TOSOH BIOSCIENCE

TOSOH The Chemistry of Innovation

1ST IFCC, EFLM, AFCB CONFERENCE "LABORATORY MEDICINE: MEETING THE NEEDS OF MEDITERRANEAN NATIONS"

Rome, Italy 02/07/2018

The diagnosis of Hemoglobinopathies

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Hemoglobinopathies are the most common monogenic, autosomal recessive hereditary disorders worldwide.

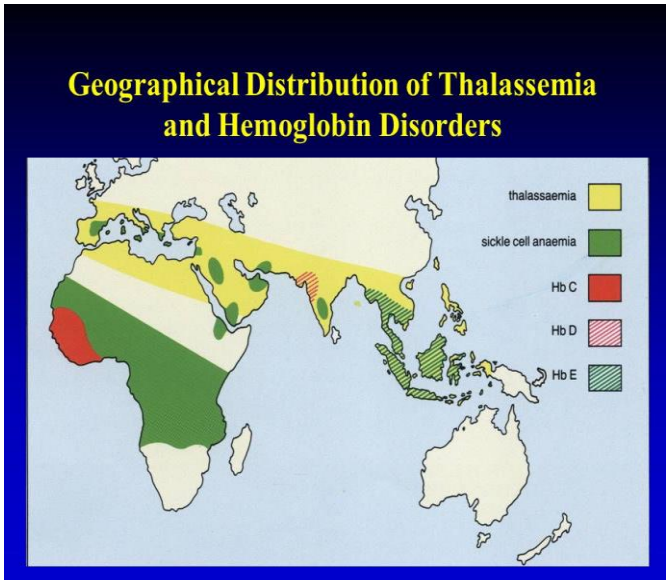
~ 4.83 % of the world population is a healthy carrier of hemoglobinopathy

1.67% carrier of α and β thalassemia

1.92% carrier of HbS

1.0% carrier of HbE

0.3% carrier of HbC



Annual births with major hemoglobin disorders	
β-thalassemia major	22,989
HbE β thalassemia	19,128
HbH disease	9568
Hb Bart's hydrops (α^0/α^0)	5183
SS disease	217,331
S β thalassemia	11,074
SC disease	54,736

A breakdown of the annual number of births with the different hemoglobin disorders

From available data (Modell and Darlison 2008; Weatherall 2010).

Mediterranean bacin	> 8.0 %
Middle East	> 10%
India	3-15%
South East Asia	> 9.0%

Thalassemia major transfusion dependent	
Europe	15.000
Italy	6.000
Sicily	2.000

β-thal carriers
8%

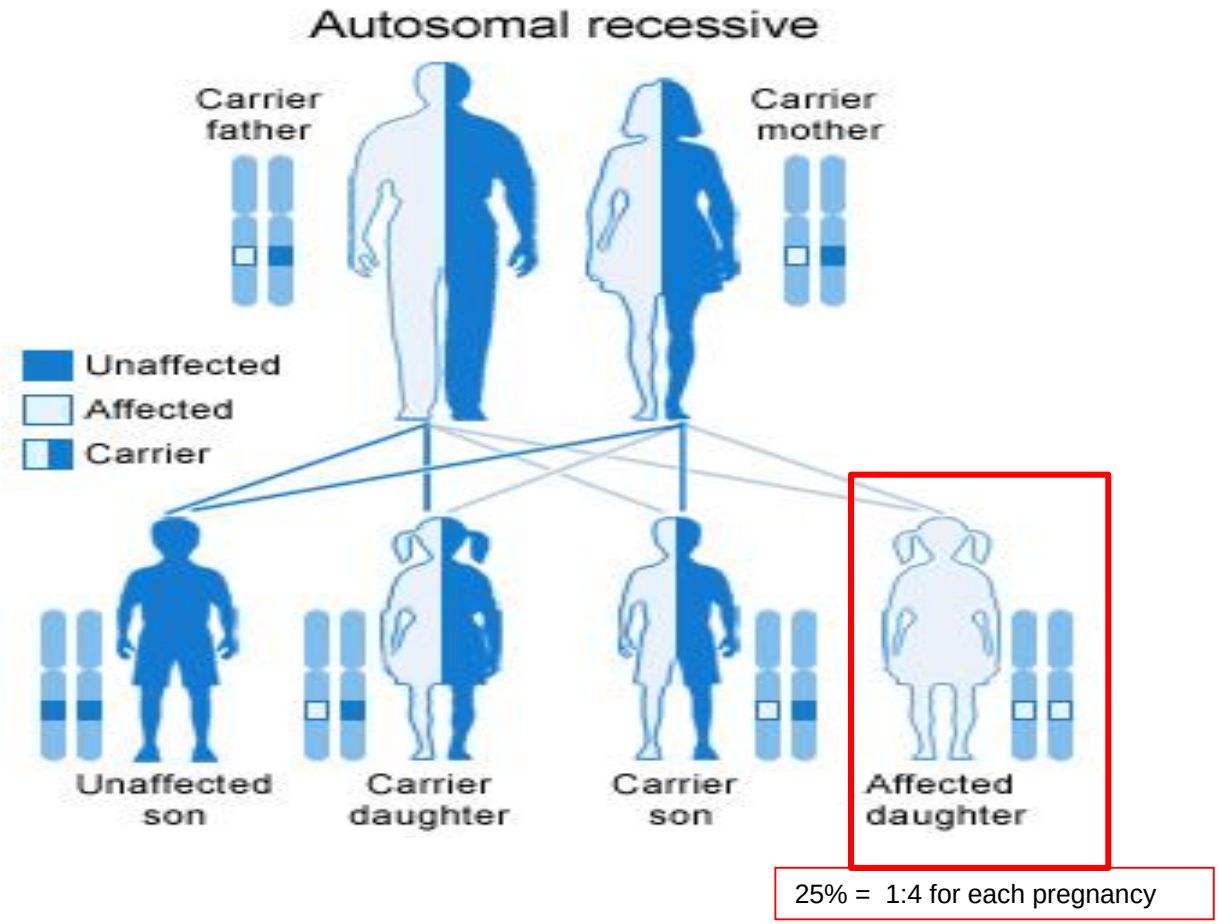
δ-thal carries
2.5%

α-thal carriers
8%

HbS
carriers 1%

Hb variant carriers
2%

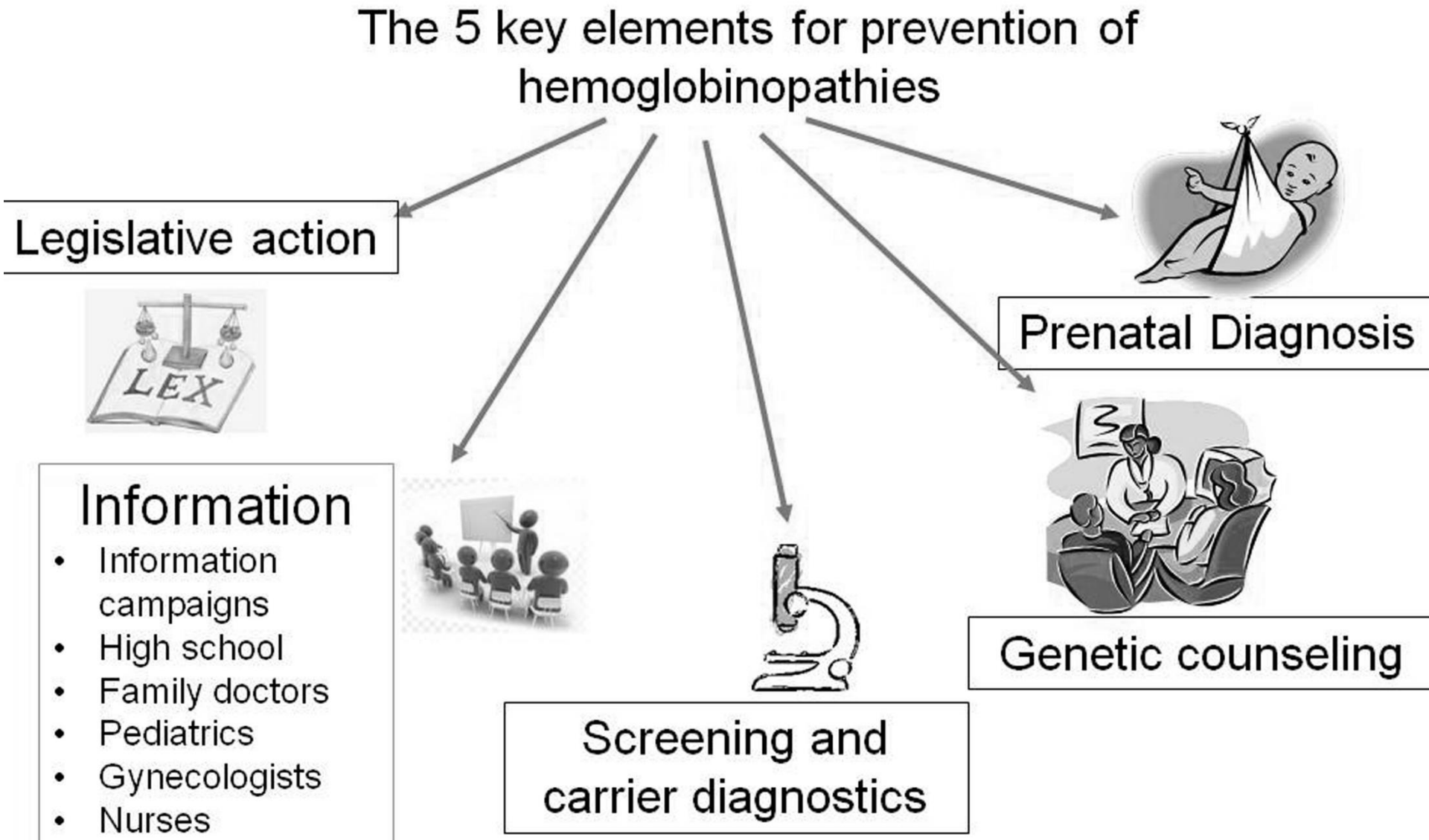
Carriers (heterozygotes) are generally healthy while the severe form can manifest in children of both genders, born from two healthy carriers.



Today, if subjects with hemoglobin disorders receive adequate therapies, have a good life expectancy but require expensive treatments:

Regular blood transfusion to maintain Hb levels above 10 g/dl. This requires adequate supplies of blood, screen to reduce reactions and to prevent the transmission of viruses and other contaminants.

- Regular expensive chelation therapy
- Other supportive therapy
- Multidisciplinary care for adult thalassemics to prevent and manage complications in vital organs (endocrine glands, liver, heart,…..)



THE INTERNATIONAL JOURNAL OF CLINICAL PRACTICE
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Incidence of haemoglobinopathies in Sicily: the impact of screening and prenatal diagnosis

A.Giambona,[1] G. Damiani,[2] M. Vinciguerra,[1] C. Jakil,[2] M. Cannata,[1] F. Cassarà,[1] F. Picciotto,[2] G. Schillaci,[2] V. Cigna,[2] D. Renda,[1] F. Leto,[1] C. Passarello,[1] A. Maggio[1]

The 5 key elements for prevention of hemoglobinopathies

Legislative action

Information

- Information campaigns
- High school
- Family doctors
- Pediatrics
- Gynecologists
- Nurses

Screening and carrier diagnostics

Prenatal Diagnosis

Genetic counseling

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Screening and carrier diagnostics: Basic hematology methods (First Level Test)

- *Complete blood count (CBC) or full blood count (FBC);*

All red cell indices are important in evaluation inclusing:

- **Hb** (Hemoglobin)
- **RBC** (Red Cell Count)
- **MCV** (mean cell volume)
- **MCH** (mean corpuscular Hb content)
- **RDW** (red cell distribution width).

- *Hemoglobin pattern analysis and hemoglobin component quantification*

- **HbA_2**
- **HbF**
- **Hb variants**

Carrier of Thalassemia: Complete blood count evaluation

- High RBC (erythrocytosis) results from a mechanism that compensates for the chronic low MCH present in thalassaemia carriers.
- Reduced Hb value than normal (1-2 gr/dl)
- MCV, MCH and MCHC are variably reduced in thalassemia carriers
- RDW value can potentially discriminate betwee thalassemia carrier and iron deficiency and sometimes between thalassemia carrier and other rare cases.

NOTE:

- Electronic measurement is recommended especially for MCV (mean cell volume) for which the measurement should be direc
- Each laboratory should establish their own cut-off ranges for these parameters, based on the ethnicity of their patient population(s) and patient age group(s).

Carrier of Thalassemia: Hemoglobin pattern analysis (1)

- ***HbA_2 quantitation***:

- HbA_2 levels above 3.5% is the standard cut-off value, above which heterozygosity for β- thalassaemia is indicated.
- Rare genetic and acquired factors may increase or reduce HbA_2 level like the presence of common or rare α-chain variants, or δ chain variants. In this last case, the HbA_2 peak can be slitted in two peaks, or coexisting δ thalassaemia, which decreases the HbA_2 peak
- On most HPLC systems, derivatives of Hb S, may co-elute with HbA_2 resulting in an overestimation of the HbA_2 level (3.5- 4.5%).
- On most HPLC systems, hemoglobin variants which elute with or close to HbA_2 on HPLC may affect HbA_2 level

- ***HbF quantitation***

- Carriers of β thalassaemia present with normal HbA and usually elevated HbA_2, while HbF levels can be normal.

- The normal amount of Hb F from two years after birth is usually less than 1%.

- The presence of elevated HbF can be associated with δβ or γδβ deletion defects, hereditary persistence of fetal haemoglobin (HPFH), point mutations in the promoters of the Gγ or Aγ genes, erythropoietic stress, treatment with certain cytotoxic agents (e.g. hydroxyurea) or pregnancy

Carrier of Thalassemia: Hemoglobin pattern analysis (2)

Note:

- HPLC is a recommended method for simultaneous automatic detection and quantitation of hemoglobin fractions.

- Since system is automated, operation of analysers is simple but interpretation of the chromatograms **requires expertise.** A careful evaluation of the graphic part of the electrochromatogram and of the retention times (elution) of the variants must be made

- Different Hb variants have a similar retection time as HbS.

- Also, attention must be paid to **quality control**, especially for measurement of HbA_2.

