Emoglobinuria Parossistica Notturna

Focus Clinico e Update Diagnostico

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Il Progetto REL-TRE: Conclusioni dello Studio

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Sistema Sanitario Regione Lombardia

Rete Ematologica Lombarda per lo screening con Test Rapido dell'EPN

REL-TRE 1° Parte:

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ORIGINAL ARTICLE

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Multicenter validation of a simplified method for paroxysmal nocturnal hemoglobinuria screening

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Abstract

Background: Paroxysmal nocturnal hemoglobinuria (PNH) diagnostic guidelines recommend single-tube five- to six-color or two-tube four-color assays. PNH clones are detectable in only a fraction of patients at risk, and screening for new PNH cases can be complex and expensive. In this multicenter study, we have validated a simplified, one-tube two-color FLAER-based assay suitable for PNH screening.

Methods: Six laboratories received samples containing spiked PNH leukocyte clones to be analyzed in parallel with a common six-color cocktail (FLAER/CD24/CD45/CD64/CD15/CD14) and a simplified two-color mixture (FLAER/CD15), a shared calibration procedure, and a common analysis protocol. Replicate precision and sensitivity tests were performed on PNH patients, from undiluted to 1:10 000. Specificity tests were performed on normal donors to identify the possible sources of artifacts.

Results: The performance comparison between six-color and two-color assays showed an excellent agreement for granulocyte PNH clones. Dilution experiments showed an accurate detectability down to 0.01% sensitivity level for granulocyte PNH clones and to 1% for monocytes. Specificity experiments disclosed that basophils and platelets can contaminate the monocyte gate and generate false PNH events.

Conclusions: A simplified two-color (FLAER/CD15) PNH screening test has been validated in a highly standardized multicenter study and proved feasible and effective in ongoing regional programs. Precision, sensitivity, and specificity of the simplified test for granulocytes were comparable to the more complex and expensive six-color assay and applicable for screening also in peripheral laboratories. The diagnostic confirmation of PNH should be always performed by a reference center using the established technique on all cell lineages.

KEYWORDS
aplastic anemia and bone marrow failure, myelodysplastic syndromes, red cell disorders

21 Laboratori di Citometria Lombardi Partecipanti alla Seconda Fase del Progetto REL-TRE

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ASST Valleolona-Gallarate	Lorella Poli
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H Mantova	Chiara Rossi
H Manzoni Lecco	Irene Guarnori
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Spedali CIvili Brescia	Marco Chiarini

REL-TRE 2° Parte:

Diffusione della metodologia semplificata ai laboratori di citometria di 21 Ematologie, Centri Trasfusionali e Laboratori Analisi Lombardi per lo screening capillare delle condizioni sospette per PNH.

I partecipanti hanno ricevuto un'aliquota di cocktail FLAER Alexa 488 con:

-CD15 APC *(15 Utilizzatori Becton-Dickinson)*
-CD15 PE-Cy5 *(6 Utilizzatori Beckman Coulter)*

Bastante per i primi 10 test di screening.

Progetto REL-TRE

L'obiettivo dello studio è la valutazione della frequenza di cloni con fenotipo da Emoglobinuria Parossistica Notturna in soggetti ad alto rischio, con l'utilizzo di uno screening citometrico 'minimalista' a due colori.
I test saranno effettuati su campioni di pazienti con diagnosi nuova o nota (ove non sia già stato eseguito il test di ricerca del clone EPN) di una o più delle seguenti condizioni:

Anemia emolitica Coombs-negativa	Citopenia refrattaria uni-, bi- o tri-lineare	Mielodisplasia a Basso Grado	Trombosi venose atipiche
▪ LDH ≥ 1,5 volte il Limite normale . ▪ E/O aumento dei Reticolociti. ▪ E/O diminuzione dell'aptoglobina.	▪ Emoglobina < 10 g/dL negli uomini e < 9 g/dL nelle donne ▪ Piastrine < 100.000/µL ▪ Leucociti < 3000/µL	▪ Definita secondo la classificazione WHO	▪ In siti atipici (vene epatiche, vena porta, vena splenica, altre vene splancniche, vene dei seni cerebrali, vene dermiche). ▪ E/O con citopenia inspiegata concomitante. ▪ E/O recidivanti in terapia anticoagulante.

CRITERI DI ESCLUSIONE :

- Test di Coombs Diretto positivo
- Osservazione nello striscio periferico di sferociti, schistociti o drepanociti
- Anemie o citopenie carenziali o da altre cause
- Trombosi arteriose

Progetto della Rete Ematologica Lombarda per lo Screening con Test Rapido dell'EPN: Progetto REL-TRE

MATERIALI E METODI

LABORATORI BD

Laboratori con strumentazione Becton Dickinson:
Pannello anticorpale a due marcatori,
composto da FLAER-Alexa 488 e CD15-APC.

LABORATORI BC

Laboratori con strumentazione Beckman Coulter:
Pannello anticorpale a due marcatori,
composto da FLAER-Alexa 488 e CD15-PE-Cy5.

Preparazione, Acquisizione e Analisi dei campioni

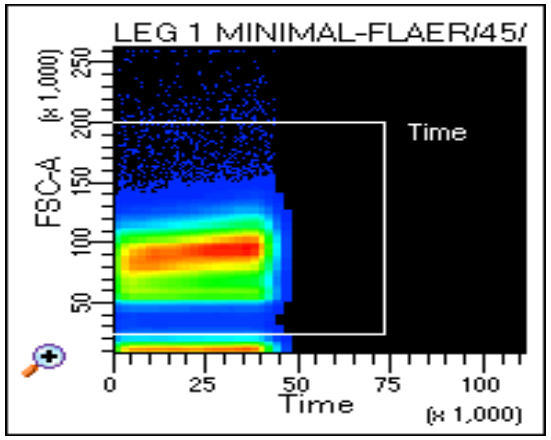
- Dispensare accuratamente 100 µl di sangue periferico con 15 µl della miscela anticorpale fornita centralmente costituita da FLAER e CD15.
- Vortexare delicatamente
- Incubare per 30 min a temperatura ambiente e al buio
- Lisare utilizzando la propria procedura
- Dopo 10 minuti circa, centrifugare a 1.500 rpm per 5min
- Allontanare delicatamente il sopranatante e risospendere in 2 mL di PBS
- Centrifugare a 1.500 rpm per 5min e risospendere in 500 µL di PBS
- Acquisire almeno 250.000 eventi, avendo cura di escludere i detriti, così da raggiungere un livello di LOD pari allo 0.01%.

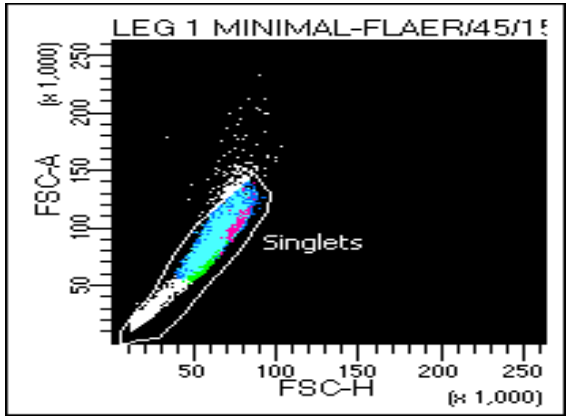
Un cluster costituito da almeno 20 eventi FLAER-negativi è considerato significativo per l' identificazione di un clone PNH. L'analisi prevede un gate FSC-A/FSC-H per la rimozione dei doppietti, un gate SSC/FSC per l'eliminazione di detriti e infine un gate SSC/CD15 con cui definire la componente granulocitaria (SSC alto/ CD15++) e il comparto monocitario (SSC intermedio/ CD15+-). Durante l'acquisizione si consiglia di verificarne la stabilità mediante l'utilizzo di un dot plot che includa il tempo.

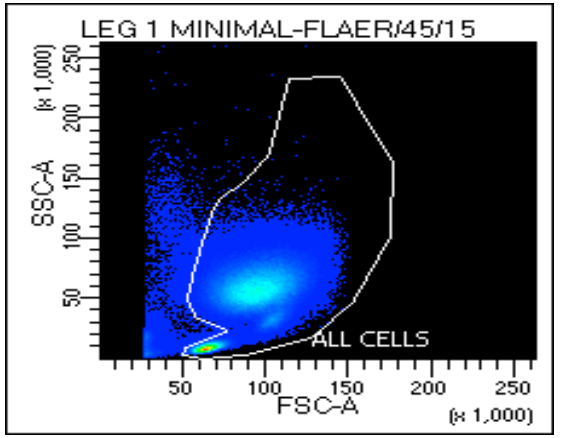
Ogni campione risultato positivo al test 'minimalista' di screening deve essere confermato con un pannello esteso presso il centro di riferimento di malattie rare (U.O. di Ematologia dell'Ospedale di Cremona)

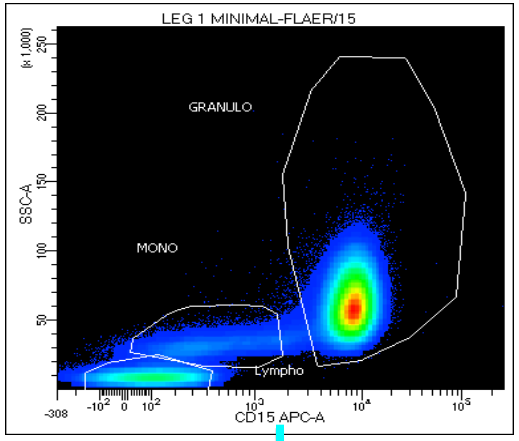
I centri inseriranno i test effettuati, sia positivi che negativi, nell'archivio elettronico Clonoteca EPN - www.clonotecaepn.it , previa registrazione.

