FORUM IN EMATOLOGIA: VERSO IL 2020

Bari, Hotel Villa Romanazzi-Carducci
21-22 ottobre 2019

DICHIARAZIONE

Relatore: NOME COGNOME

Come da nuova regolamentazione della Commissione Nazionale per la Formazione Continua del Ministero della Salute, è richiesta la trasparenza delle fonti di finanziamento e dei rapporti con soggetti portatori di interessi commerciali in campo sanitario.

- Posizione di dipendente in aziende con interessi commerciali in campo sanitario **(NIENTE DA DICHIARARE)**
- Consulenza ad aziende con interessi commerciali in campo sanitario **(NIENTE DA DICHIARARE)**
- Fondi per la ricerca da aziende con interessi commerciali in campo sanitario **(NIENTE DA DICHIARARE)**
- Partecipazione ad Advisory Board **(NIENTE DA DICHIARARE)**
- Titolarietà di brevetti in compartecipazione ad aziende con interessi commerciali in campo sanitario **(NIENTE DA DICHIARARE)**
- Partecipazioni azionarie in aziende con interessi commerciali in campo sanitario **(NIENTE DA DICHIARARE)**
- Altro

UNIVERSITÀ DEGLI STUDI FIRENZE

Azienda Ospedaliero Universitaria Careggi

NGS nelle neoplasie mieloproliferative: dalla diagnosi agli score prognostici

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Diagnostic Criteria of MPN

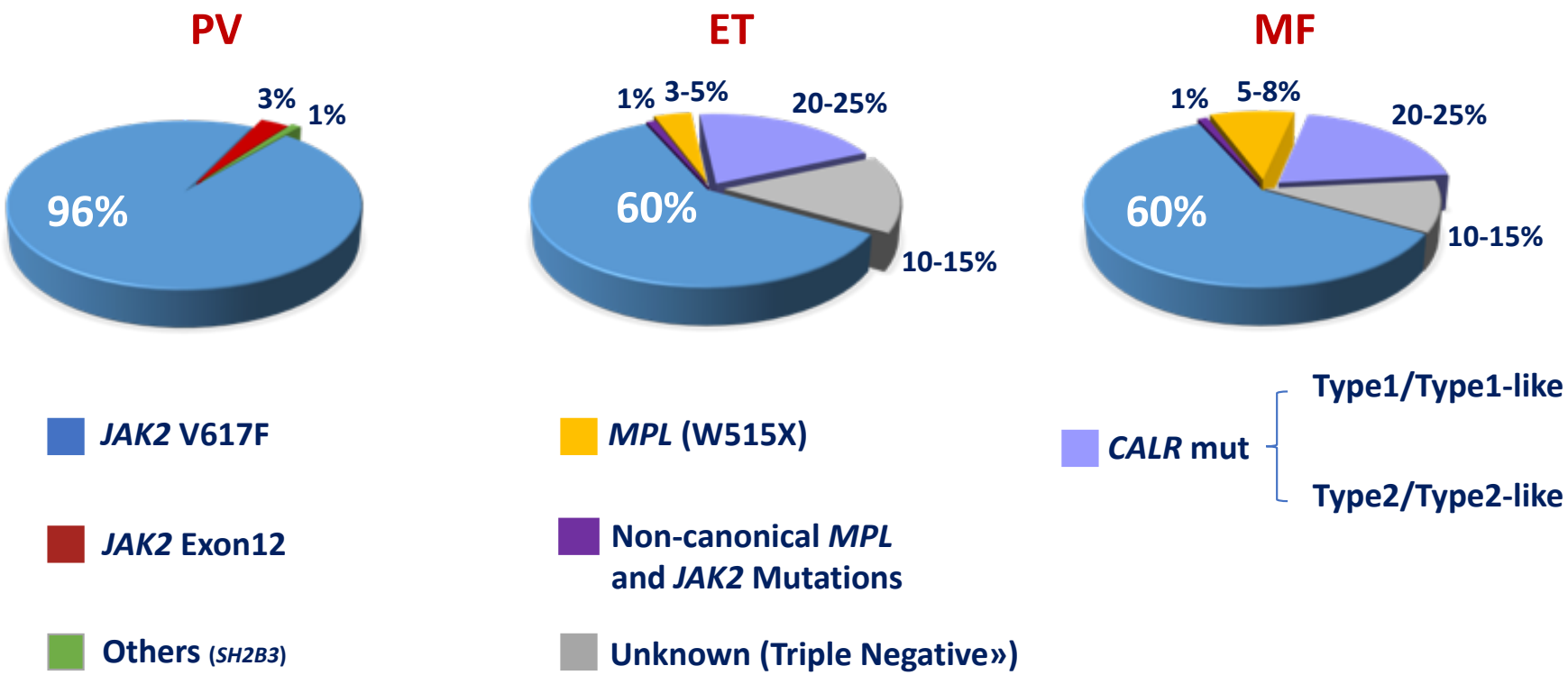
WHO2016

PV	ET	Pre-PMF	Overt PMF
Major criteria **1.** Hb ≥16.5g/dL in men, or 16.0 g/dL in women, or Hct ≥49% and 48%, or increased RCM **2.** BM biopsy with hyper-cellularity with panmyelosis and Mk proliferation with pleomorphic Mks **3. Presence of *JAK2*V617F or *JAK2* ex12 mutation** **Minor criteria** **1.** Subnormal sEPO levels	**Major criteria** **1.** Platelet count >450x10^9/L **2.** BM biopsy with proliferation mainly of the Mk lineage with mature enlarged Mk with hyperlobulated nuclei **3.** Not meeting WHO criteria for other myeloid neoplasms **4. Presence of *JAK2*V617F, CALR or *MPL* mutation** **Minor criteria** **1.** Presence of a clonal marker, or absence of evidence of reactive thrombocytosis	**Major criteria** 1. BM biopsy with Mk proliferation and atypia, w/o reticulin fibrosis >G1; with incr. cellularity, granulocytic prolifer. and often decreased erythr'iesis **2.** Not meeting WHO criteria for other myeloid neoplasms **3. Presence of *JAK2*V617F, *CALR* or *MPL* mutation, or in the absence of these mutations, presence of another clonal marker,** or absence of minor reactive BM reticulin **Minor criteria** 1. Anemia 2. Leucocytosis >11x10^9/L 3. Palpable splenomegaly 4. Increased LDH	**Major criteria** **1.** BM biopsy with Mk proliferation and atypia with either reticulin fibrosis G2-3 and/or collagen **2.** Not meeting WHO criteria for other myeloid neoplasms **4. Presence of *JAK2*V617F, *CALR* or *MPL* mutation, or in the absence of these mutations, presence of another clonal marker,** or absence of reactive myelofibrosis **Minor criteria** 1. Anemia 2. Leucocytosis >11x10^9/L 3. Palpable splenomegaly 4. Increased LDH 5. Leukoerythroblastosis
3 major or first 2 major + minor	**4 major or first 3+ minor**	**3 major + ≥1 minor**	**3 major + ≥1 minor**

In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (eg, ***ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1***) are of help in determining the clonal nature of the disease.

