How to apply NGS based tests to Myeloid malignancies biomarker

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Hematology Research Laboratory of National and Kapodistrian University of Athens

- Research and diagnostic laboratory

- Monitoring of patients with hematological malignancies:

  - ✓ Myeloproliferative neoplasms (MPN)
  - ✓ Myeloid/lymphoid neoplasms
  - ✓ Myelodysplastic syndromes (MDS)
  - ✓ Acute myeloid leukemia (AML) and related neoplasms
  - ✓ B/T lymphoblastic leukemia/lymphoma

- 8.000-9.000 samples per year from 94 centers in Greece

Techniques

- PCR
- Real-time PCR
- Fragments Analysis
- Sanger sequencing
- Next generation sequencing

Certifications

- ISO 9001:2015 certification (TUV HELLAS/ TUV NORD) (041160004)
- BCR-ABL monitoring with Real-Time PCR, by ***European Leukemia Net (ELN)****
- TP53 mutation detection with Sanger Sequencing, by ***European Research Initiative on CLL (ERIC)****
- IGVH mutation detection with Sanger Sequencing, by ***European Research Initiative on CLL (ERIC)****

The laboratory is in the process of accreditation with the ISO 15189:2012 standard

*Out laboratory is one of the two in Greece that have these certifications

Precision medicine

The laboratory is a member of:

✓ the National Network of Precision Medicine

✓ the new department of the National and Kapodistrian University of Athens:

<<Center for new biotechnologies and precision medicine, School of Medicine>>

The base of precision medicine is ***Next Generation Sequencing (NGS)***

Our experience in NGS

We use NGS for biomarker detection:

➢ Myeloid Malignancies

- acute myeloid leukemia (AML),
- myeloid dysplastic syndrome (MDS),
- myeloproliferative neoplasms (MPN),
- chronic myeloid leukemia (CML),
- chronic myelomonocytic leukemia (CMML),
- juvenile myelomonocytic leukemia (JMML)

→ **Oncomine Myeloid Research Assay (Thermo Fisher Scientific)**

➢ Lymphoid Malignancies

- B-acute lymphoblastic leukemia (ALL)/lymphoma
- T-acute lymphoblastic leukemia (ALL)/lymphoma

→ **LymphoTrack *IGH* FR3 Assay Panel**
LymphoTrack *TCRG* Assay Panel
(Invivoscribe)

NGS Platform: Ion GeneStudio™ S5 System

Myeloid Malignancies - Why NGS

- Myeloid malignancies are often difficult to classify
- Many genes are involved (with driver or acquired mutations)
- Previously biomarker detection was the sequential gene testing → expensive and time consuming for the patient
- NGS gave the opportunity of high-throughput sequencing with high sensitivity (~3-5%)

Oncomine™ Myeloid Research Assay

It interrogates all relevant DNA mutations and fusion transcripts associated with myeloid disorders

Table 1. Oncomine Myeloid Research Assay gene targets.

Hotspot genes (23)		Full genes (17)		Fusion driver genes (29)			Expression genes (5)	Expression control genes (5)
ABL1	*KRAS*	*ASXL1*	*PRPF8*	*ABL1*	*HMGA2*	*NUP214*	*BAALC*	*EIF2B1*
BRAF	*MPL*	*BCOR*	*RB1*	*ALK*	*JAK2*	*PDGFRA*	*MECOM*	*FBXW2*
CBL	*MYD88*	*CALR*	*RUNX1*	*BCL2*	*KMT2A*	*PDGFRB*	*MYC*	*PSMB2*
CSF3R	*NPM1*	*CEBPA*	*SH2B3*	*BRAF*	*(MLL)*	*RARA*	*SMC1A*	*PUM1*
DNMT3A	*NRAS*	*ETV6*	*STAG2*	*CCND1*	*MECOM*	*RBM15*	*WT1*	*TRIM27*
FLT3	*PTPN11*	*EZH2*	*TET2*	*CREBBP*	*MET*	*RUNX1*		
GATA2	*SETBP1*	*IKZF1*	*TP53*	*EGFR*	*MLLT10*	*TCF3*		
HRAS	*SF3B1*	*NF1*	*ZRSR2*	*ETV6*	*MLLT3*	*TFE3*		
IDH1	*SRSF2*	*PHF6*		*FGFR1*	*MYBL1*			
IDH2	*U2AF1*			*FGFR2*	*MYH11*			
JAK2	*WT1*			*FUS*	*NTRK3*			
KIT								