Numerical part

Barcode: 0002 - 02 Theor. Plate: 956

Operator: SUPERUSER Analyzer: G8 Date of analysis: 27/02/2013

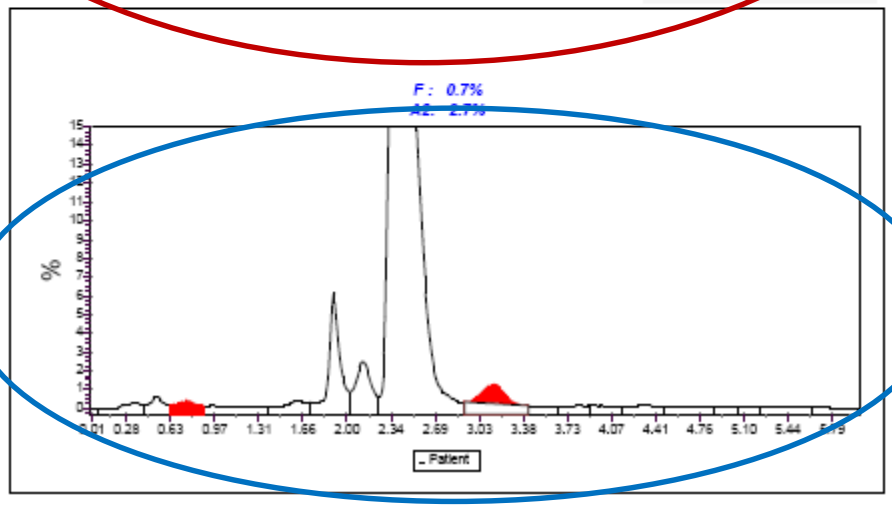
Sample Numb: 02270 Flag & Comment:

Parameter	Value %	Time min.	Area	Total Area
P00	0.5	0.34	15.76	3.427.2
P01	0.7	0.5	25.27	
F	0.7	0.73	22.67	
P02	0.4	0.94	14.3	
P03	0.7	1.61	25.53	
P04	5.7	1.89	196.58	
P05	3.4	2.12	117.22	
A0	83.7	2.38	2.868.75	
A2	2.7	3.13	68.67	
P06	0.3	3.8	9.57	
P07	0.3	3.91	9.42	
P08	0.4	4.35	13.17	
P09	0.4	4.68	12.77	

Y=(Ax+B)

Element	Factor-A	Factor-B
1	1.0214	0.0000
2	1.3096	0.0000

Analyzer: G8
Serial Nb.: 11479905
Soft. Version: 4.00
UIN: Analyzer UIN



The graphics

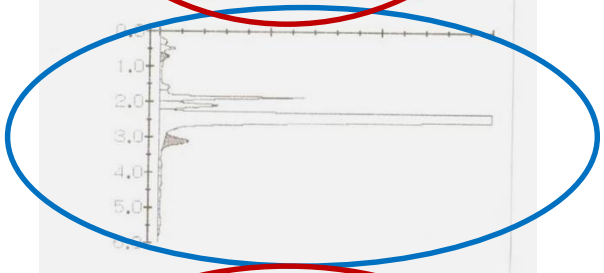
** THALASSEMIA REPORT **

2013/02/27 13:53
TOSOH CORPORATION V05.02
NO: 0004 SL 0002 - 02
ID: 0002 - 02
CALIB F Y= 1.0214X
A2 Y= 1.3096X

TP 956

NAME	%	TIME	AREA
F	0.7	0.73	22.67
A0	83.7	2.38	2868.75
A2	2.7	3.13	68.67
D+	0.0	0.00	0.00
S+	0.0	0.00	0.00
C+	0.0	0.00	0.00
	TOTAL	AREA	3427.17

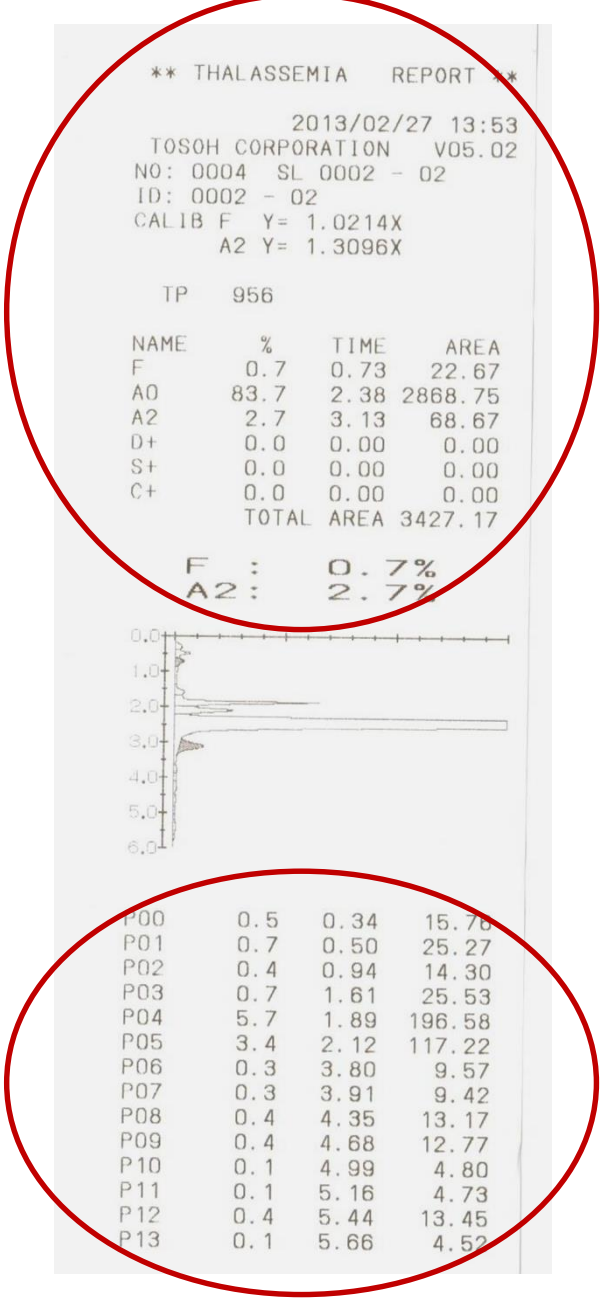
F : 0.7%
A2 : 2.7%



P00	0.5	0.34	15.76
P01	0.7	0.50	25.27
P02	0.4	0.94	14.30
P03	0.7	1.61	25.53
P04	5.7	1.89	196.58
P05	3.4	2.12	117.22
P06	0.3	3.80	9.57
P07	0.3	3.91	9.42
P08	0.4	4.35	13.17
P09	0.4	4.68	12.77
P10	0.1	4.99	4.80
P11	0.1	5.16	4.73
P12	0.4	5.44	13.45
P13	0.1	5.66	4.52

Numerical part:

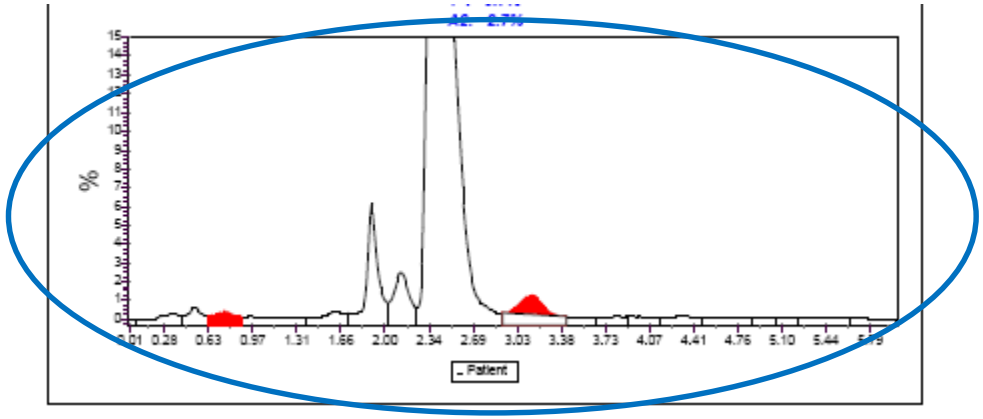
- *Name of the peak (window)*
- *Retention time*
- *Percentage of the analyte*

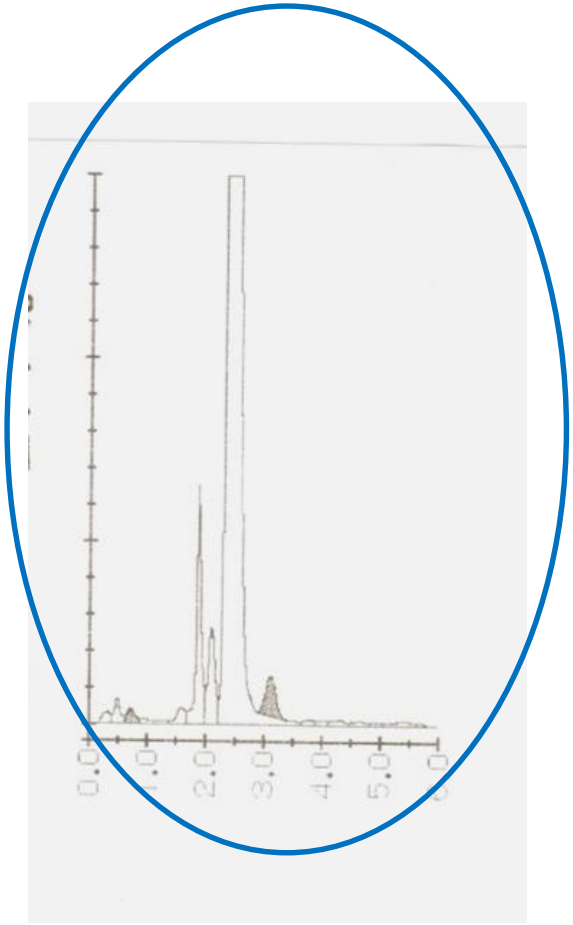


"Window" refers to the time interval within which the most common hemoglobin variants may elute

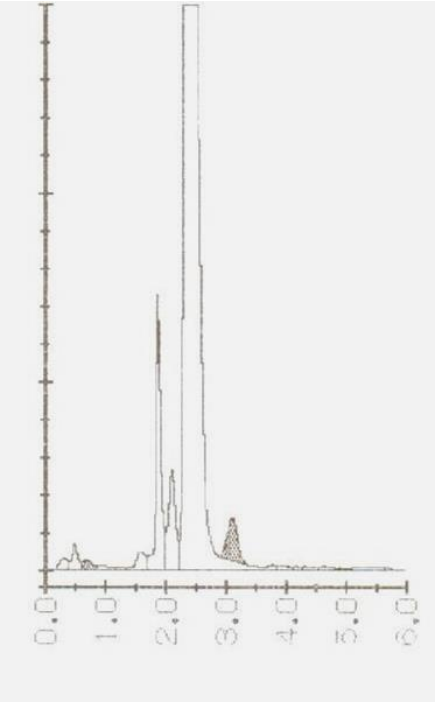
The graphics

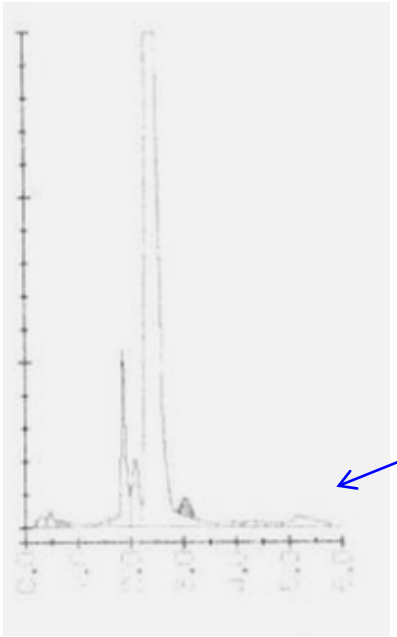
- *Baseline*
- *Presence of anomalous peak*
- *Morphology of the peak*

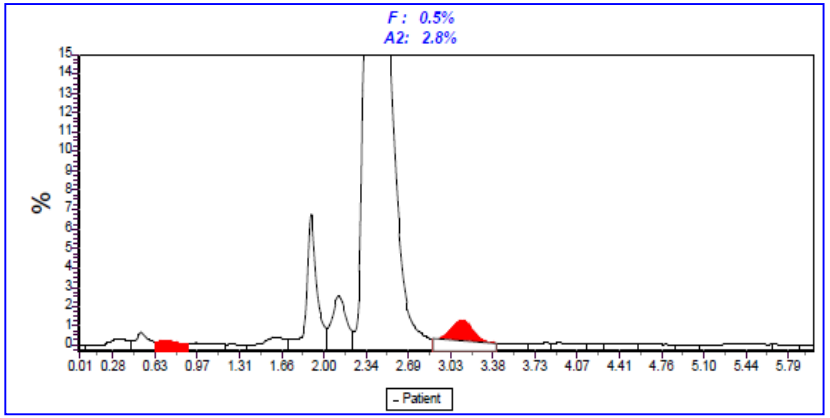


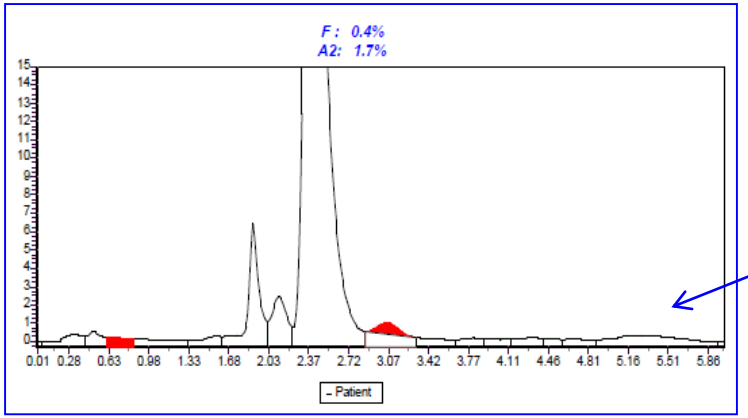


•*baseline*

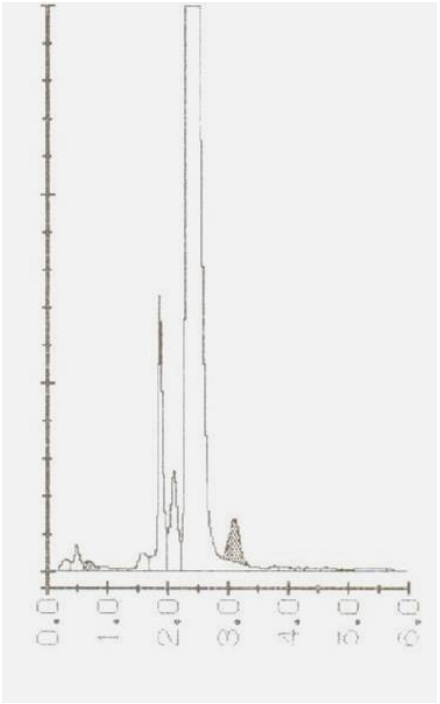


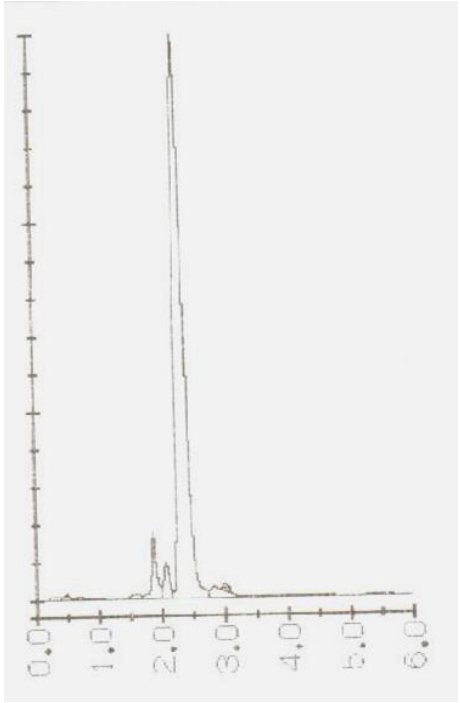


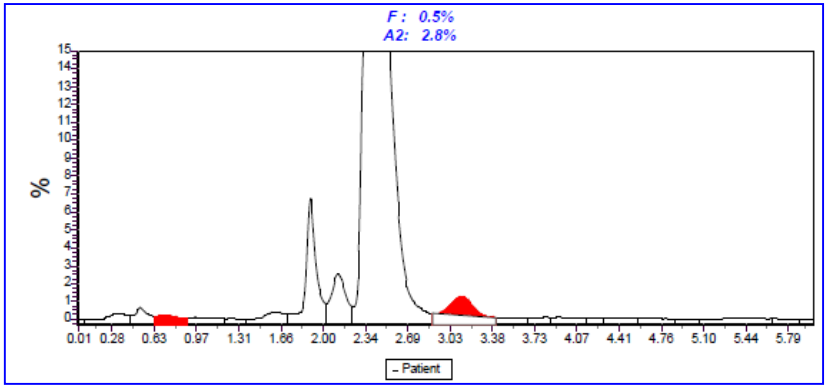

F : 0.5%
A2: 2.8%
%
0.01 0.28 0.63 0.97 1.31 1.66 2.00 2.34 2.69 3.03 3.38 3.73 4.07 4.41 4.76 5.10 5.44 5.79
- Patient