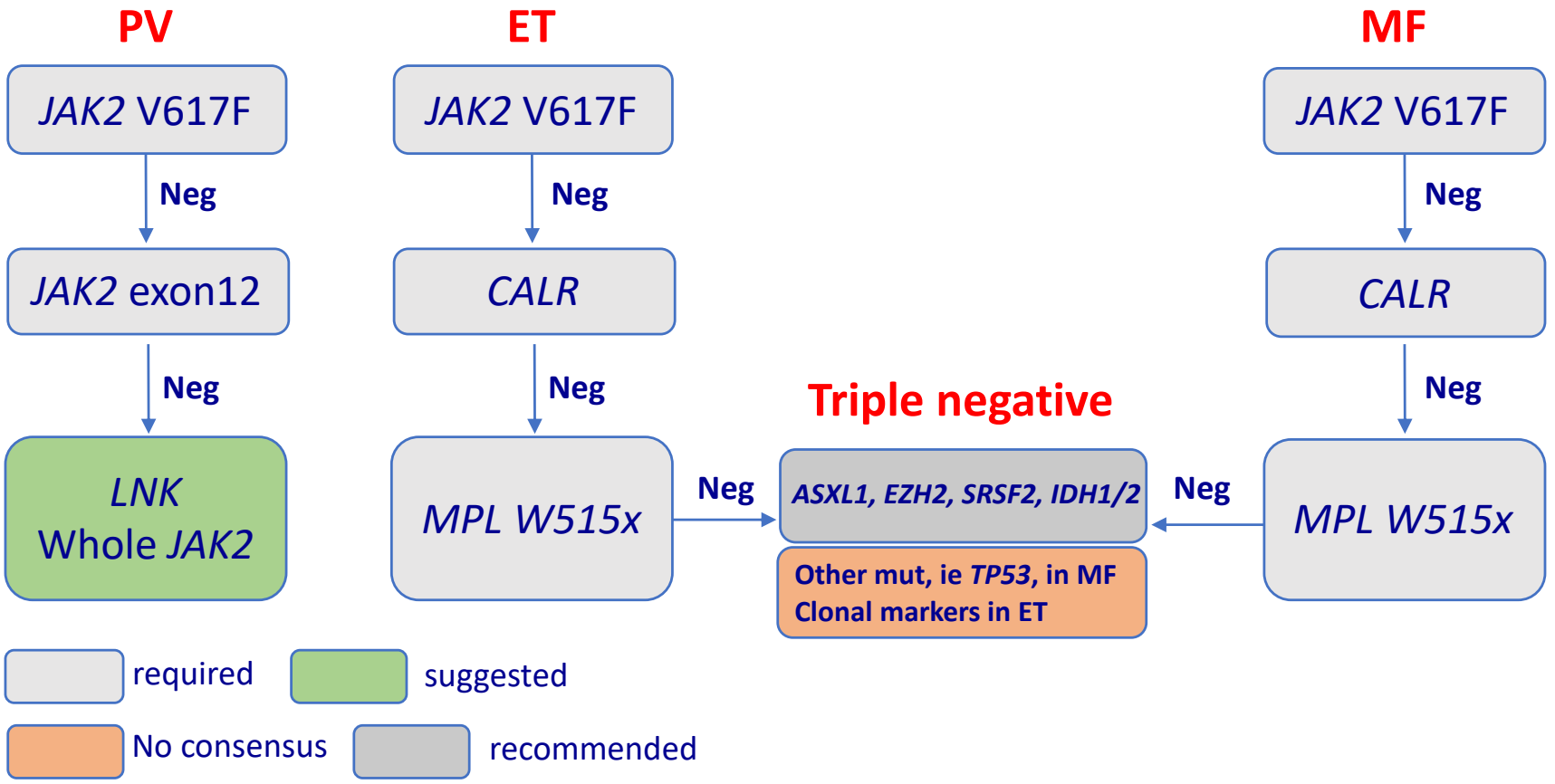
Arber DA et al, Blood 2016; 127:2391-405

Phenotypic Driver Mutations in MPNs



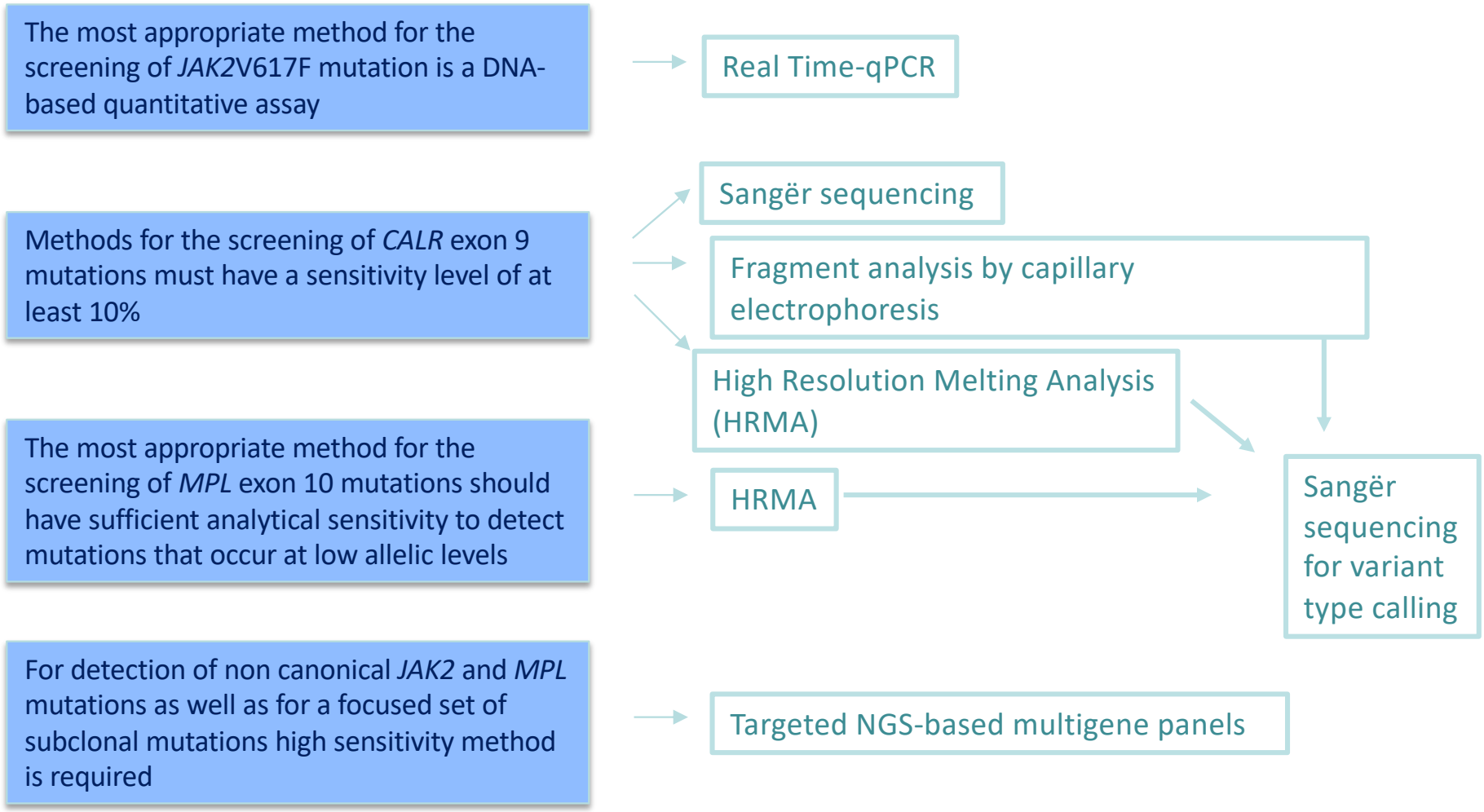
Klampfl T, et al. *NEJM* 2013;369(25):2379-90;
Nangalia J, et al. *NEJM* 2013;369(25):2391-405; Milosevic JD et al. Blood 2016;127:325-332; Cabagnols J et al. Blood 333-342

