Introduction to Next-Generation Sequencing for Oncology Applications

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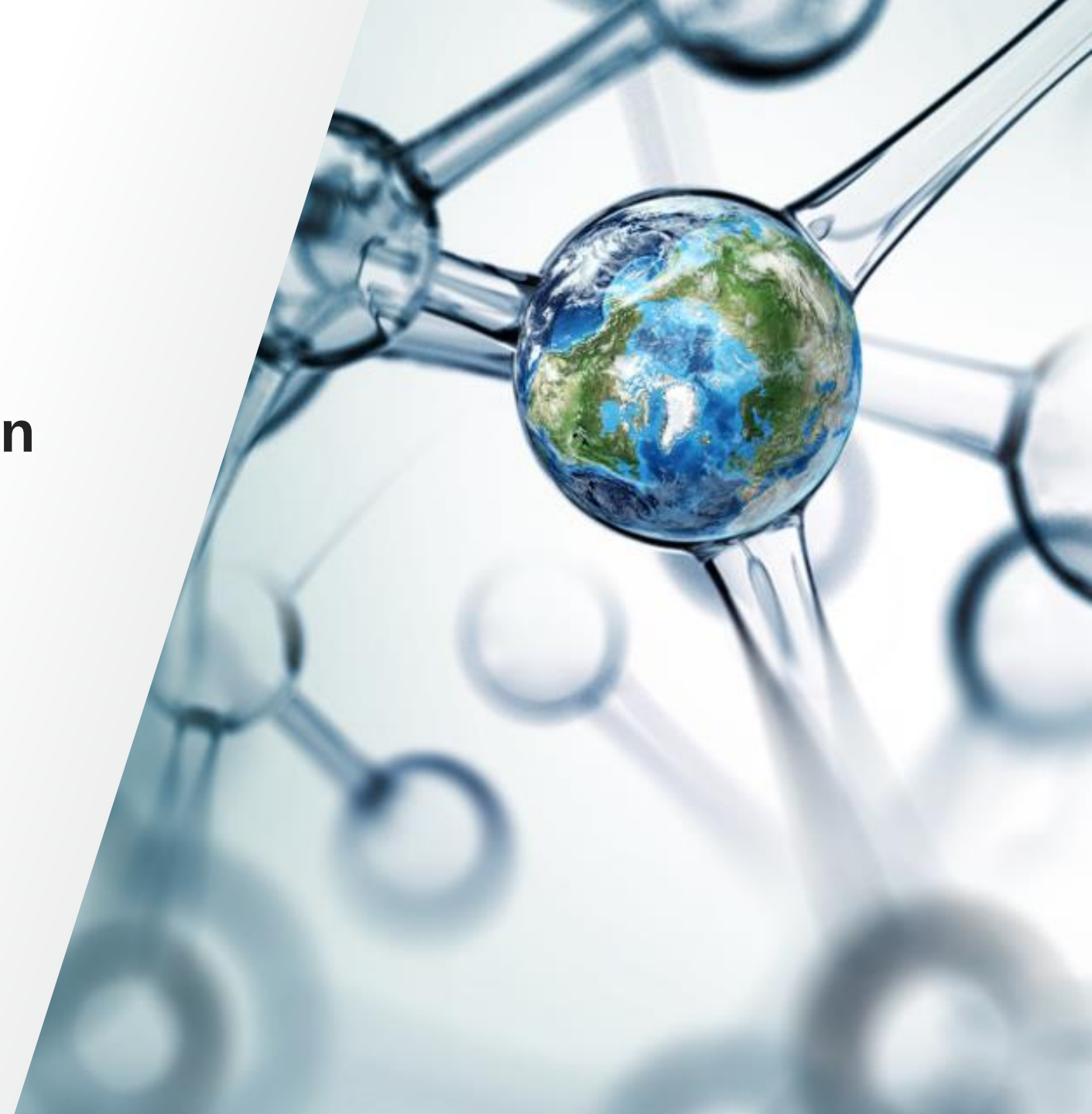
Clinical Next-Generation Sequencing