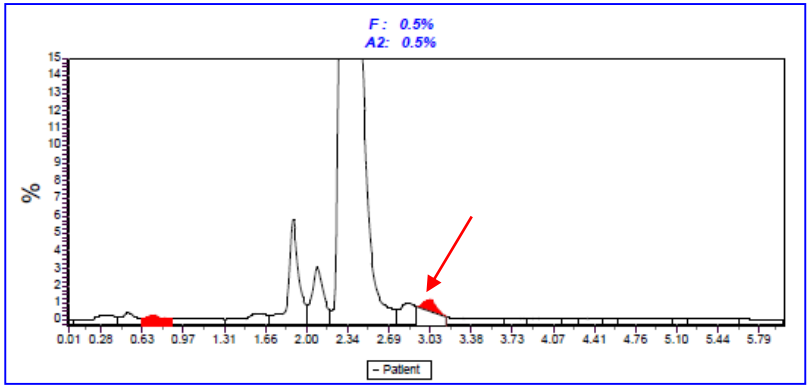

F : 0.4%
A2: 1.7%
0.01 0.28 0.63 0.98 1.33 1.68 2.03 2.37 2.72 3.07 3.42 3.77 4.11 4.46 4.81 5.16 5.51 5.86
- Patient

- *Morphology of the peak*

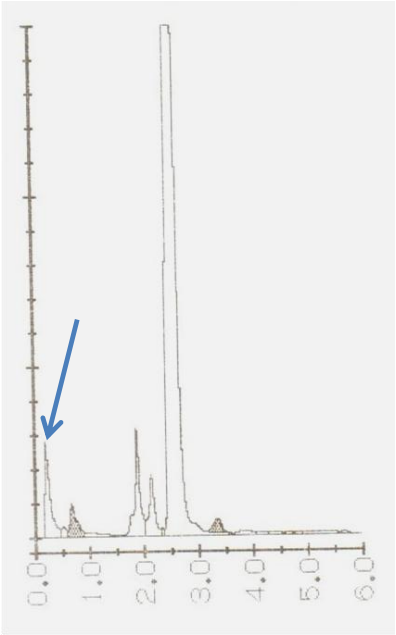


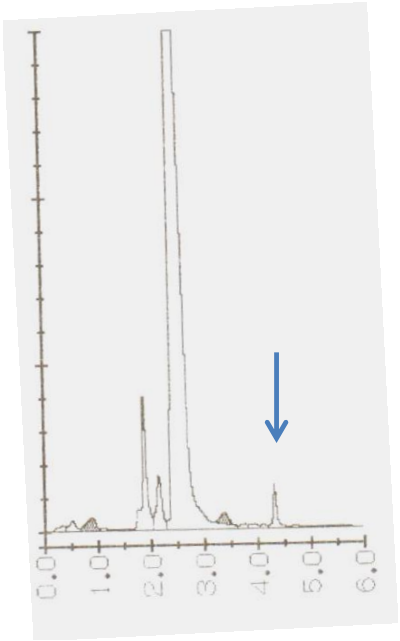


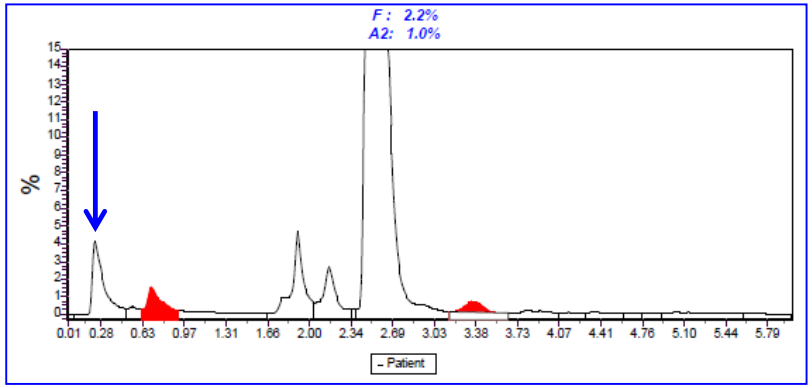


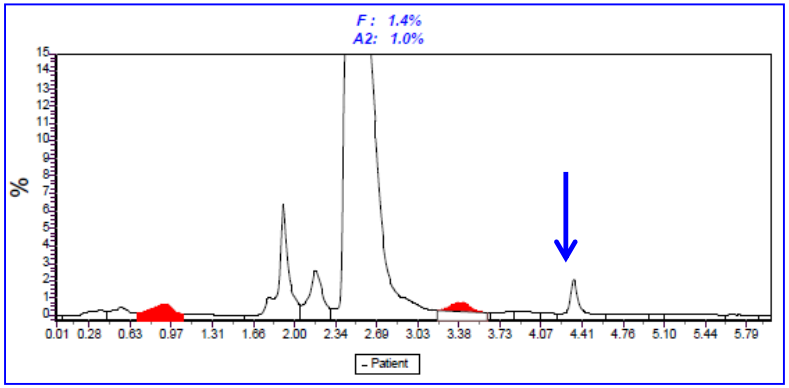


•*presence of anomalous peak*



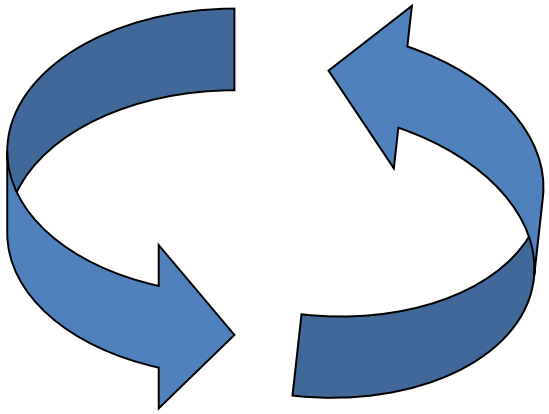






Hemoglobinopathies are the only hereditary diseases in which it is possible identify healthy carriers with blood tests (screening of the first level) rather than molecular analysis.

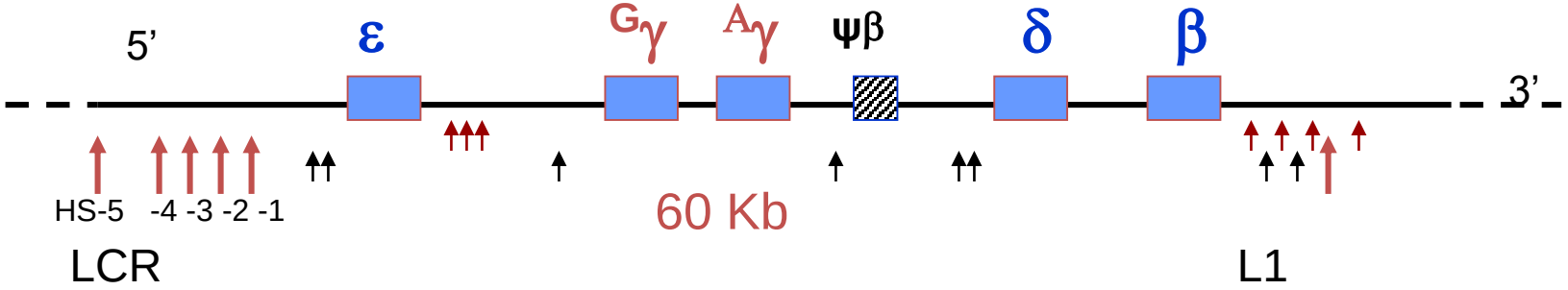
From Phenotype



To Genotype

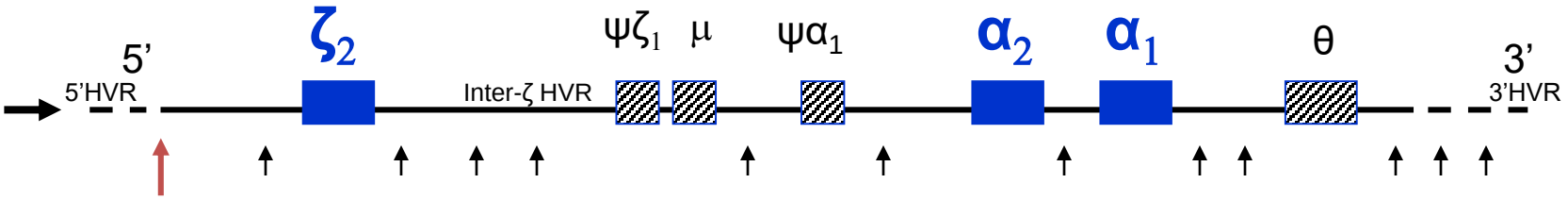
11 p15.5

Non-alpha Cluster (β–Cluster)



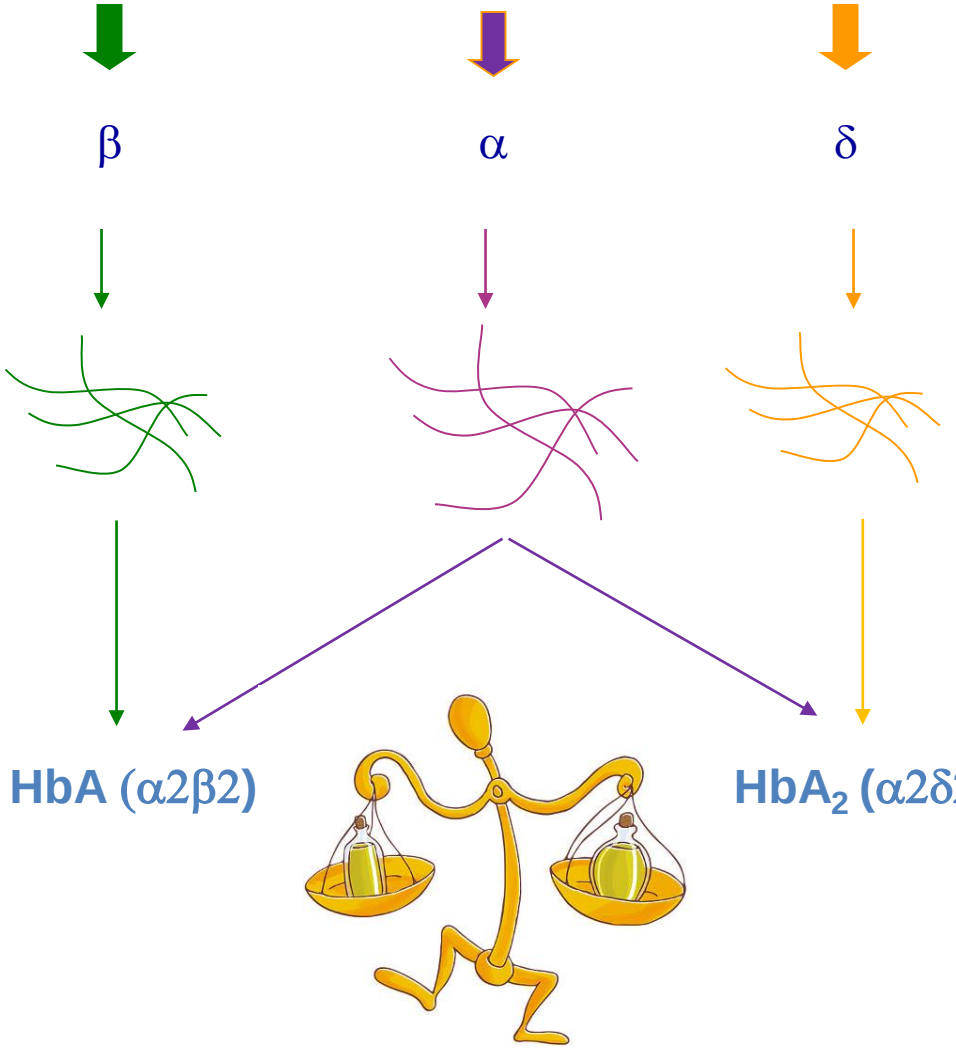
Alpha Cluster

16pter-p13.3



α–MRE
HS-40

BETA, DELTA AND ALPHA GLOBIN SYNTHESIS



HEMOGLOBINOPATHIES HEREDITARY

- MICROCYTHEMIA or THALASSEMIA

1) lack of- or reduction in the synthesis of the corresponding globin chain.

2) altered amino acid sequence with the production of highly unstable globin chains and / or low affinity for other chains.

Cause: UNBALANCED ratios of the normal bio-synthetic α/β or α/δ.

- HEMOGLOBIN VARIANTS

1) altered amino acid sequence (HbS, HbC, HbD ... .)

2) synthesis of a normal amount of the corresponding globin chain.

Cause: an alteration of the normal physiology of the globin chain produced.