40 genes in DNA

27 fusion genes in RNA

NGS - 3 days workflow

PCR-Library preparation

6 hours

Automated template preparation on Ion Chef Systems

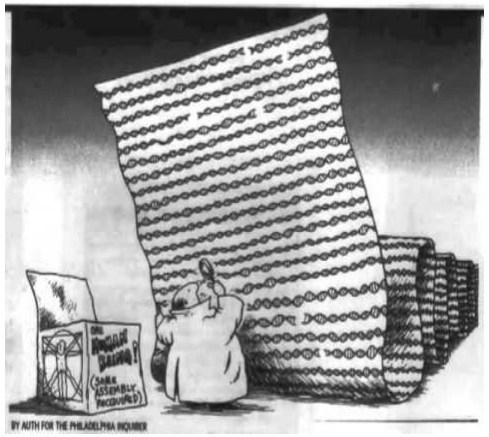
16 hours

Sequencing on Ion S5 Systems

5 hours

End-to-end bioinformatics solution developed for particular assays and applications

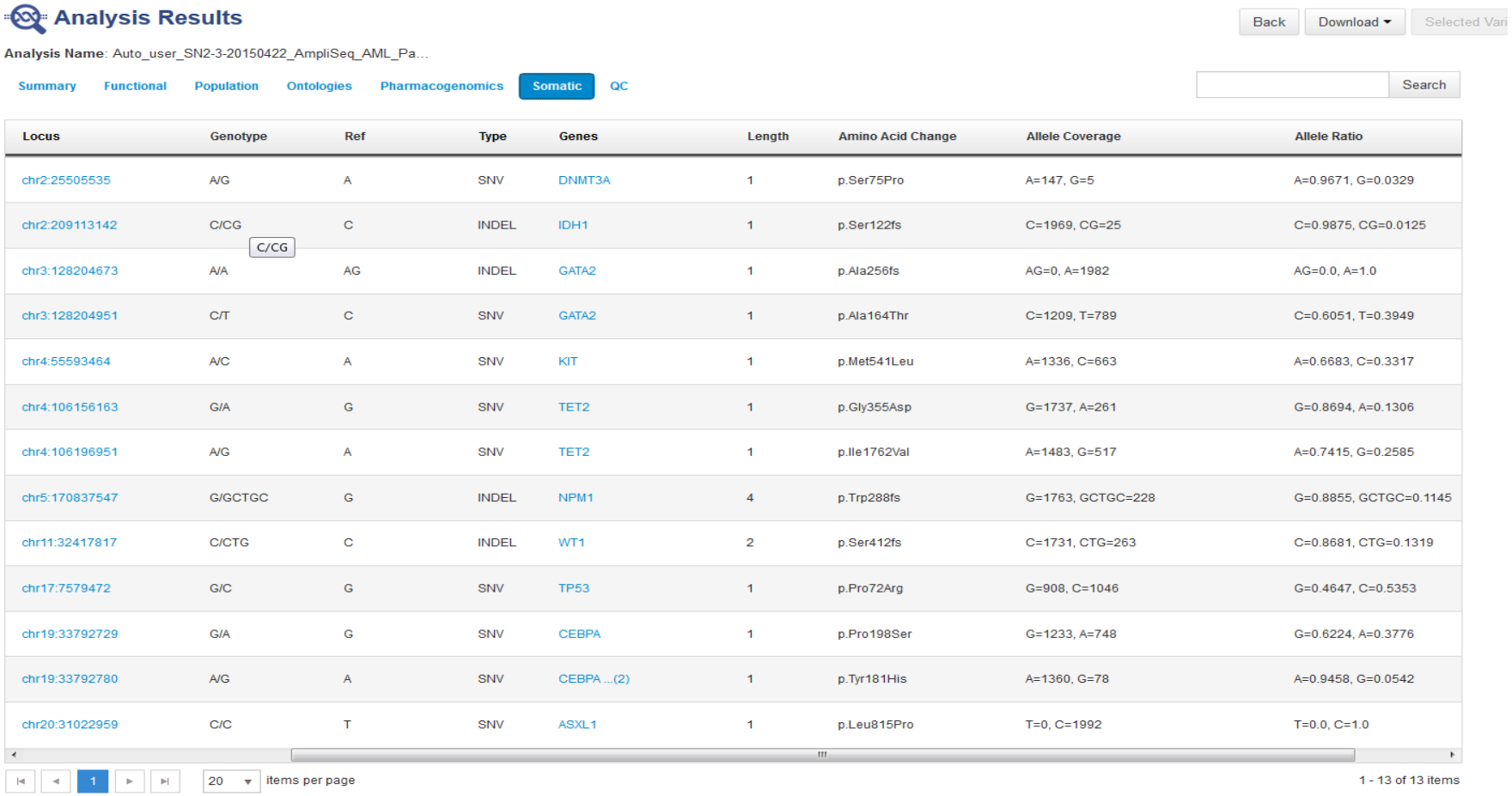
3 hours



Evaluation of the results

.....hours

Example of analysis results



Analysis Results

Back | Download | Selected Vari

Analysis Name: Auto_user_SN2-3-20150422_AmpliSeq_AML_Pa...

