LA DIAGNOSI DELLE EMOGLOBINOPATIE

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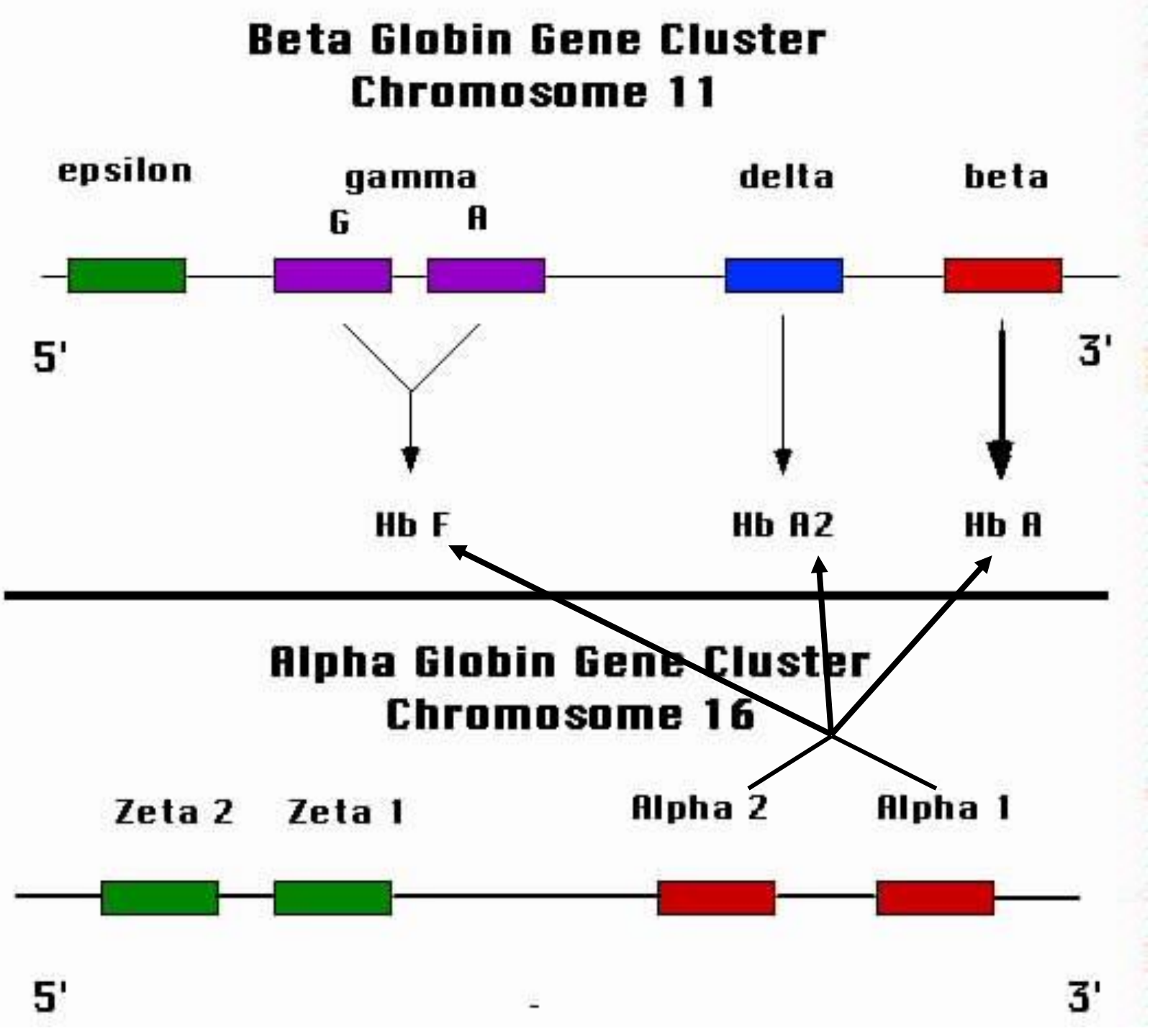
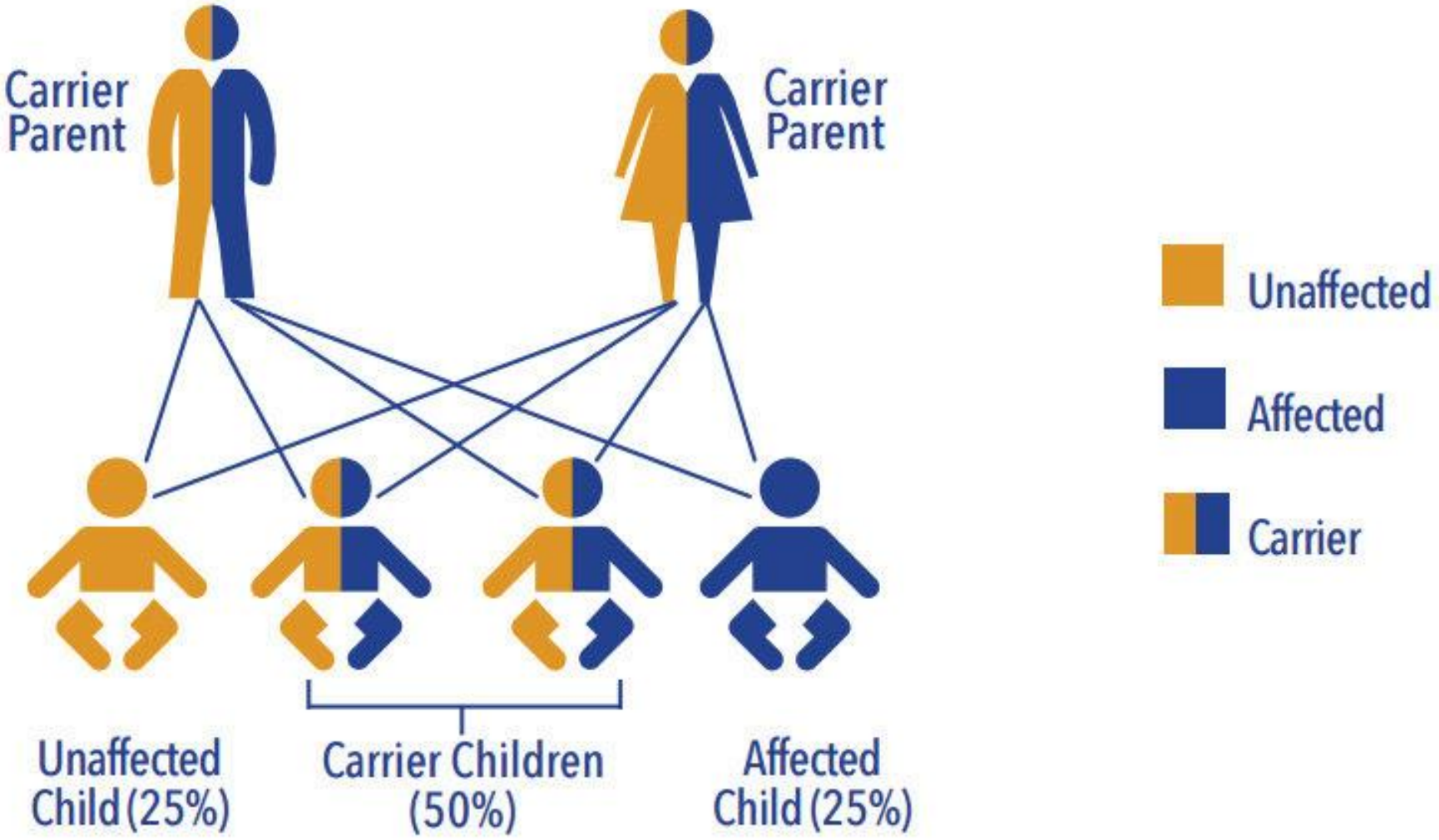

Beta Globin Gene Cluster
Chromosome 11
epsilon
gamma
G
A
delta
beta
5'
3'
Hb F
Hb A2
Hb A
Alpha Globin Gene Cluster
Chromosome 16
Zeta 2
Zeta 1
Alpha 2
Alpha 1
5'
3'

Autosomal Recessive Inheritance Pattern



European Journal of Human Genetics (2015) 23, 426–437

www.nature.com/ejhg

EJHG Open

POLICY

EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies

Ematochimici

Diagnostica di I e II livello delle Emoglobinopatie

Buone Pratiche SITE

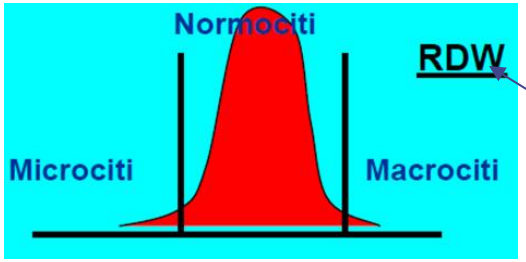
Genetici

SITE SOCIETA' ITALIANA TALASSEMIE ED EMOGLOBINOPATIE

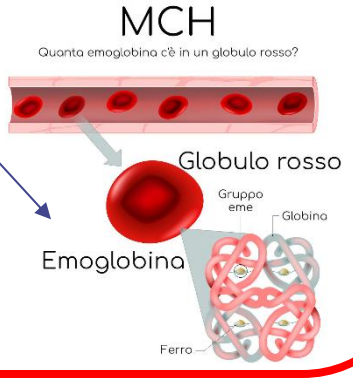
- «M00» emocromo e separazione Hb

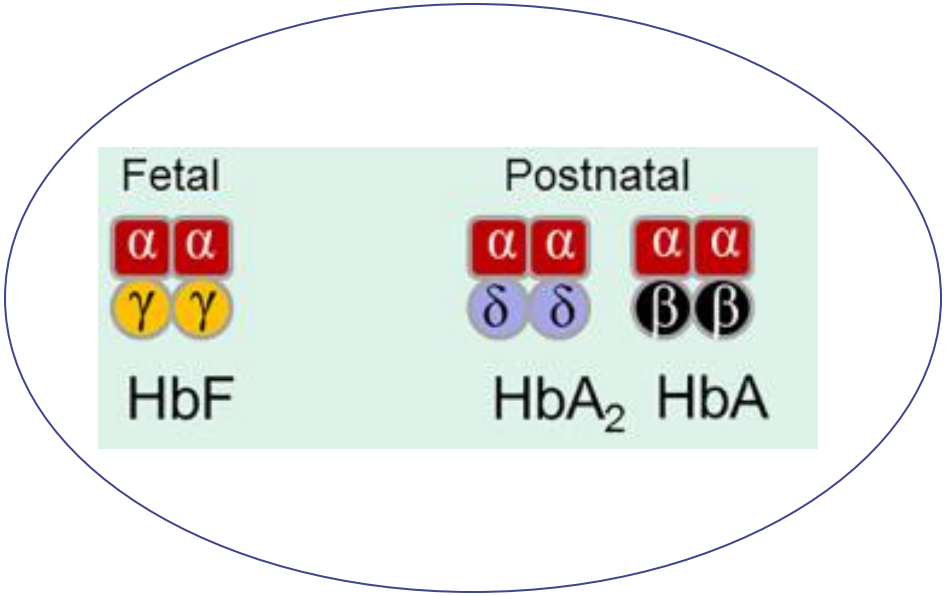
(Supplemento ordinario alla "Gazzetta Ufficiale„ n. 65 del 18 marzo 2017 - Serie generale)

- HbA2 cod 90.66.3
- Hb anomale cod 90.66.5
- HbF cod 90.66.4


Normociti
RDW
Microciti
Macrociti

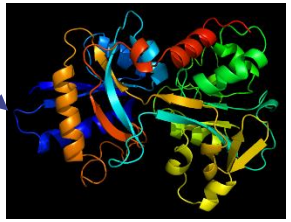

MCV tra
80-100 fl

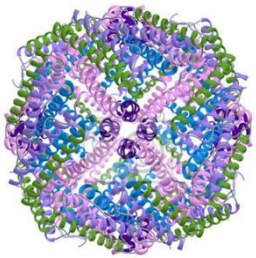

MCH
Quanta emoglobina c'è in un globulo rosso?
Globulo rosso
Gruppo eme
Globina
Emoglobina
Ferro