PHENOTYPIC CLASSIFICATION OF HEMOGLOBINOPATHIES

❑ *Typical Phenotypes*

the typical parameters of α– , β– or δ- thalassemia or variant hemoglobin carrier' phenotype are present

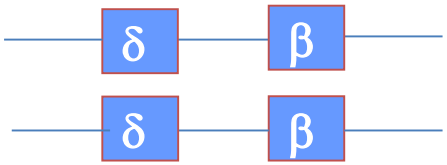
❑ *Atypical Phenotypes*

the typical parameters of α, β–thalassemia or variant hemoglobin carrier' phenotype are not present

Typical Phenotypes

TYPICAL PHENOTYPE OF β-THALASSEMIA TRAIT

- Microcytosis (reduced values of MCV ed MCH)
- Increased levels of HbA_2



β°

absence of beta-chains
Marked microcytosis (MCV < 65 fl)
High value of HbA_2 (>5.0%)

β+

modest presence of beta-chains
Marked microcytosis (MCV 65-68 fl)
High value of HbA_2 (4.5-5.0%)

β++ (mild)

low presence of beta chains
mild microcytosis (MCV 68-75 fl)
HbA_2 borderline or slightly above the normal levels (3.5-4.5% or >5,5%)

β+++ (silent)

low presence of beta chains
slight microcytosis or normal (MCV >78-80 fl)
HbA_2 borderline (3,3-3,9%)

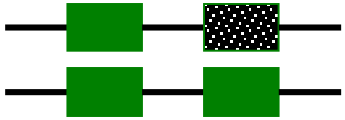
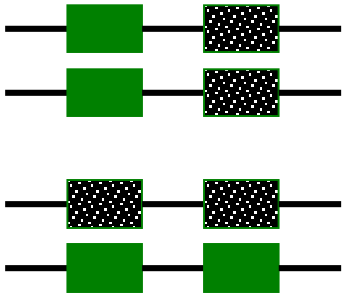
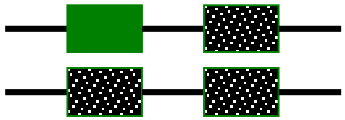
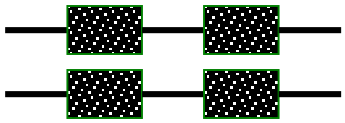
β-Thalassemia:

In most cases, first-level analysis is used to define the status of a healthy carrier

RBC	5.60	5.46	4.96
HB	13.8	13.9	12.8
HCT	41.4	42.9	41.4
MCV	**61.5**	**64.9**	**70.5**
MCH	**22.1**	**20.1**	**22.6**
MCHC	32.1	34.1	32.0
RDW	13.4	13.4	13.4
HbA_2	**5.4%**	**4.9%**	**4.0%**

Presumptive Diagnosis of: | β^0-carrier | β^+-carrier | β^{++}-carrier |

Alpha-Thalassemia

Phenotypic classification	Functional genes	Hematologic status	Hemoglobin status
α^{+} Thal		almost normal	Normal
α^{o} Thal		altereted	Normal
HbH (β_4) desease		altereted	Presence of Hb H
Fetal Hydrops with Hb Bart's (γ_4)		This condition is not compatible with life	

Region	β-Thalassemia	α°-Thalassemia	α⁺-Thalassemia
Americas	0–3	0–5	0–40
Eastern Mediterranean	2–18	0–2	1–60
Europe	0–10	1–2	0–12
Southeast Asia	0–11	1–30	3–40
Sub-Saharan Africa	0–12	0	10–50
Western Pacific	0–13	0	2–60

Global Distribution of Thalassemia: carrier frequencies of α–thalassemia alleles (%)

α^+

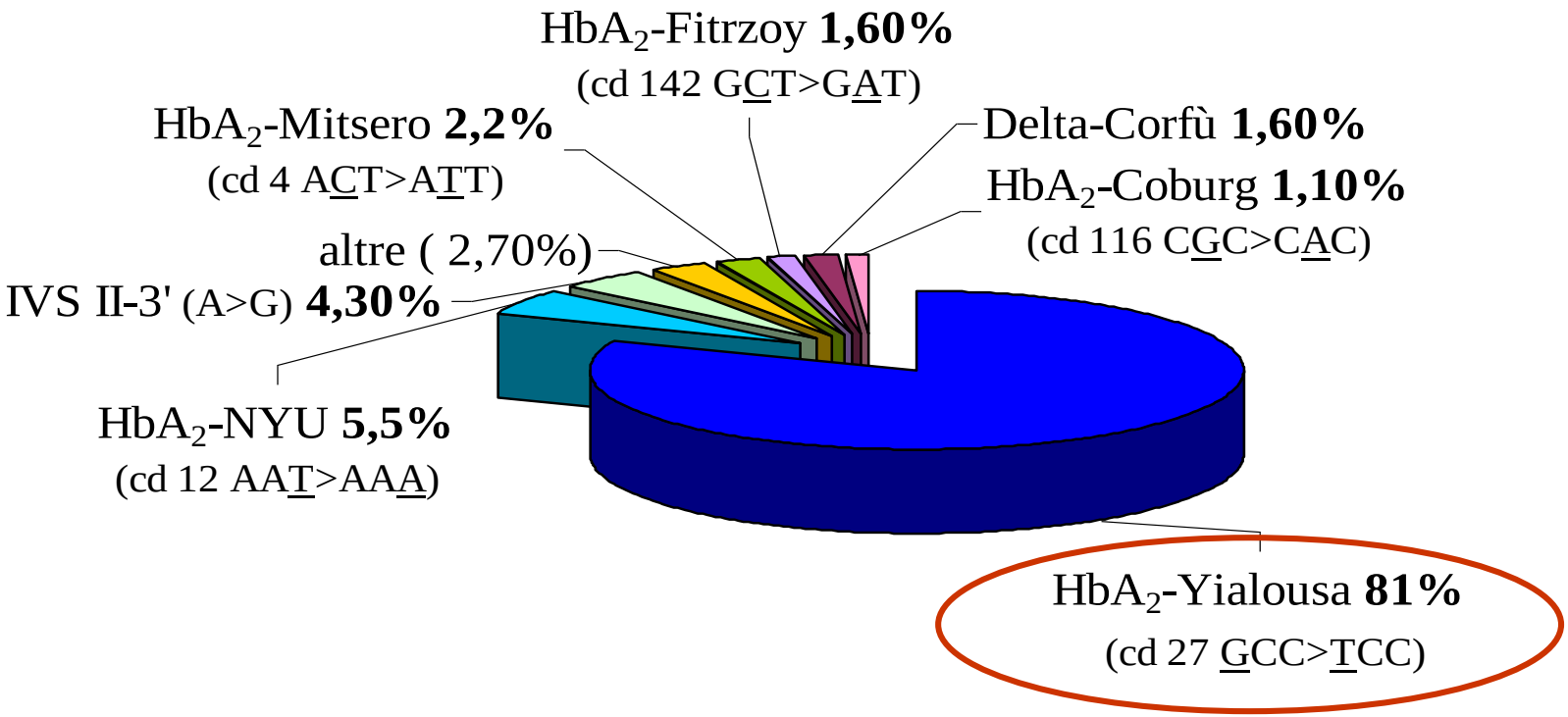
RBC	4.94	5.14	5.55	5.65
HB	13.7	13.7	13.5	14.4
HCT	40.8	41.8	41.3	43.7
MCV	**80.6**	**79.5**	**74.0**	**77.3**
MCH	**27.1**	**26.7**	**24.2**	**25.5**
HbA_2	**2.9%**	**2.7%**	**2.7%**	**2.6%**
	$\alpha^{-3.7I}/\alpha\alpha$	$\alpha^{-4.2}/\alpha\alpha$	$\alpha^{NcoI}\alpha/\alpha\alpha$	$\alpha^{HphI}\alpha/\alpha\alpha$

α°

RBC	5.53	5.47	6.24	5.59	6.39
HB	12.6	11.0	13.2	12.3	13.3
HCT	38.8	38.3	41.0	38.5	40.4
MCV	**70.0**	**63.0**	**67.0**	**68.8**	**63.0**
MCH	**22.9**	**20.1**	**21.1**	**22.1**	**20.7**
HbA_2	**2.8**	**2.7**	**2.4**	**2.7%**	**2.4%**
	$\alpha^{3.7I}/\alpha^{3.7I}$	$\alpha^{3.7I}/\alpha^{4.2}$	$\alpha^{3.7I}/\alpha^{HphI}\alpha$	--med/αα	--20.5/αα

Presumptive Diagnosis of α-thalassemia

δ-Thalassemia in Sicily

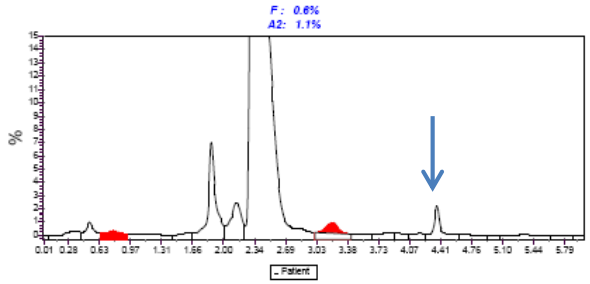


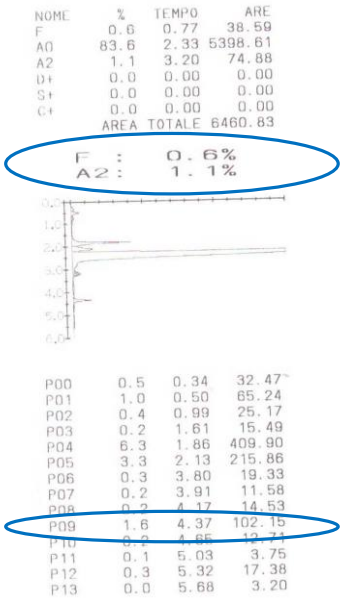
Giambona A et al., Haematologica. 2006 Dec;91(12):1681-4.

RBC	4.60	5.14	5.25	5.65
HB	13.5	14.2	14.5	14.4
HCT	45.8	41.8	41.3	43.7
MCV	90.6	89.5	88.0	87.3
MCH	27.1	27.7	28.2	27.5
HbA_2	**2.0%**	**1.8%**	**1.9%**	**1.3%** ←
Slow Hb	**---**	**---**	**---**	**1.5%** ←

Presumptive Diagnosis of δ-thalassemia

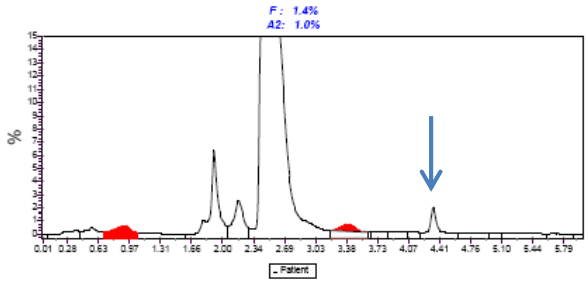
RBC	4.62
Hb	13.0
HCT	38.3
MCV	84.0
MCH	28.1
RDW	12.3

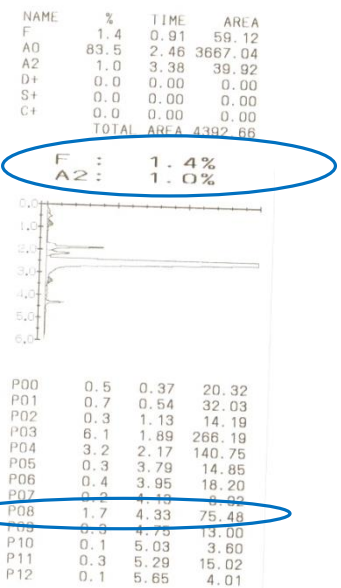




HbA_2 NYU (delta 12(A9) Asn>Lys)

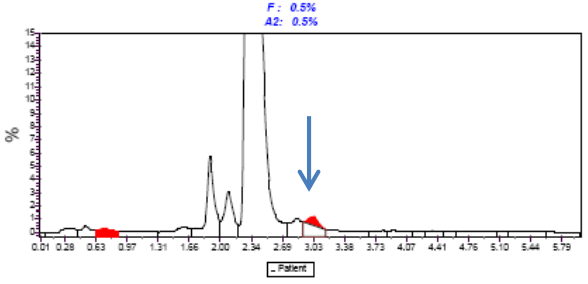
RBC	4.02
Hb	13.7
HCT	41.8
MCV	94.0
MCH	29.1
RDW	11.0

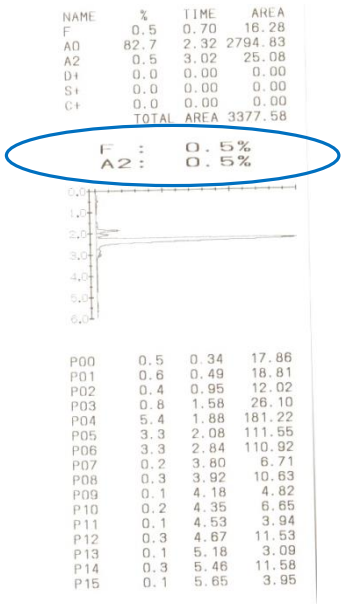




Hb A_2' (delta 16(A13) Gly>Arg)

RBC	5.13
Hb	14.7
HCT	43.6
MCV	85.0
MCH	28.6
RDW	12.1





HbA_2-Coburg (delta 116(G18) Arg>His)

Atypical Phenotypes

ATYPICAL PHENOTYPES

-1- HbA_2 borderline with normocytosis or microcytosis

-2- Microcytosis with normal levels of HbA_2 ed HbF

-3- Microcytosis with very high level of HbA_2

-4- Increased levels of HbF

ATYPICAL PHENOTYPES

-1- HbA_2 borderline with normocytosis or microcytosis

-2- Microcytosis with normal levels of HbA_2 ed HbF

-3- Microcytosis with very high level of HbA_2

-4- Increased levels of HbF

HBA_2 BORDERLINE WITH NORMOCYTOSIS OR MICROCYTOSIS

3.1%< ***HbA_2 borderline*** < 4.0%

Causes:

- Silent mutations in the β-globin gene
- Presence of triple-alpha allelic structure
- Co-inheritance of β mutations and δ mutations
- Co-inheritance of β mutations and α mutations
- β-globin variants
- Other: Hyperthyroidism
  Use of anti-HIV retroviral drugs (AZT)
  Dosage of the HbA_2 value

β promoter mutations (silent mutations)

	β^{-92}/β		β^{-101}/β	
RBC	4.92	4.91	4.10	5.01
HB	15.3	12.8	12.0	14.0
MCV	87.0	82.3	86.0	84.0
MCH	31.2	27.5	29.2	28.0
RDW	12.8	12.9	13.3	12.2
Hb A_2	3.6	3.8	3.6	4.0
Hb F	0.2	0.4	1.7	1.3

β^{-101}/β^{+}

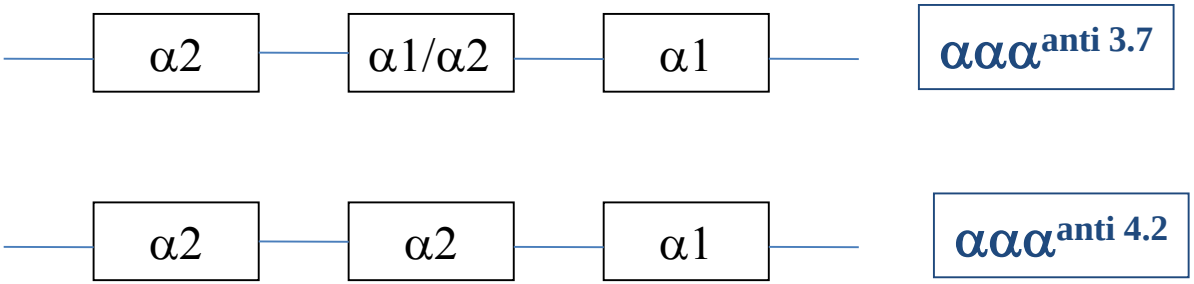
RBC	5.21	6.11	5.33	5.39
HB	9.5	11.2	9.4	9.9 ←
MCV	57.7	61.0	57.0	58.0 ←
MCH	18.2	18.4	17.6	18.4 ←
RDW	22.9	17.4	22.4	16.1
Hb A_2	7.3	7.2	6.6	5.4 ←
Hb F	5.3	5.3	14.1	3.1

β^{-101}/β^{0}

RBC	6.57	5.13	3.96
HB	11.3	9.5	9.8 ←
MCV	56.0	60.0	83.2 ←
MCH	17.2	18.6	25.6 ←
RDW	20.3	20.4	19.9
Hb A_2	7.8	6.5	4.8 ←
Hb F	3.2	20.4	8.7

ααα/αα

RBC	4.26	4.46	3.78	4.26	3.82
HB	11.7	12.4	11.4	12.2	11.4
MCV	83.0	83.0	86.0	83.0	88.0
MCH	27.5	27.8	30.2	28.7	29.8
RDW	14.6	13.4	12.8	13.4	12.8
Hb A_2	3.2	3.3	3.3	3.5	3.4
Hb F	0.2	0.5	0.8	0.3	0.6



triple-alpha allelic structure

HBA_2 BORDERLINE WITH NORMOCYTOSIS OR MICROCYTOSIS

3.1%< ***HbA_2 borderline*** < 3.9%

Borderline HbA_2 is not a rare event and it should be more investigate, specially in presence of reduced MCV value and if partner is an healty carrier of β–thalassemia.