Common Template of Analysis

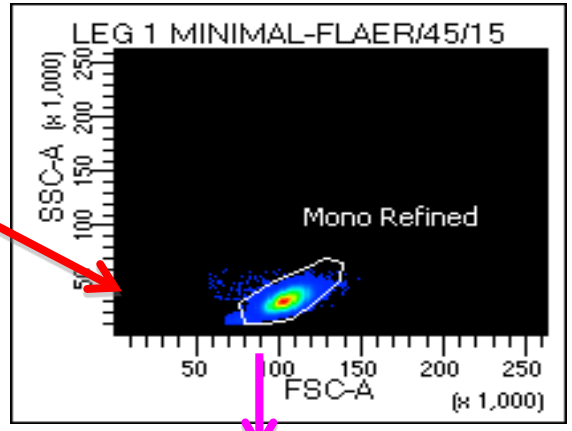


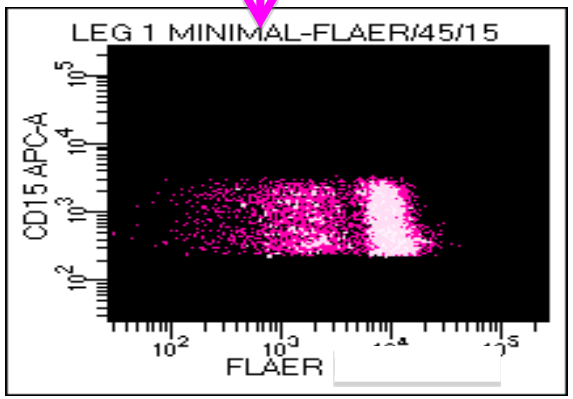


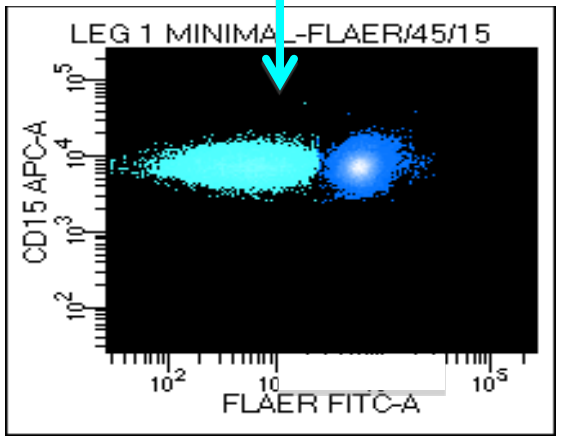




Backgating on monocytes to refine the gate







Tube: FLAER/15

Population	#Events	%Parent	%Total
All Events	250,000	####	100.0
Time	222,392	89.0	89.0
Singlets	202,294	91.0	80.9
ALL CELLS	192,938	95.4	77.2
GRANULO	142,735	74.0	57.1
PNH Granulo	27,691	19.4	11.1
MONO	143	0.1	0.1
Mono Refined	98	68.5	0.0
PNH Mono	13	13.3	0.0
Lympho	28,653	14.9	11.5

Clonoteca Data Collection 2018

REL-TRE Clonoteca Data Collection 2018

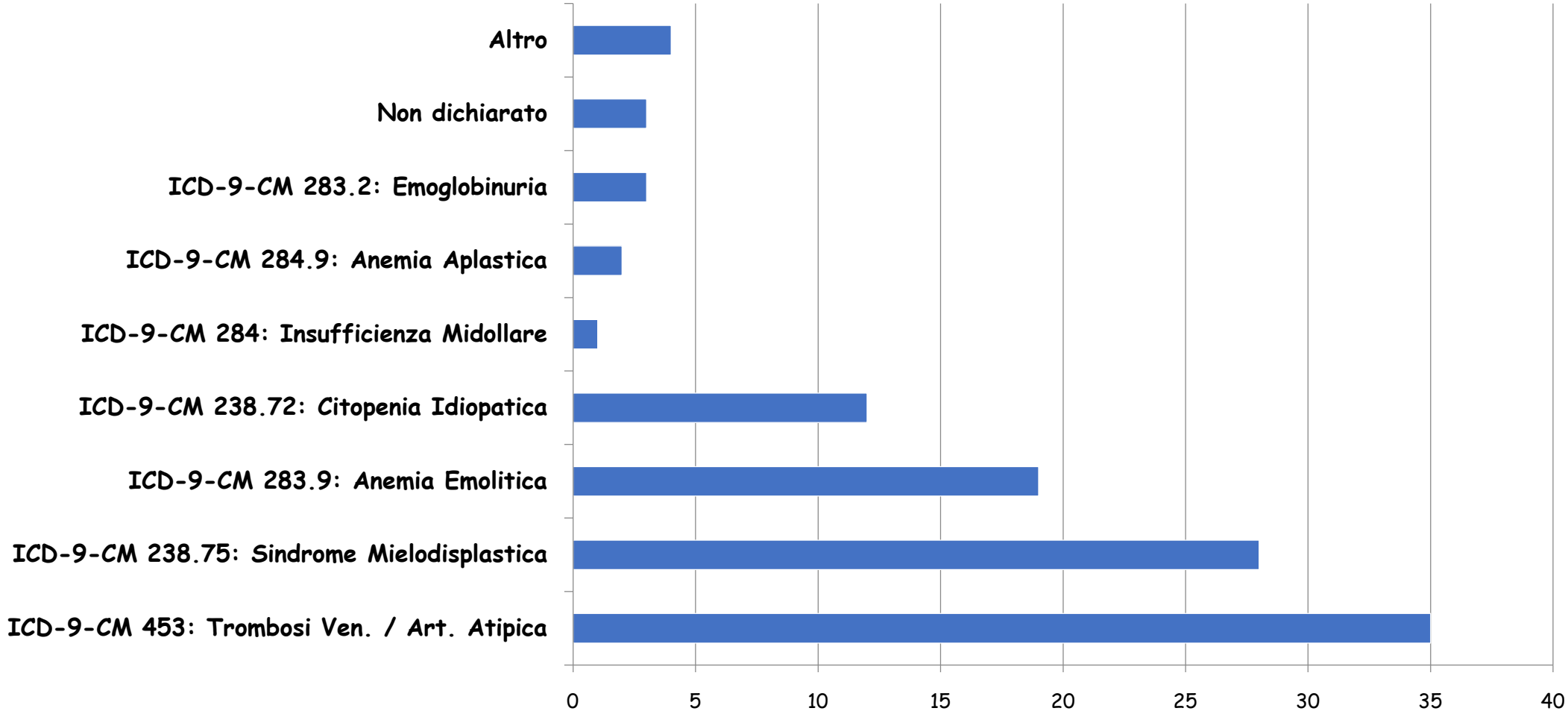
21 Laboratori di Citometria Lombardi Partecipanti alla Seconda Fase del Progetto REL-TRE...

Adesione di 9 centri all'inserimento in Clonoteca "Progetto speciale REL-TRE "

Data Collection

- **9** laboratories
- **107** new **reasons for testing** included into "Clonoteca Progetto Speciale REL-TRE"
- **8** retests
- **13** cases were **new PNH clones** (12.1%)

107 New Reasons For Testing

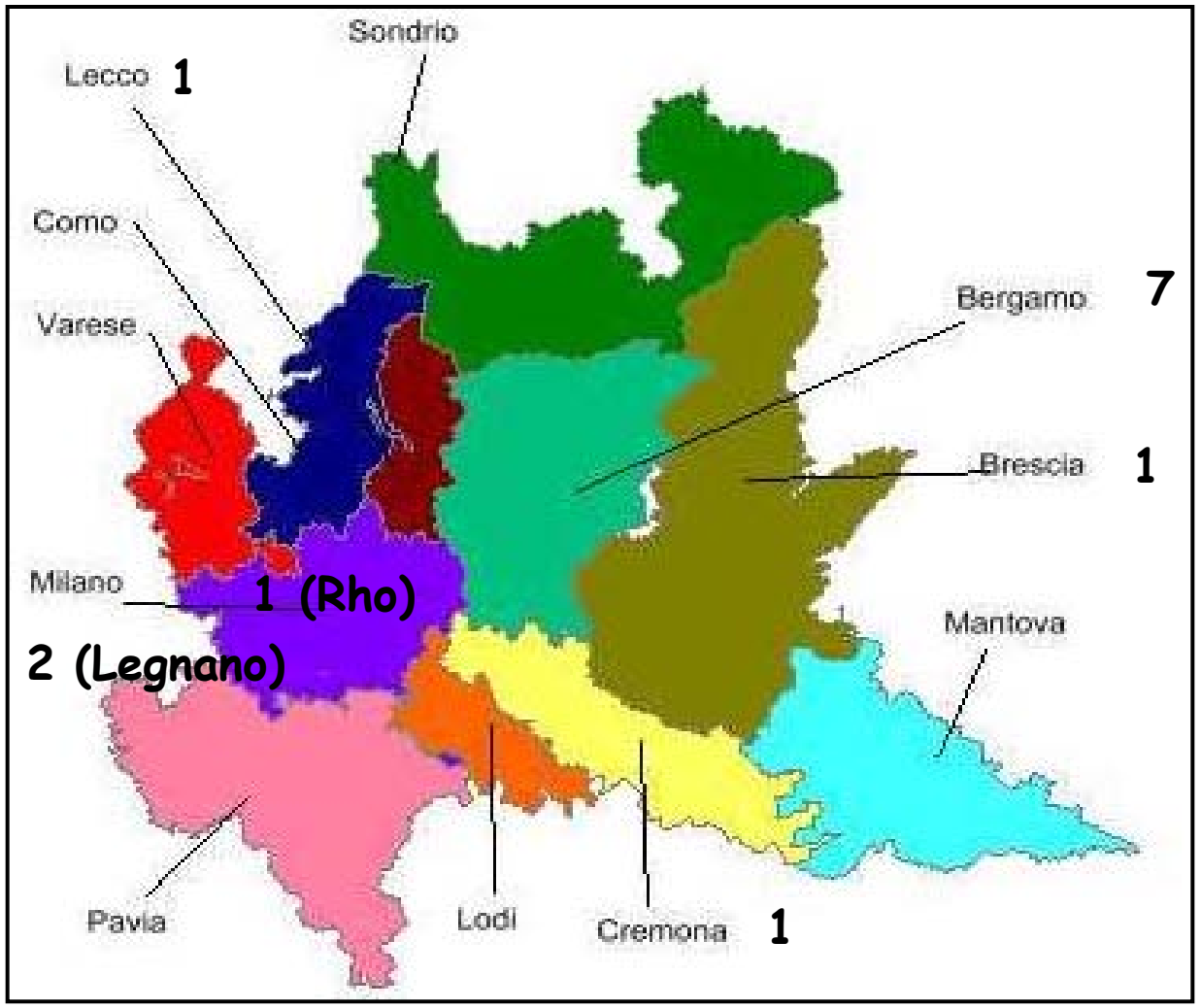


Frequency of PNH positive cases according to the reason for testing

PNH+ Reasons for Testing	
ICD-9-CM 238.75: Sindrome Mielodisplastica	5
ICD-9-CM 238.75: Sindrome Mielodisplastica,ICD-9-CM 284.9: Anemia Aplastica	1
ICD-9-CM 238.75: Sindrome Mielodisplastica,ICD-9-CM 284: Insufficienza Midollare	1
ICD-9-CM 284: Insufficienza Midollare	2
ICD-9-CM 238.72: Citopenia Idiopatica	1
ICD-9-CM 284.9: Anemia Aplastica	1
ICD-9-CM 283.2: Emoglobinuria	1
ICD-9-CM 283.9: Anemia Emolitica	1

Patients with hematological disorders (85%)

Distribution of PNH clones



Frequency of PNH positive cases according to the Medical indications that triggered for PNH Screening in Spanish laboratories