Fetal
Postnatal
HbF
HbA2
HbA


Serum Iron Test


26
Fe
Ferro
55.845





Hb	13,9
MCV	71,6
MCH	24,1
RDW	17,6
Serum Iron	36
Transferrin	335
Transferrin sat	7%
Ferritin	4
HbA2	2,6
HbF	0,1

Hb	16,6
MCV	88
MCH	27,6
RDW	16,3
Serum Iron	123
Transferrin	276
Transferrin sat	33%
Ferritin	56
HbA2	2,6
HbF	0,1

Globin separation

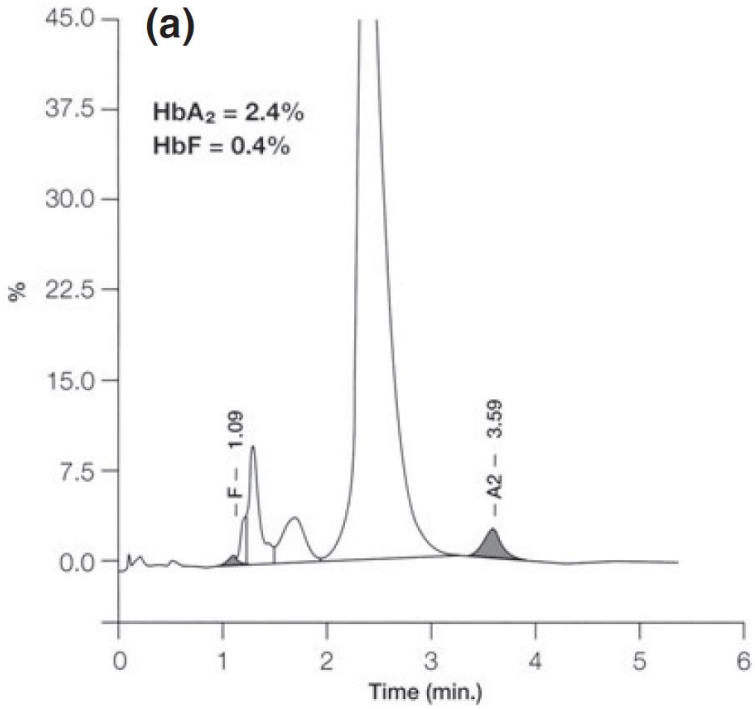
High Performance Liquid Chromatography (HPLC)

Column with small silica spheres, weakly cationic.

Haemolisates is injected in the column

gradient of increasing ionic strenght

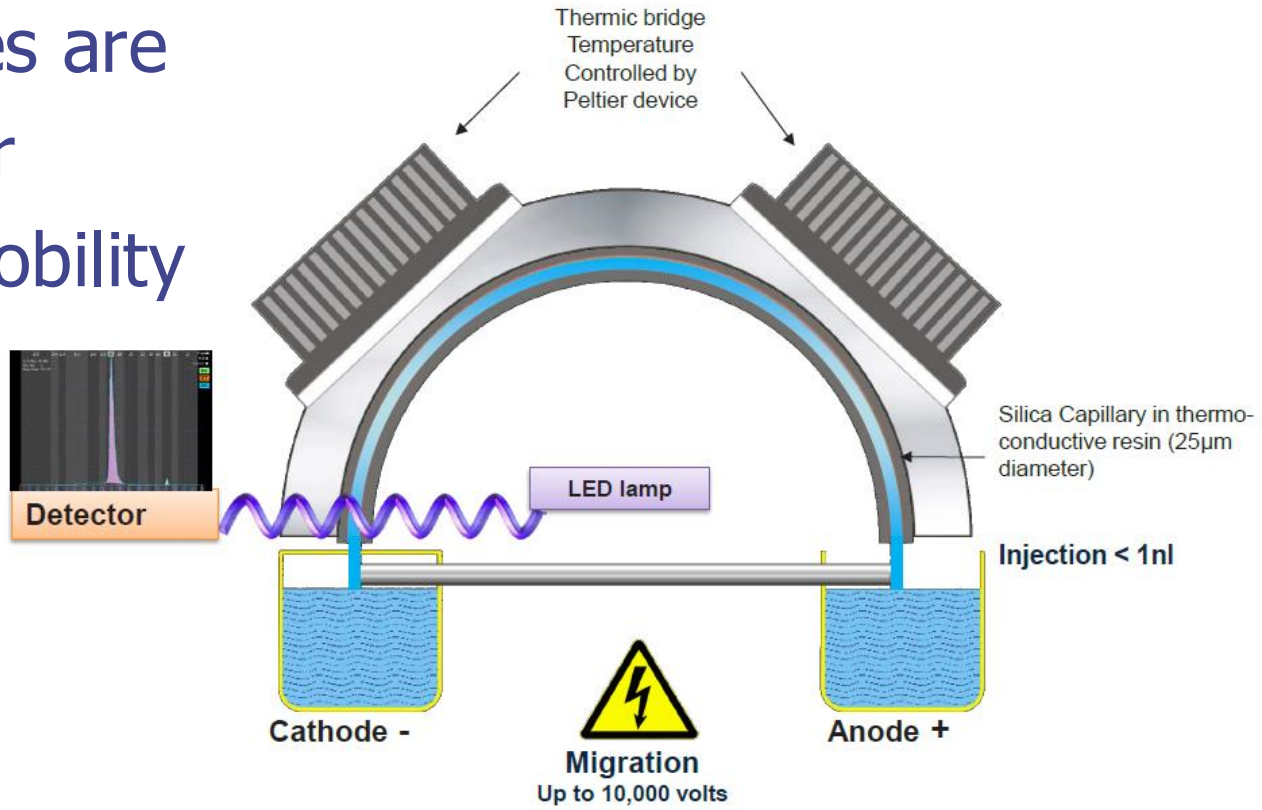
Photometer reads the absorbance



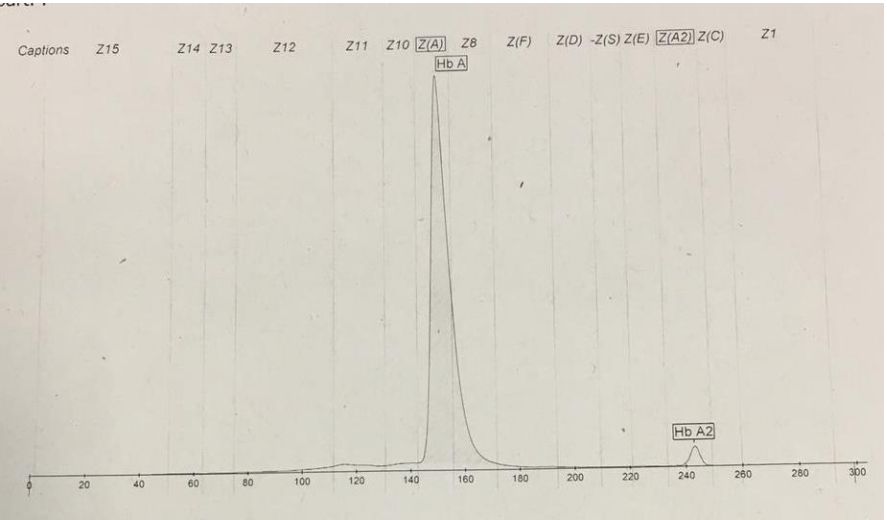
Globin separation

Capillary electrophoresis (CE)

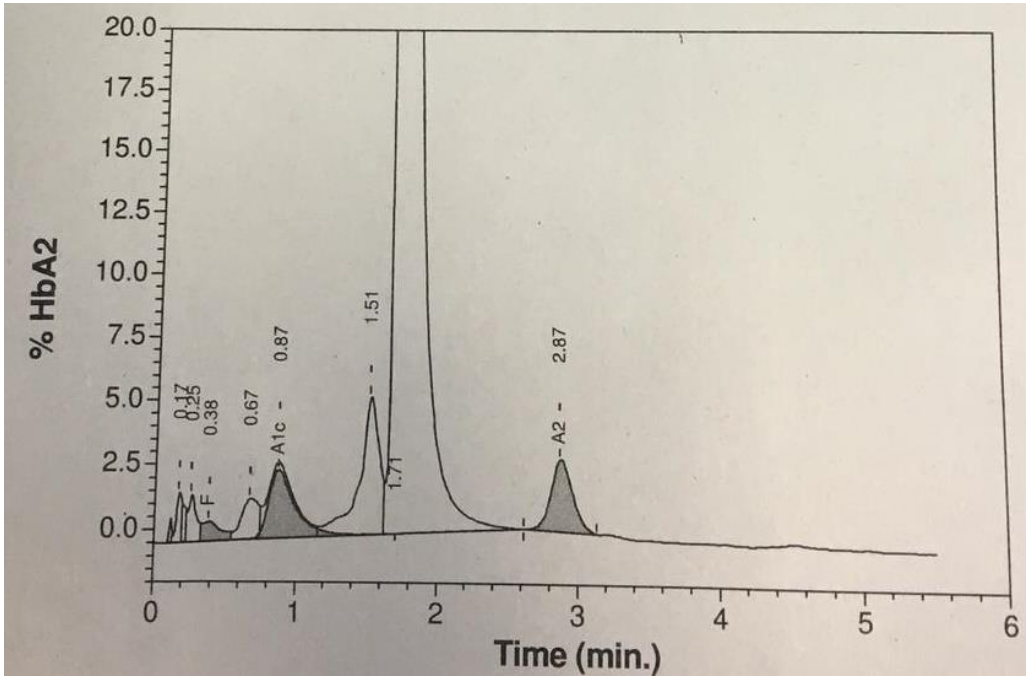
Charged molecules are separated by their electrophoretic mobility