Recommendations for Molecular Testing in Ph1neg MPN. 2018 Revised Management Recommendations from ELN



Vannucchi AM et al, Ann Oncol. 2015 Sep;26 Suppl 5:v85-99; Guglielmelli P et al., Leuk Res. 2017 Jul;58:63-72

Appropriateness of the methods for diagnostic genetic testing in MPNs



Guglielmelli P et al., Leuk Res. 2017 Jul;58:63-72

Source of sample and DNA template for genetic testing in MPNs

- Peripheral blood granulocytes should be the preferred source of cells for genetic testing in MPNs. However, use of whole blood, leukocytes or bone marrow aspirate for diagnostic testing is not inappropriate

- Peripheral blood granulocytes must be used when low levels of mutation allele burden are expected, like in the monitoring of patients' response to specified treatments.

- Genomic DNA should be the preferred template for genetic testing in MPNs.

- Either automatic or manual, commercial or in-house methods of DNA purification may be employed, following protocol recommendation.

- Either spectrophotometric or fluorimetric methods of DNA quality assurance may be employed. Both controls and sample should be processed using the same approach. DNA quantity should be carefully assessed before testing. This is particularly important for comparative assays such as high resolution melting (HRM), and are fundamental for NGS analysis, in which fluorometric-based assessments are mandatory.

Guglielmelli P et al., Leuk Res. 2017 Jul;58:63-72

Genetic testing for MONITORING MPNs

- High-sensitivity quantitative monitoring of driver somatic mutations is not recommended for assessing the molecular response to the currently available potentially disease modifying drugs in patients with MPNs.

- The Panel argued that in PV patients treated with the putative disease-modifying agent interferon alpha, molecular monitoring should be considered in a controlled use of the drug (i.e. clinical trial or registry) as well as in any prospective trial with novel drugs.

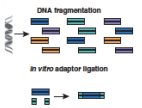
- High-sensitivity quantitative monitoring of *JAK2*V617F and *CALR* mutations is recommended in all patients after allo-SCT for minimal residual disease (MRD) assessment. This could inform early evaluation of treatment outcome, and help to adopt a preemptive donor lymphocyte infusion treatment of impending relapses.

- Molecular monitoring after allo-SCT is recommended also in patients with triple negative genotype provided they have a clonal marker that can be quantitatively assayed

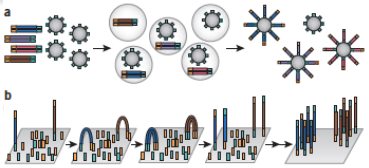
Guglielmelli P et al., Leuk Res. 2017 Jul;58:63-72

Next generation sequencing procedures

Massive parallel sequencing



Library preparation
(DNA fragmentation + adapter ligation)



Clonal Amplifications
(Emulsion PCR or Bridge PCR)

Sequencing (Synthesis or ligation)

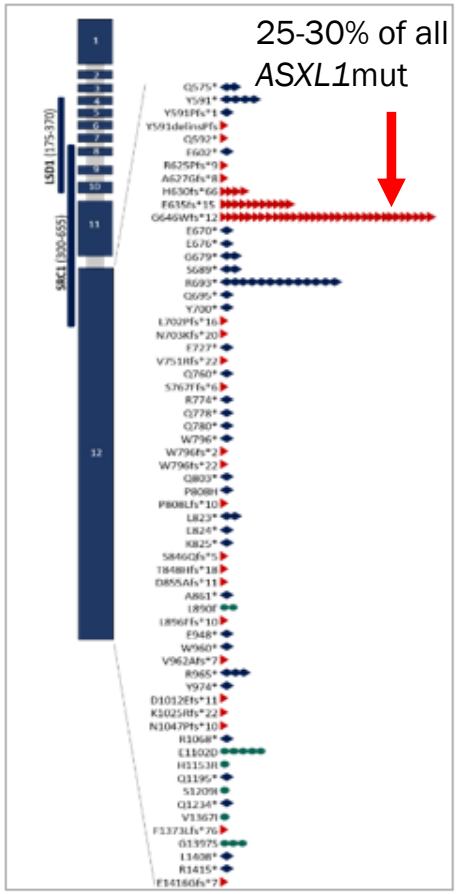
↓

Data analysys (Genome alignement)

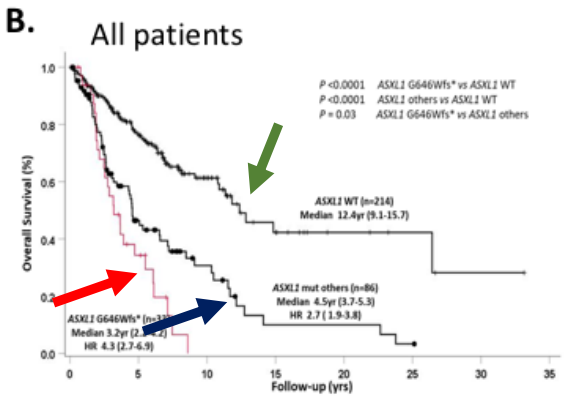
- small amout of DNA
- high throughput
- short time
- low costs

- Short reads (verify the accuracy of variant calls)
- Complexity of algorithms
- Necessity of data storage and a powerful CPU (from 10Gb to Tb of raw data for experiment)
- Analysis/interpretation

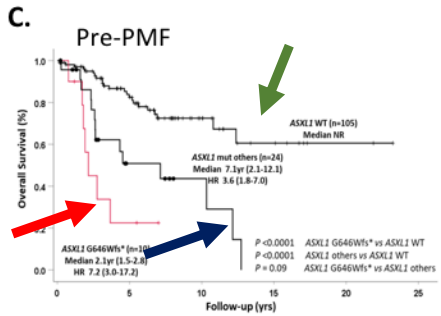
Prognostic impact of p.Gly646Trpfs*12 in PMF

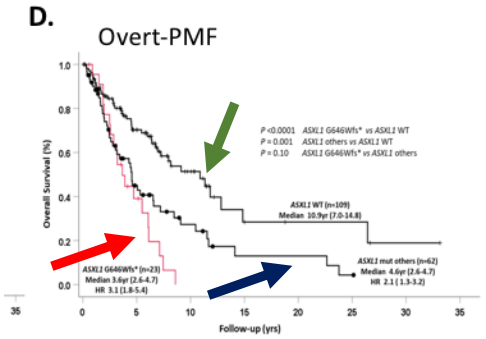


- ***ASXL1* G646Wfs*12**: Considered an artifact of NGS
- Homopolymeric region (8 guanine strand); Low coverage
- Variably included in reports and analyzed for prognosis
- Easily detected, and validated, by Sanger



WT
ASXL1 mut all other
ASXL1 6467





Rotunno et al, Blood 2019;133(26):2802-2808

Appropriateness of the methods for diagnostic genetic testing - NON DRIVER mut -

WHO 2016: In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (eg, *ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1*) are of help in determining the clonal nature of the disease.