Summary | Functional | Population | Ontologies | Pharmacogenomics | Somatic | QC

Search

Locus	Genotype	Ref	Type	Genes	Length	Amino Acid Change	Allele Coverage	Allele Ratio
chr2:25505535	A/G	A	SNV	DNMT3A	1	p.Ser75Pro	A=147, G=5	A=0.9671, G=0.0329
chr2:209113142	C/CG	C	INDEL	IDH1	1	p.Ser122fs	C=1969, CG=25	C=0.9875, CG=0.0125
chr3:128204673	A/A	AG	INDEL	GATA2	1	p.Ala256fs	AG=0, A=1982	AG=0.0, A=1.0
chr3:128204951	C/T	C	SNV	GATA2	1	p.Ala164Thr	C=1209, T=789	C=0.6051, T=0.3949
chr4:55593464	A/C	A	SNV	KIT	1	p.Met541Leu	A=1336, C=663	A=0.6683, C=0.3317
chr4:106156163	G/A	G	SNV	TET2	1	p.Gly355Asp	G=1737, A=261	G=0.8694, A=0.1306
chr4:106196951	A/G	A	SNV	TET2	1	p.Ile1762Val	A=1483, G=517	A=0.7415, G=0.2585
chr5:170837547	G/GCTGC	G	INDEL	NPM1	4	p.Trp288fs	G=1763, GCTGC=228	G=0.8855, GCTGC=0.1145
chr11:32417817	C/CTG	C	INDEL	WT1	2	p.Ser412fs	C=1731, CTG=263	C=0.8681, CTG=0.1319
chr17:7579472	G/C	G	SNV	TP53	1	p.Pro72Arg	G=908, C=1046	G=0.4647, C=0.5353
chr19:33792729	G/A	G	SNV	CEBPA	1	p.Pro198Ser	G=1233, A=748	G=0.6224, A=0.3776
chr19:33792780	A/G	A	SNV	CEBPA ...(2)	1	p.Tyr181His	A=1360, G=78	A=0.9458, G=0.0542
chr20:31022959	C/C	T	SNV	ASXL1	1	p.Leu815Pro	T=0, C=1992	T=0.0, C=1.0

C/CG

1 | 20 items per page

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It can filter out the mutations that are:

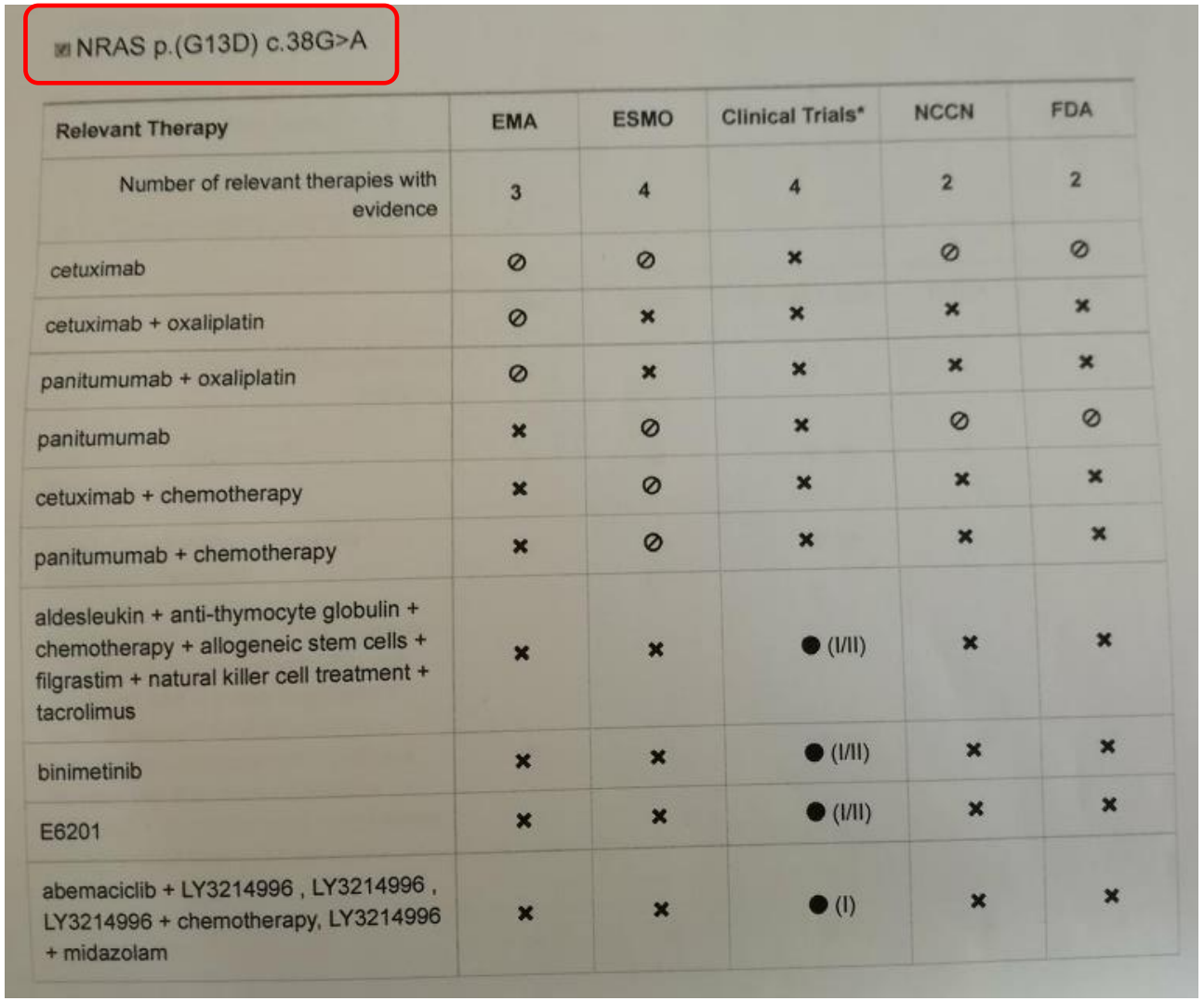
- polymorphisms,
- synonymous,
- in introns etc

So it can discriminate the mutations with a clinical significance according to gene databases

Variant report

Mutation that has been found

Relevant substances or clinical studies



☑ NRAS p.(G13D) c.38G>A

Relevant Therapy	EMA	ESMO	Clinical Trials*	NCCN	FDA
Number of relevant therapies with evidence	3	4	4	2	2
cetuximab	⊘	⊘	×	⊘	⊘
cetuximab + oxaliplatin	⊘	×	×	×	×
panitumumab + oxaliplatin	⊘	×	×	×	×
panitumumab	×	⊘	×	⊘	⊘
cetuximab + chemotherapy	×	⊘	×	×	×
panitumumab + chemotherapy	×	⊘	×	×	×
aldesleukin + anti-thymocyte globulin + chemotherapy + allogeneic stem cells + filgrastim + natural killer cell treatment + tacrolimus	×	×	● (I/II)	×	×
binimetinib	×	×	● (I/II)	×	×
E6201	×	×	● (I/II)	×	×
abemaciclib + LY3214996 , LY3214996 , LY3214996 + chemotherapy, LY3214996 + midazolam	×	×	● (I)	×	×

NGS performances evaluation

- Completed within a month
- We checked samples with known mutations and in serial dilutions
- Sanger sequencing *vs* NGS → same results, even in difficult targets, such as CEBPA (GC rich region) or FLT3 internal tandem duplications (ITDs)
- Sensitivity about 3-5%
- Mean depth 1500x

Limitations

- You need to collect many sample in order to have a cost-effective run - it depends on the capacity of the chip
  - chip 520→ 6 samples
  - chip 530 → 12 samples

- The cost (initial cost of the equipment and consumables for each run)
  - But cheaper than sequential gene testing with sanger sequencing

- Samples should be analyzed in a central laboratory in each country in order to minimize the:
  - time to response
  - cost

NGS in clinical research

- We have already analyzed 230 samples within 1,5 years that we use NGS
- NGS allows a rapid carachterization of AML and MDS samples
- NGS use has the potential to to evaluate response to therapy
- According to plan our laboratory will handle all the new cases of AML in Greece for molecular analysis with NGS

NGS testing – examples of cases

Acute Myeloid Leukemia (AML)

Current stratification

Table 5. 2017 ELN risk stratification by genetics

Risk category*	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}†
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}†
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}† (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*‡
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype,§ monosomal karyotype‖
	Wild-type *NPM1* and *FLT3*-ITD^{high}†
	Mutated *RUNX1*¶
	Mutated *ASXL1*¶
	Mutated *TP53*#