Medical indications for PNH screening	Spanish laboratories (n = 1,718)
Individuals with clinical & biological signs/symptoms of PNH in the absence of a previous hematological disorder	**4.0%**
Hemoglobinuria	25.0%
Hemolytic anemia	7.8%
Subtotal hemolysis	*9.4%*
Unexplained cytopenias including anemia	5.6%
Unexplained cytopenias without anemia	3.2%
Anemia, not otherwise specified	1.4%
Subtotal cytopenia	*3.6%*
Thrombosis with non-hemolytic anemia and/or other cytopenias	7.3%
Thrombosis without anemia and/or other cytopenia	0.8%
Subtotal thrombosis	*1.7%*
Iron deficiency	0%
Other	0%
Subtotal others	*0%*
Patients with hematological disorders	**21.7%**
Aplastic/hypoplastic anemia	35.2%
Myelodysplastic syndrome	9.7%
Subtotal BM failure	*25.2%*
Chronic myeloproliferative neoplasm	5.3%
Other hematological and/or immunological disorders	0%
Subtotal other non-BM failure disorders	*1.1%*
Total	**10.1%**

M. Morado et al. Cytometry Part B 2017; 92B: 361- 370

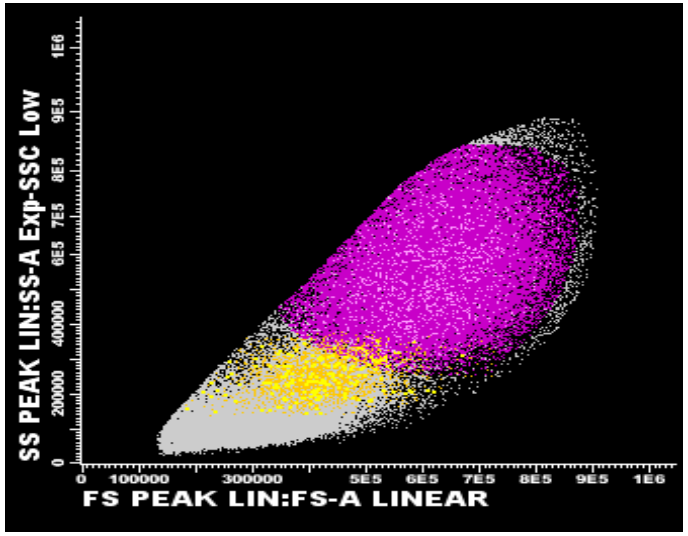
The Size of PNH clones

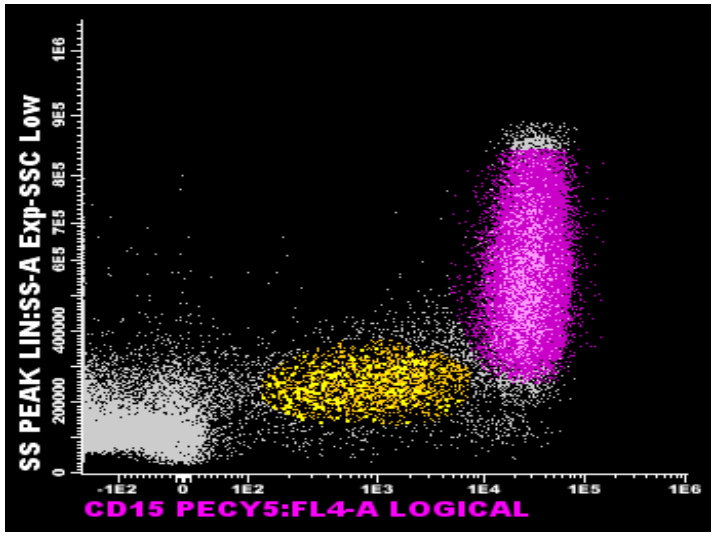
Reasons for Testing PNH+	PNH clone type III (%)
ICD-9-CM 284: Insufficienza Midollare	5
ICD-9-CM 238.75: Sindrome Mielodisplastica	0,25
ICD-9-CM 238.75: Sindrome Mielodisplastica	0,1
ICD-9-CM 238.72: Citopenia Idiopatica	2,9
ICD-9-CM 284: Insufficienza Midollare	10,2
ICD-9-CM 283.9: Anemia Emolitica	60
ICD-9-CM 238.75: Sindrome Mielodisplastica	37
ICD-9-CM 238.75: Sindrome Mielodisplastica,ICD-9-CM 284.9: Anemia Aplastica	0,1
ICD-9-CM 284.9: Anemia Aplastica	2,7
ICD-9-CM 238.75: Sindrome Mielodisplastica,ICD-9-CM 284: Insufficienza Midollare	0,015
ICD-9-CM 284: Insufficienza Midollare	71,8
ICD-9-CM 283.2: Emoglobinuria	98
ICD-9-CM 238.75: Sindrome Mielodisplastica	70

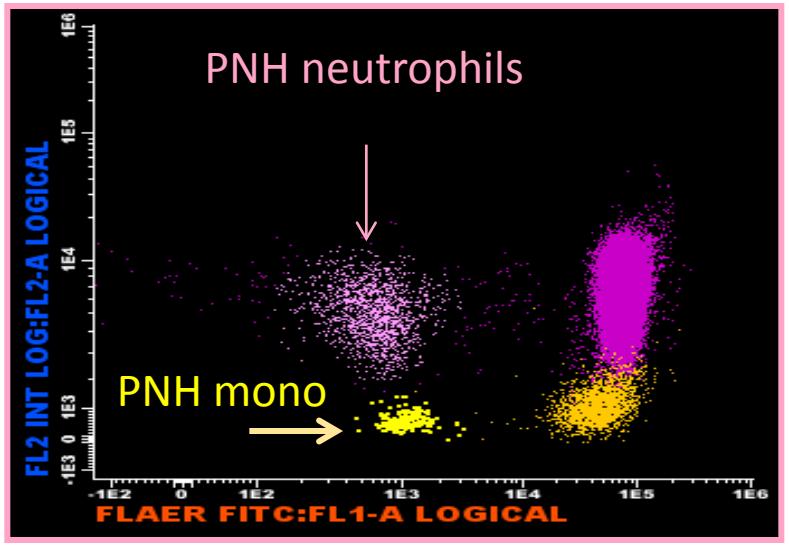
2 Colors Screening approach (REL-Tre) versus MFC (6 colors) analysis in 5 PNH+ cases

	2 color screening		MFC (6 color)	
	granulo%	mono%	granulo%	mono%
ICD-9-CM 238.75: Sindrome Mielodisplastica	0,4	2,1	0,5	2,1
ICD-9-CM 238.75: Sindrome Mielodisplastica	0,1	0,5	0,1	0,2
ICD-9-CM 238.72: Citopenia Idiopatica	2.9	7.54	2,9	3,4
ICD-9-CM 284: Insufficienza Midollare	5	4,30	5	4,30
ICD-9-CM 238.75: Sindrome Mielodisplastica	0,7	63,2	0,3	70

Case n° SAGI1966M (ASST Spedali Civili di Brescia)





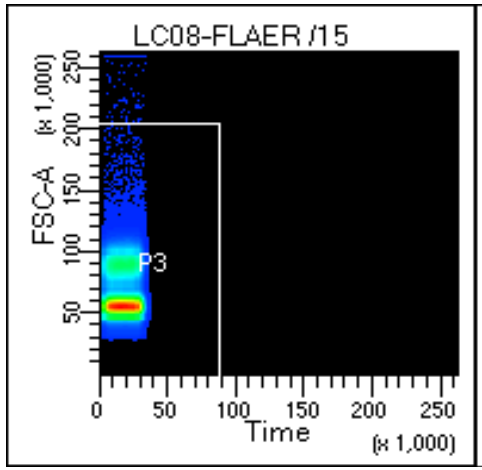


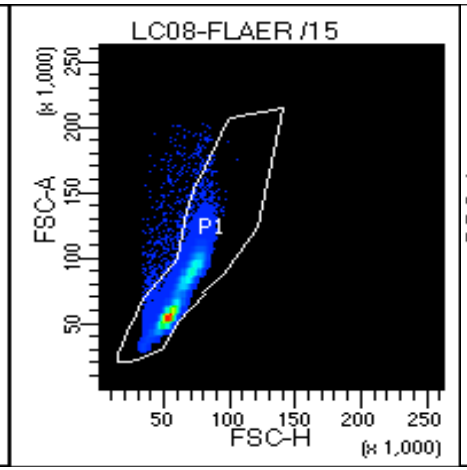
Infinicyt Strategy Analysis

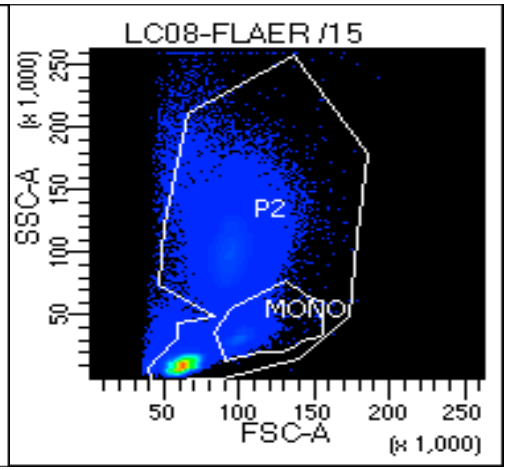
Neutrophils events: 47363
PNH%: 2.94 (1433 events)

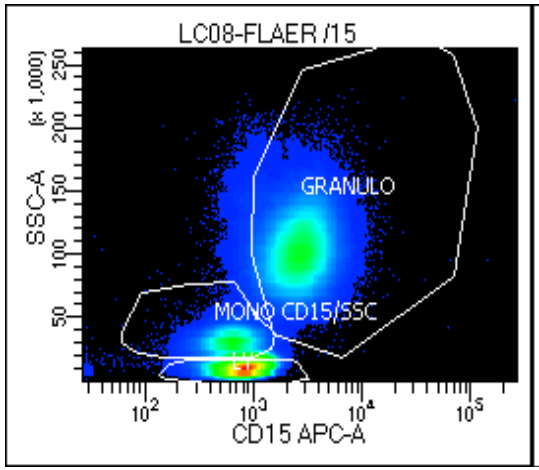
Monocytes events: 2164
PNH%: 7.76% (168 events)

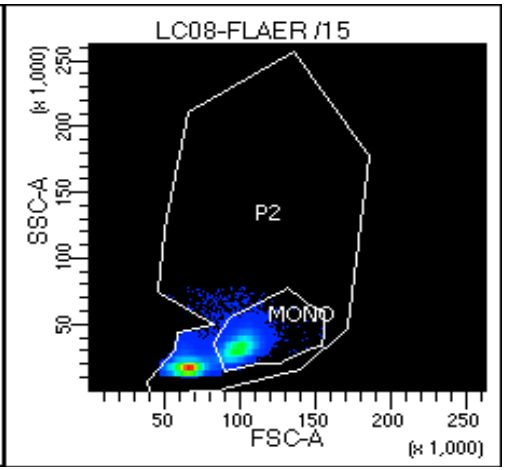
Case n° LCO81946M (Ospedale A. Manzoni Lecco) 2 colors Screening Analysis