ATYPICAL PHENOTYPES

-1- HbA_2 borderline with normocytosis or microcytosis

-2- Microcytosis with normal levels of HbA_2 ed HbF

-3- Microcytosis with very high level of HbA_2

-4- Increased levels of HbF

MICROCYTOSIS WITH NORMAL LEVELS OF HBA_2 AND HBF

- Reduced values of MCV and MCH (< 80 fl, e < 26 pg)
- $HbA_2 \leq 3.2\%$, $HbF \leq 2\%$

Causes:

- α° thalassemia (two alpha genes mutated)
- $\alpha^{+/\circ}$ thalassemia (one alpha gene alterated)
- α^{+} thalassemia (one alpha gene alterated)
- β mild or slight mutations in eterozygosis with δ mutations
- Hemoglobin Variants
- Iron deficiency
- Age

INTERACTIONS BETWEEN BETA AND DELTA MUTATIONS

β α δ

reduction

HbA_2

MCV, MCH =

δ+β°

RBC	6.10	6.01	5.58	5.54
HB	12.3	12.2	11.9	11.2
HCT	37.3	37.1	37.3	36.1
MCV	61.2	61.8 ←	62.0	59.6 ←
MCH	20.0	20.2	21.3	20.2
MCHC	32.7	32.7	31.9	32.0
RDW	14.3	14.3	13.9	15.1
Hb A_2	5.6%	3.5% ←	5.2%	3.5% ←
Hb F	0.6%	1.3%	2.6%	0.5%
	β^{Cd30}/β	β^{Cd30}_{+}/β δ^{Cd27}/δ	β^{Cd39}/β	β^{Cd39}_{+}/β δ^{Cd27}/δ

MICROCYTOSIS WITH NORMAL LEVELS OF HBA_2 AND HBF

$\delta+\beta^{+}$

RBC	5.81	5.64	6.05	6.62
HB	12.1	11.6	12.4	13.1
HCT	37.0	37.0	39.6	41.5
MCV	64.0	65.6 ←	65.0 ←	62.7 ←
MCH	20.9	20.6	20.6	19.9
MCHC	32.8	31.4	31.5	31.7
RDW	14.8	14.5	13.2	14.5
Hb A_2	4.8%	3.5% ←	3.2% ←	3.1% ←
Hb F	<0.5%	0.9%	0.0%	0.5%

$\beta^{IVSI\ nt\ 110}/\beta$

$\beta^{IVSI\ nt\ 110}/\beta$

+

δ^{Cd27}/δ

MICROCYTOSIS WITH NORMAL LEVELS OF HBA_2 AND HBF

$\delta+\beta^{+}$

RBC	5.81	5.65	6.39
HB	12.1	11.9	13.3
HCT	37.0	34.7	40.4
MCV	64.0	61.4 ←	63.0
MCH	20.9	19.5	20.7
MCHC	32.8	31.7	32.9
RDW	14.8	15.3	13.8
A_2	4.8%	2.8% ↓	2.6% ↓
F	<0.5%	1.0%	<0.5%

$\beta^{IVSI\ nt110}/\beta$

$\beta^{IVSI\ nt110}/\beta$

δ^{Cd142}/δ

HbA_2 Fitrzoy

$\alpha^{-20.5}/\alpha\alpha$

MICROCYTOSIS WITH NORMAL LEVELS OF HBA_2 AND HBF

δ+β^{++}

RBC	5.27	5.02	5.24	6.25
HB	11.9	13.4	12.3	14.2
HCT	37.6	36.9	38.5	44.3
MCV	71.3	73.5	73.5	70.9
MCH	22.5	24.8	23.5	22.7
MCHC	31.6	33.8	31.9	32.1
RDW	14.0	13.3	14.0	13.5
Hb A_2	4.1%	2.6%	2.6%	2.8%
Hb F	0.9%	0.5%	0.0%	0.5%

$\beta^{IVSI\ nt\ 6}/\beta$

$\beta^{IVSI\ nt\ 6}/\beta$ + δ^{Cd27}/δ

$\alpha^{Ncol}\alpha/\alpha\alpha$

MICROCYTOSIS WITH NORMAL LEVELS OF HBA_2 AND HBF

ATYPICAL PHENOTYPES

-1- HbA_2 borderline with normocytosis or microcytosis

-2- Microcytosis with normal levels of HbA_2 ed HbF

-3- Microcytosis with very high level of HbA_2

-4- Increased levels of HbF

MICROCYTOSIS WITH VERY HIGH LEVEL OF HBA_2

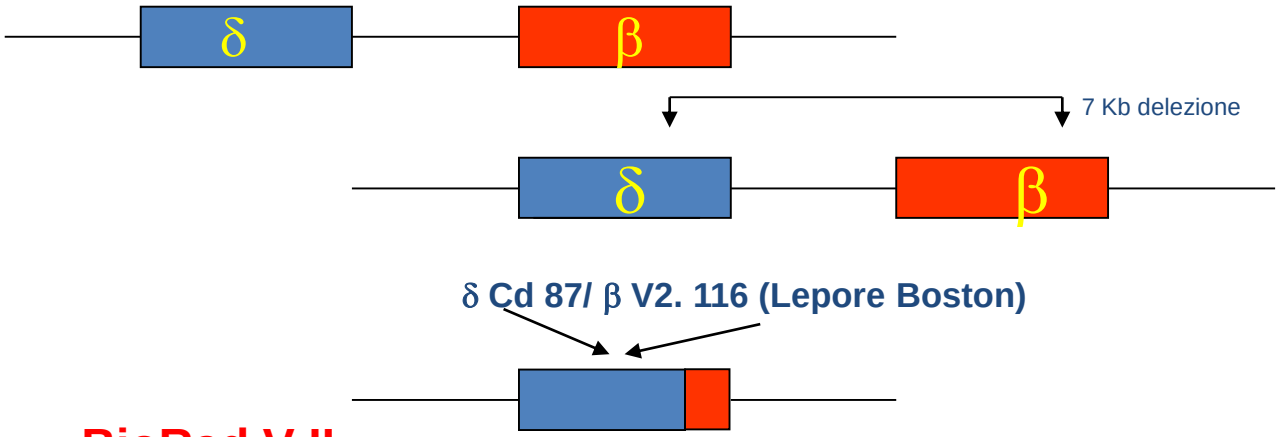
- Reduced values of MCV ed MCH (< 80 fl, e < 26 pg)
- HbA_2 > 6.0%

Causes:

- Hb Lepore
- Hemoglobin Variants : Hb E (Cd 26 Glu →Lys)
- β–cluster deletions

Hb-Lepore

Hollandia:	δ22 - β50
Baltimora:	δ50 - β86
Boston:	δ87 - β116



RBC	6.00 +/- 0.5
Hb	12.5 +/- 0.7
MCV	**70.0 +/- 5.0**
MCH	22.0 +/- 1.5
HbA_2(HPLC)	**10-12% (2% HbA_2)**

BioRad V II

Patient Data
Sample ID: PENNINO ANNA
Patient ID:
Name:
Physician:
Sex:
DOB:
Comments:

Analysis Data
Analysis Performed: 18/09/2017 1
Injection Number: 7571R
Run Number: 316
Rack ID: 0006
Tube Number: 5
Report Generated: 16/02/2018 1
Operator ID:

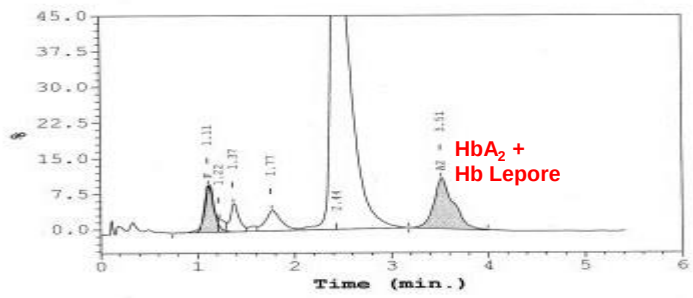
Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
F	5.6*	---	1.11	106934
Unknown	---	1.0	1.22	19848
P2	---	2.9	1.37	58327
P3	---	3.7	1.77	75578
Ao	---	75.5	2.44	1530939
A2	10.8*	---	3.51	235846

Total Area: 2,027,473

F Concentration = 5.6*%
A2 Concentration = 10.8*%

*Values outside of expected ranges

Analysis comments:



Tosoh G 11

Patient Chromatogram Date: 16/02/2018

Last Name: Result
Barcode: 15526
Rack: 0006
Position: 07
Sample Numb: 16340

First Name: Unknown
Theor. Plate: 1061
Operator: SUPERUSER
Analyzer: G11
Flag & Comment:

Date of birth:
PUI:
Version: 4.41.0.0 Rev. I
Date of analysis: 19/09/2017
Time of analysis: 16.22.08

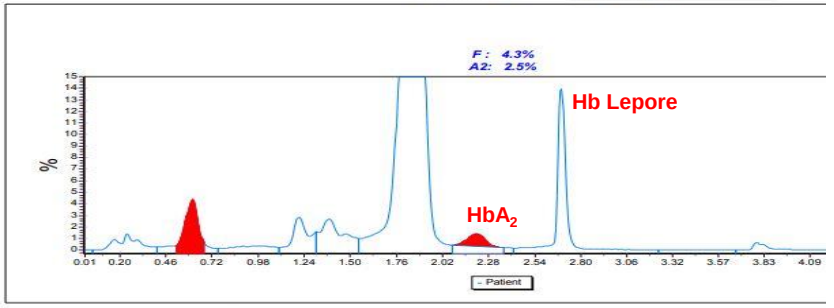
Parameter	Value %	Time min.	Area	Total Area
P00	2.4%	0.23	72.69	3.008
P01	0.4%	0.41	13.1	
F	4.3%	0.61	136.7	
P02	0.3%	0.67	7.78	
P03	1.2%	0.99	35.08	
P04	3.5%	1.21	103.84	
P05	4.9%	1.37	147.63	
A0	70.8%	1.84	2.130.7	
A2	2.5%	2.21	46.97	
P06	9.3%	2.69	279.38	
P07	0.1%	3.33	4.48	
P08	0.9%	3.81	25.73	
P09	0.0%	4.46	1.08	
P10	0.0%	4.58	1.25	
P11	0.1%	4.68	1.54	

Y=(Ax+B)

Element	Factor-A	Factor-B
1	0.9552	-0.0536
2	1.3852	0.3157

B-Thalassemia

Analyzer: G11
Serial Nb.: demo
Soft. Version: demo
UIN: Analyzer UIN



TOSOH EUROPE Transportstraat 4 3980 Tessenderlo

MICROCYTOSIS WITH VERY HIGH LEVEL OF HBA_2

- Reduced values of MCV ed MCH (< 80 fl, e < 26 pg)
- HbA_2 > 6.0%

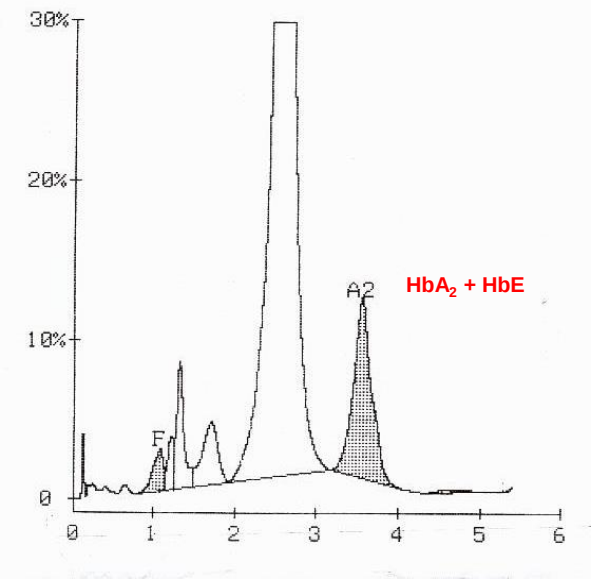
Causes:

- Hb Lepore
- Hemoglobin Variants : Hb E (Cd 26 Glu →Lys)
- β–cluster deletions

Haemoglobin AE Variant

Bio-Rad Variant II

ANALYTE ID	%	TIME	AREA
F	1.5	1.08	26705
Unknown 1	1.1	1.22	18320
P2	3.4	1.32	57143
P3	3.5	1.71	59075
A0	76.6	2.54	1285109
A2	12.8	3.55	191981
	TOTAL AREA		1638333
F	1.5% A2		12.8%



Tosoh G8

Patient Chromatogram — Date: 18/06/2018

Last Name: Result — First Name: Unknown — Date of birth:
Barcode: BEGUM RIPA — Theor. Plate: 1547 — PUI:
Rack: 0005 — Operator: SUPERUSER — Version: 4.41.0.0 Rev. I
Position: 08 — Analyzer: G8 — Date of analysis: 19/09/2017
Sample Numb: 09190 — Flag & Comment: — Time of analysis: 17.42.49