Survival curves based on current stratification and chemotherapy

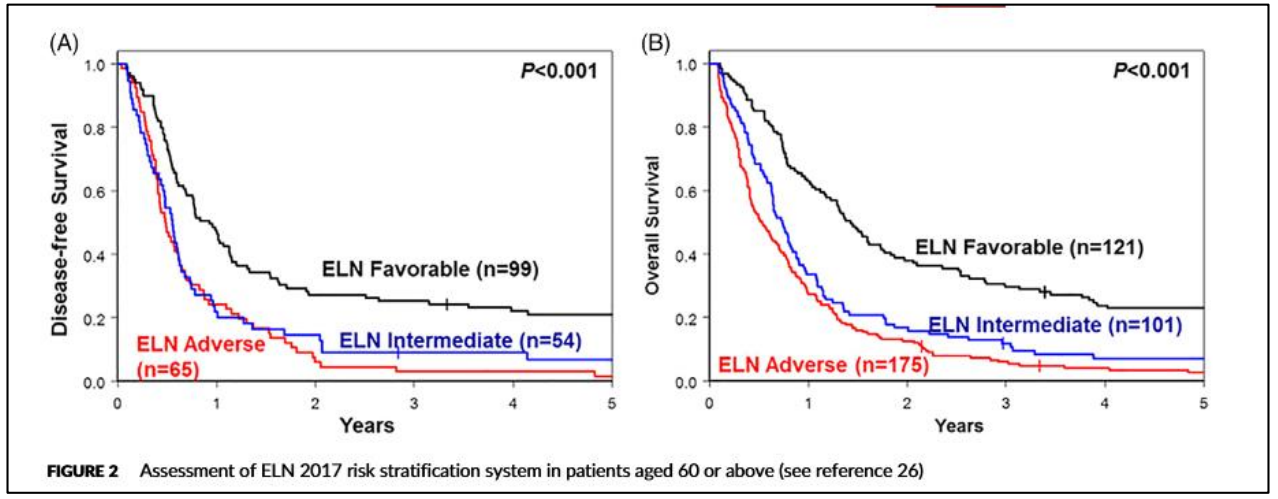


FIGURE 2 Assessment of ELN 2017 risk stratification system in patients aged 60 or above (see reference 26)

What's new in AML

Genomic heterogeneity of AML

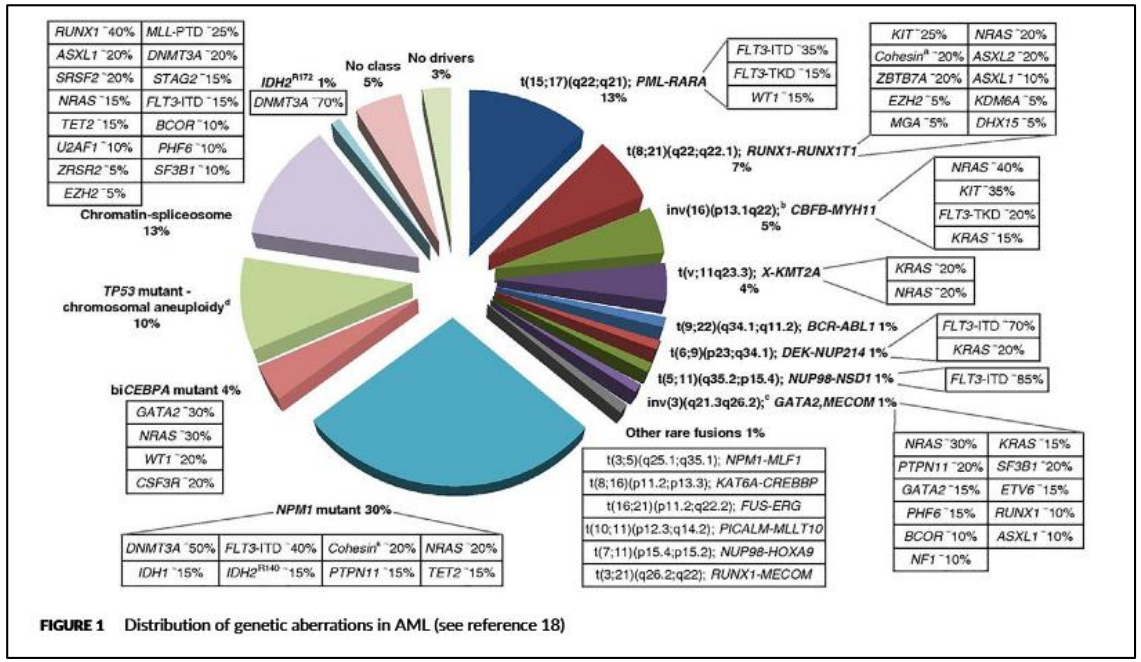


FIGURE 1 Distribution of genetic aberrations in AML (see reference 18)