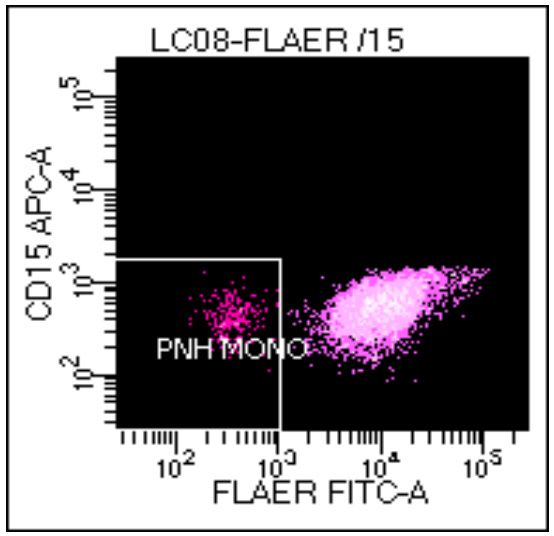


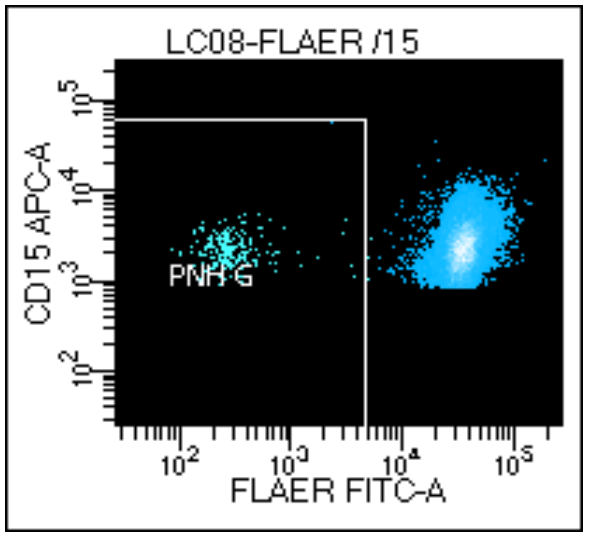








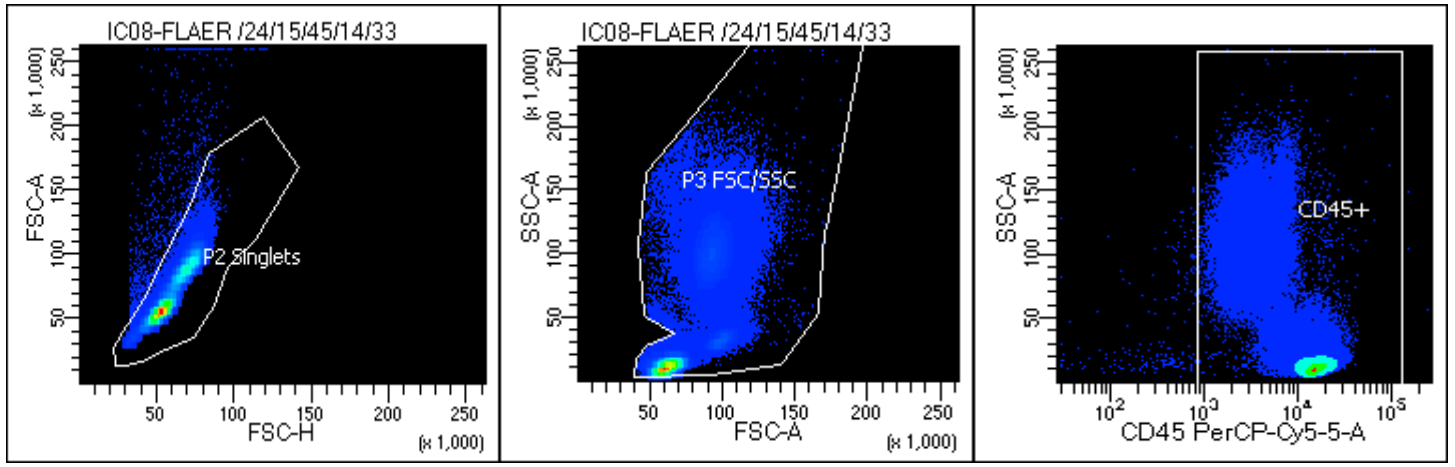


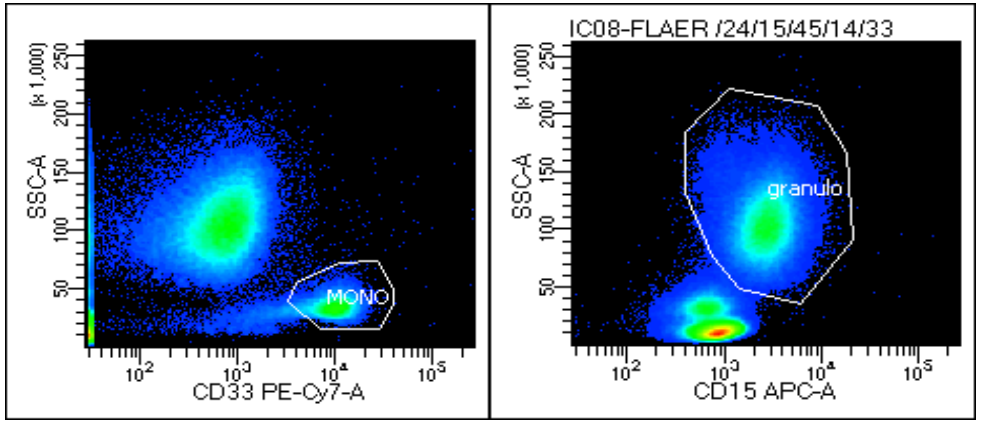


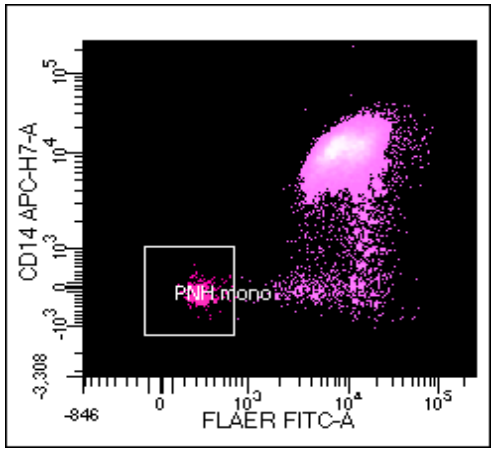
Tube: FLAER /15

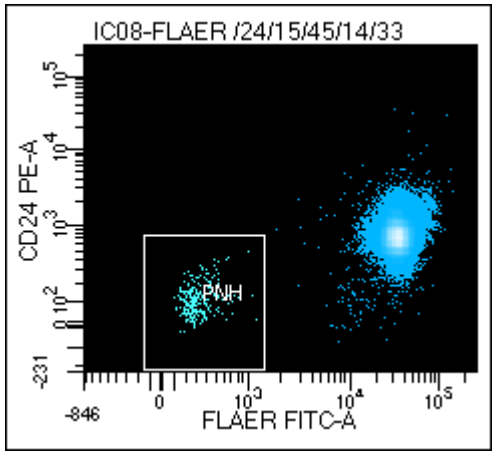
Population	#Events	%Parent	%Total
All Events	161,391	####	100.0
P3	161,215	99.9	99.9
P1	160,597	99.6	99.5
P2	153,534	95.6	95.1
GRANULO	50,035	[illegible]	31.0
PNH G	223	0.4	0.1
LY	64,057	[illegible]	39.7
MONO CD15/SSC	24,894	16.2	15.4
MONO	11,101	44.6	6.9
PNH MONO	234	2.1	0.1

Case n° LCO81946M (Ospedale A. Manzoni Lecco) 6 colors MFC Analysys









Tube: FLAER /24/15/45/14/33

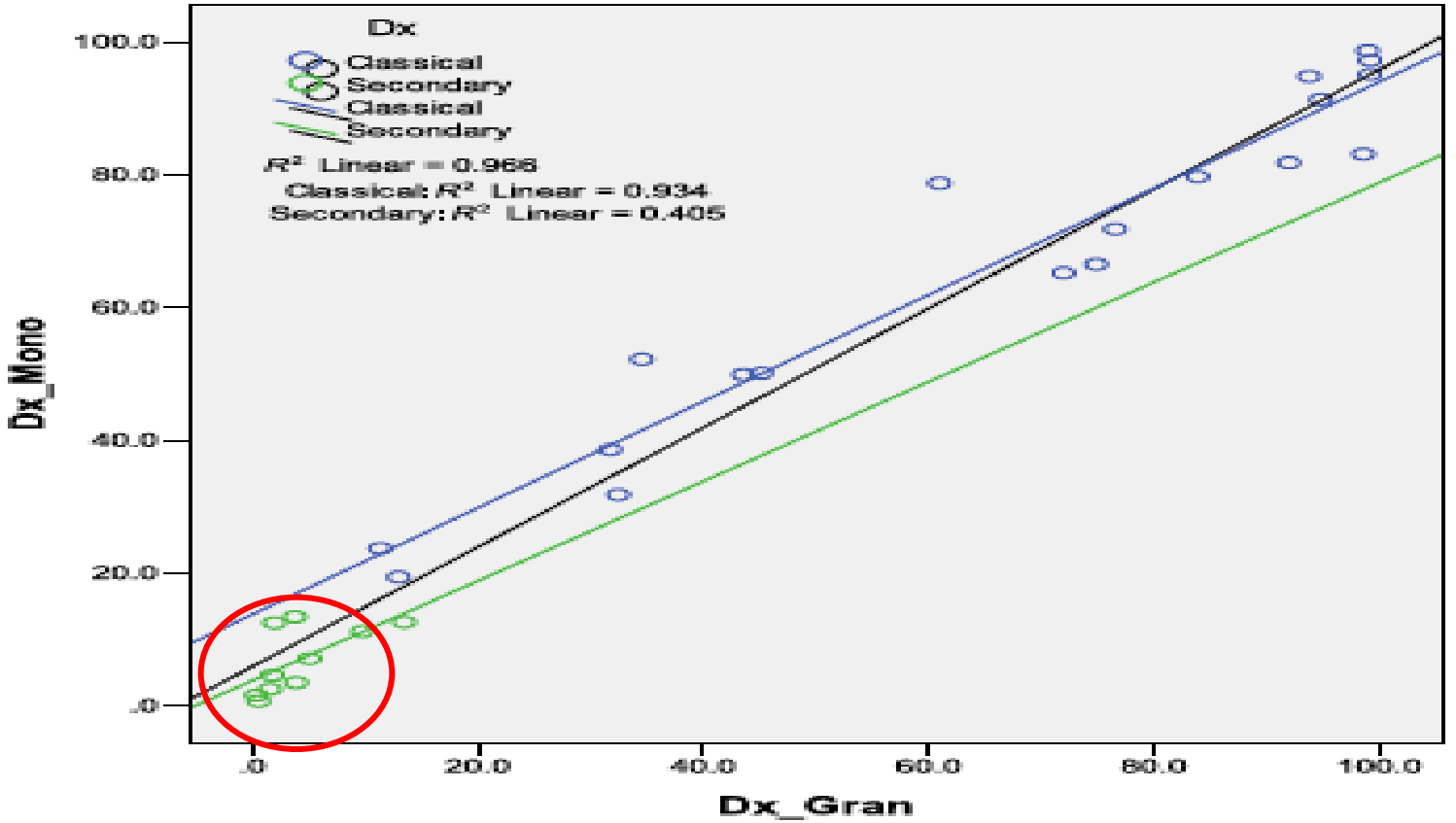
Population	#Events	%Parent
All Events	161,391	####
P2 Singlets	160,265	99.3
P3 FSC/SSC	155,523	97.0
CD45+	155,294	99.9
granulo	52,355	33.7
PNH	257	0.5
MONO	10,072	6.5
PNH mono	209	2.1