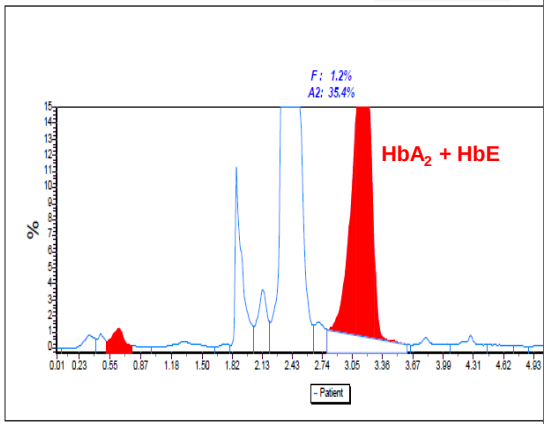
Parameter	Value %	Time min.	Area	Total Area
P00	0.6%	0.33	29.53	4.981.8
P01	0.4%	0.45	21.04	
F	1.2%	0.63	50.89	
P02	0.1%	0.78	5.94	
P03	0.8%	1.32	38.92	
P04	0.2%	1.73	8.94	
P05	5.5%	1.85	271.87	
P06	2.4%	2.13	119.04	
A0	57.0%	2.36	2.841.39	
P07	4.6%	2.7	227.68	
A2	35.4%	3.16	1.224.97	
P08	0.8%	3.8	39.61	
P09	0.8%	4.27	39.09	
P10	0.3%	4.5	13.95	
P11	0.1%	4.75	5.97	
P12	0.1%	4.98	7.12	
P13	0.2%	5.18	8.03	
P14	0.3%	5.33	15.87	
P15	0.2%	5.75	11.95	

Y=(Ax+B)

Element	Factor-A	Factor-B
1	1.1435	0.0000
2	1.3274	0.0000

B-Thalassemia

Analyzer: G8
Serial Nb.: 11819110
Soft. Version: 5.24
UIN: Analyzer UIN



Tosoh G11

Patient Chromatogram — Date: 18/06/2018

Last Name: Result — First Name: Unknown — Date of birth:
Barcode: 15536 — Theor. Plate: 1130 — PUI:
Rack: 0005 — Operator: SUPERUSER — Version: 4.41.0.0 Rev. I
Position: 08 — Analyzer: G11 — Date of analysis: 19/09/2017
Sample Numb: 15490 — Flag & Comment: — Time of analysis: 15.37.08

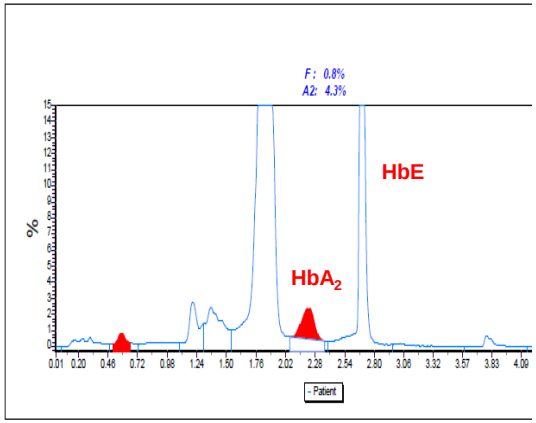
Parameter	Value %	Time min.	Area	Total Area
P00	1.4%	0.23	44.48	3.192.2
P01	0.1%	0.48	1.68	
F	0.8%	0.57	27.86	
P02	0.1%	0.66	4.09	
P03	1.0%	0.87	31.89	
P04	3.2%	1.2	103.51	
P05	4.9%	1.36	155.49	
A0	60.9%	1.84	1.944.04	
A2	4.3%	2.22	94.86	
E+	23.0%	2.69	733.56	
P06	0.6%	3.02	17.36	
P07	0.8%	3.81	26.01	
P08	0.2%	4.23	7.56	

Y=(Ax+B)

Element	Factor-A	Factor-B
1	0.9552	-0.0536
2	1.3852	0.3157

B-Thalassemia

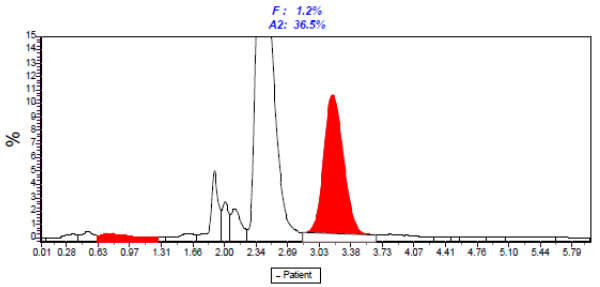
Analyzer: G11
Serial Nb.: demo
Soft. Version: demo
UIN: Analyzer UIN

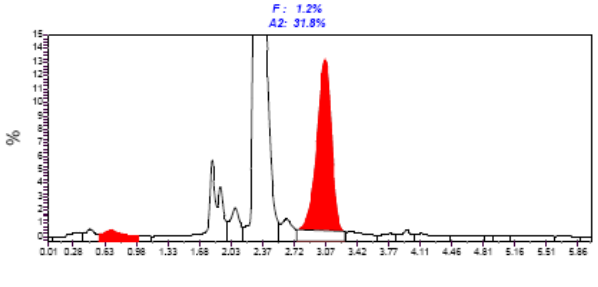


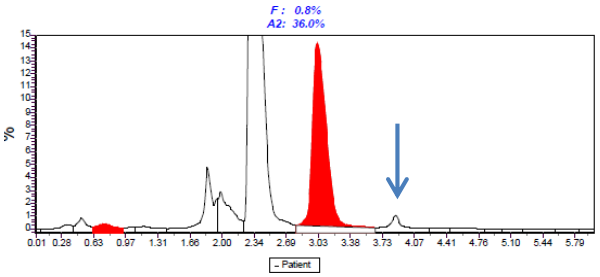
TOSOH EUROPE Transportstraat 4 3980 Tessenderlo

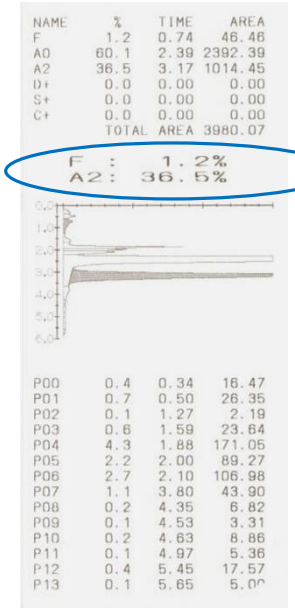
Contextual assessment of hematological data: same window, similar percentage and differente complete blood count

RBC	4.76	**RBC**	4.52	**RBC**	4.48
Hb	12.0	**Hb**	12.6	**Hb**	13.1
HCT	35.8	**HCT**	38.4	**HCT**	41.6
MCV	75.0	**MCV**	85.0	**MCV**	92.0
MCH	24.6	**MCH**	27.8	**MCH**	29.4
RDW	13.7	**RDW**	12.7	**RDW**	12.8

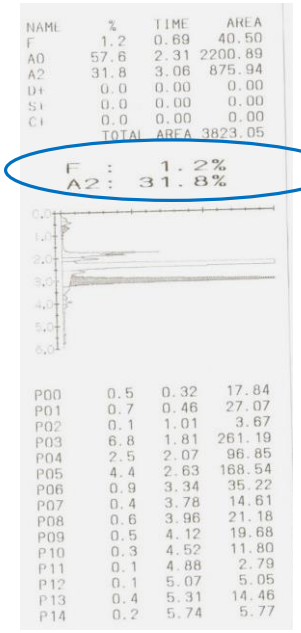




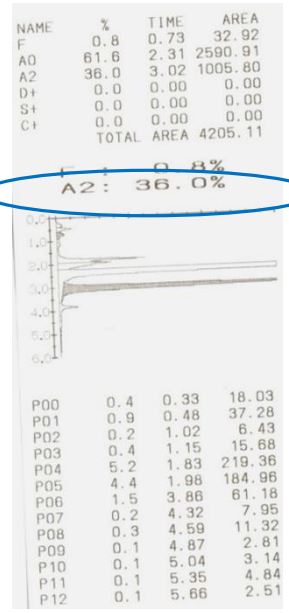




F 1.2 %
A_2 36.5 %



F 1.2 %
A_2 31.8 %



F 0.8 %
A_2 36.0 %
P06 1.5%
(r.t.=3.86)

Hb E (beta 26(B8) Glu>Lys)

Hb E (beta 26(B8) Glu>Lys) + $\alpha^{3.7}$ / $\alpha\alpha$

Hb St.Truiden ($alpha_2$ 68(E17) Asn>His)

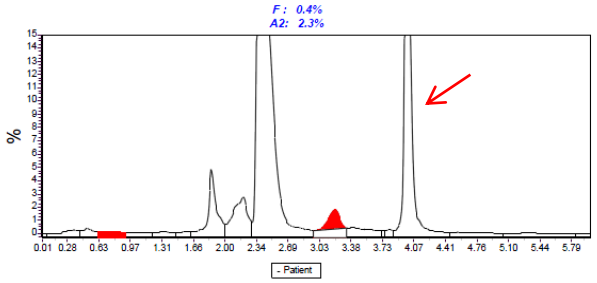
Abnormal Hemoglobin

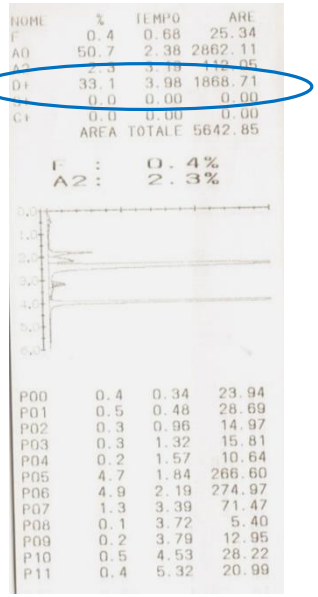
Careful observation of the hematology (complete blood count) and hemoglobin (HPLC graph / retention time and percentage of peak) state may be suspected the presence of a particular hemoglobin variant.

In presence of an abnormal hemoglobin, the results obtained by first-level testing remains a *presumptive* data and the molecular analysis must be performed.

Abnormal peaks in windows dedicated to the most frequent variants

RBC	4.20
Hb	12.1
HCT	37.5
MCV	88.0
MCH	28.8
RDW	12.8

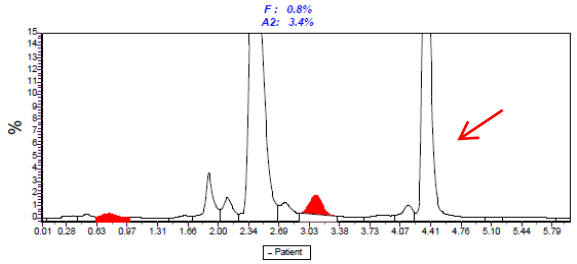


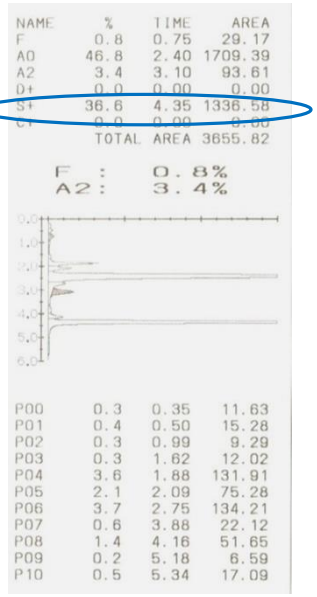


Hb F 0.4 %
Hb A_2 2.3 %
HbD 33.1%

Hb D-Los Angeles (beta 121(GH4) Glu>Gln)

RBC	5.02
Hb	15.8
HCT	45.4
MCV	90.5
MCH	31.4
RDW	14.1

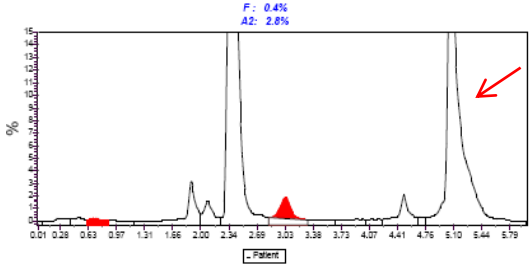


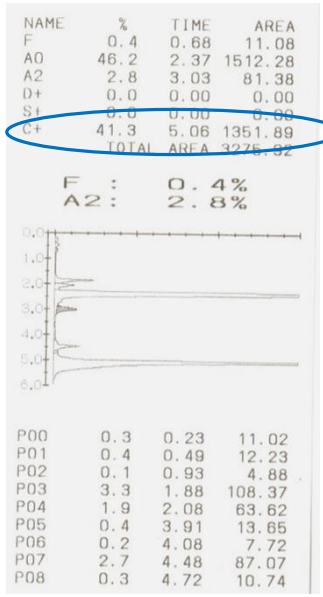


Hb F 0.8 %
Hb A_2 3.4 %
HbS 36.6%

HbS (beta 6(A3) Glu>Val)

RBC	4.65
Hb	13.5
HCT	40.8
MCV	88.0
MCH	29.1
RDW	11.8



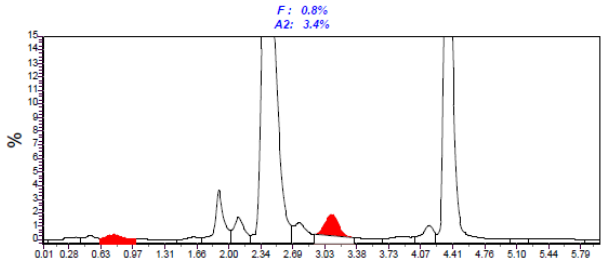


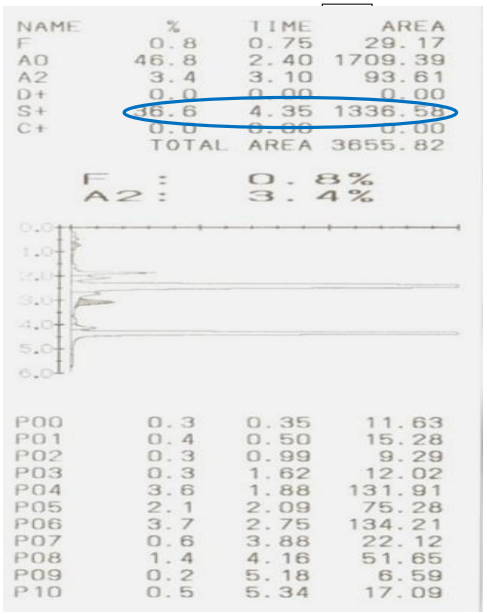
Hb F 0.4 %
Hb A_2 2.8 %
HbC 41.3%

HbC (beta 6(A3) Glu>Lys)

Contextual assessment of hematological data: same window, same percentage but differente complete blood count

RBC	5.02
Hb	15.8
HCT	45.4
MCV	90.5
MCH	31.4
RDW	14.1





NAME	%	TIME	AREA
F	0.8	0.75	29.17
A0	46.8	2.40	1709.39
A2	3.4	3.10	93.61
D+	0.0	0.00	0.00
S+	36.6	4.35	1336.58
C+	0.0	0.00	0.00
TOTAL		AREA	3655.82