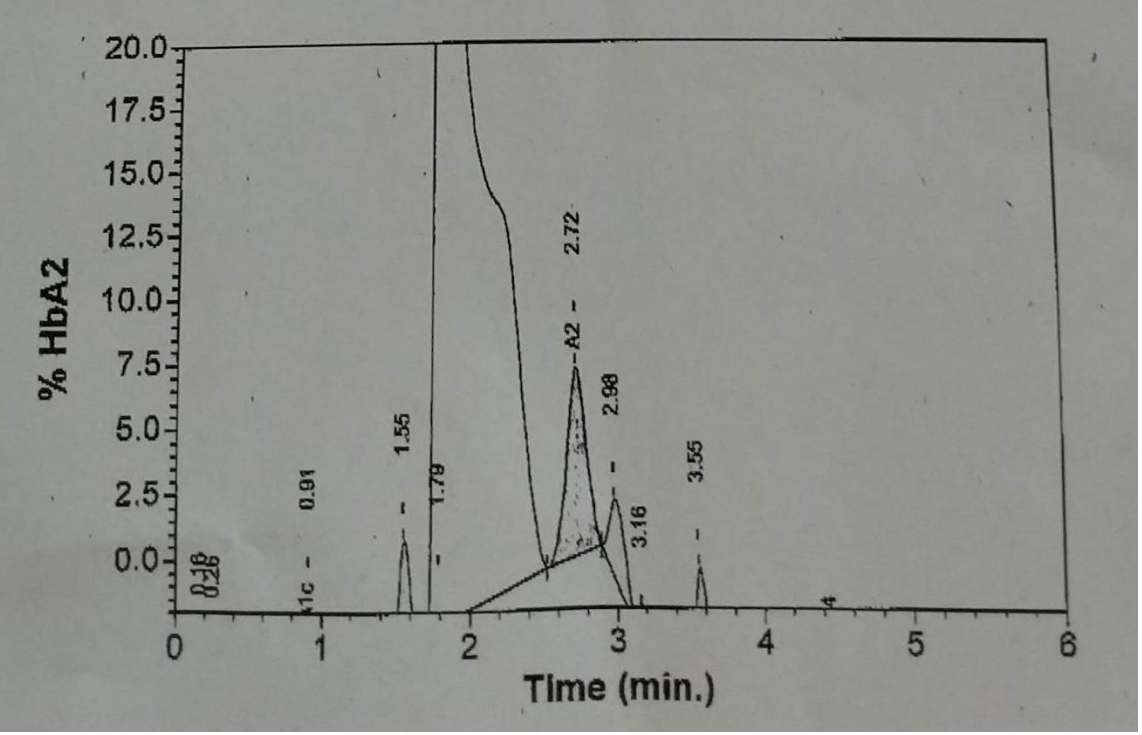


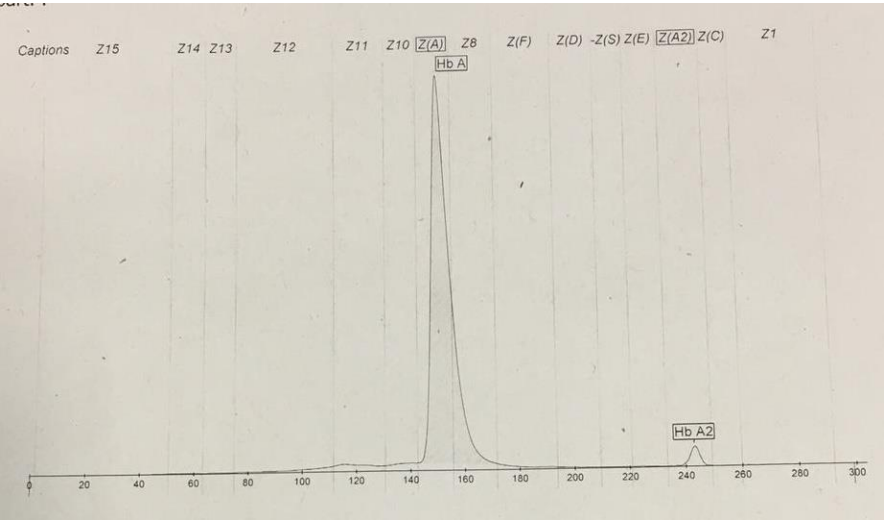
CE

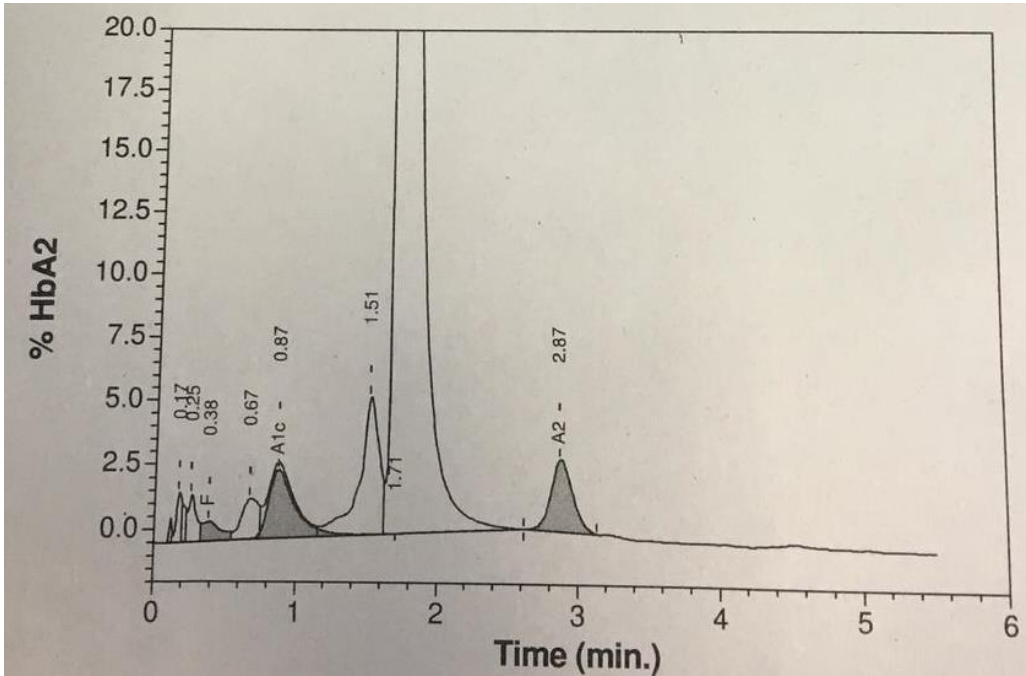

Captions
Z15
Z14
Z13
Z12
Z11
Z10
Z(A)
Z8
Z(F)
Z(D)
Z(S)
Z(E)
Z(A2)
Z(C)
Z1
Hb A
Hb A2
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300

HPLC


% HbA2
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.17
0.25
0.38
F
0.67
A1c
0.87
1.51
1.71
A2
2.87
0
1
2
3
4
5
6
Time (min.)


% HbA2
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.91
1.55
1.79
2.72
A2
2.98
3.16
3.55
A1c
0 1 2 3 4 5 6
Time (min.)


Captions Z15 Z14 Z13 Z12 Z11 Z10 Z(A) Z8 Z(F) Z(D) -Z(S) Z(E) Z(A2) Z(C) Z1
Hb A
Hb A2
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300


% HbA2
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.38
0.67
A1c
0.87
1.51
1.71
2.87
A2
0 1 2 3 4 5 6
Time (min.)

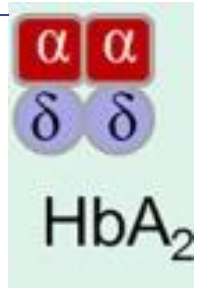
Normal

12 – 15 g/dl
13 – 17 g/dl

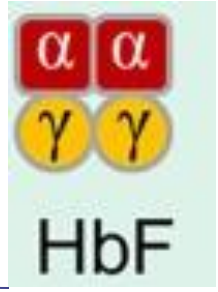
MCV 80 – 100 fl

MCH 27 – 31 pg

In adults
AGE DEPENDENT!



2 – 3,2 %



<1,5 %

Delta thalassemia carrier

12 – 15 g/dl
13 – 17 g/dl

1 – 1,5 %

MCV 80 – 100 fl

MCH 27 – 31 pg

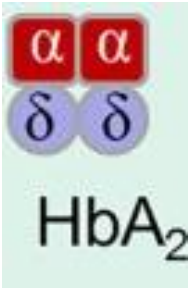
0 – 1,5 %

Hereditary Persistance Fetal Hb (HPFH)

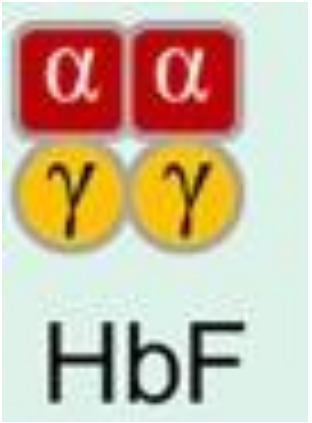
12 – 15 g/dl
13 – 17 g/dl

MCV 80 – 100 fl

MCH 27 – 31 pg



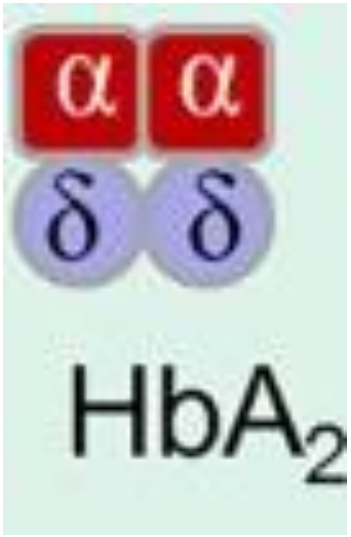
2 – 3,2 %



>1,5 %

Beta thalassemia carrier

Mutation dependent



>3,5%

MCV 60 - 75 fl

MCH 18 – 26 pg

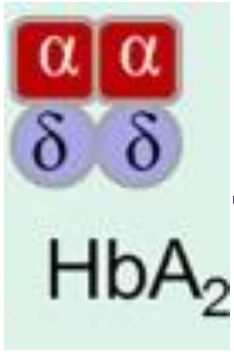
>1,5 %

Beta +

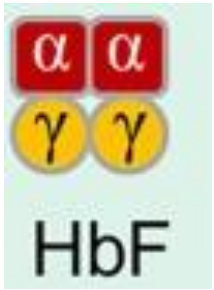
Mutation dependent

MCV 80 – 100 fl

MCH 27 – 31 pg



3,2 – 3,5 %



0 – 1,5 %

Known causes underlying Hb A_2 levels outside the normal range

Increase (excluding β-thalassaemia variants)

Genetic

KLF1 variants

Triplicated α gene

Some unstable variants

Hb variants eluting with or close Hb A_2

Acquired

Hyperthyroidism

Megaloblastic anaemia

Aplastic crisis in HS

Antiretroviral drugs

Pseudoanthoma elasticum

Reduction

Genetic

δ-thalassaemia

δ-chain variants

α-chain variants

Hb Lepore[1]

α-thalassaemia[2]

δβ and γδβ thalassaemia;

some mild β thalassaemia variants

Acquired

Severe iron deficiency anaemia

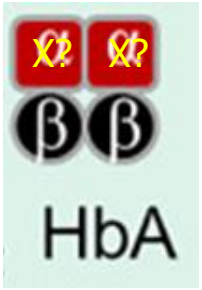
Sideroblastic anaemia

Lead poisoning

Leukaemia, aplastic anaemia

EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies

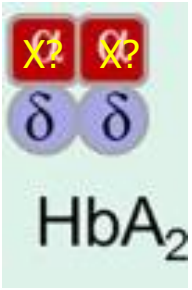
Alpha thalassemia carrier



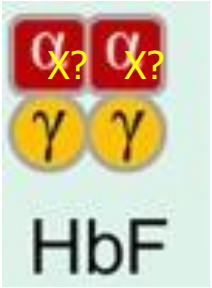
12 – 15 g/dl
13 – 17 g/dl

MCV ≤ 80 fl

MCH <27 pg



2 – 3,2 %



0 – 1,5 %

Alpha thalassemia carrier

12 – 15 g/dl

13 – 17 g/dl

MCV ≤ 80 fl

MCH <27 pg

4 alpha genes!!

2 – 3,2 %

0 – 1,5 %

Standardizzazione dei valori di laboratorio per la diagnosi di portatore di alfa talassemia

Vincenzo Voi[1], Lidia Cereda[1], Emanuele Pivetta[2], Antonio Piga[1]

[1] Centro per le Emoglobinopatie, Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Ospedale S. Luigi Gonzaga, Regione Gonzole 10, 10043 Orbassano (Torino), Italia

[2] Epidemiologia dei tumori e CRPT U, Medicina d'Urgenza – MECAU, Dipartimento Scienze Mediche, Università di Torino, AOU Città della Salute e della Scienza di Torino – Presidio Molinette

Introduzione: La maggior parte delle linee guida ritengono un MCV < 79 fl e un MCH < 27 pg utili allo screening diagnostico di portatore sano di alfa talassemia e suggeriscono l'identificazione di valori propri di riferimento.

Obiettivo dello Studio: verificare la sensibilità/specificità di tali indicazioni nella nostra realtà ed eventualmente individuare nuovi valori soglia di MCV e MCH ottimali per l'orientamento diagnostico di portatore sano di alfa talassemia nella nostra attività di prevenzione.

Metodi: abbiamo incluso nell'analisi solo i soggetti studiati direttamente a livello molecolare in quanto partner di portatori sani di beta talassemia e HbS afferenti all'ambulatorio di diagnosi e prevenzione delle emoglobinopatie, nel periodo 2011-2018.

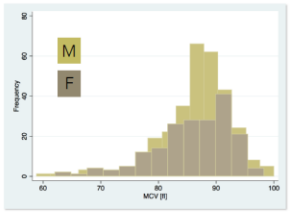
La diagnosi molecolare è stata effettuata con reverse dot blot o sequenziamente del gene.

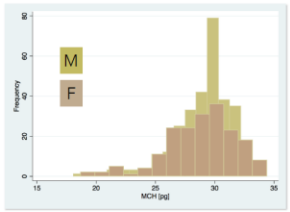
Criteri di esclusione: soggetti selezionati per lo studio molecolare in base agli indici globulari o a criteri clinici; soggetti portatori (o con associazione di) di altra emoglobinopatia o di triplo alfa; soggetti con valori di saturazione della transferrina < 15%, o ferritina < 30 ng/dl; soggetti con patologie croniche.