Thermo Fisher Scientific



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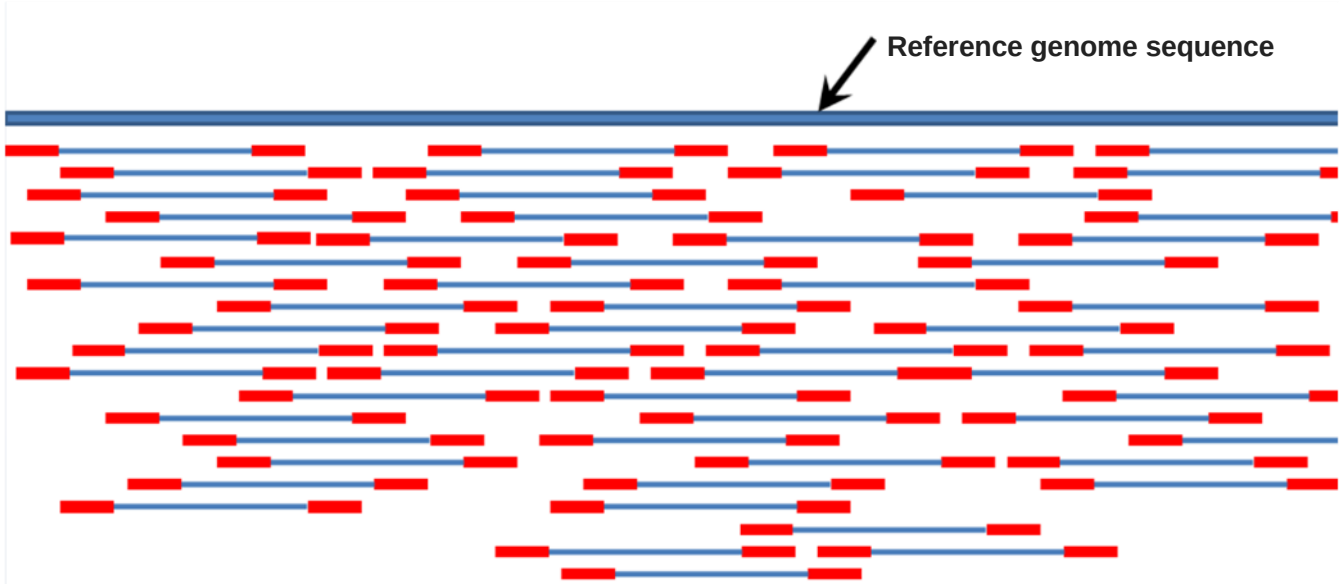
What is next-generation sequencing?



Next-generation sequencing (NGS) is a technology for determining the sequence of DNA or RNA to study genetic variation associated with diseases or other biological phenomena.

NGS is also called "**massively parallel sequencing**" because millions of DNA strands are sequenced simultaneously.

Source: Wikimedia Commons—by Suspencewl [CC0]

Typical NGS workflow

Nucleic acid extraction and purification

Nucleic acid (DNA or RNA) is first extracted and purified from a biological specimen such as FFPE tissue or plasma.

DNA and RNA of sufficient yield and quality are crucial for clinical applications.

Library preparation

Prior to sequencing, a library must be constructed. DNA or cDNA is fragmented, and adaptors and barcodes are ligated to enable binding to surfaces and sequencing. Specific genomic regions may be enriched during this process, depending on the application.

Sequencing

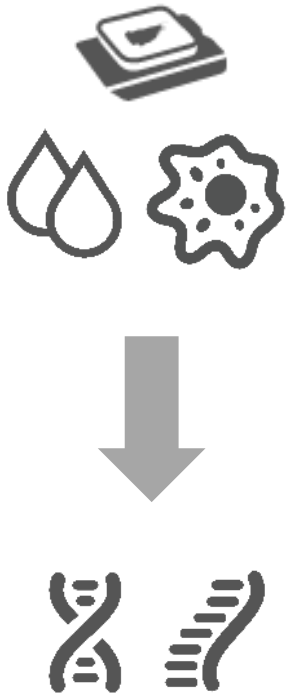
Libraries are pooled and sequenced to determine individual bases as they are incorporated.