- Search for mutations in the entire MPL gene and for complementary clonal markers is recommended in suspected ET and MF TN patients. An extended mutation panel including at least ASXL1, EZH2, IDH1/2,SRSF2 or in addition TP53, TET2, DNMT3A and CBL, is appropriate
- The use of tests addressing additional somatic, non-driver, subclonal mutations for diagnostic purposes should be reserved to cases with consistent suspicion of MPN, who are triple-negative, and in whom other diagnostic criteria do not allow a firm differential diagnosis with a reactive condition.

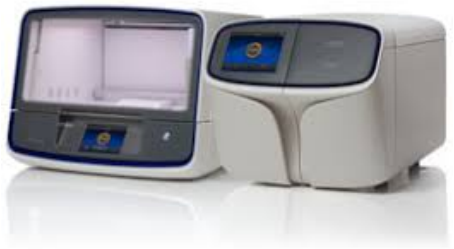
⇒ no specific recommendations can be made on the design of the panel of genetic mutations to analyze.

NON – driver mutations

- myeloid panel
- Erythrocytosis/Thrombocytosis/thrombocytopenia panel

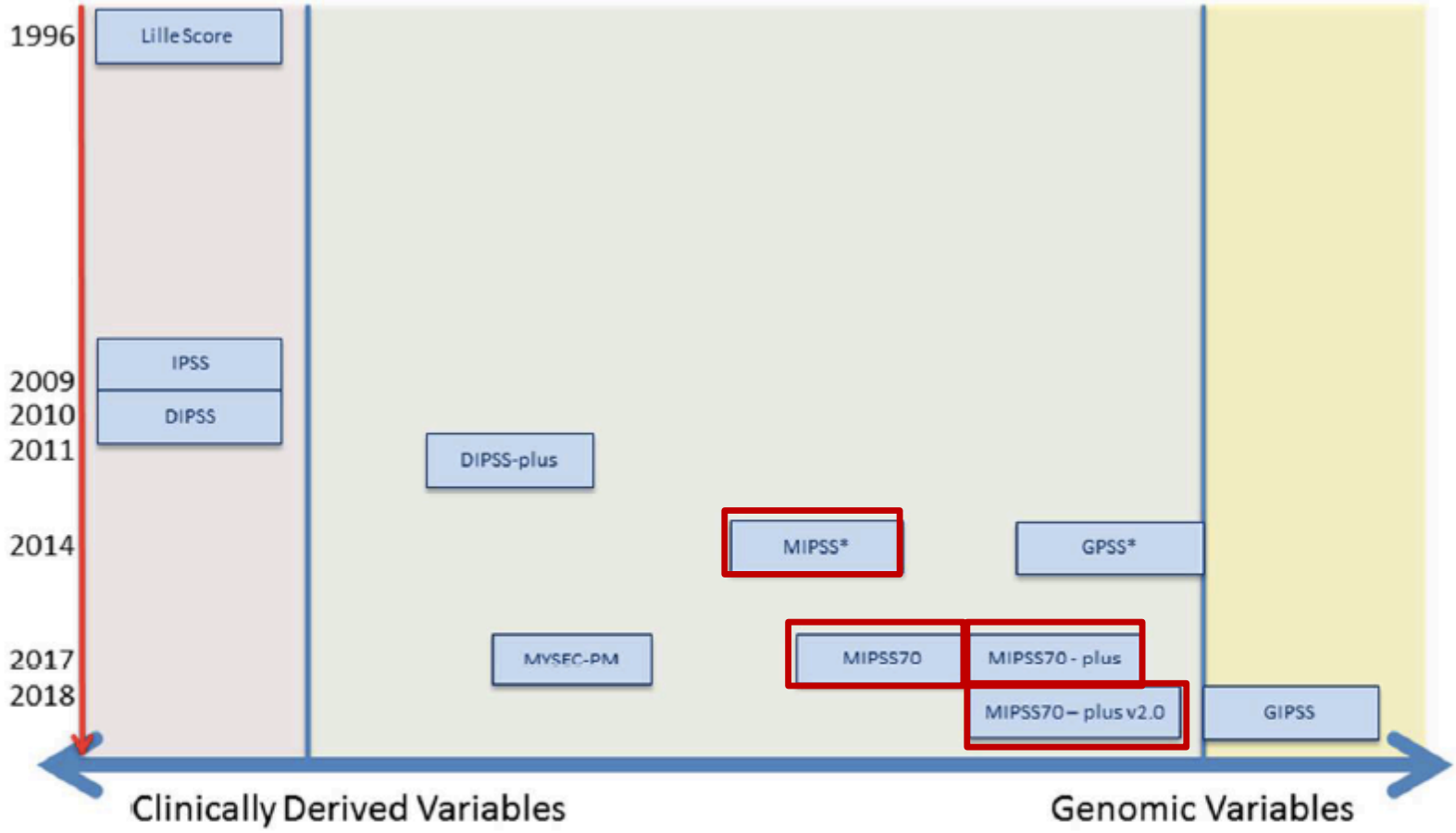
NGS technology

- Sensitivity 1%
- For reporting 5%



Guglielmelli P et al., Leuk Res. 2017 Jul;58:63-72

The Evolution of Prognostic Models in MF



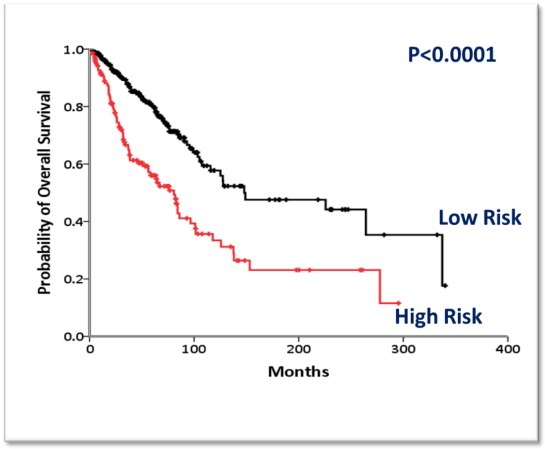
Genetic testing for the PROGNOSIS of MPNs

- Testing with an extended mutation panel including at least *ASXL1*, *EZH2*, *IDH1/2*, *SRSF2* and *TP53*, is appropriate for prognostic prediction in selected patients with PMF.