Percentage of GPI-deficient Neutrophils and Monocytes in PNH+ cases

	% PNH nuetrophils	% PNH monocytes
Hemolysis	72%±30%	76%±28%
Unexplained Cytopenias	35%±41%	39%±41%
MDS	27%±36%	31%±38%
AA	19%±29%	21%±30%

M. Morado et al. Cytometry Part B 2017; 92B: 361- 370

A linear correlation can be found between granulocyte and monocyte PNH clone size at diagnosis



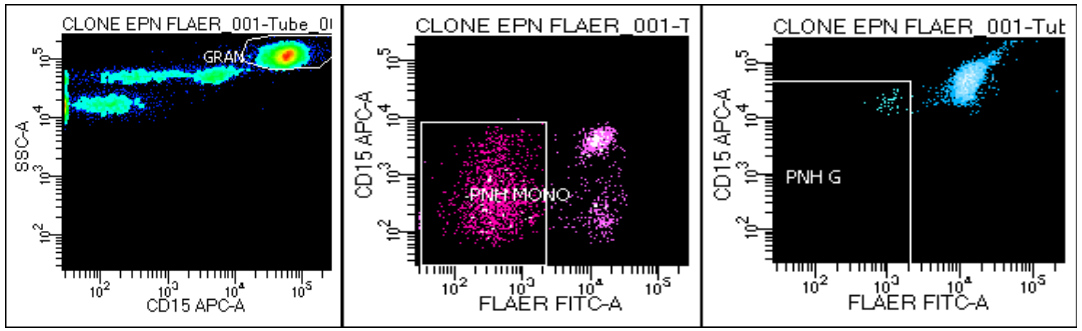
In AA/MDS the clone size was usually <10%

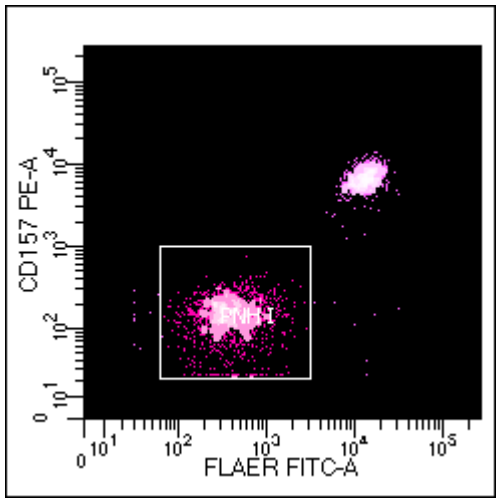
Why testing monocytes?

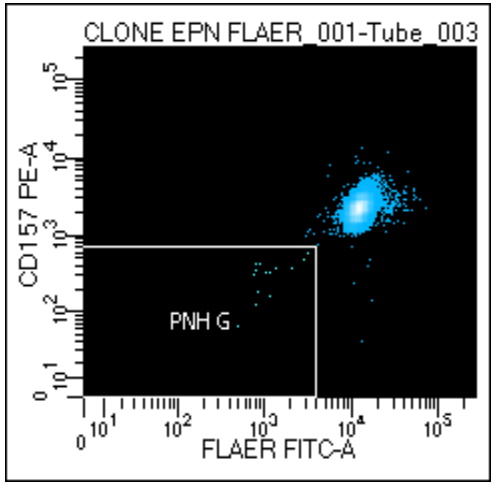
Agarwal et al. Int. Jnl. Lab. Hem 2015; 37: 14-21

Case n° GAMA2304M (Lab Ematologia Paolo Belli Bergamo)

2 colors vs 6 colors MFC Analysys







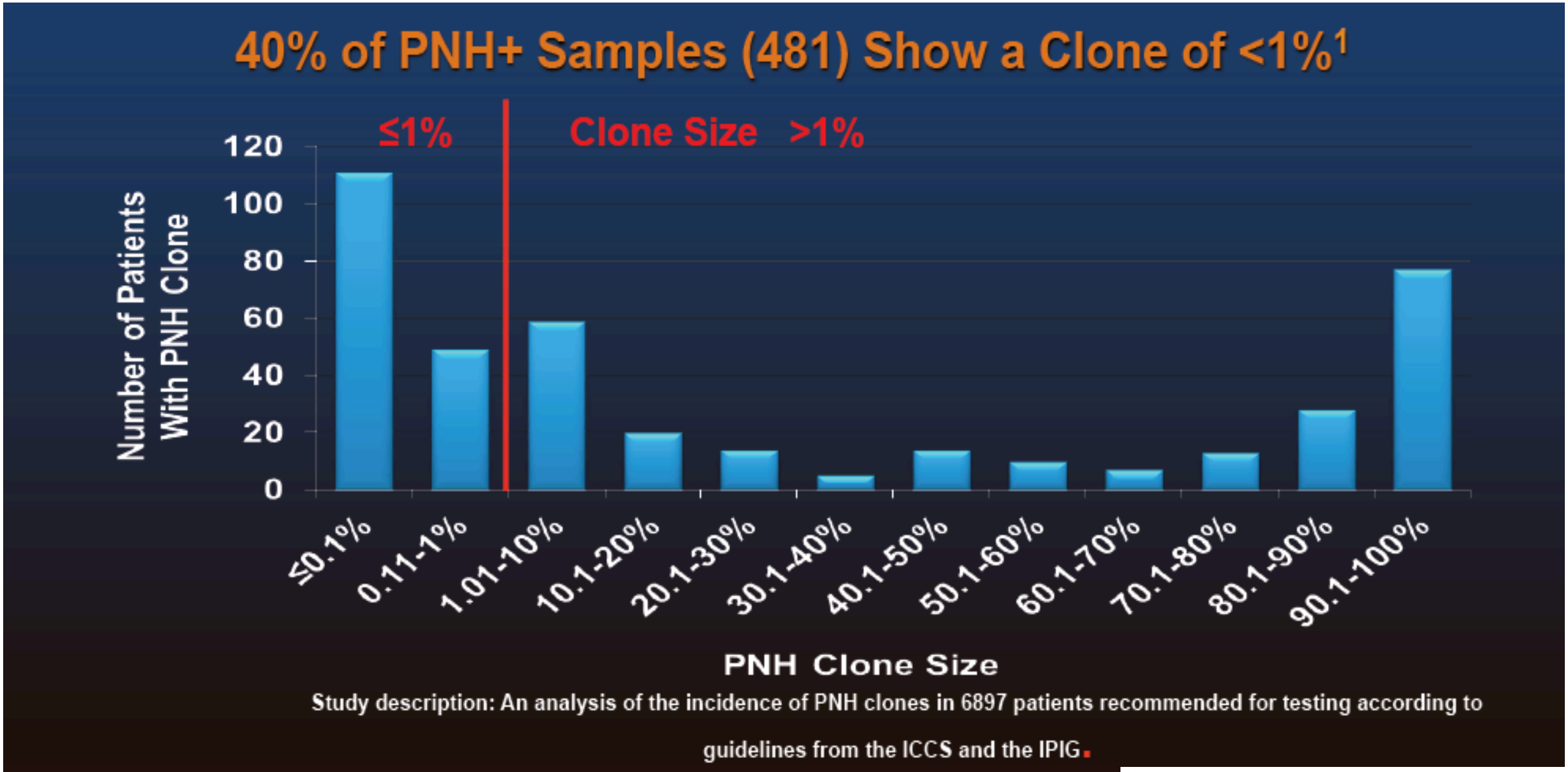
Tube: Tube_003

Population	#Events	%Parent	%Total
All Events	14,025	####	100.0
P3	13,958	99.5	99.5
P1	13,875	99.4	98.9
P2	13,870	100.0	98.9
GRANULO	5,142	37.1	36.7
PNH G	38	0.7	0.3
LY	5,483	39.5	39.1
MONO CD15/SSC	1,986	14.3	14.2
MONO	1,923	96.8	13.7
PNH MONO	1,216	63.2	8.7

Tube: Tube_003

Population	#Events	%Parent
All Events	14,025	####
P2 Singlets	13,821	98.5
P3 FSC/SSC	13,438	97.2
CD45+	13,347	99.3
GRAN	4,927	36.9
PNH G	14	0.3
LY	5,176	38.8
MONO	2,046	15.3
PNH I	1,397	68.3

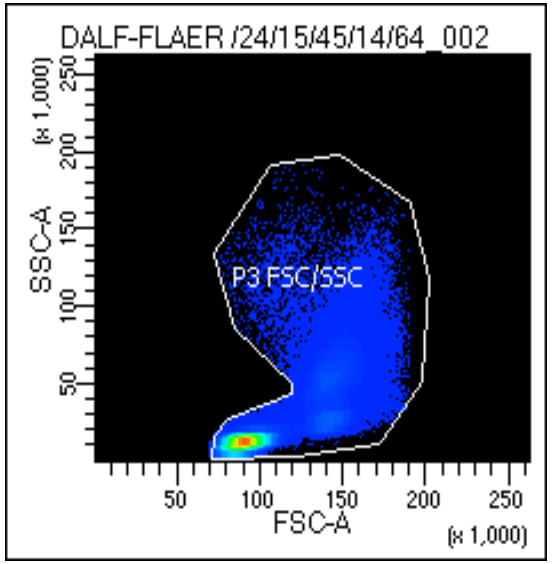
High-Sensitivity Flow Cytometry (0.01%)

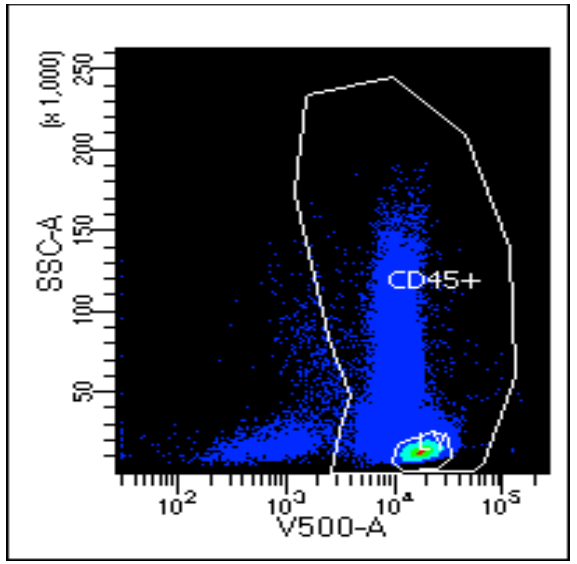


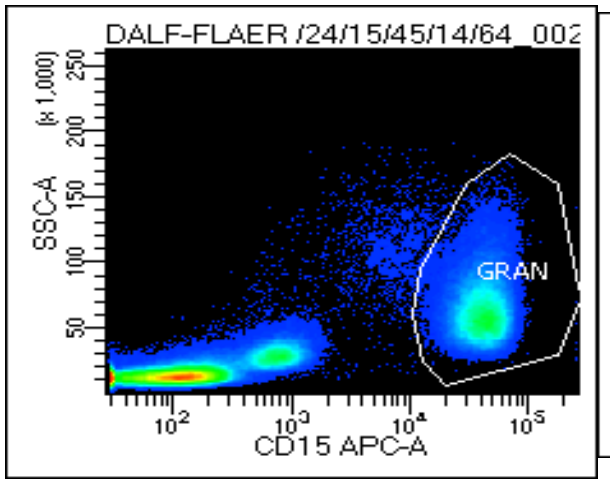
Study description: An analysis of the incidence of PNH clones in 6897 patients recommended for testing according to guidelines from the ICCS and the IPIG.