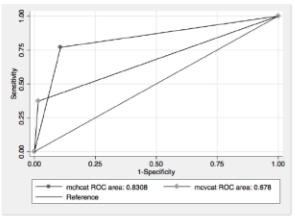
È stata calcolata l'accuratezza diagnostica per MCV < 80 fl e MCH < 27 pg.

Per individuare i valori di MCV e MCH per massimizzare sensibilità e specificità dei parametri abbiamo applicato il test di Youden e il metodo di Liu).

Risultati: da un totale di 489 pazienti analizzati, sono state incluse 394 osservazioni: l'età mediana era di 33,5 anni (iqr 8,4 anni). I casi portatori di un difetto di tipo alfa talassemico sono risultati 83; 78 delezioni o mutazioni degli alfa geni e 5 portatori di triplicazione del gene che non sono stati inclusi nell'analisi dei cut-off (Vedi grafici a lato). L'MCV era inferiore a 80 fl in 29 casi (37,2%) e 60 avevano un MCH < 27 pg (76,9%). La sensibilità e la specificità, applicando uno dei due parametri, sono rispettivamente per l'MCV: 37,2% (intervallo di confidenza al 95%, IC, 26,5% - 48,9%) e 98,4% (IC 96,3% - 99,5%); per MCH: 76,9% (IC 66% - 85,7%) e 89,2% (IC 85,3% - 92,4%). Le curve ROC mostrano un valore di area sotto la curva (AUC) di 0,678 per l'MCV e di 0,831 per l'MCH (p<0,001).







Il valore massimo di AUC di MCV e di MCH per l'identificazione di un difetto alfa è risultato essere rispettivamente di 84,25 fl e di 27,55 pg, con un' AUC di 0,82 per l'MCV e di 0,84 per l'MCH (utilizzando sia lo Youden index che il metodo di Liu).

Discussione: le soglie identificate per il nostro laboratorio si discostano dalle linee guida internazionali per l'MCV, risultando essere più sensibile e specifico il valore di 84 fl mentre l'MCH < 27 pg si conferma essere un valore adeguato. In teoria il nostro risultato potrebbe avere diverse spiegazioni. Possiamo escludere bias di rilevamento, data l'alta affidabilità dei counter di ultima generazione. Abbiamo posto la massima cura nel prevenire bias di selezione, molto comuni in questo tipo di studi, escludendo tutti i soggetti che potessero essere stati studiati in base agli indici globulari. Limitando lo studio alla nostra attività per le coppie abbiamo voluto indirettamente, ma efficacemente limitare il bias per età, non includendo quindi la ben nota variabilità di MCV/MCH dei soggetti pediatrici (ed in parte degli anziani). Alcuni degli studi che supportano le linee guida includono soggetti in età pediatrica. Di nuovo diversi studi di riferimento per le linee guida sono stati fatti su popolazioni in cui la prevalenza di difetti alfa è maggiore, con indici globulari mediamente più bassi.

In conclusione, i nostri dati confermano l'utilità di individuare i propri valori di riferimento per migliorare la propria accuratezza diagnostica.

Incidenza dei difetti alfa analizzati nello studio

Mutazione	n	%
Eterozigote -3,7	52	66,7
Eterozigote α2 init cd (T>C)	5	6,4
- - MED	1	1,3
cluster α -25kb	1	1,3
Omozigote -3,7/-3,7	9	11,5
Eterozigote IVSI (-5nt)	5	6,4
-4,2	1	1,3
-20,5	2	2,6
Omozigote α2 init cd (T>C)	1	1,3
Eterozigote c.91G>A	1	1,3
TOTALE	78	

SITE 2018

Standardizzazione dei valori di laboratorio per la diagnosi di portatore di alfa talassemia

Vincenzo Voi[1], Lidia Cereda[1], Emanuele Pivetta[2], Antonio Piga[1]

[1] Centro per le Emoglobinopatie, Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Ospedale S. Luigi Gonzaga, Regione Gonzole 10, 10043 Orbassano (Torino), Italia

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MCH<27

Introduzione: La maggior parte delle linee guida ritengono un MCV < 79 fl e un MCH < 27 pg utili allo screening diagnostico di portatore sano di alfa talasser

Obiettivo dello Studio: verifi valori soglia di MCV e MCH prevenzione.

Metodi: abbiamo incluso nel beta talassemia e HbS afferen

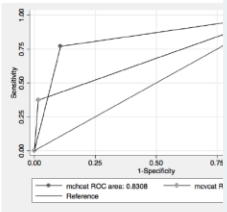
La diagnosi molecolare è stat

Criteri di esclusione: soggetti con associazione di) di altra e 30 ng/dl; soggetti con patolo

È stata calcolata l'accuratezza

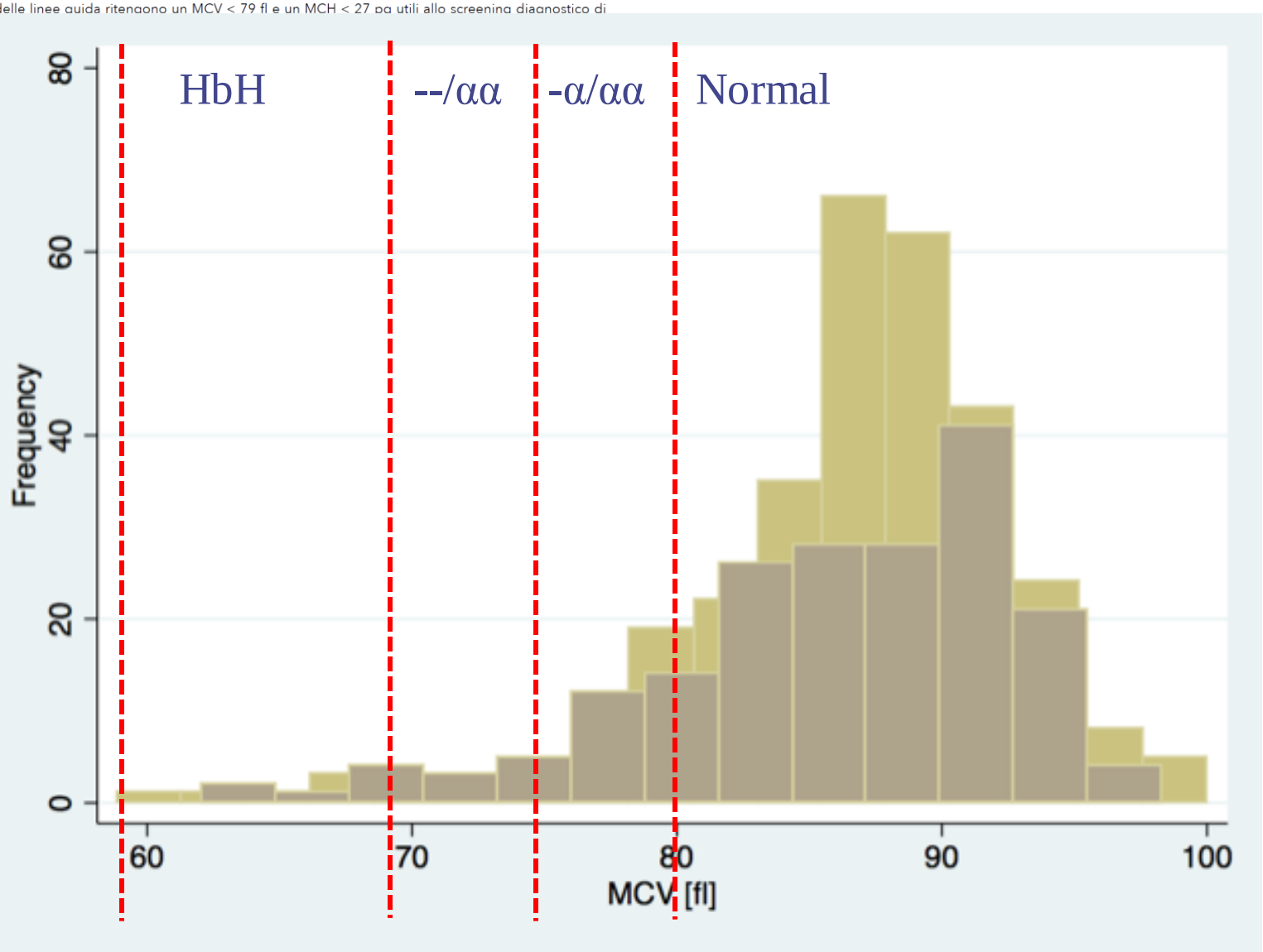
Per individuare i valori di MC il metodo di Liu).

Risultati: da un totale di 489 state incluse 394 osservazio 33,5 anni (iqr 8,4 anni). I casi tipo alfa talassemico sono ris mutazioni degli alfa geni e 5 del gene che non sono stati i off (Vedi grafici a lato). L'MCV casi (37,2%) e 60 avevano un sensibilità e la specificità, a



specifico il valore di 84 fl mer nostro risultato potrebbe ave l'alta affidabilità dei counter c bias di selezione, molto com essere stati studiati in base coppie abbiamo voluto indir quindi la ben nota variabilità degli studi che supportano l studi di riferimento per le lir alfa è maggiore, con indici gl

In conclusione, i nostri dati migliorare la propria accuratezza diagnostica.