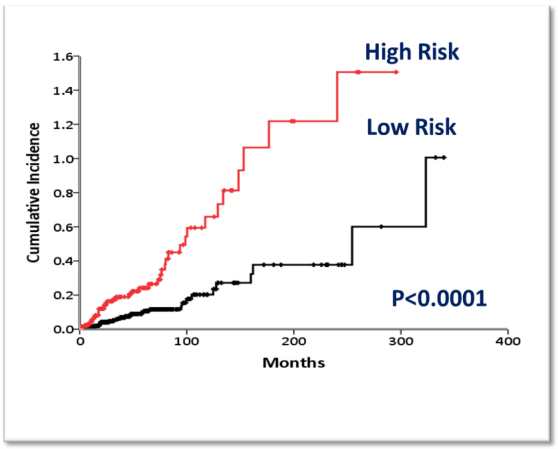
PMF High Molecular Risk Prognostic Category

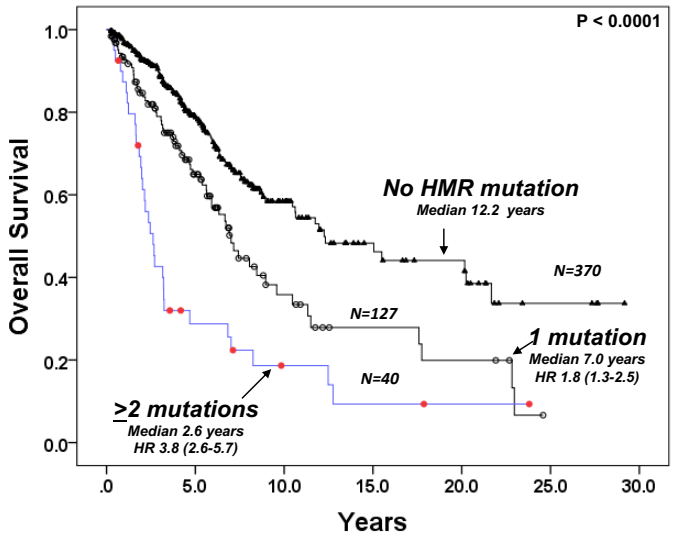
harboring ≥1 mutation in any one of *ASXL1, EZH2, SRSF2, IDH1/2*

Overall Survival



Blast Transformation





A HMR status is associated with reduced OS and increased risk of blast transformation in PMF patients independent of IPSS/DIPPS-plus

- Of the HMR mutations,*SRSF2* mutation was significant for reduced OS in PET-MF.

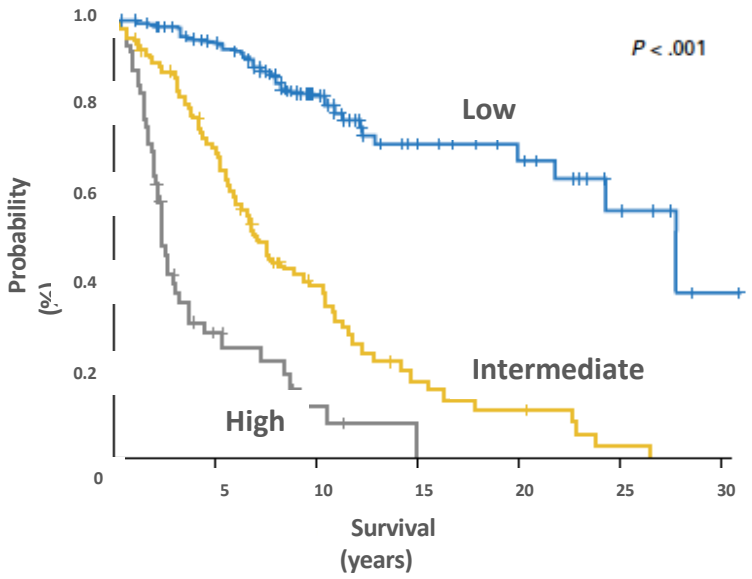
Guglielmelli P et al., Leuk Res. 2017 Jul;58:63-72;
Vannucchi AM, et al. Leukemia. 2013;27:1861-9; Guglielmelli P, et al. Leukemia. 2014 Sep;28(9):1804-10

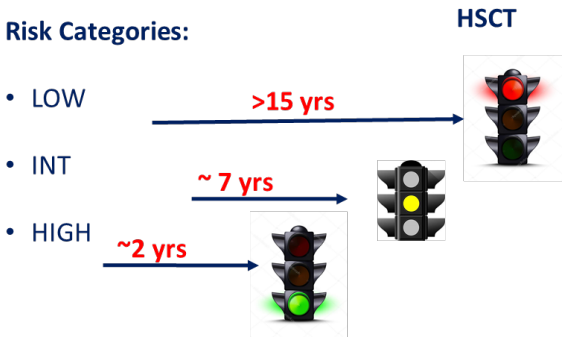
MIPSS70: Mutation Enhanced Prognostic Score System for Transplant-Age Patients with PMF

Variables	Weighted value
Hb <100g/L	1
WBC >25x10^9/L	2
PLT <100x10^9/L	2
PB blasts ≥2%	1
Constitutional Symptoms	1
Grade ≥2 BM fibrosis	1
Absence *CALR* Type1	1
HMR category*	1
≥2 HMR mutations	2

Risk category	Score	OS (y)	HR
Low	0-1	27.7	1
Intermediate	2-4	7.1	5.5 (3.8-8.0)
High	≥5	2.3	16.0 (10.2-25.1)

* HMR category= any mutation in: *ASXL1*, *EZH2*, *SRSF2*, *IDH1/2*





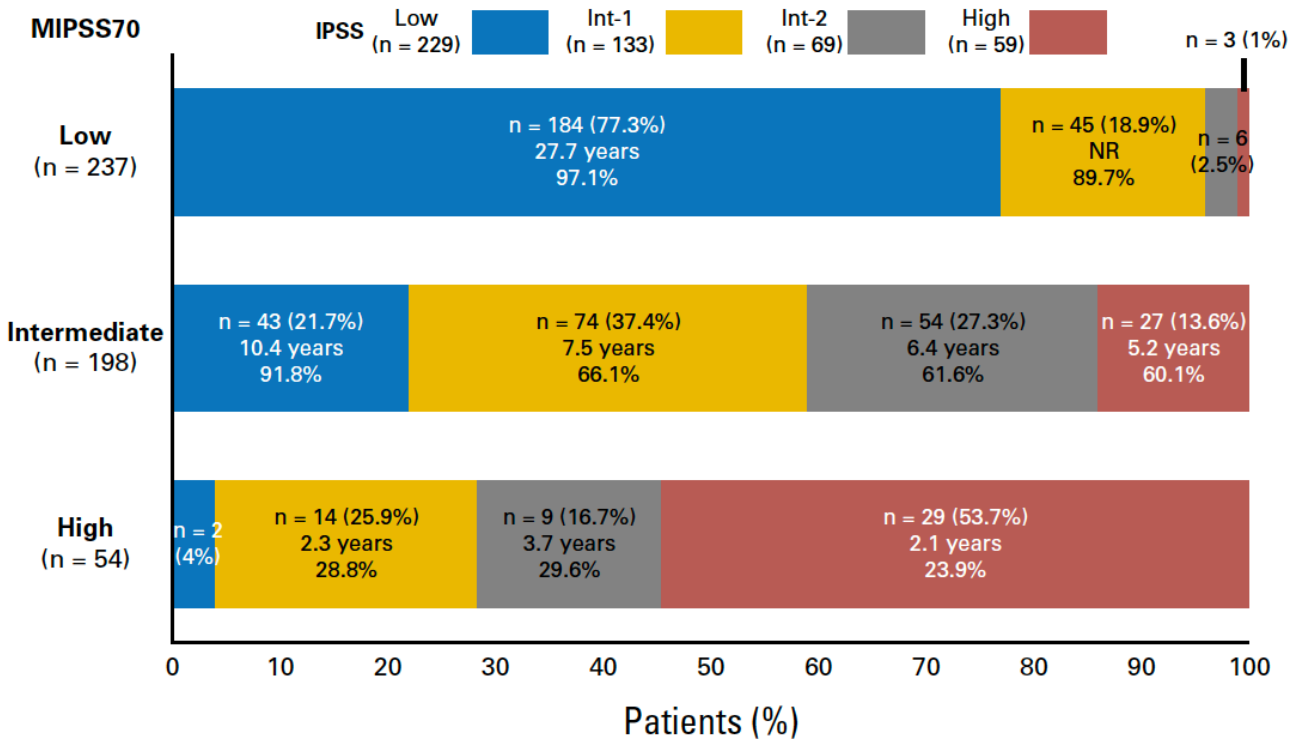
Learning cohort: Italian cohort.