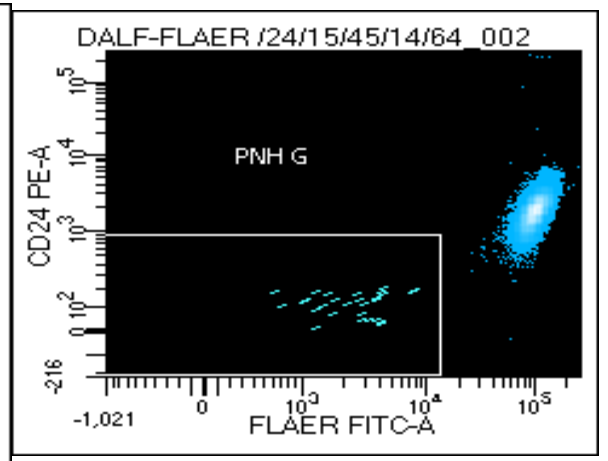
. Movalia MK et al. Poster presented at 53rd Annual meeting of the ASH, San Diego, CA. 2011.

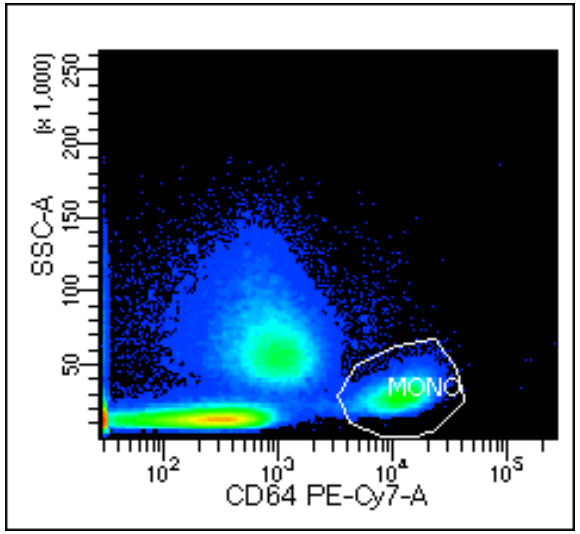
Case n° DALF1941F (ASST Ovest Milanese) 6 colors MFC Analysys

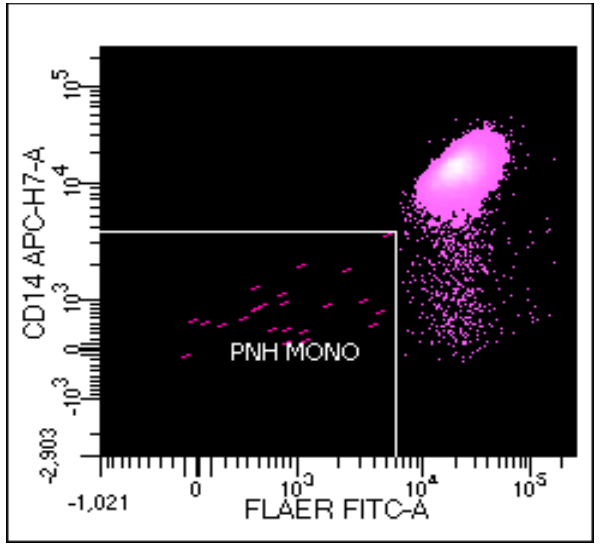










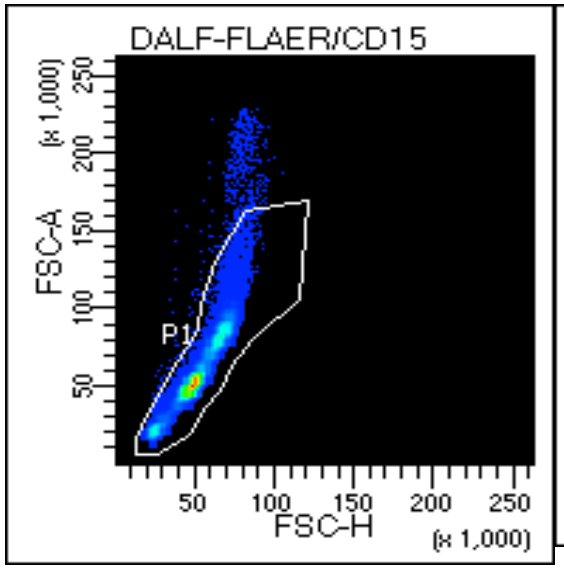


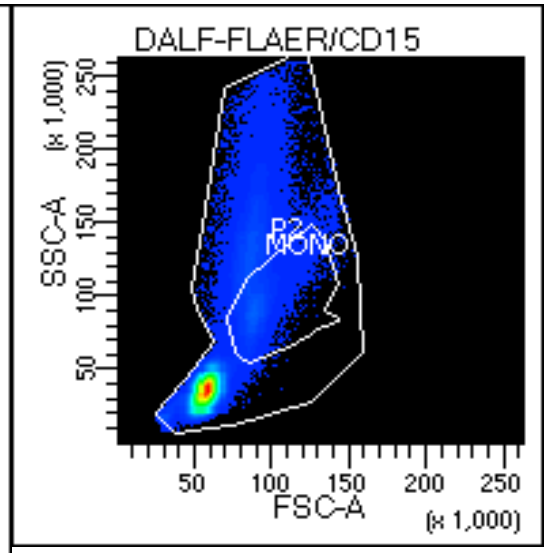
Tube: FLAER /24/15/45/14/64_002

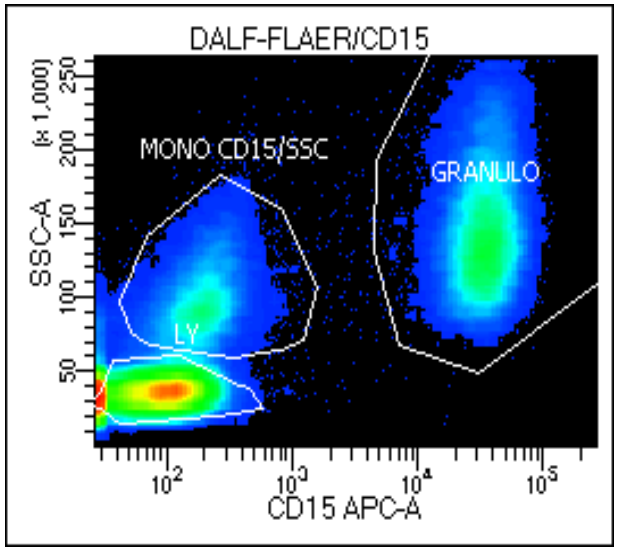
Population	#Events	%Parent
All Events	658,125	####
P2 Singlets	147,957	22.5
P3 FSC/SSC	112,152	75.8
CD45+	110,182	98.2
GRAN	26,333	23.9
PNH G	29	0.1
MONO	10,648	9.7
PNH MONO	23	0.2
LY	65,206	59.2

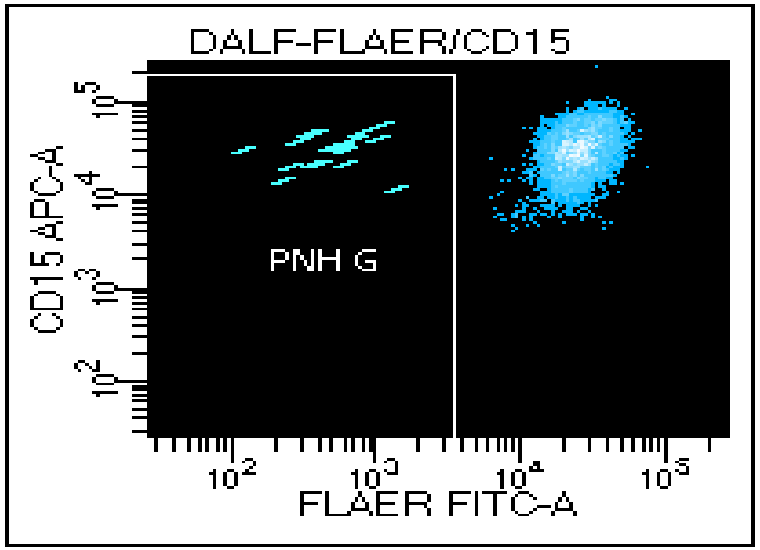
Let's Know Our Limits!