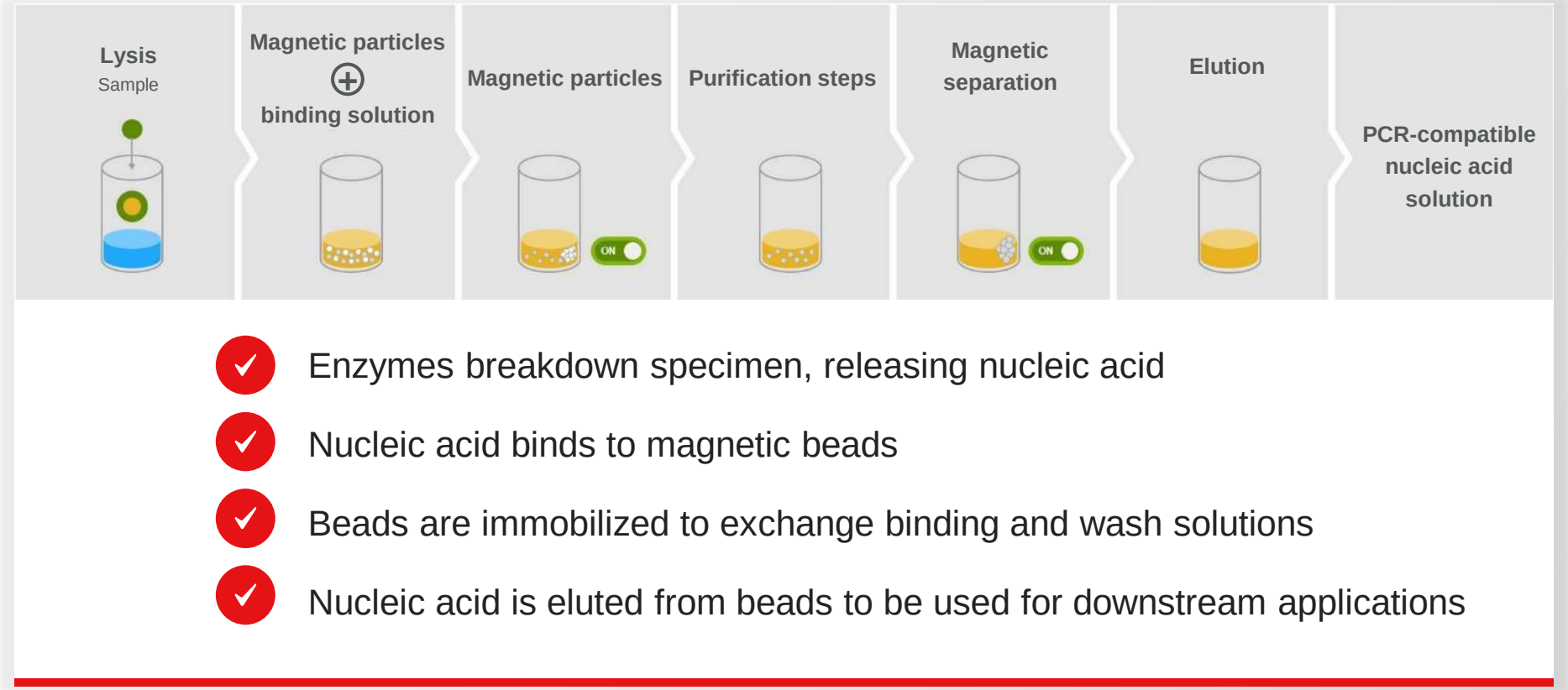
Raw signals are digitized into base calls and assembled into sequencing reads, which represent library fragments.

Data analysis

Bioinformatics tools and applications are used for quality control, alignment to reference genomes, and identification of genetic alterations.