The higher categories included 30% of patients originally classified as low or intermediate IPSS risk; conversely, 6 patients (3.5%) included in intermediate-2 IPSS group were downgraded to the lowest risk categories of MIPSS70. In MIPSS70 plus Version 2.0 ***U2AF1*** mutations were included in HMR status.

http://www.mipss70score.it/

Guglielmelli P, et al. JCO 2018; ;36(4):310-318; Tefferi A JCO 2018 ; 36 (17): 1769

MIPSS70 is more performant for predicting survival in PMF than IPSS

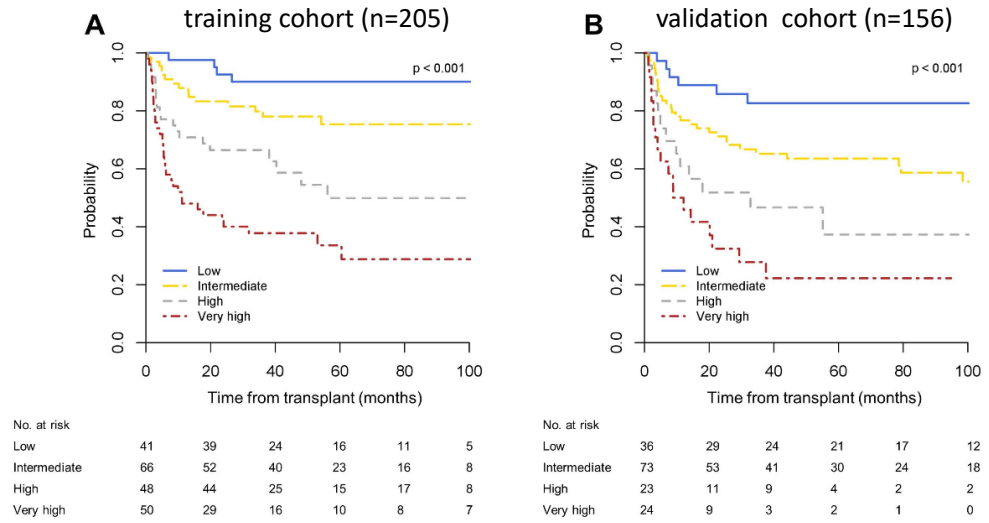


The higher categories included 30% of patients originally classified as low or intermediate IPSS risk; conversely, 6 patients (3.5%) included in intermediate-2 IPSS group were downgraded to the lowest risk categories of MIPSS70.

Guglielmelli P, et al. JCO 2018; ;36(4):310-318.

Comprehensive clinical-molecular transplant scoring system for MF undergoing HSCT (MTSS).

Covariates	HR (95% CI)	P	weighted score	Our pt
age ≥ 57 years	1.65 (1.15-2.36)	0.006	1	0
Karnofsky performance status <90%	1.50 (1.06-2.13)	0.021	1	0
non-CALR/MPL driver mutation genotype	2.40 (1.30-4.71)	0.012	2	2
*ASXL1*mutation	1.42 (1.01-2.01)	0.041	1	1
HLA-mismatch unrelated donor	2.08 (1.45-2.97)	<0.001	2	0
WB count >25x10^9/L	1.57 (1.16-2.41	0.007	1	1
Platelet count <150x10^9/L	1.67 (1.16-2.40)	0.006	1	0

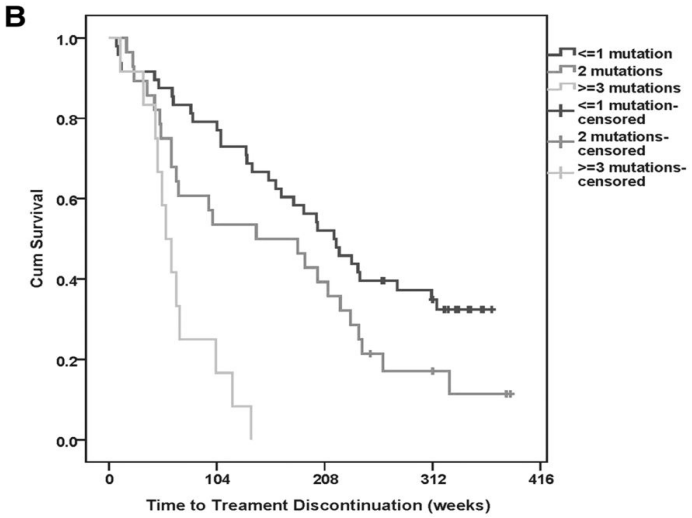


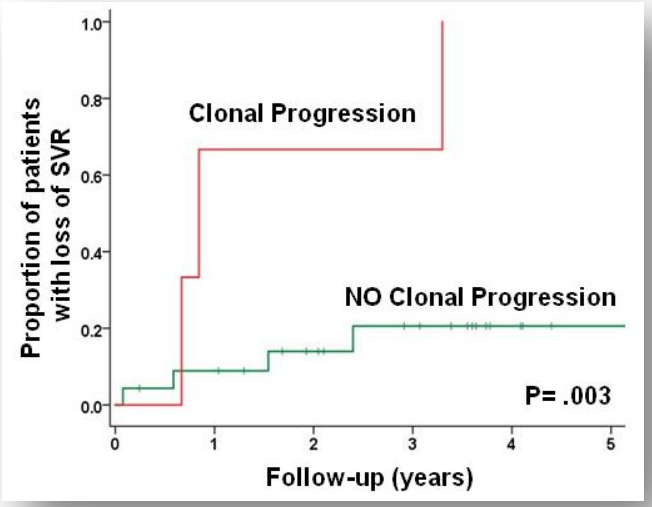
LR = 0-2
IR = 3-4
HR = 5
vHR >5

The 5-year survival were 90% (low), 77% (intermediate), 50% (high), and 34% (very high) in the training cohort (n = 205) ($p < 0.001$, respectively).

Gagelman N et al, Blood. 2019 May 16;133(20):2233-2242.

Correlation of the Number of Mutations and Response in Long-Term Ruxolitinib-Treatment





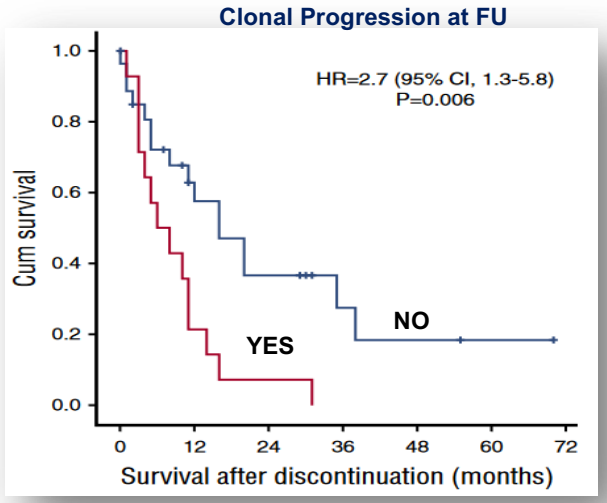
- MF Patients with ≤2 mutations had nine-fold higher odds of a spleen response than those with ≥3 mutations (odds ratio = 9.37; 95% confidence interval, 1.86-47.2).
- MF Patients with ≥3 mutations also had a shorter time to treatment discontinuation and shorter overall survival than those with fewer mutations.