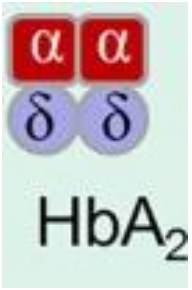


Eterozigote c.91G>A	1	1,3
TOTALE	78	

SITE 2018

Alpha thalassemia carrier

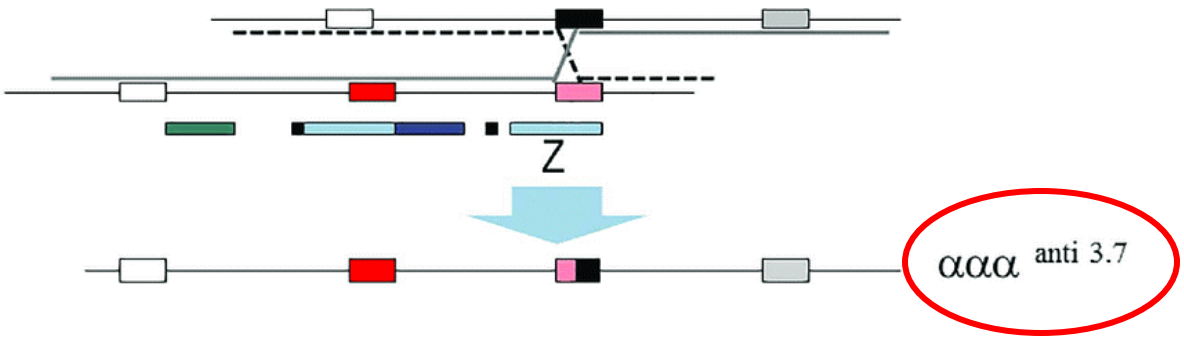
12 – 15 g/dl
13 – 17 g/dl



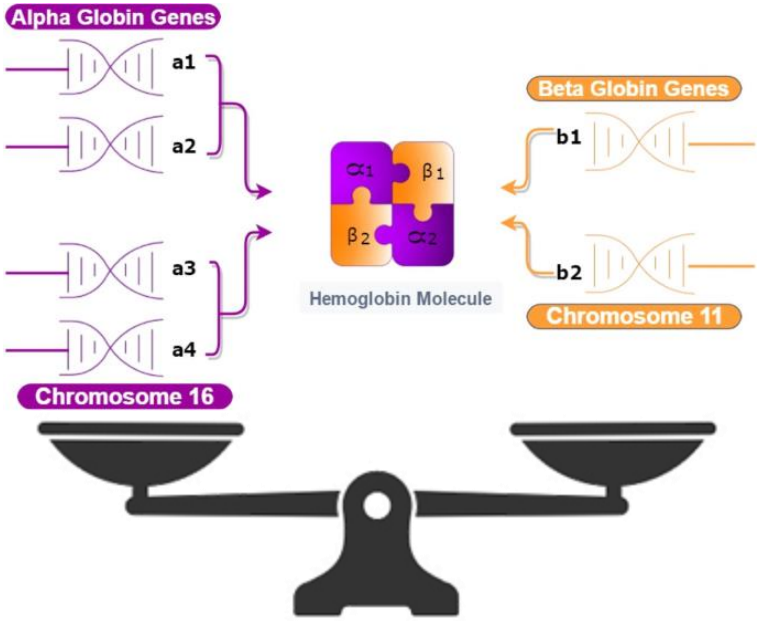
2 – 3,2 %

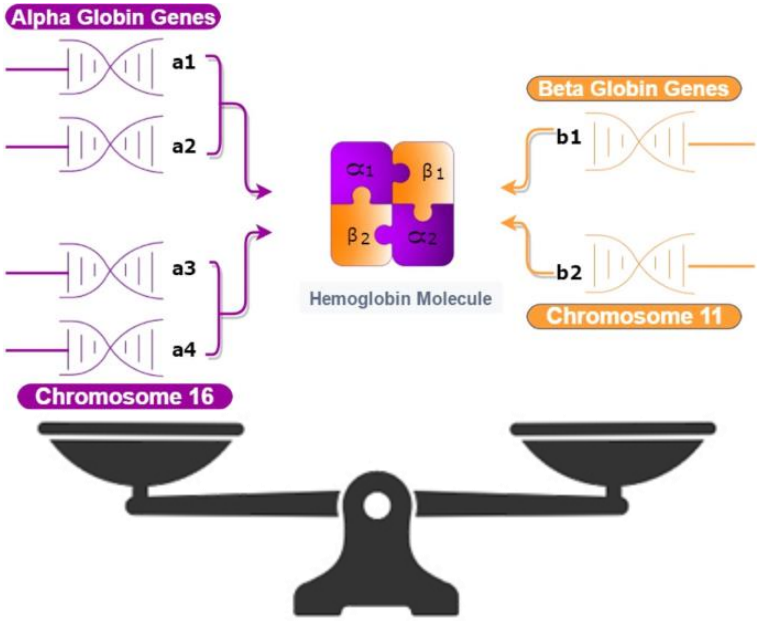
MCV 80 – 100 fl

MCH 27 – 31 pg



0 – 1,5 %


Alpha Globin Genes
a1
a2
a3
a4
Chromosome 16
Beta Globin Genes
b1
b2
Chromosome 11
α1
β1
β2
α2
Hemoglobin Molecule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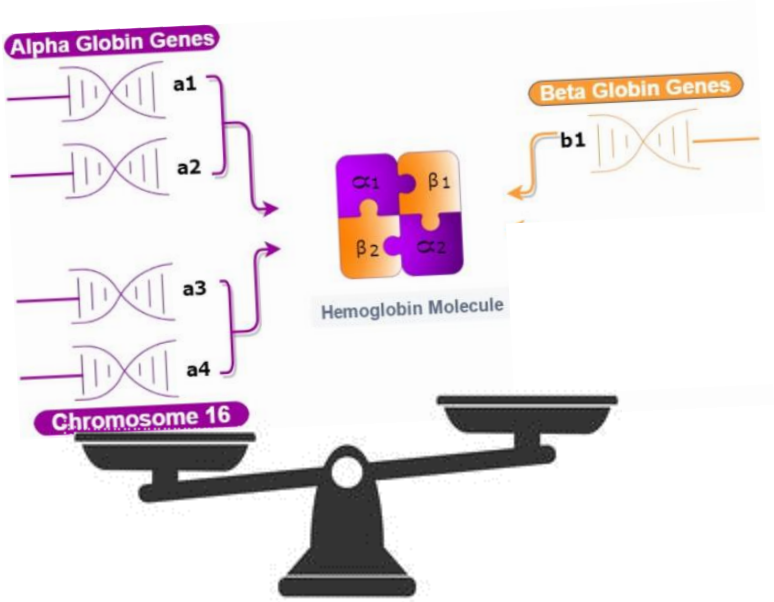
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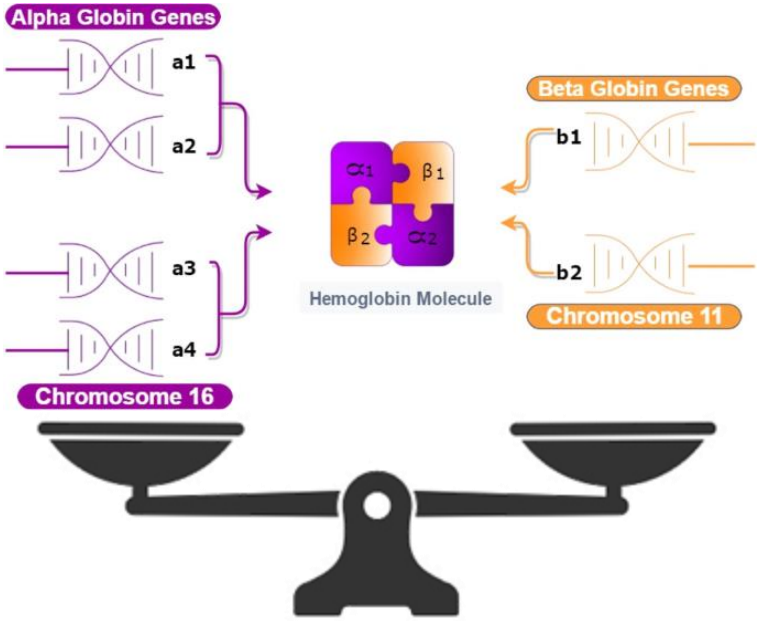
Beta Globin Genes
b1
Hemoglobin Molecule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b2
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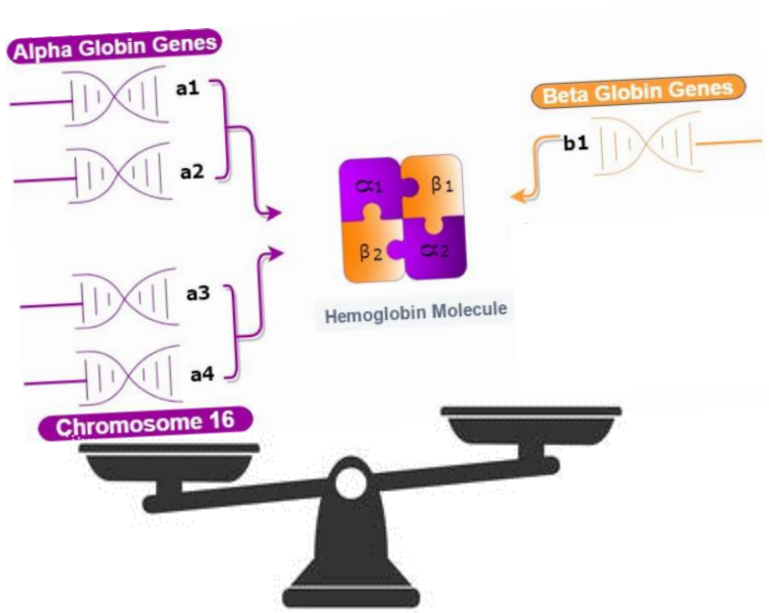
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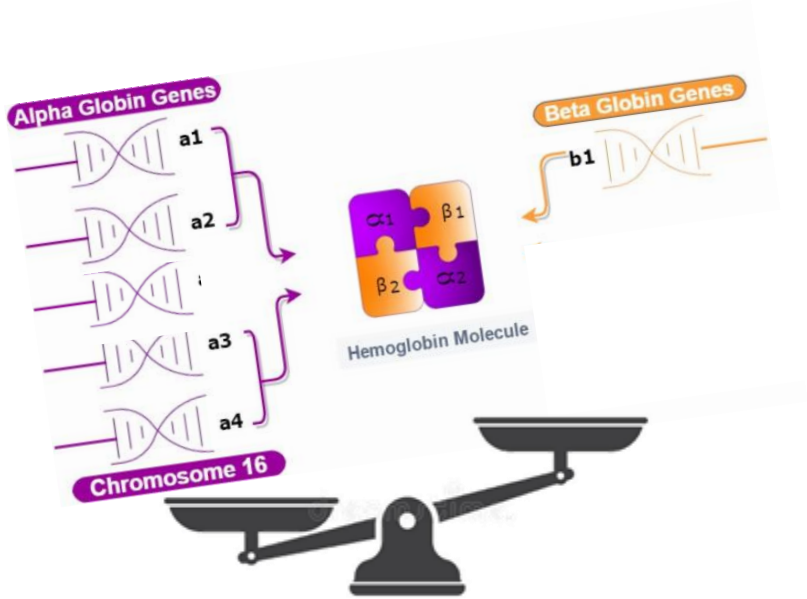
b1


Alpha Globin Genes
a1
a2
a3
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Hemoglobin Molecule
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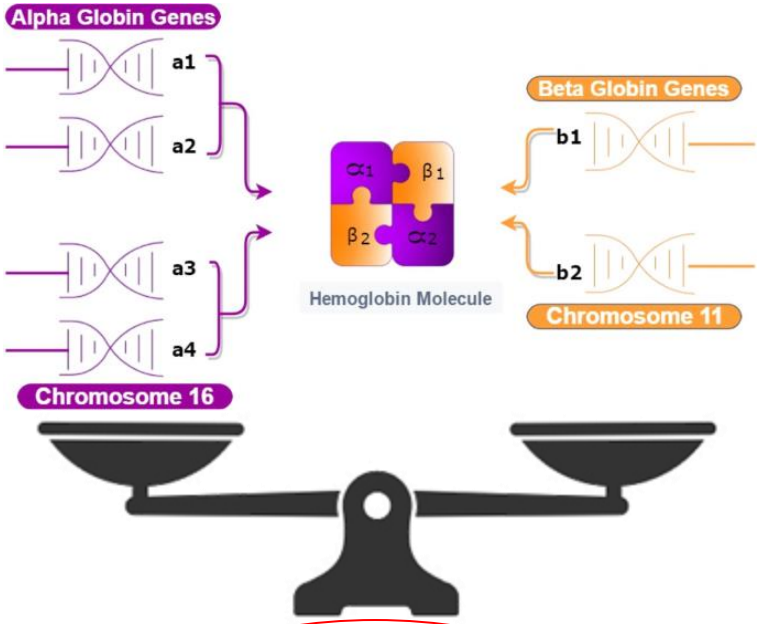
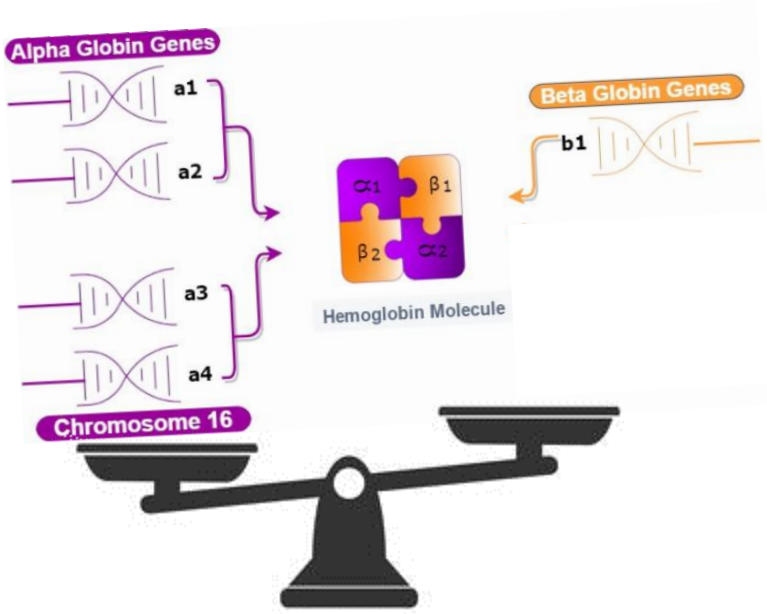
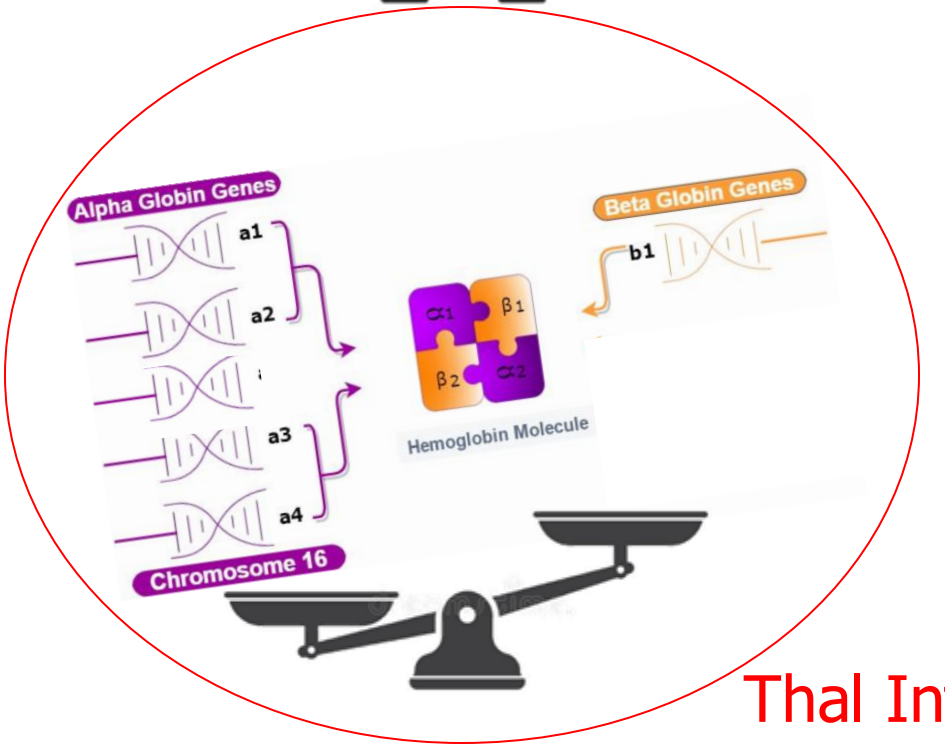
b1