Step 1: Nucleic Acid Isolation

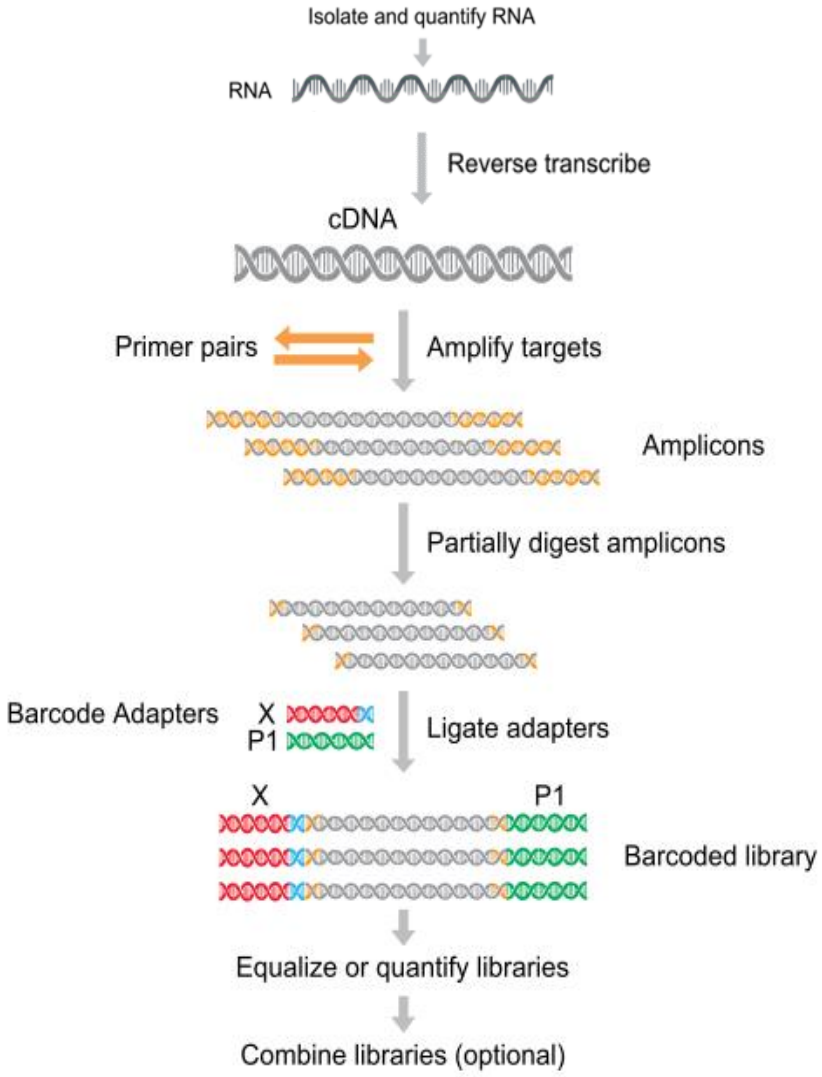




- Enzymes breakdown specimen, releasing nucleic acid
- Nucleic acid binds to magnetic beads
- Beads are immobilized to exchange binding and wash solutions
- Nucleic acid is eluted from beads to be used for downstream applications

Extraction of DNA and/or RNA from tissue or blood specimens

Step 2: Targeted library preparation

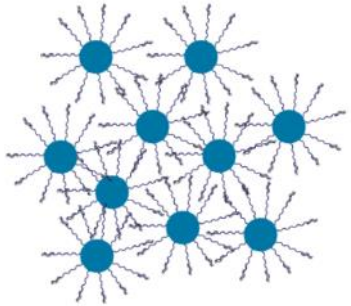


- If sequencing RNA, perform reverse transcription to create complimentary DNA
- DNA/cDNA is amplified through PCR with primers targeting genomic regions of interest
- Sequencing adapters are ligated to amplified targets with molecular barcode
- Barcoded libraries can be pooled to sequence multiple samples simultaneously

Enrichment of genes of interest and incorporation of sequencing adapters

Step 3: Ion semiconductor sequencing

1



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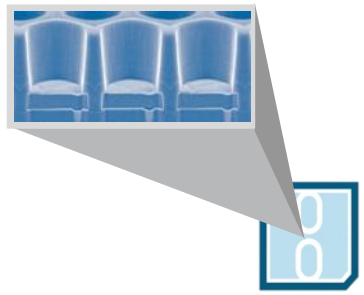

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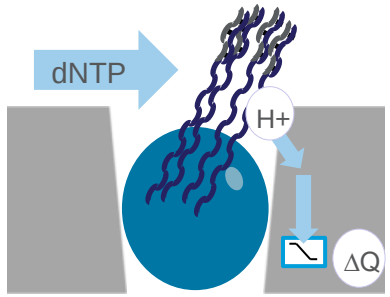