- Median duration of SVR of 10 mo *vs* to not-reached in pts with or w/o clonal progression.
- none of the 7 patients who showed decrease of ≥20% from baseline *JAK2*V617F VAF lost SVR compared to 6 out of 13 (46.1%) who showed stable or increased *JAK2*V617F VAF (HR=61.8,95% CI 1.01–870.2)

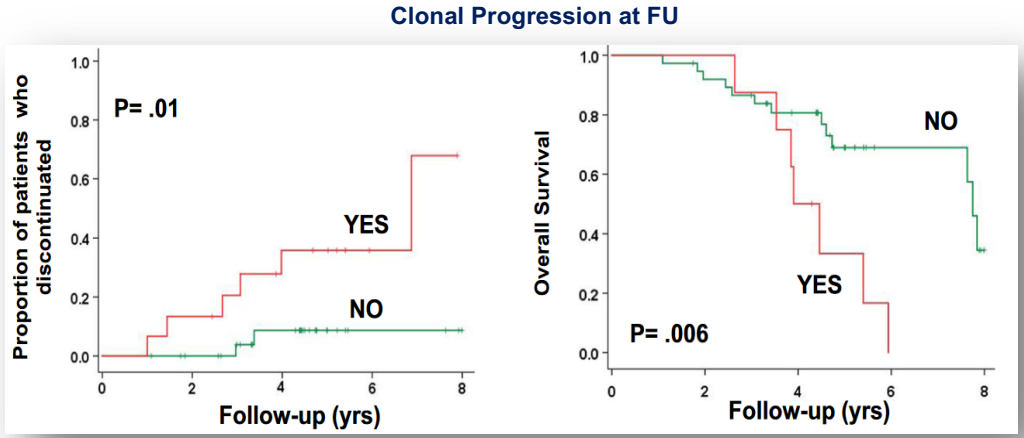
K. P. Patel et al. *Blood* 2015;126:790-797; Pacilli et al Blood Cancer J. 2018 Nov 22;8(12):122

Acquisition of new mutations while on JAk1/2 inhibitor treatment correlated with higher rate of dismal outcome in MF

Patients with clonal evolution had significantly shorter survival after discontinuation[1,2]



Transfusion dependency was the only clinical variable associated with clonal evolution.



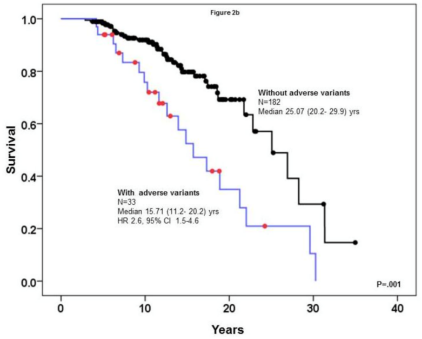
- Clonal progression was also associated with a higher rate of treatment discontinuation (38% vs 13%, HR 3,9; P=.05)

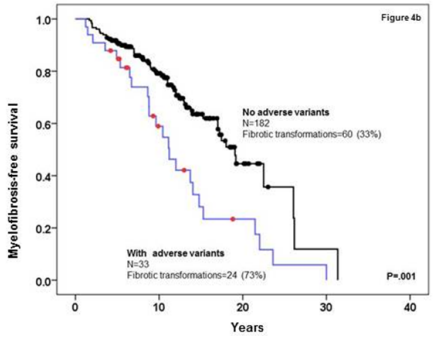
Modifications of mutation profile during follow-up were substantially similar in patients receiving ruxolitinib or hydroxyurea[2]

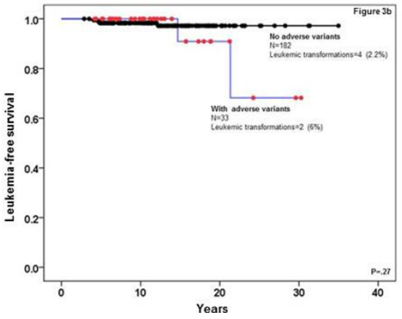
1. Newberry KJ et al. Blood. 2017; 31;130(9):1125-1131; 2, ; Pacilli et al Blood Cancer J. 2018 Nov 22;8(12):122

Impact of Mutations on Outcome in PV and ET

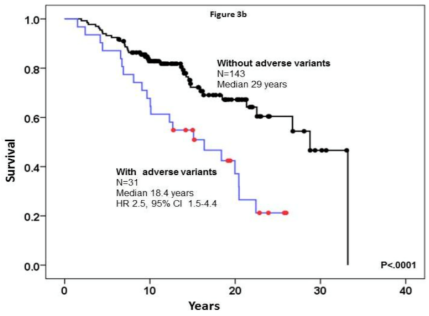
PV:
ASXL1, SRSF2, IDH.

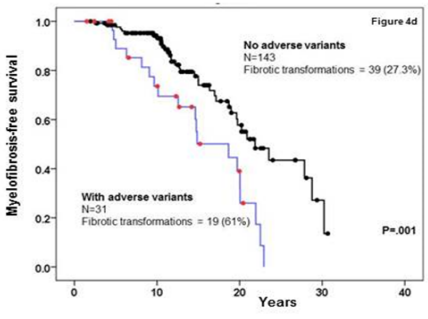


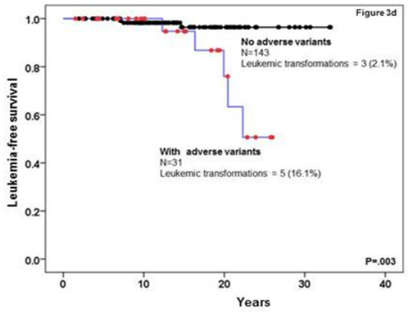




ET:
SH2B3, IDH2, SF3B1, U2AF1, EZH2, TP53







MIPSS-ET is based on age >60y, male gender, leukocyte count ≥11 x 10^9/L and adverse mutations (*SRSF2*, *SF3B1*, *U2AF1* and *TP53*)

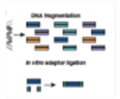
MIPSS-PV is based on age >67y, leukocyte count ≥15 x 10^9/L, thrombosis history and *SRSF2* mutations

Tefferi A et al. Blood Adv 2016;1:21-30; Tefferi A et a. BJH, accepted for publication.