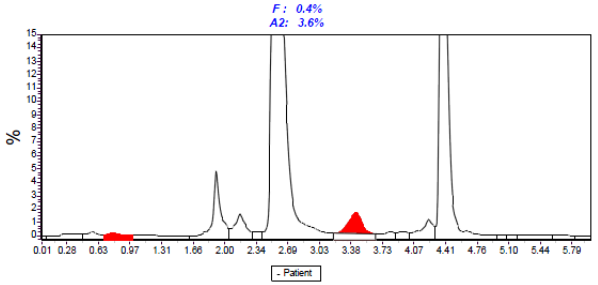
F : 0.8%
A2 : 3.4%

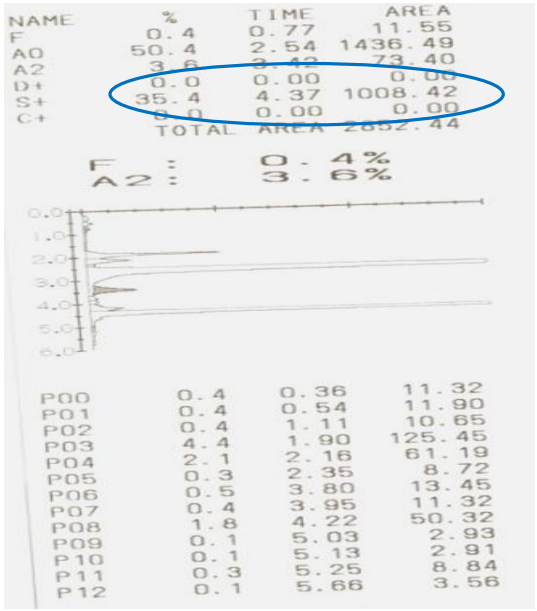
P00	0.3	0.35	11.63
P01	0.4	0.50	15.28
P02	0.3	0.99	9.29
P03	0.3	1.62	12.02
P04	3.6	1.88	131.91
P05	2.1	2.09	75.28
P06	3.7	2.75	134.21
P07	0.6	3.88	22.12
P08	1.4	4.16	51.65
P09	0.2	5.18	6.59
P10	0.5	5.34	17.09

Hb F 0.8 %
Hb A_2 3.4 %
Sw 36.6%

HbS (beta 6(A3) Glu>Val)

RBC	4.62
Hb	12.4
HCT	38.4
MCV	81.0
MCH	28.9
RDW	11.9





NAME	%	TIME	AREA
F	0.4	0.77	11.55
A0	50.4	2.54	1436.49
A2	3.6	3.42	73.40
D+	0.0	0.00	0.00
S+	35.4	4.37	1008.42
C+	0.0	0.00	0.00
TOTAL		AREA	2852.44

F : 0.4%
A2 : 3.6%

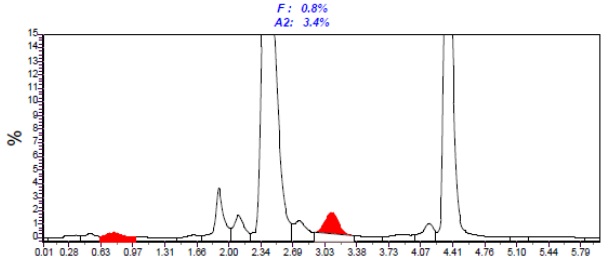
P00	0.4	0.36	11.32
P01	0.4	0.54	11.90
P02	0.4	1.11	10.65
P03	4.4	1.90	125.45
P04	2.1	2.16	61.19
P05	0.3	2.35	8.72
P06	0.5	3.80	13.45
P07	0.4	3.95	11.32
P08	1.8	4.22	50.32
P09	0.1	5.03	2.93
P10	0.1	5.13	2.91
P11	0.3	5.25	8.84
P12	0.1	5.66	3.56

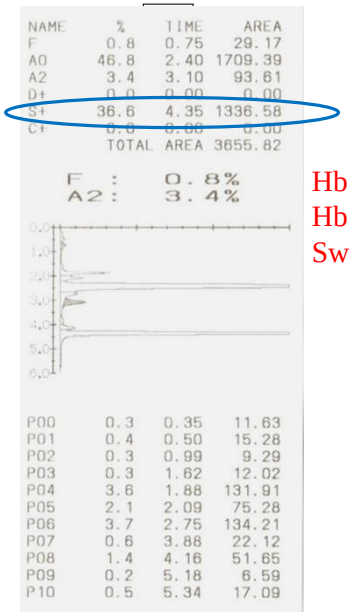
Hb F 0.4 %
Hb A_2 3.6 %
Sw 35.4%

Hb G-San José (beta 7(A4) Glu>Gly)

Contextual assessment of hematological data: same window, differente percentage and differente complete blood count

RBC	5.02
Hb	15.8
HCT	45.4
MCV	90.5
MCH	31.4
RDW	14.1

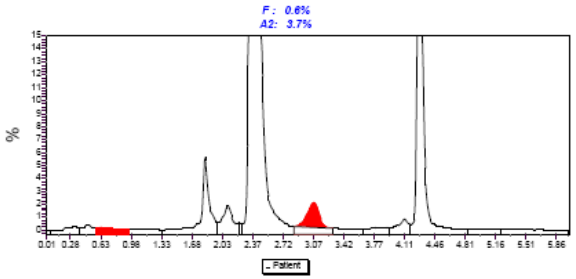




Hb F 0.8 %
Hb A_2 3.4 %
Sw 36.6%

HbS (beta 6(A3) Glu>Val)

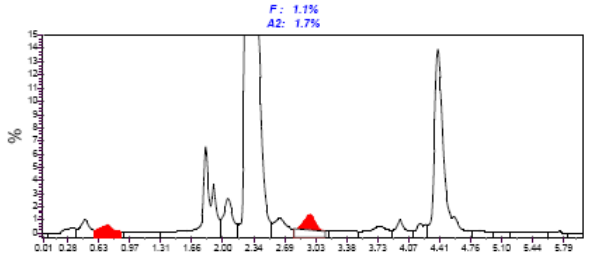
RBC	6.75
Hb	14.4
HCT	46.3
MCV	69.0
MCH	21.3
RDW	14.9

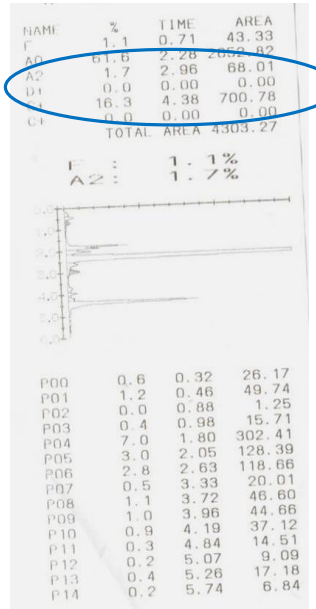


Hb F 0.6 %
Hb A_2 3.7 %
Sw 26.3%

HbS (beta 6(A3) Glu>Val) + α $^{3.7}$ / α $^{3.7}$

RBC	4.17
Hb	11.3
HCT	34.5
MCV	83.0
MCH	27.0
RDW	13.9





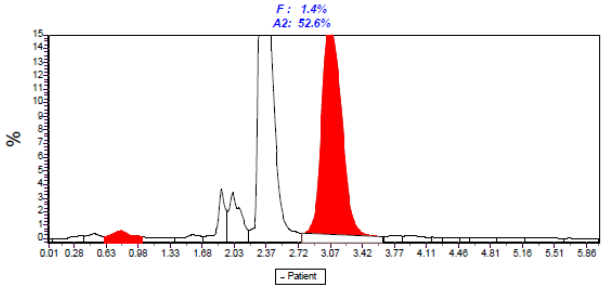
Hb F 1.1 %
Hb A_2 1.7 %
Sw 16.3%

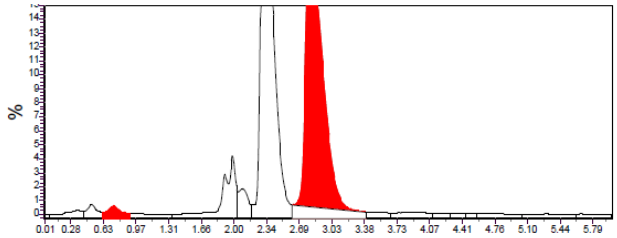
Hb Setif (alpha2 94(G1) Asp>Tyr)

Contextual assessment of hematological data: same window, same percentage and same complete blood count

RBC	4.99
Hb	12.3
HCT	38.2
MCV	86.0
MCH	29.8
RDW	13.9

RBC	4.44
Hb	12.4
HCT	37.4
MCV	84.0
MCH	27.8
RDW	14.7





NAME	%	TIME	AREA
F	1.4	0.78	60.94
A0	48.9	2.33	2181.01
A2	52.6	3.05	1690.47
D+	0.0	0.00	0.00
S+	0.0	0.00	0.00
C+	0.0	0.00	0.00
	TOTAL AREA		4458.73

F : 1.4%
A2 : 52.6%

P00	0.3	0.34	13.91
P01	0.6	0.49	25.40
P02	0.3	1.02	12.61
P03	0.5	1.57	22.62
P04	3.1	1.88	140.13
P05	4.8	2.00	214.20
P06	0.4	3.79	17.75
P07	0.6	3.92	24.95
P08	0.1	4.18	5.06
P09	0.2	4.34	8.29
P10	0.1	4.53	4.03
P11	0.2	4.68	10.84
P12	0.2	4.97	9.40
P13	0.3	5.42	12.88
P14	0.1	5.66	4.25

F 1.4 %
A_2 52.6 %

NAME	%	TIME	AREA
F	0.9	0.72	39.64
A0	48.6	2.31	2241.30
A2	58.9	2.80	1835.90
D+	0.0	0.00	0.00
S+	0.0	0.00	0.00
C+	0.0	0.00	0.00
	TOTAL AREA		4610.47

F : 0.9%
A2 : 58.9%

P00	0.4	0.34	19.65
P01	0.8	0.48	36.66
P02	0.3	0.90	12.30
P03	5.4	1.98	250.86
P04	2.0	2.09	93.57
P05	0.6	3.96	29.10
P06	0.2	4.17	8.24
P07	0.2	4.34	7.29
P08	0.1	4.46	4.29
P09	0.3	4.63	14.20
P10	0.3	5.28	13.57
P11	0.1	5.66	3.91

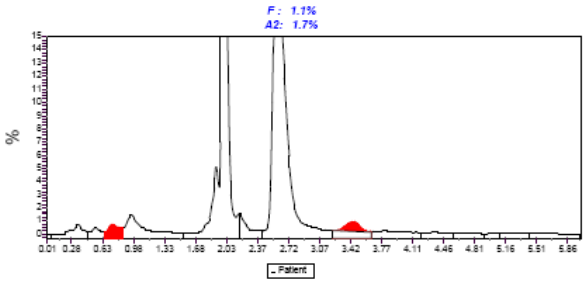
F 0.9 %
A_2 58.9 %

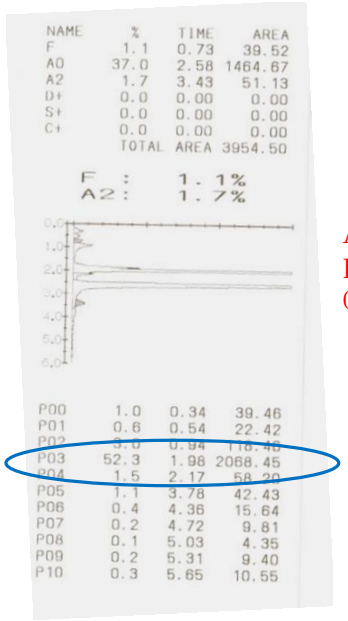
Hb G-Copenhagen (beta 47(CD6) Asp>Asn)

Hb G-Coushatta (beta 22(B4) Glu>Ala)
o Hb G-Saskatoon

Contextual assessment of hematological data: similar window, similar percentage and same complete blood count

RBC	4.66
Hb	13.5
HCT	41.2
MCV	88.0
MCH	28.9
RDW	12.7

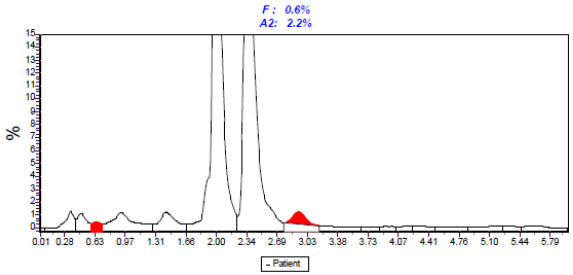


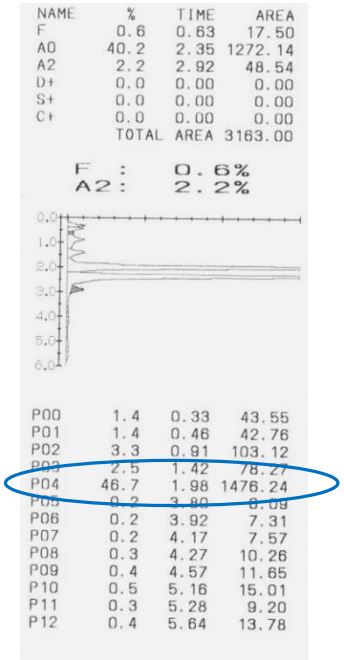


F 1.1 %
A_2 1.7 %
P03 52.3%
(r.t.=1.98)

Hb Camden (beta 131(H9) Gln>Glu)

RBC	5.04
Hb	14.9
HCT	46.1
MCV	91.5
MCH	29.6
RDW	13.5

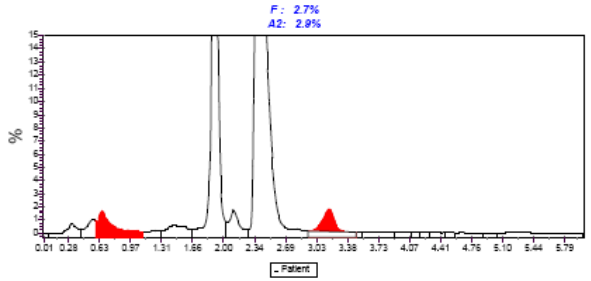


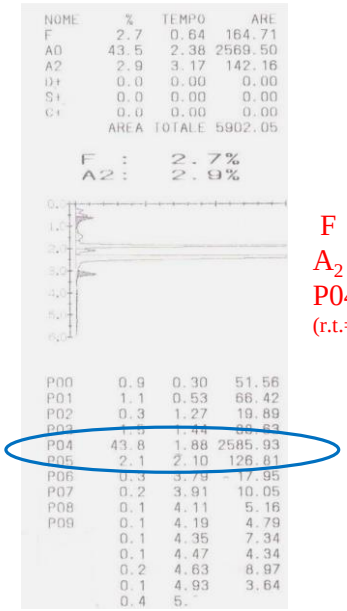


F 0.6 %
A_2 2.2 %
P04 46.7%
(r.t.=1.98)

Hb Santa Clara (beta 97(FG4) His>Asn)

RBC	5.05
Hb	16.1
HCT	47.2
MCV	89.0
MCH	31.0
RDW	11.7





F 2.7 %
A_2 2.9 %
P04 43.8%
(r.t.=1.88)

Hb Camperdown (beta 104(G6) Arg>Ser)

ATYPICAL PHENOTYPES

-1- HbA_2 borderline with normocytosis or microcytosis

-2- Microcytosis with normal levels of HbA_2 ed HbF

-3- Microcytosis with very high level of HbA_2

-4- Increased levels of HbF

INCREASED LEVEL OF HBF

- Normal HbA_2 levels, Increased HbF levels (2% - 30%)

Phenotypic classification

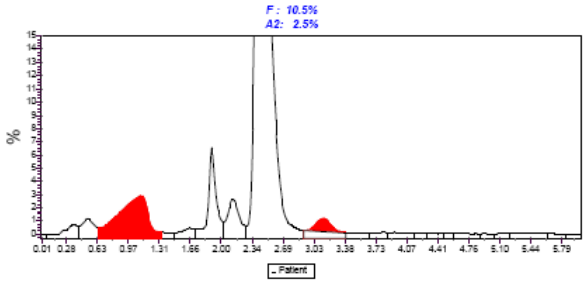
- " **Thalassemic mutations**" with reduced MCV and MCH values and heterocellular HbF distribution.
- " **Hereditary persistence of HbF (HPFH)**" with normal MCV and MCH values and pancellular HbF distribution.