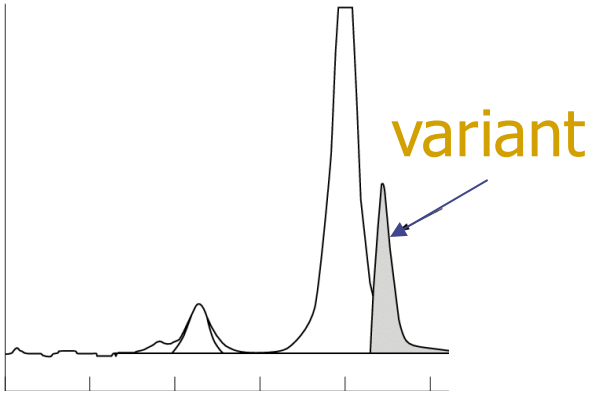




Thal Intermedia - NTDT

Variant haemoglobin

- Stability
- O_2 affinity



α 4 genes $\rightarrow$ 25%

β 2 genes $\rightarrow$ 50%

δ 2 genes $\rightarrow$ 50%

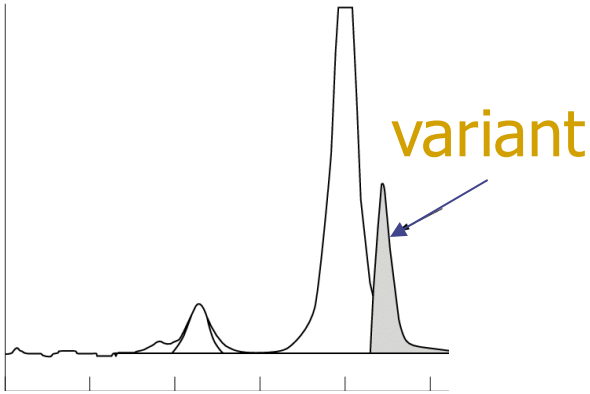
Variant haemoglobin

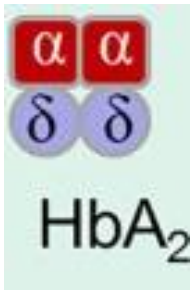
12 – 16 g/dl
13 – 17 g/dl

MCV 80 – 100 fl

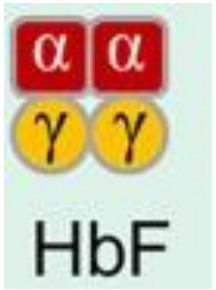
MCH 27 – 31 pg

p50





2 – 3,2 %



0 – 1,5 %

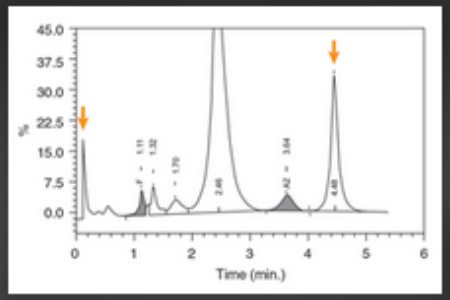
https://hemoglobins.bio-rad.com/Pages/ChromatogramPage.aspx?labid=15

BIO-RAD

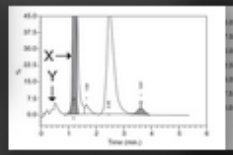
Information | Library of Variants | Founding Contributors | Bio-Rad Support Centers | Institutional Resources

1 / 17

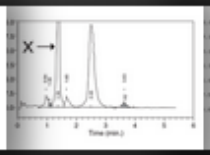
Double-click for more information



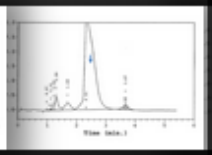
H Disease and HbS, 0.15



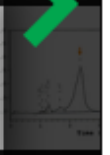
J Iran, 1.16



Hope, 1.35



San Diego, 2.31



HbS/La De
2.3

Case Type

Educational References

Platform

VARIANT™ II

Retention time

Hb name

Hb classes

Frequency / Occurence

Search

Reset

European Journal of Human Genetics (2015) 23, 426–437

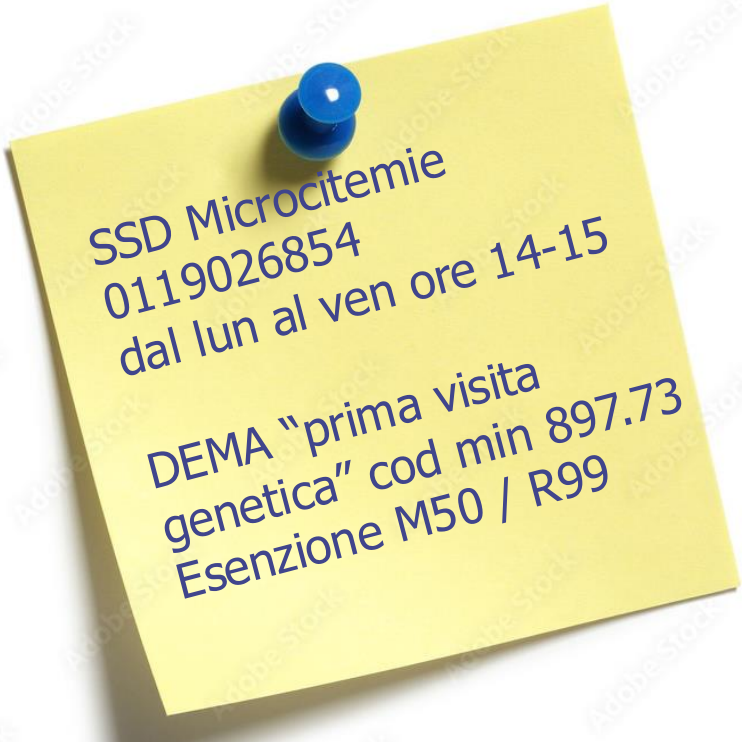
www.nature.com/ejhg

EJHG Open

POLICY

EMQN Best Practice Guidelines for molecular and haematology methods for carrier identification and prenatal diagnosis of the haemoglobinopathies

Molecular analysis is a second level test



Diagnostica di I e II livello delle Emoglobinopatie

Buone Pratiche SITE

SOCIETA' ITALIANA TALASSEMIE ED EMOGLOBINOPATIE

- First level test not conclusive
  - variant Hb characterization
  - α talassemia carrier (α+)
  - β+/silent mutations (e.g. borderline HbA2)

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- «double carrier»: suspicion of α and β thalassemia carrier, in order to correctly define the recurrence risk

- First level test not conclusive
  - variant Hb characterization
  - α talassemia carrier (α+)
  - β+/silent mutations (e.g. borderline HbA2)

- «double carrier»: suspicion of α and β thalassemia carrier, in order to correctly define the recurrence risk

- To define the mutation in a carrier (gen-phen correlation)

Table 5 Genetic variations associated with normal/borderline Hb A2 levels—a guideline of related haematological and biosynthetic characteristics

Variation HGVS nomenclature NM_000518.4 (HBB)	*Variation traditional nomenclature*	*MCV fl*	*MCH pg*	*Hb A_2*	*α/β ratio*
c.−151C>T	β −101 (C→T)	88.5±7.8	30.1±1.0	3.1±1.0	1.3±0.4
c.−142C>T	β −92 (C(T)	83.0±6.0	28.3±2.0	3.5±0.4	1.3±0.8
c.−18C>G	β +33 (C(G)	82.0±9.2	27.1±3.4	2.5±1.4	1.3±0.6
c.316-7C>G	βIVS2-844 (C→G)	96.0±4.0	30.3±1.8	3.2±0.2	1.0±0.6
c.*6C>G	β +1480 (C→G)	88.3±9.5	27.9±6.0	2.7±0.8	1.6±0.4
	$\alpha\alpha\alpha/\alpha\alpha$	85.5±7.8	30.4±5.0	2.8±0.6	1.2±0.4
	KLF1 variants (29)	82.7±5.7	27.8±2.2	3.6±0.2	
c.−50A>C	Cap+1 (A(C)	23–26*	75–80*	3.4–3.8*	—
c.92+6T>C	β IVS1-6 (T→C)	71.0±4.0	23.1±2.2	3.4±0.2	1.9±1.0
	$\delta+\beta$ thalassaemia	64.3±4.0	20.9±1.4	3.6±0.2	1.7±0.6

Values (mean ± 2SD or range (*)) are a guideline and represent those reported in various studies on carriers of these variants (prepared by R Galanello).
Note: It is recommended that subjects with borderline Hb A2 levels, particularly if their partner is a typical β-thalassaemia carrier, should be extensively investigated (α and β gene analysis, globin biosynthesis), although the majority usually have normal HBB and HBA genes. Borderline-raised Hb A2 levels in normal individuals are usually explained as the extreme distribution of the normal range of the Hb A2.
Furthermore, in couples where one partner is heterozygous for a severe α-thalassaemia defect and the other is a β-thalassaemia carrier, it is recommended that the *HBA* gene cluster be fully characterized in the β-thalassaemia carrier in order to preclude any risk of offspring with severe Hb H disease or Hb Bart's hydrops.