Case n° DALF1941F (ASST Ovest Milanese) 2 colors MFC Analysys



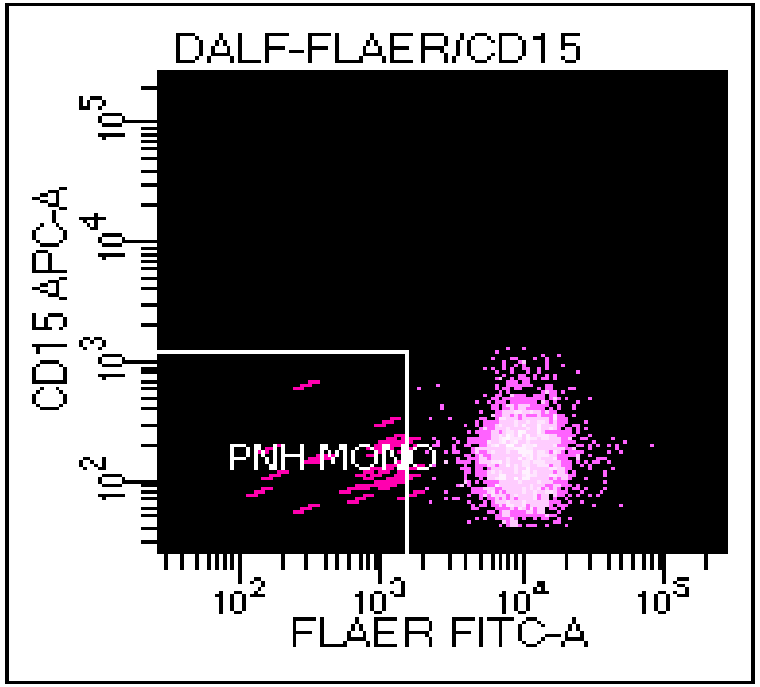






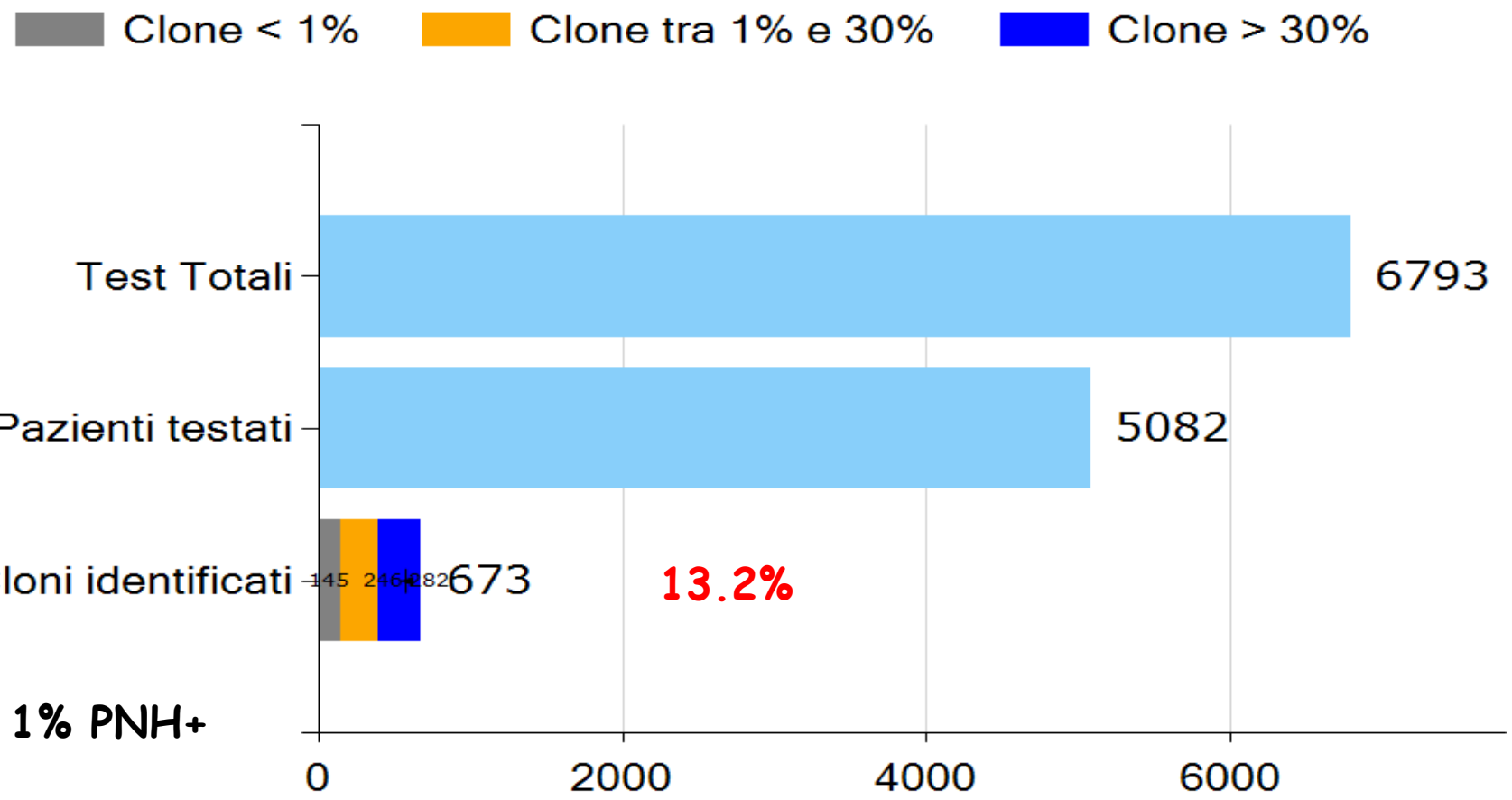
Tube: FLAER/CD15

Population	#Events	%Parent	%Total
All Events	459,195	####	100.0
P3	79,411	17.3	17.3
P1	78,788	99.2	17.2
P2	67,857	86.1	14.8
GRANULO	15,497	22.8	3.4
PNH G	17	0.1	0.0
LY	33,911	50.0	7.4
MONO CD15/SSC	7,886	11.6	1.7
MONO	6,396	81.1	1.4
PNH MONO	29	0.5	0.0