Novel targeted therapies

Table 10. Novel therapies in clinical development in AML

Novel therapies in clinical development	
Protein kinase inhibitors	• FLT3 inhibitors (midostaurin, quizartinib, gilteritinib, crenolanib) • KIT inhibitors • PI3K/AKT/mTOR inhibitors • Aurora and polo-like kinase inhibitors, CDK4/6 inhibitors, CHK1, WEE1, and MPS1 inhibitors • SRC and HCK inhibitors
Epigenetic modulators	• New DNA methyltransferase inhibitors (SGI-110) • HDAC inhibitors • IDH1 and IDH2 inhibitors • DOT1L inhibitors • BET-bromodomain inhibitors
Chemotherapeutic agents	• CPX-351 • Vosaroxin • Nucleoside analogs
Mitochondrial inhibitors	• Bcl-2, Bcl-xL, and Mcl-1 inhibitors • Caseinolytic protease inhibitors
Therapies targeting oncogenic proteins	• Fusion transcripts targeting • EVI1 targeting • NPM1 targeting • Hedgehog inhibitors
Antibodies and immunotherapies	• Monoclonal antibodies against CD33, CD44, CD47, CD123, CLEC12A • Immunoconjugates (eg, GO, SGN33A) • BiTEs and DARTs • CAR T cells or genetically engineered TCR T cells • Immune checkpoint inhibitors (PD-1/PD-L1, CTLA-4) • Anti-KIR antibody • Vaccines (eg, WT1)
Therapies targeting AML environment	• CXCR4 and CXCL12 antagonists • Antiangiogenic therapies

Why we need NGS in AML

- ✓Better classification of the risk, based on analysis of big number of genes, necessary to stratify risk group and take decisions about the proper therapeutic approach

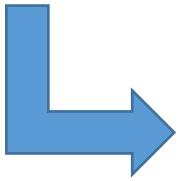
- ✓Detection of critical mutations that are sensitive to specific inhibitors (e.g. on FLT3 inhibitors, IDH inhibitors and more are coming…)

NGS gave the opportunity of high-throughput sequencing of all the genes involved, in one run

Example of AML case

Sample of a female with AML

- ✓ Normal karyotype
- ✓ NPM1 gene negative for mutations (Fragments analysis)
- ✓ FLT3 gene negative for mutations (Fragments analysis)
- ✓ CEBPA gene negative for mutations (Sanger sequencing)



- Typical screening for AML new cases
- Based on this screening the samples is classified as intermediate risk

Example of AML case

NGS screening with Oncomine™ Myeloid Research Assay (Thermo Fisher Scientific) Ion GeneStudio™ S5 System

NGS AML	**28/2/2018**	
Gene	**Amino Acid Change**	**Allele Frequency %**
NRAS	p.Gly12Asp	4.21
WT1	p.Val371fs	43.40
RUNX1	p.Arg204Gln	49.51

NGS FOLLOW UP	**27/9/2018**	
-		

NGS FOLLOW UP – ~~END OF TREATMENT~~	**27/11/2018**	
-		

ALLOGENEIC BONE MARROW TRANSPLANTATION

NGS MOLECULAR RELAPSE AFTER ALLO-MAK	**29/1/2019**	
Gene	**Amino Acid Change**	**Allele Frequency %**
RUNX1	p.Arg204Gln	3.20

NGS ~~CLINICAL~~ RELAPSE AFTER ALLO-MAK	**5/3/2019**	
Gene	**Amino Acid Change**	**Allele Frequency %**
WT1	p.Val371fs	20.00
RUNX1	p.Arg204Gln	20.40

Classification based on NGS testing:
High risk (RUNX1 pos)→ allogeneic bone marrow transplantation

Sequential test and MRD analysis with NGS:

<u>Pros</u>

- ✓ Monitoring the kinetics of the subclones
- ✓ Detection of resistant subclones
- ✓ Detection of clonal evolution that may be missed by flow cytometry