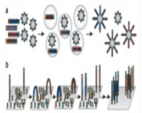
CONGENITAL or FAMILIAL ERYTHROCYTOSIS / THROMBOCYTOSIS

Hereditary disorders with germline defects affecting the erythroid/megakaryocyte lineage with mendelian inheritance, high penetrance, polyclonality of hematopoiesis

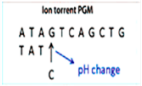
	GENE	DETTAGLIO DELLA REGIONE SEQUENZIATA
PANNELLO FORME RARE ERITROCITOSI PIASTRINOSI TROMBOCITOPENIE	HBB	CDS
	BPGM	EX3
	HBA1	CDS
	HBA2	CDS
	SH2B3 (ERITROCITOSI)	EX2-4
	EPOR	EX5-8
	VHL	CDS
	EGLN1 (PHD2)	CDS
	EPAS1 (HIF2A)	EX9, EX12
	JAK2	EX12-14, EX17, EX20-21
	THPO	INTR2, INTR3, 5UTR
	MPL (PIASTRINOSI)	CDS
	MPL INTR	INTR1-2
	GELSOLIN	EX4, EX5, EX10, EX13, EX15
	GATA1	CDS
	WAS (TROMBOCITOPENIE)	CDS
	RUNX1	EX3-EX7
	SRC	EX14



Library preparation
(DNA fragmentation + adapter ligation)



Clonal Amplifications
(Emulsion PCR or Bridge PCR)



Sequencing
(Synthesis or ligation)

Data analysys (Genome alignement)

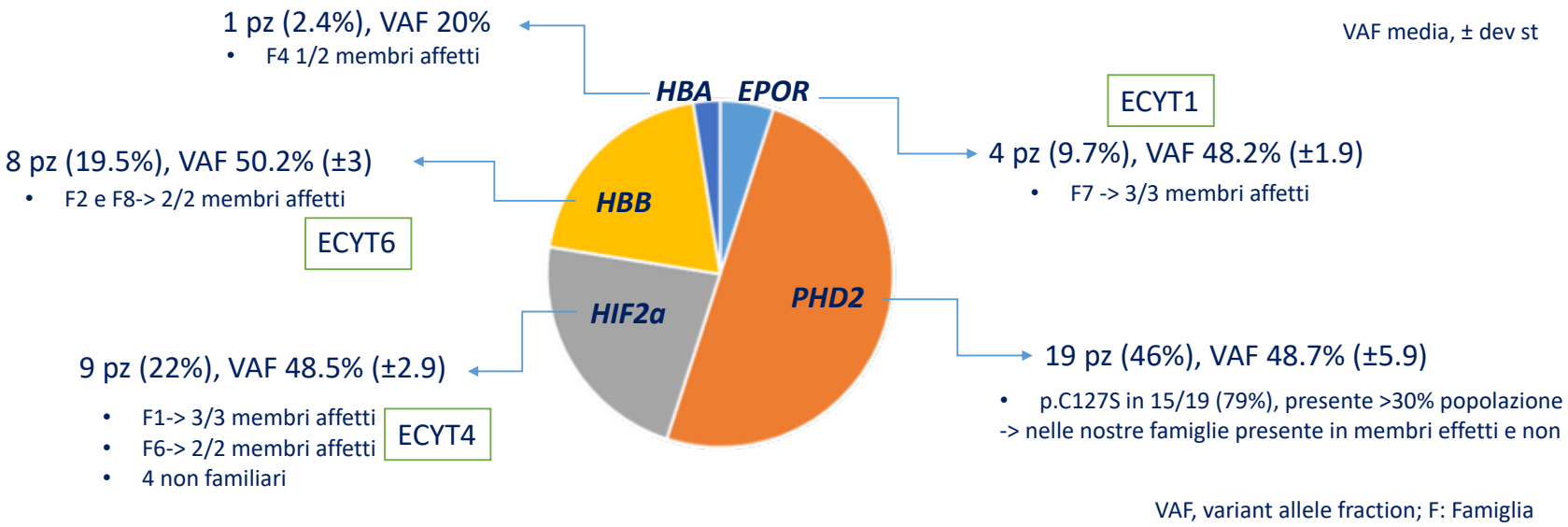
Variant filtering and annotation

CONGENITAL or FAMILIAL ERYTHROCYTOSIS : CRIMM Experience

SCREENING PER ERITROCITOSI

N° = 94 pts → 2 ***JAK2*** V617F media VAF 4.08% (±1.2)
1 ***JAK2*** EX12 H538_K539>L VAF 5.2% → **MPN**

N = 91 inclusi nell'analisi → 41 pz (45%) almeno 1 variante



VAF, variant allele fraction; F: Famiglia

Rotunno G et al. Abstrac C016, 47° Congresso Nazionale SIE 2019

Facts, challenges, actions

to make NGS clinically relevant

Challenges	Current knowledge	Actions
• Discriminating leukemia-associated mutations from **polymorphisms and passenger mutations**	• Usually at higher VAF than passenger • Predicted to be functionally significant	• Cancer-specific databases • Newer powerful bioinfo algorithms • Dynamic monitoring of VAF
• Discriminating leukemia-associated mutations **from CHIP**	• CHIP is age-dependent	• Better understanding of CHIP • Dynamic assessment of VAF
• Discriminating leukemia-associated mutations from **pathogenic germline variants**	• «Familial» predisposition genes	• Familial history/analysis • Appropriate germline controls • Stable VAF around 50%
• Discriminating »true» from **«false» negative MDR**	• Known mutations may be re-identified at 1-2% VAF	• Depth, internal controls • Other complementary approaches
• Discriminating «true» leukemia-associated mutations from **errors**	• Seq errors homopolimers • Analysis/interpretation	• Sanger validation • Cancer-specific databases • Bioinfo algoritms

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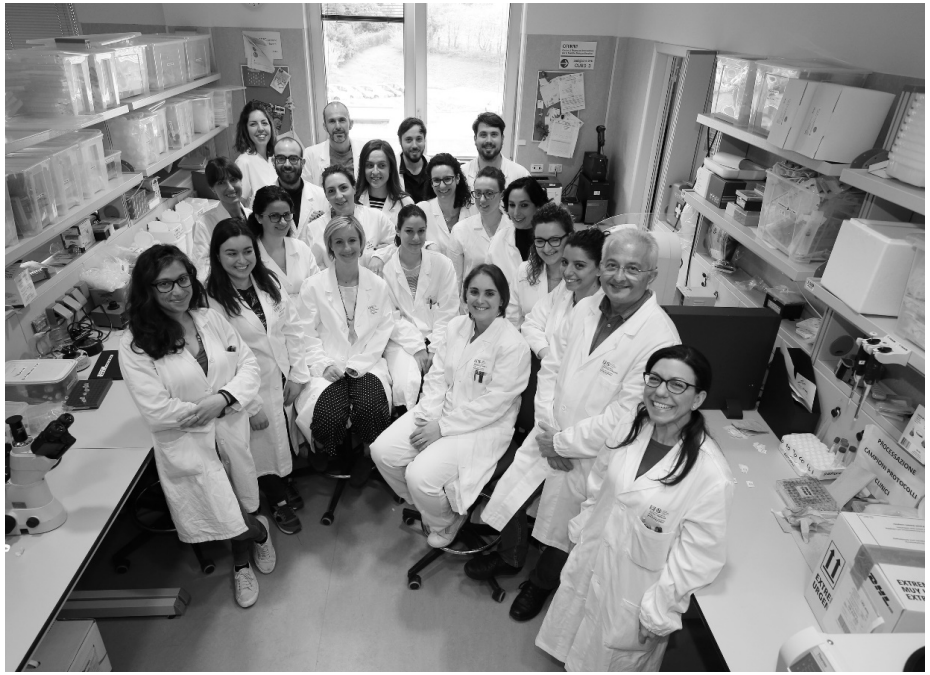

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