient for statistical evaluation; however, they do offer some preliminary indications: for example, of the three family groups found to be carriers of the IVS II 844 C→G mutation of the β gene, two are originally from Puglia even though the subjects originating from Puglia are only 6% in the population of Latium.

Unlike the classic β thalassemias which have – as is known – a very different distribution between costal areas and internal areas as a result of earlier endemic malaria, the distribution of the silent varieties is practically uniform. There is only a slight tendency (Table 8) toward higher values for the α^+ thalassemia due to the α_2^{HphI} mutation in the province of Frosinone (which recalls the analogous increase in frequency already described for β thalassemia due to the IVS II 745 mutation)[57] and for the –101 C→T mutation in the province of Rieti. These data might be the expression of a remote *founder effect*.

In conclusion, it is evident that there are β and α thalassemias with a silent or sub-silent phenotype. These varieties are numerous and some of them are also frequent. In Latium β^+ thalassemia due to –101 C→T mutation has an incidence of 1.14% of all β thalassemias,[25] the deletion and non deletion α thalassemias have an incidence in the total population of Latium of about 13%, and the triplication of an α gene locus has an incidence of 1.13%.[52]

Therefore it is extremely important for the operators in the field of thalassemia diagnosis not only to be aware of the existence of these varieties of silent forms but also to recognize the detailed picture of the minimal phenotypic alterations these thalassemias present, which can lead one to suspect their presence even before molecular studies are performed.

For this reason we carried out the present, analytical study of the phenotypic characteristics of the main silent thalassemias found in Italy.

This analysis revealed that:

a) the –101 C→T mutation, that is in the distal CACCC box of the β globin gene promoter, and the α gene triplication on one chromosome have in common a phenotype that is sometimes silent and sometimes sub-silent. In the latter case the minimal β thalassemic alterations that the subject shows, can orient the physician toward a direct diagnosis of α gene triplication;

b) in the area of α thalassemias, α^+ thalassemia due to the $-\alpha^{3.7\,I}$ deletion always has a silent phenotype and thus cannot be recognized at the hematological level, while in the thalassemias due to the $-\alpha^{3.7\,II}$ deletion and $-\alpha^{4.2}$ deletion a sub-silent phenotype seems to be more frequent. The two α thalassemias varieties not caused by deletions, $\alpha^{Nco\,I}$ and $\alpha^{Hph\,I}$, have phenotypes that are sometimes silent and sometimes sub-silent, characterized in the latter case by slight α thalassemic alterations that, analogously to what occurs for the silent β thalassemias, can lead the physician to immediately suspect the presence of an α thalassemia.

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