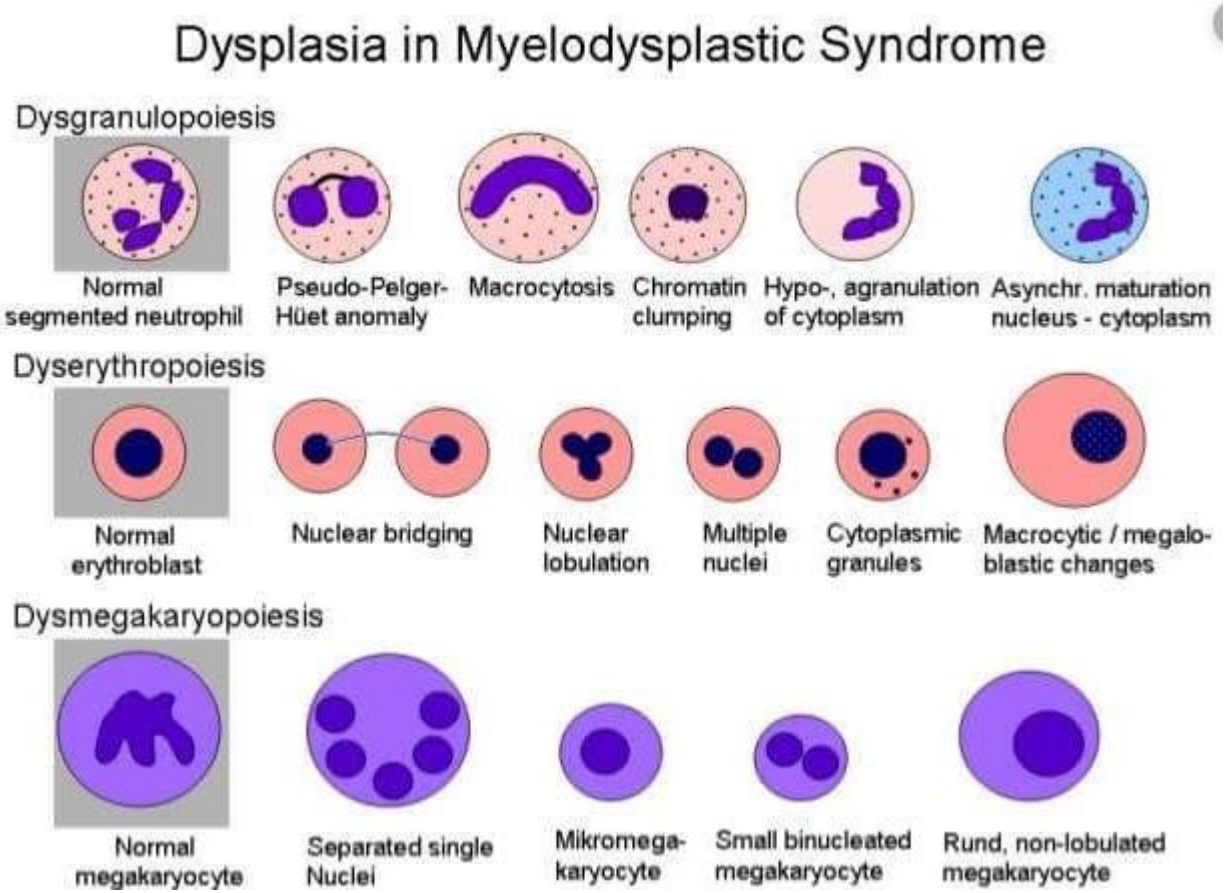
<u>Cons</u>

- ✓ Low sensitivity (~1%)- not indicated for MRD analysis for the moment

Until now we have analyzed 85 samples with AML and we have submitted an abstract, based on these results, at the Hellenic Hematology Congress (7-10/11/2019)

"Acute Myelogenous Leukemia with RUNX1 Gene Mutations: clinical characteristics and prediction" Oral presentation No 34

Myelodysplastic Syndromes (MDS)



- Many cell lines are affected due to mutations in the hematopoietic stem cells
- Dysplasia in different cell lines
- Cytopenias that cause sensitivity to infections
- Treatment is based on clinical and morphological features
- Molecular markers are not used in clinical practice in MDS

Do we need NGS in MDS?