- Sideropenia in pregnant couples:

α and β globin genes analysis if the partner is a carrier in order to avoid PND delay

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α and β globin genes analysis if the partner is a carrier in order to avoid PND delay

- Exclude alpha globin gene triplication/quadruplication in the partner of a β thalassemia carrier to rule out NTDT risk

- Sideropenia in pregnant couples:

α and β globin genes analysis if the partner is a carrier in order to avoid PND delay

- Exclude alpha globin gene triplication/quadruplication in the partner of a β thalassemia carrier to rule out NTDT risk

- Before prenatal diagnosis:
  - Parental DNA must be known
  - PND 50 is possible but residual risk

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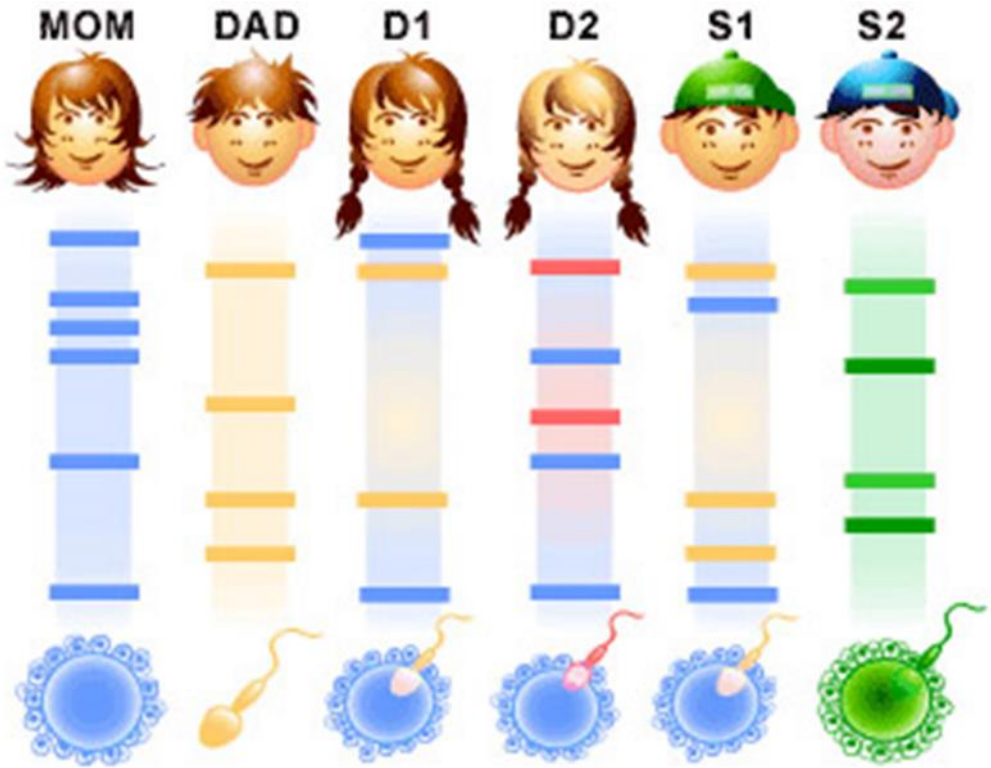
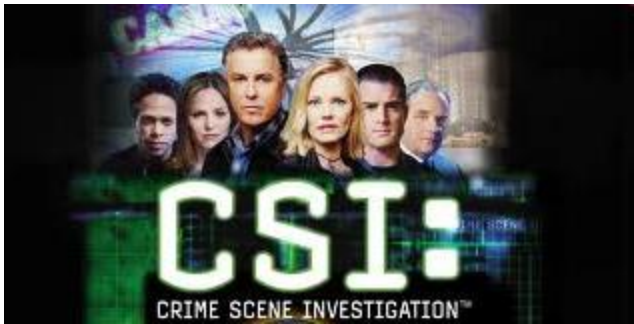
  - Parental DNA must be known
  - PND 50 is possible but residual risk

(2 different techniques – maternal cell contamination)

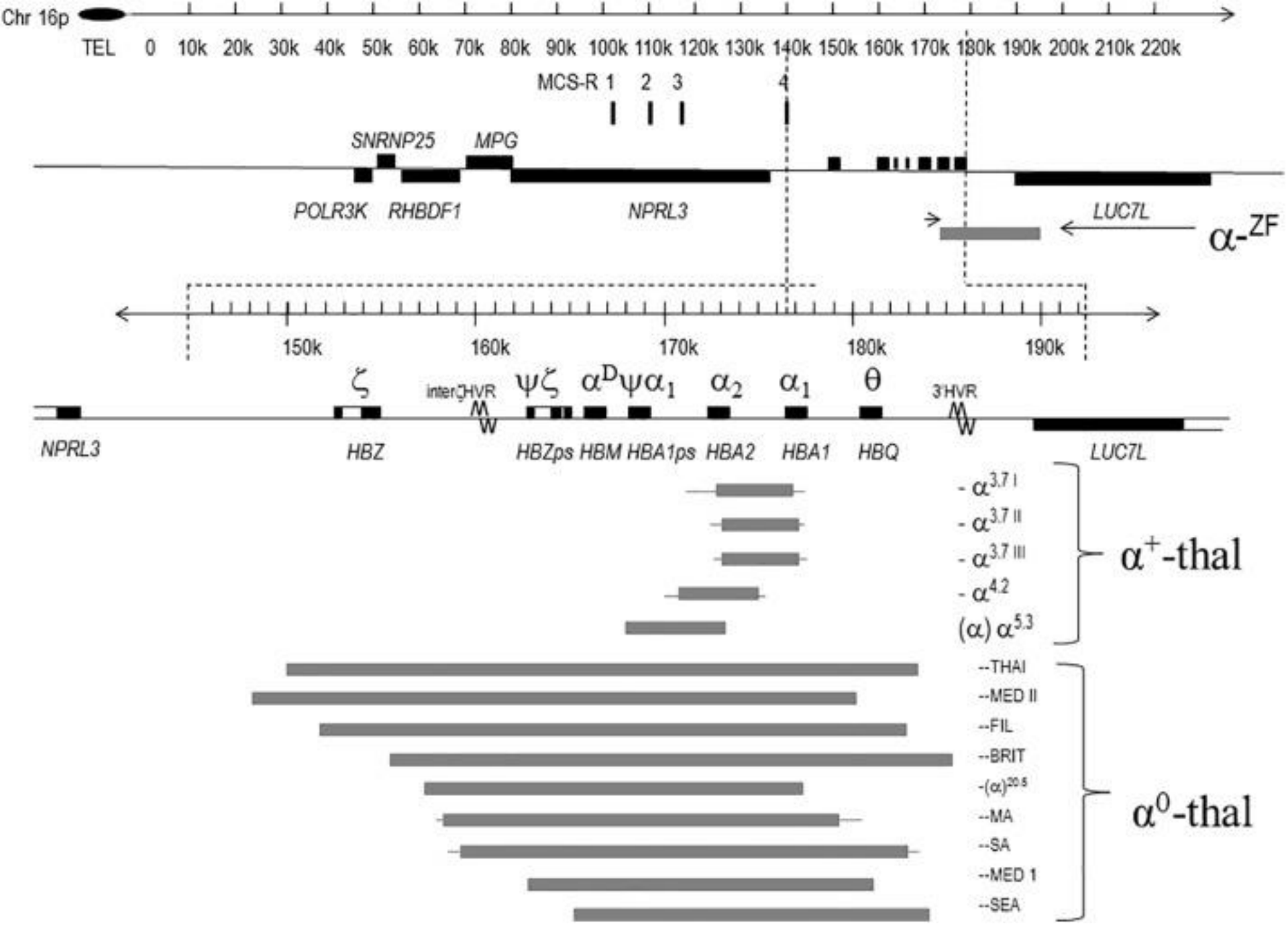
Paternity – maternal contamination

Microsatellite= short repeated DNA sequences, non coding, highly polymorphic



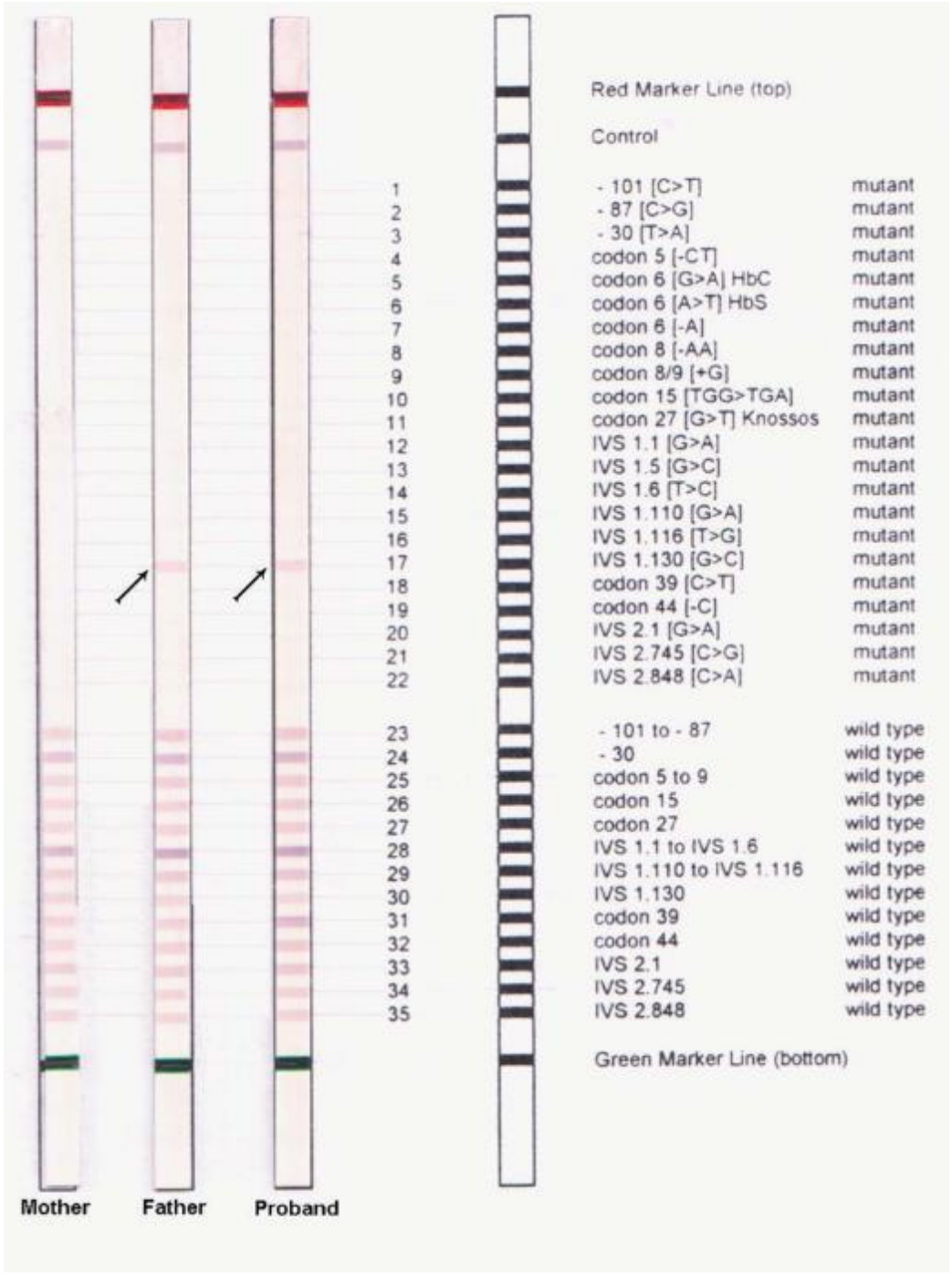


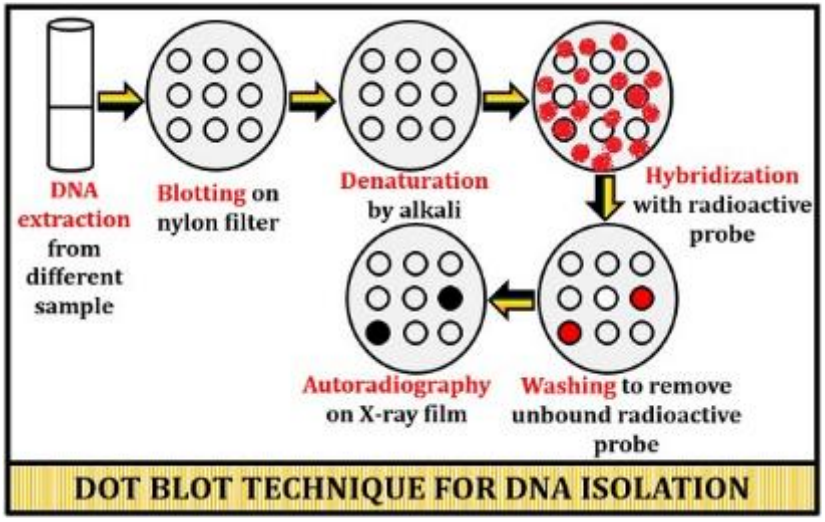
- α-globin genes more prone to deletion/duplication