	$^{G}\gamma\ ^{A}\gamma$HPFH	$^{G}\gamma\ ^{A}\gamma(\delta\beta^{0})$talassemia
Erythrocytes morphology	normal	reduced
MCH	almost normal	reduced
HbF	15-30%	4-18%
Distribution of HbF	pancellular	heterocellular

- There are about 130 known point mutations of γ-globin genes
- About 20 affected the γ-globin gene promoter resulting in increased HbF level

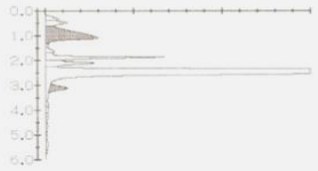
Mutazione	Gene	Popolazioni interessate	Catene Gγ	Catene Aγ	Distribuzione Hb	Hb F nell'eterozigote
delezione da -225 a -222 (delez. di 4 bp)	Aγ	Africani	66%	—		6-7%
delezione da -225 a -222 (delez. di 4 bp)	AγT	Sardi				
da -203 a –200 +CCCC	Gγ	Tunisini				
-202 C→G	Gγ	Africani	100%	—	pancellulare	15-20%
-202 C→T	Aγ	Africani	10%	90%		3%
-198 T→C	Aγ	Inglesi	10%	90%	eterocellulare	4-12%
-196 C→T	Aγ	Italiani (Sardi) *Cinesi*	5%	95%	pancellulare	10-15%
-195 C→G	Aγ	Brasiliani	14%	86%		4-5%
-175 T→C	Aγ	*Afro Americani*	—	*100%*	*pancellulare*	17-38%
-175 T→C	Gγ	Afro Americani *Inglesi* Italiani (Sardegna sett.)	100%	—		28-29%
-161 G→A	Gγ	Africani				1-2%
-158 C→T	Gγ	Arabi Sauditi Afro Americani	100%	—	eterocellulare	non aumentata
-158 C→T	GGγ	Afro Americani di Atlanta	100%	—	eterocellulare	2-5%
-117 G→A	Aγ	Greci Italiani (Sardegna sett.)	10%	90%	pancellulare	8-10%
-114 C→G	Gγ	Australiani				8.6%
-114 C→T	Gγ	Giapponesi				11-14%
-114 C→T (tipo Georgia)	Aγ	*Afro Americani*	—	100%		3-6%
delezione da -114 a -102 (delez. 13 bp)	Aγ	*Africani*	—	100%		30%
mutazione sconosciuta		*Svizzeri*	*40%*	*60%*	eterocellulare	3-8%
3 mutazioni dell'enhancer 3' gene Aγ	Aγ	Afro Americani di Seattle	50%	50%	eterocellulare	4-8%
traslocazione di segmenti dei cromosomi 6, 9, 11, 20 con un breakpoint nella regione del locus β (nessuna delezione)		?	36%	64%	eterocellulare	5-8%
-110 A→C	Gγ	Cecoslovacchi	95%	5%		0.8-1.0%

RBC	5.5
Hb	12.5
HCT	39.3
MCV	65.0
MCH	22.5
RDW	16.3



NAME	%	TIME	AREA
F	10.5	1.10	275.47
A0	72.1	2.43	1939.00
A2	2.5	3.13	50.13
D+	0.0	0.00	0.00
S+	0.0	0.00	0.00
C+	0.0	0.00	0.00
	TOTAL	AREA	2687.53

F : 10.5%
A2: 2.5%



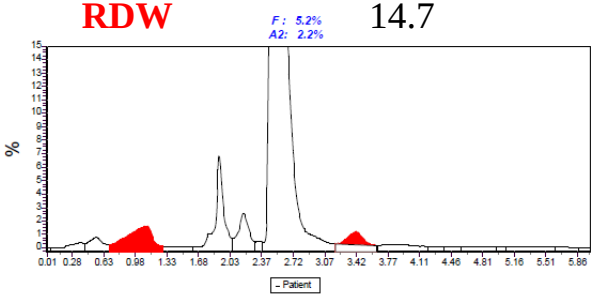
P00	1.0	0.36	26.15
P01	1.9	0.51	51.21
P02	0.2	1.33	4.38
P03	0.7	1.64	19.76
P04	6.2	1.88	165.40
P05	4.0	2.13	107.71
P06	0.2	3.80	6.06
P07	0.3	3.96	8.24
P08	0.1	4.18	3.67
P09	0.2	4.35	4.29
P10	0.1	4.57	2.19
P11	0.2	4.68	5.87
P12	0.1	4.93	2.60
P13	0.1	5.18	3.11
P14	0.4	5.47	9.56
P15	0.1	5.66	2.74

Sicilian Delta-beta

(deletion of 13378 nts from the delta gene to beta gene)

♀pregnant

RBC	3.89
Hb	11.3
HCT	33.9
MCV	91.0
MCH	30.1
RDW	14.7



NAME	%	TIME	AREA
F	5.2	1.09	166.73
A0	78.6	2.49	2679.04
A2	2.2	3.40	57.46
D+	0.0	0.00	0.00
S+	0.0	0.00	0.00
C+	0.0	0.00	0.00
	TOTAL	AREA	3406.45

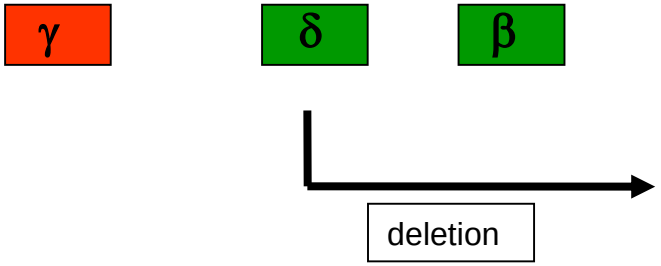
F : 5.2%
A2: 2.2%

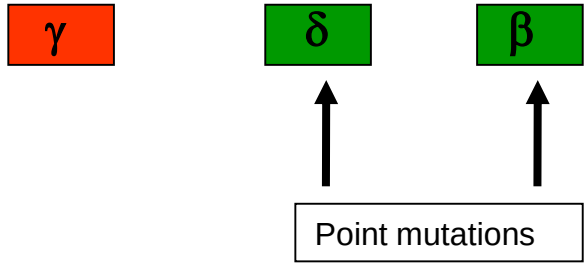
P00	0.6	0.37	21.10
P01	1.3	0.54	43.51
P02	0.2	1.28	8.42
P03	6.8	1.89	230.24
P04	3.3	2.16	113.43
P05	0.4	2.34	14.48
P06	1.0	3.78	33.23
P07	0.2	4.27	5.71
P08	0.1	4.44	4.84
P09	0.3	4.75	10.66
P10	0.1	5.03	2.22
P11	0.3	5.28	11.91
P12	0.1	5.65	3.46

γ^G -158 etero

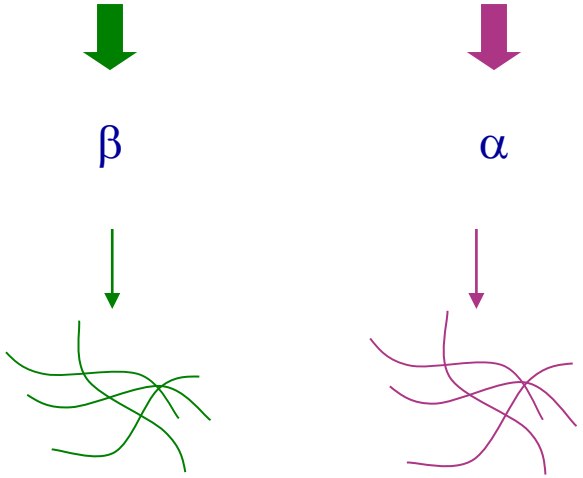
δβ-Microcitemie

	Detetion δβ–Thal	δ+β Thalassemia
RBC	5.50	5.50
Hb	13.5	13.5
MCV	→ 75.0	→ 64.0
HbA_2	→ 2.40%	→ 3.0-3.8%
HbF	→ 12-15%	→ < 1%





INTERACTIONS BETWEEN BETA AND ALPHA MUTATIONS



Reducing the imbalance between the chains α/β

↑ **MCV**

= HbA_2

$\alpha+\beta^{+}$

RBC	5.81	5.77
HB	12.1	12.6
HCT	37.0	38.7
MCV	64.5	68.6 ↑
MCH	20.9	21.8
MCHC	32.8	32.5
RDW	14.8	15.4
Hb A_2	5.1%	5.3% =
Hb F	<0.1%	<0.1%

$\beta^{IVSI\ nt110}/\beta$

$\beta^{IVSI\ nt110}/\beta$ + $\alpha^{Hphl}\alpha/\alpha\alpha$

RBC	5.07	5.61
HB	12.4	15.3
HCT	39.0	45.3
MCV	73.4	80.8 ↑
MCH	22.4	27.3
MCHC	32.4	33.8
RDW	13.1	13.2
Hb A	6.1%	6.0% =
Hb F	1.4%	0.7%

β^{-87G}/β

β^{-87G}/β + $\alpha^{3.7\ I}/\alpha\alpha$

$\alpha+\beta^{++}$

RBC	5.27	4.26	4.13	4.77
HB	11.9	11.6	11.9	12.9
HCT	37.6	34.6	35.1	35.3
MCV	71.3	81.2 ↑	84.9	82.3
MCH	22.5	27.1	28.7	28.5
MCHC	31.6	33.4	33.9	31.5
RDW	14.0	14.3	12.7	12.9
Hb A_2	4.0%	3.9% =	3.8%	3.9%
Hb F	0.9%	0.7%	1.9%	1.5%

$\beta^{\text{IVSI nt 6}}/\beta$ | $\beta^{\text{IVSI nt 6}}/\beta$ + $\alpha^{3.7\ I}/\alpha\alpha$ | β^{-101}/β | β^{-92}/β

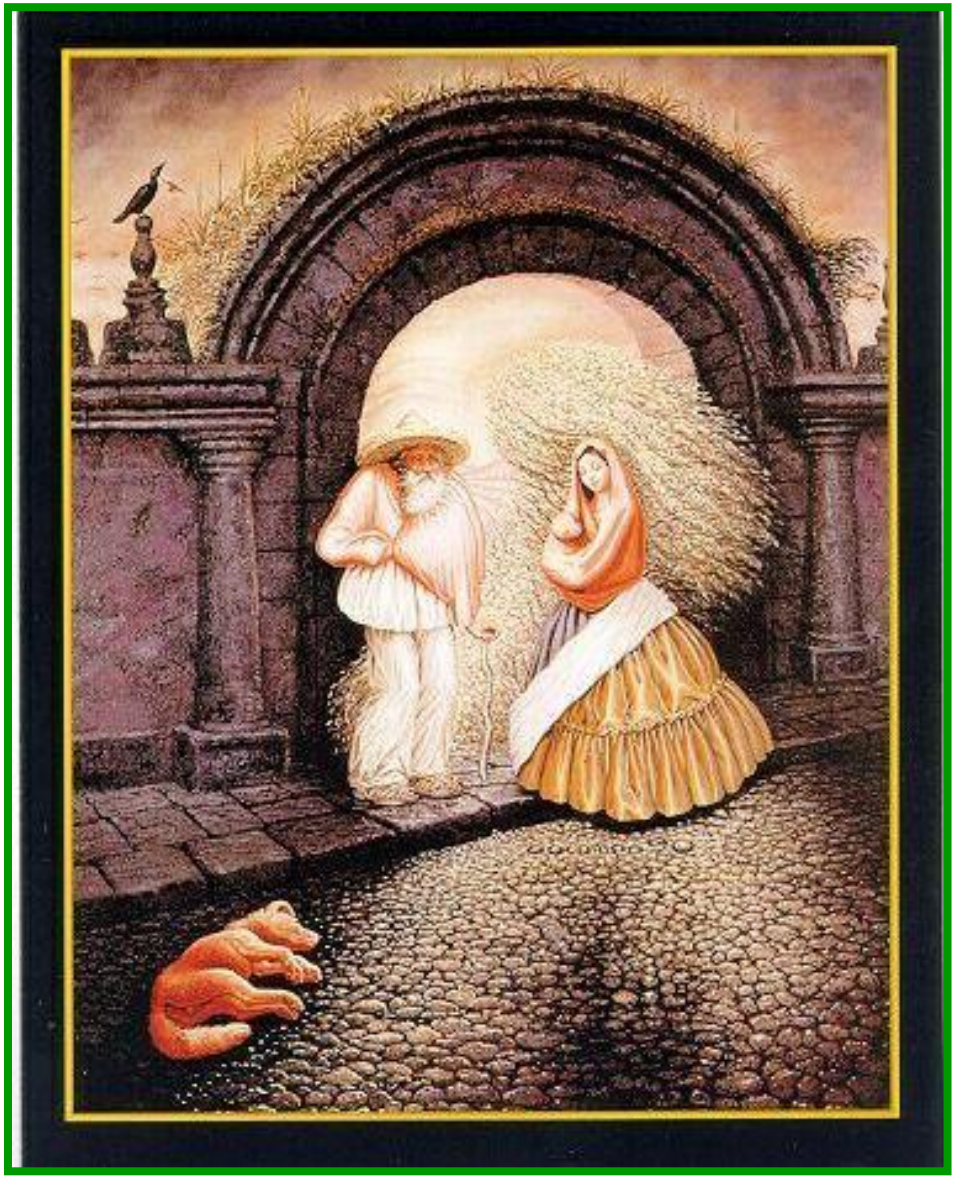
- From the integration of the hematological and hemoglobinic (HPLC) status, the first level study makes it possible to obtain a diagnosis, in most cases, "***presumptive***".

- In the case of classical carriers of beta-thalassemia and delta-thalassemia, the first level study is «***exhaustive»***.

- In the case of alpha-thalassemia carriers, the first-level study provides only a "***suspect***" that must then be confirmed with the molecular.

- In the case of variants the molecular analysis must always be performed for the definitive diagnosis, even if the careful observation of the blood count and of the HPLC graph (Retention Time and Peak Percentage) is possible to suspect the presence of a given variant.

Concluding ….



«The whole is more than the sum of the individual parts»