- Helpful in better risk stratification (low, intermediate and high risk)
- Prognostic for risk of transformation to AML
- Not in clinical practice yet

Example of MDS case

- Male MDS sample
- MDS low risk based on morphological and clinical aspects
- NGS analysis with Oncomine Myeloid Research Assay (Thermo Fisher Scientific): Ion GeneStudio™ S5 System

Gene	Amino Acid Change	Coding	Exon	Variant ID	Locus	Allele Frequency %	Transcript	Variant Effect
IDH1	p.Arg132Cys	c.394C>T	4	COSM28747	chr2:209113113	35.95	NM_005896.3	missense
SRSF2	p.Pro95Arg	c.284C>G	1	COSM211661	chr17:74732935	36.94	NM_003016.4	missense
ASXL1	p.Ser989Ter	c.2966_2967delCT	12		chr20:31023478	4.41	NM_015338.5	nonsense

Table 1. Summary of driver mutations in myelodysplastic syndromes (MDS)

Mutated genes	Associated phenotypes	MDS types	Other disease	Frequency in MDS (%)	Effect on outcome	Application to treatment
RNA splicing (mutually exclusive)				60–70		None
SF3B1	Ring sideroblasts	RARS, RCMD-RS	RARS-T	15–30	Good	
SRSF2		RCMD, RAEB	CMML	10–20	Poor	
U2AF1		RCMD, RAEB	CMML	5–10	Poor	
ZRSF2		RCMD, RAEB	CMML	5–10	None	
DNA methylation (*TET2* and *IDH1/2* are exclusive)				40–50		DNA methyltransferase inhibitors
TET2	Myeloid dominancy	All MDS, normal karyotype	CMML	20–30	None	IDH1/2 inhibitors
IDH1/2		RCMD, RAEB	CMML	5	Poor (*IDH2*)	
DNMT3A		All MDS	AML	10	None	
Chromatin modification				20–30		Deacetylase inhibitors
ASXL1		RCMD, RAEB	CMML	15–20	Poor	
EZH2	-7/7q-	RCMD, RAEB	CMML	5	Poor	
BCOR		RCMD, RAEB		5	Poor	
Transcriptional factor				20–30		None
RUNX1	Thrombocytopenia	RCMD, RAEB	CMML, AML	10	Very poor	
CEBPA		RCMD, RAEB	AML	<5	None–poor	
ETV6		RCMD, RAEB		<5	Poor	
Signal transduction (mutually exclusive)				20–30		Kinase inhibitors
NRAS/KRAS		All MDS	JMML, CMML	10	Poor	
CBL		All MDS	JMML, CMML	5	Poor	
JAK2	Megakaryocytosis	All MDS	RARS-T, MPN	5	None	JAK inhibitors
NF1		All MDS	JMML	<5	Poor	
FLT3		All MDS	AML	<5	Poor	FLT3 inhibitors
Cohesin complex (mutually exclusive)				10		None
STAG2		RCMD, RAEB	AML, CMML	5–10	None–poor	
TP53	Complex karyotype	RAEB, isolated del(5q)		10	Very poor	None

- ✓ 3 mutated genes with poor prognosis
- ✓ Related with transformation to AML

- IDH1 35,95% VAF
- SRSF2 36,94% VAF
- ASXL1 4,41% VAF

- ✓ MDS High risk according to molecular profile
- ✓ Closer monitoring
- ✓ Searching for donor for allogenic transplantation if patient has good clinical status (age, comorbidities etc)

Until now we have analyzed 82 samples with MDS and some of the results were published in the following paper in a collaboration with the Hellenic MDS Study Group.

Annals of Hematology (2019) 98:1383–1392
https://doi.org/10.1007/s00277-019-03650-w

ORIGINAL ARTICLE

Bone marrow PARP1 mRNA levels predict response to treatment with 5-azacytidine in patients with myelodysplastic syndrome

Panagiotis T. Diamantopoulos[1] · Christina-Nefeli Kontandreopoulou[1] · Argiris Symeonidis[2] · Ioannis Kotsianidis[3] · Vassiliki Pappa[4] · Athanasios Galanopoulos[5] · Theodoros Vassilakopoulos[1] · Maria Dimou[6] · Eleni Solomou[2] · Marie-Christine Kyrtsonis[6] · Marina Siakantaris[1] · Maria Angelopoulou[1] · Alexandra Kourakli[2] · Sotirios Papageorgiou[4] · Georgia Christopoulou[2] · Maria Roumelioti[6] · Panayiotis Panayiotidis[6] · Nora-Athina Viniou[1] · On behalf of the Hellenic MDS Study Group

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Future applications of NGS

- Ion AmpliSeq HD technology → ultra high sensitivity technology that finds variants with a low limit of detection—down to 0.1%

- We have designed primers for the detection of TP53 mutations

- TP53 mutations are prognostic and predictive marker for the patients with Chronic Lymphocytic Leukemia (CLL)

- European Research Initiative on CLL (ERIC) is already starting a new collaborative project in which we are interested in participating:

‘’MULTICENTER STUDY ON PROGNOSTIC AND PREDICTIVE IMPACT OF TP53 VARIANTS BELOW 10% VAF (variant allelic frequency)’’