doi.org/10.1016/j.bcmd.2017.09.004

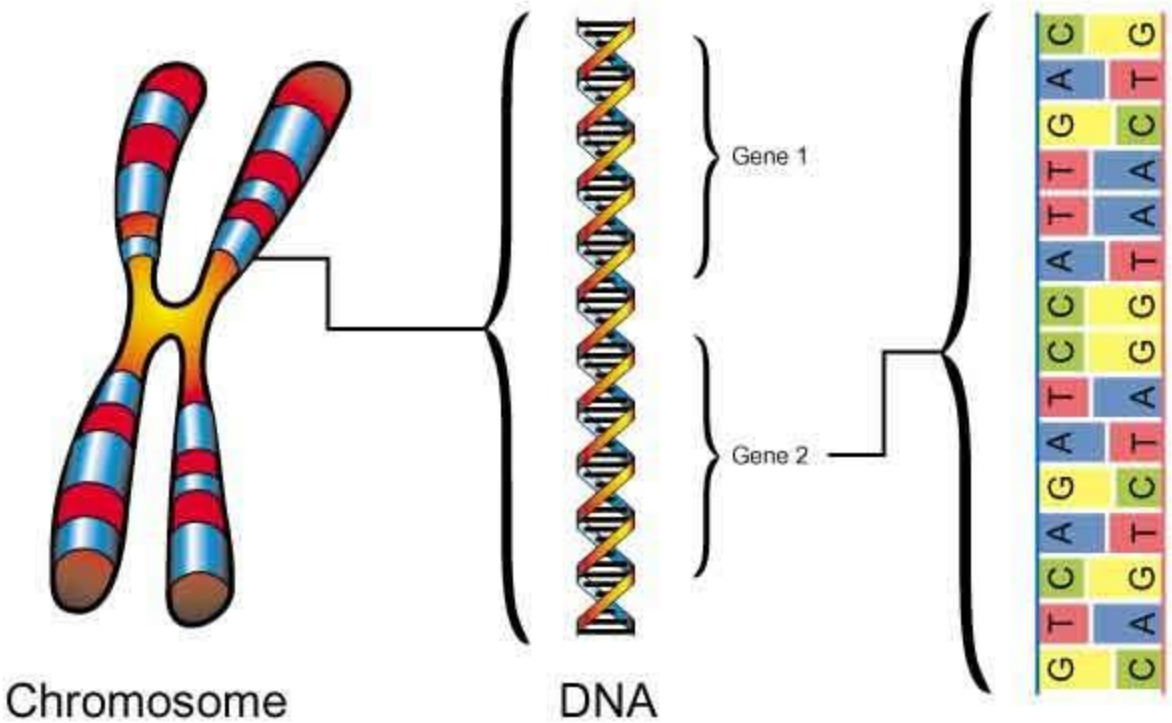
- α-globin genes more prone to deletion/duplication
- β-globin gene more prone to point mutations

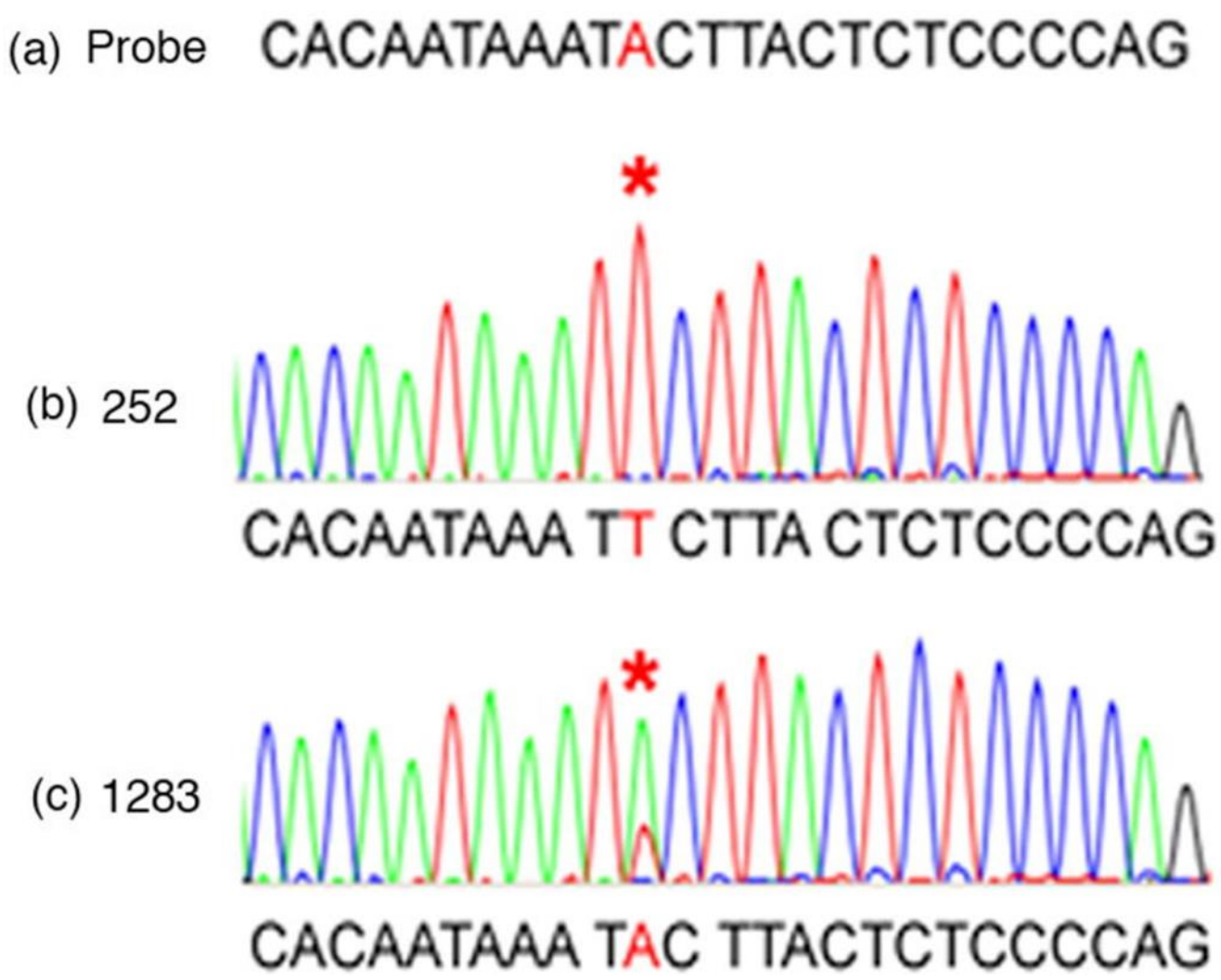

Mother
Father
Proband
Red Marker Line (top)
Control
1 - 101 [C>T] mutant
2 - 87 [C>G] mutant
3 - 30 [T>A] mutant
4 codon 5 [-CT] mutant
5 codon 6 [G>A] HbC mutant
6 codon 6 [A>T] HbS mutant
7 codon 6 [-A] mutant
8 codon 8 [-AA] mutant
9 codon 8/9 [+G] mutant
10 codon 15 [TGG>TGA] mutant
11 codon 27 [G>T] Knossos mutant
12 IVS 1.1 [G>A] mutant
13 IVS 1.5 [G>C] mutant
14 IVS 1.6 [T>C] mutant
15 IVS 1.110 [G>A] mutant
16 IVS 1.116 [T>G] mutant
17 IVS 1.130 [G>C] mutant
18 codon 39 [C>T] mutant
19 codon 44 [-C] mutant
20 IVS 2.1 [G>A] mutant
21 IVS 2.745 [C>G] mutant
22 IVS 2.848 [C>A] mutant
23 - 101 to - 87 wild type
24 - 30 wild type
25 codon 5 to 9 wild type
26 codon 15 wild type
27 codon 27 wild type
28 IVS 1.1 to IVS 1.6 wild type
29 IVS 1.110 to IVS 1.116 wild type
30 IVS 1.130 wild type
31 codon 39 wild type
32 codon 44 wild type
33 IVS 2.1 wild type
34 IVS 2.745 wild type
35 IVS 2.848 wild type
Green Marker Line (bottom)


DNA extraction from different sample
Blotting on nylon filter
Denaturation by alkali
Hybridization with radioactive probe
Washing to remove unbound radioactive probe
Autoradiography on X-ray film
DOT BLOT TECHNIQUE FOR DNA ISOLATION

Singol gene analysis

- DNA extraction
- PCR
- Sequencing




(a) Probe CACAATAAATACTTACTCTCCCCAG
*
(b) 252
CACAATAAA TT CTTA CTCTCCCCAG
*
(c) 1283
CACAATAAA TAC TTACTCTCCCCAG

Next generation sequencing

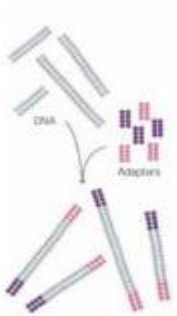


Figure 1
Randomly fragment genomic DNA and ligate adapters to both ends of the fragments.

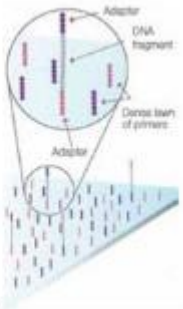


Figure 2
Bind single-stranded fragments randomly to the inside surface of the flow cell channels.

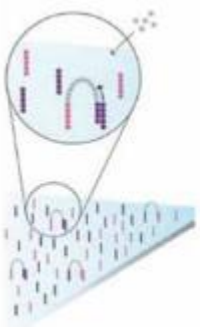


Figure 3
Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

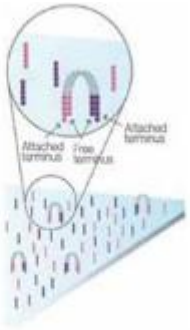
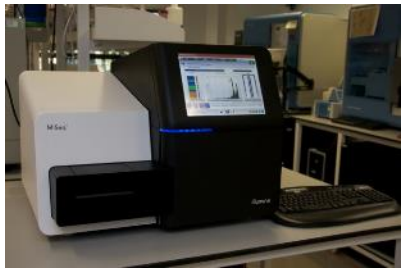


Figure 4
The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate.



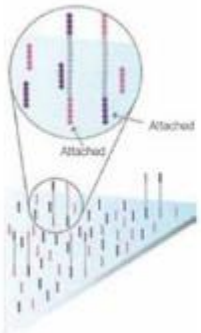


Figure 5
Denaturation leaves single-stranded templates anchored to the substrate.

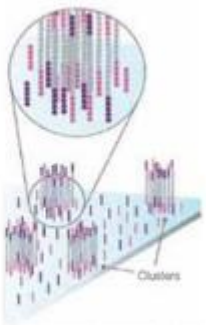


Figure 6
Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.

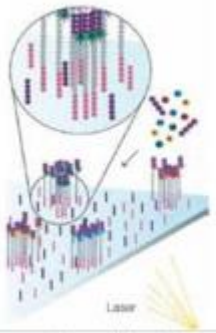


Figure 7
The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase.

Figure 8
After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified.

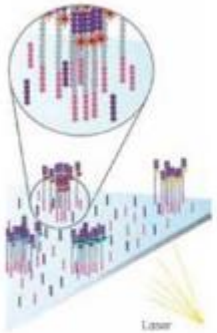


Figure 9
The next cycle repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase.

Figure 10
After laser excitation, the image is captured as before, and the identity of the second base is recorded.

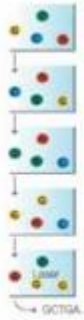


Figure 11
The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

Figure 12
The data are aligned and compared to reference, and sequencing differences are identified.