Clonoteca EPN

Data Collection 2018

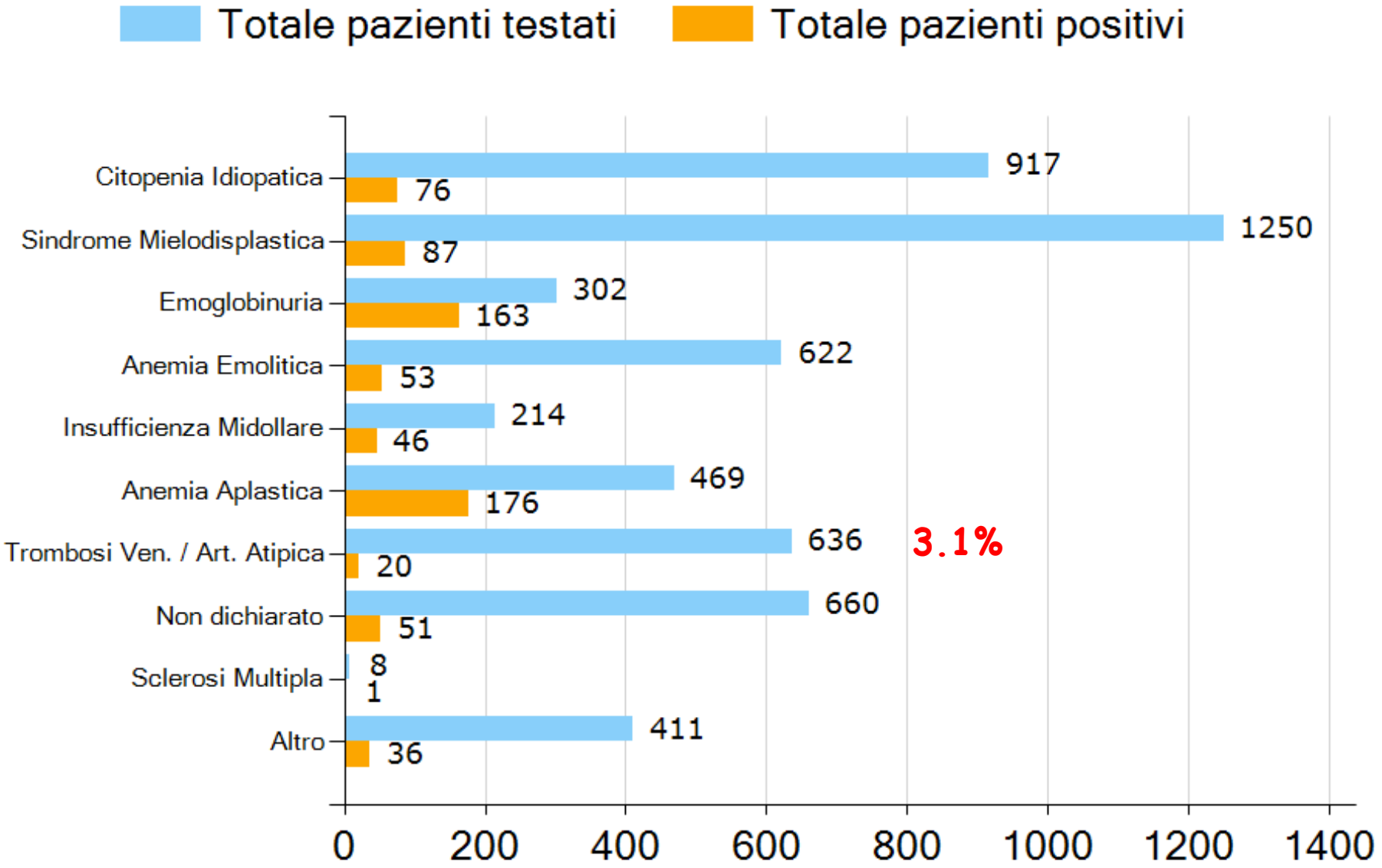


13.2%

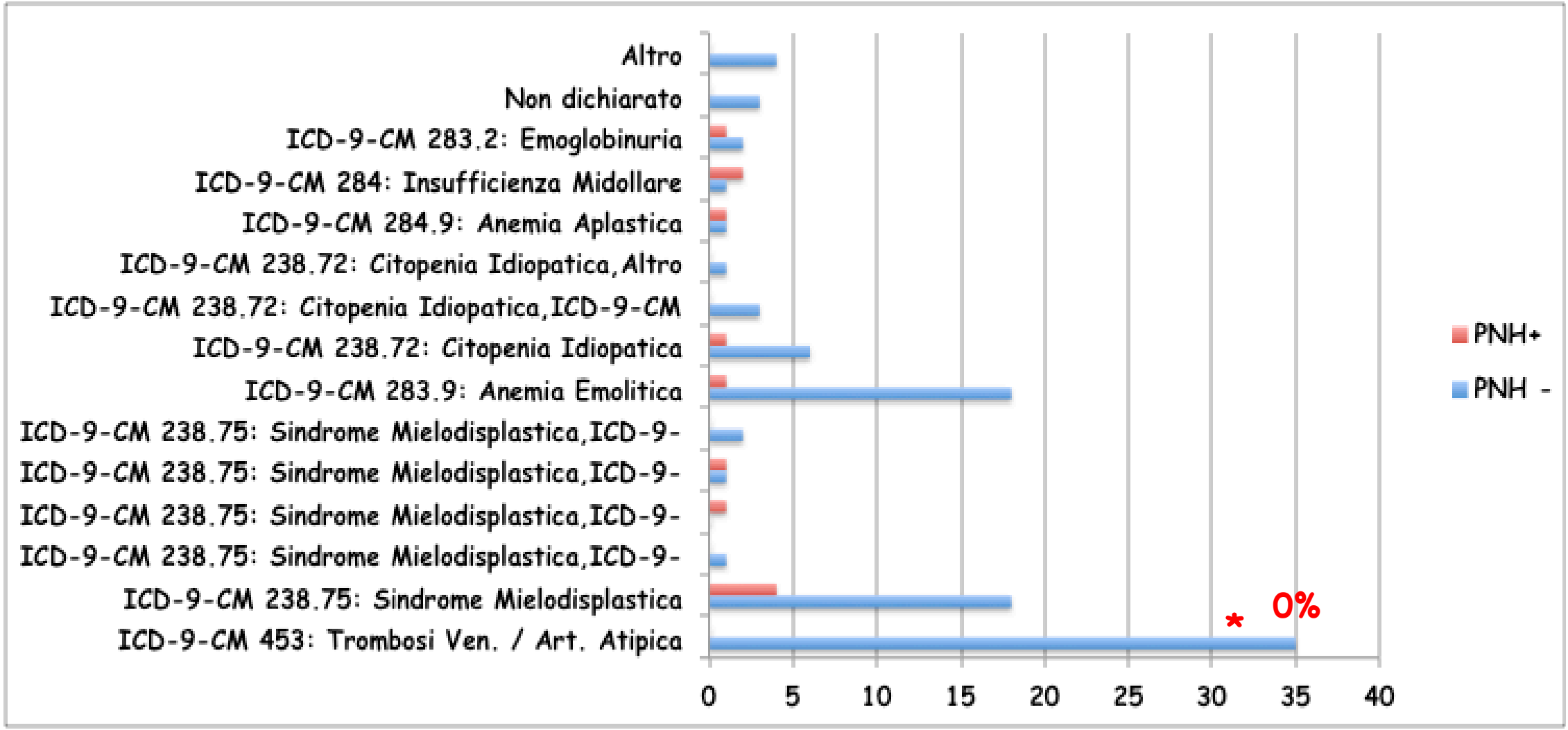
21,5% < 1% PNH+

Clonoteca EPN

Data Collection 2018



Reason for testing distribution in 94 PNH negative cases and in 13 PNH positive cases



* Thrombosis is a life-threatening complication of PNH, but its frequency is relatively low, particularly among patients without hemolysis.

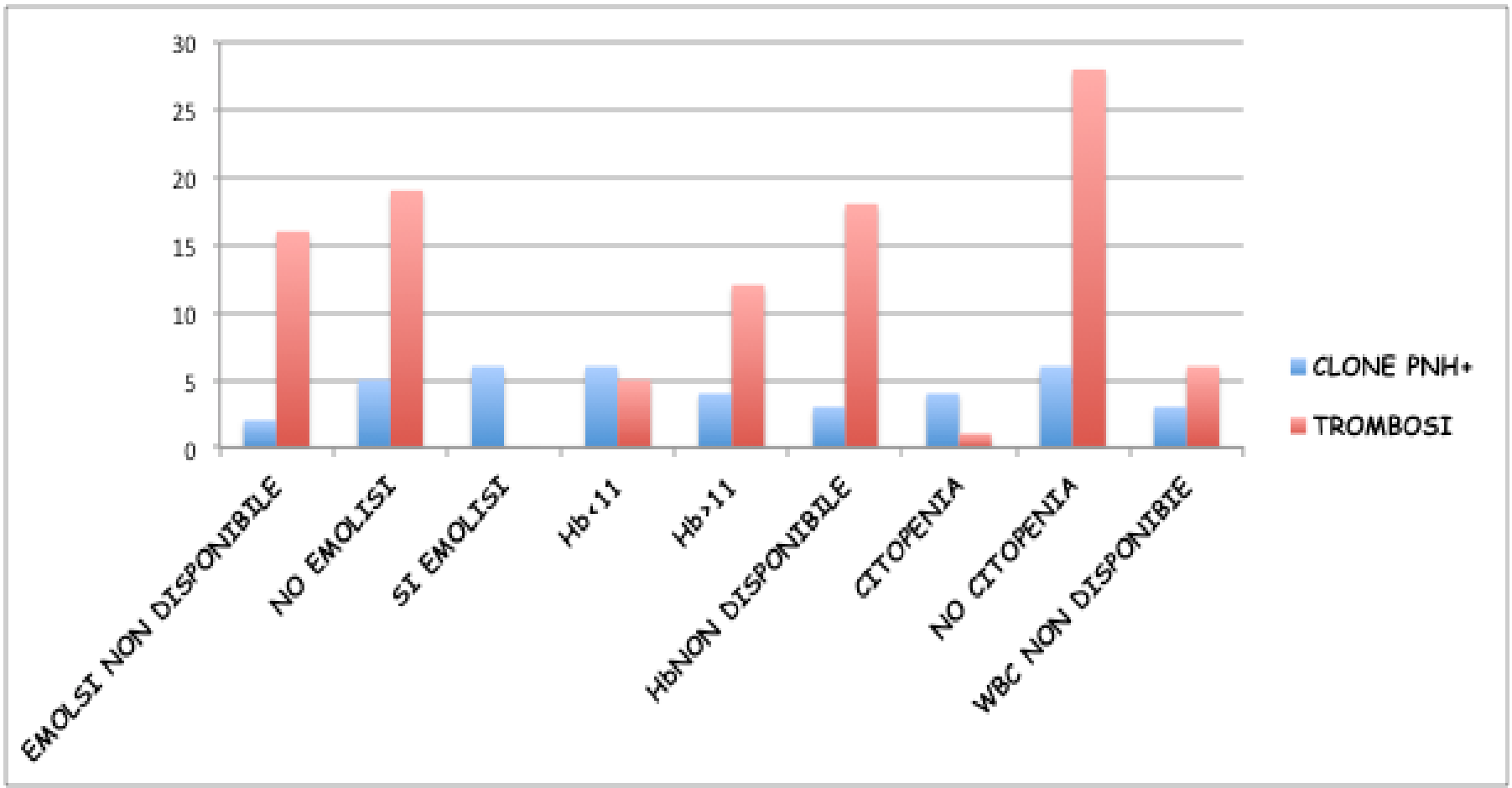
Frequency of PNH positive cases according to the Medical indications that triggered for PNH Screening

Medical indications for PNH screening	Frequency of PNH+ cases
Individuals with clinical and biological signs/symptoms of PNH in the absence of a previous hematological disorder (*n* = 3,032)	**8.7%**
Hemoglobinuria (*n* = 73)	47.9%
Hemolytic anemia (*n* = 382)	18.6%
Subtotal hemolysis (n = 455)	*23.3%*
Unexplained cytopenias including anemia (*n* = 393)	22.4%
Unexplained cytopenia without anemia (*n* = 772)	5.1%
Anemia, not otherwise specified (*n* = 468)	3.6%
Subtotal cytopenia (n = 1,633)	*8.8%*
Thrombosis with nonhemolytic anemia and/or other cytopenias (*n* = 73)	* 13.7%
Thrombosis without anemia and/or other cytopenia (*n* = 800)	* 0.4%
Subtotal thrombosis (n = 873)	*1.5%*
Iron deficiency (*n* = 57)	0%
Other (*n* = 14)	0%
Subtotal others (n = 71)	*0%*
Patients with hematological disorders (*n* = 906)	**29.8%**
Aplastic/hypoplastic anemia (*n* = 541)	44.9%
Myelodysplastic syndrome (*n* = 261)	9.8%
Subtotal BM failure (n = 802)	*33.3%*
Chronic myeloproliferative neoplasm (*n* = 21)	4.8%
Other hematological and/or immunological disorders (*n* = 78)	0%
Subtotal other non-BM failure disorders (n = 99)	*1.0%*
Total (*n* = 3,938)	**14.3%**

Patients with thrombosis who had normal blood cell counts typically tested negative for PNH

M. Morado et al. Cytometry Part B 2017; 92B: 361- 370

REL-TRE: Hemolysis and Cytopenia in PNH- patients with thrombosis and in PNH+ cases



No patients with thrombosis show hemolysis

Use of Simplified Method in PNH UK NEQAS SCHEME

UK NEQAS PNH Scheme	Consensus Neqas	Lower Q	Upper Q	%	events PNH	Total events
1718_105 ne	0,00	0,00	0,00	0,00	14	56847
1718_105 mo	0,00	0,00	0,00	0,90	39	4172
1718_106 ne	10,70	10,00	12,00	9,80	4675	47405
1718_106 mo	10,40	8,60	11,50	9,90	389	3906
1718_107 ne	0.00	0.00	0,00	0	5	195927
1718_107 mo	0.00	0.00	0,00	0,1	25	16992
1718_108 ne	9,90	9,02	10,42	10	21562	215707
1718_108 mo	6,00	5	6,72	4,6	1049	37096
1718_109 ne	3,06	2	2,8	2,3	3398	146840
1718_109 mo	3,06	2,5	3,72	3,1	595	26739
1718_110 ne	2,00	1,4	2,4	2,1	3735	176486
1718_110 mo	2,33	2,1	5,57	1,9	574	45646
1718_111 ne	0.00	0.00	0,00	0	5	335160
1718_111 mo	0.00	0.00	0,00	20	32519	0,1
1718_112 ne	5,30	3,93	6,2	5,7	5043	89065
1718_112 mo	5,20	4,92	5,6	4,3	388	12170
1718_113 ne	1,20	1,1	1,3	1,5	2971	192930
1718_113 mo	0,60	0,12	0,92	1,6	185	14165
1718_114 ne	0.00	0.00	0,00	0	1	123310
1718_114 mo	0.00	0.00	0,00	0,2	10	12424
1718_115 ne	1,80	1,63	1,9	2	79	64614
1718_115 mo	2,90	2,3	3,3	2,4	134	6357
1718_116 ne	0.00	0.00	0,00	0	0	99339
1718_116 mo	0.00	0.00	0,00	8	0,1	14114

2-COLOR FCM ASSAY FOR PNH SCREENING : Conclusions 1

- The simplified 2-color screening assay for PNH is able to disclose small clones (i.e. 0.1% PMN).
- Despite the limitations in Monocyte definition using CD15 only, if an isolated Monocyte clone is present, it can be detected with sufficient accuracy.
- Discordant Monocyte/Granulocyte PNH clones can be present!
- Monocyte sensitivity is > 0.1% (5000 monocytes are acquired)
- Thrombosis alone, even if in atypical sites, without hemolysis or cytopenias is a questionable reason for testing (<0.5%).
- The 2017 experience by the REL-Tre consortium in Lombardy has disclosed a series of new cases at an incidence of 12.1% of the reasons for testing (as compared to Morado 2017).
- The LOD and LOQ rules should be strictly applied in high-resolution PNH analysis.

2-COLOR FCM ASSAY FOR PNH SCREENING : Conclusions 2

- "Clonoteca " is a powerful tool to study PNH clones.
- In most cases hemolysis and cytopenia data are not reported.
- It is more probable to find PNH clone if multiple reasons for testing are present.
- 2 color PNH screening may be applied to PNH UK Neqas scheme (work in progress).

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Marco Chiarini (Spedali Civili Brescia)