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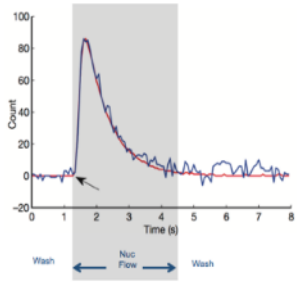

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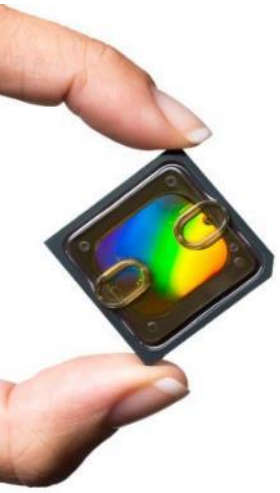


Template libraries are bound to beads and distributed into wells of sequencing chip

Nucleotides flow over chip one at a time to build a complementary strand

When a base is incorporated, a hydrogen ion is released, changing pH

Sequential changes in pH are detected by a sensor, building an output sequence

Base incorporation leads to changes in pH, which are translated into a sequence

Step 4: Bioinformatic Analysis

- Reads are complied and filtered by analysis software
- Reads are aligned to reference genome
- Software identifies variants and provides results in report
- Reports are typically annotated with clinical significance

Reads are compiled and variants identified through bioinformatic software

EGFR L858R Mutation (Chromosome 7)

Reference sequence { …CAGATTTTGGGCTGGCCAAACTGCT…

Read pileup {

TTTGGGCTGGCCAAACTG
TTTGGGCTGGCCAAAC
TTTGGGCTGGCCAA
TTTGGGCTGGCC
TTTGGGCGGGCC
TTTGGGCTGGC
TTTGGGCTGGCCAA
TTTGGGCGGGCCAA
TTTGGGCTGGCCAAAC
TTTGGGCTGGCCAAACTG

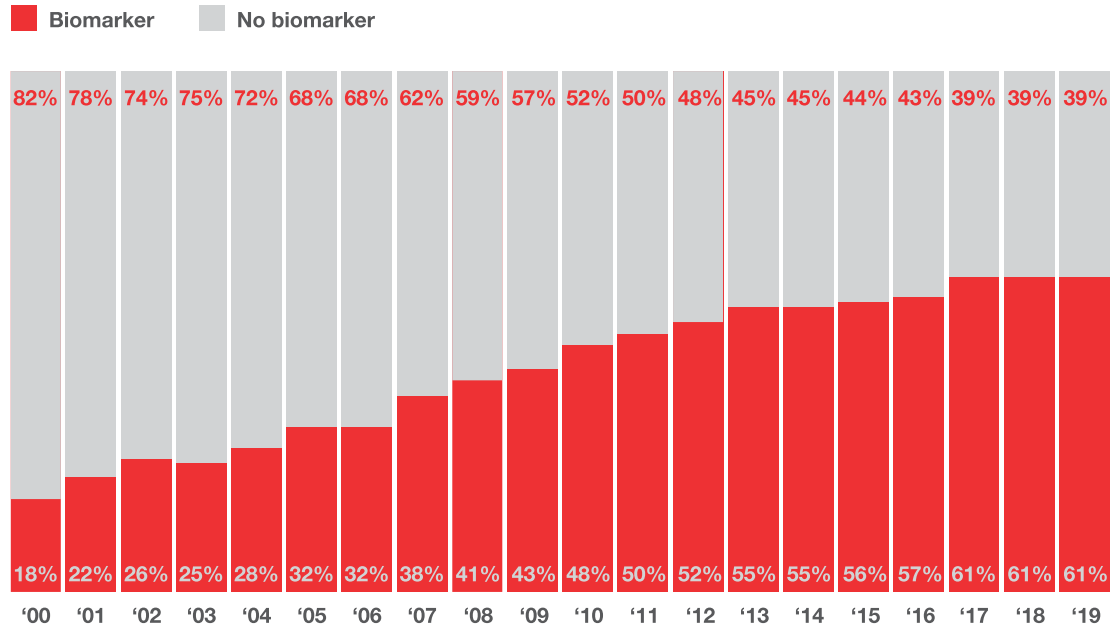
Variant reads

andrew.hutchison@thermofisher.com | September-2022

How does NGS apply to oncology?

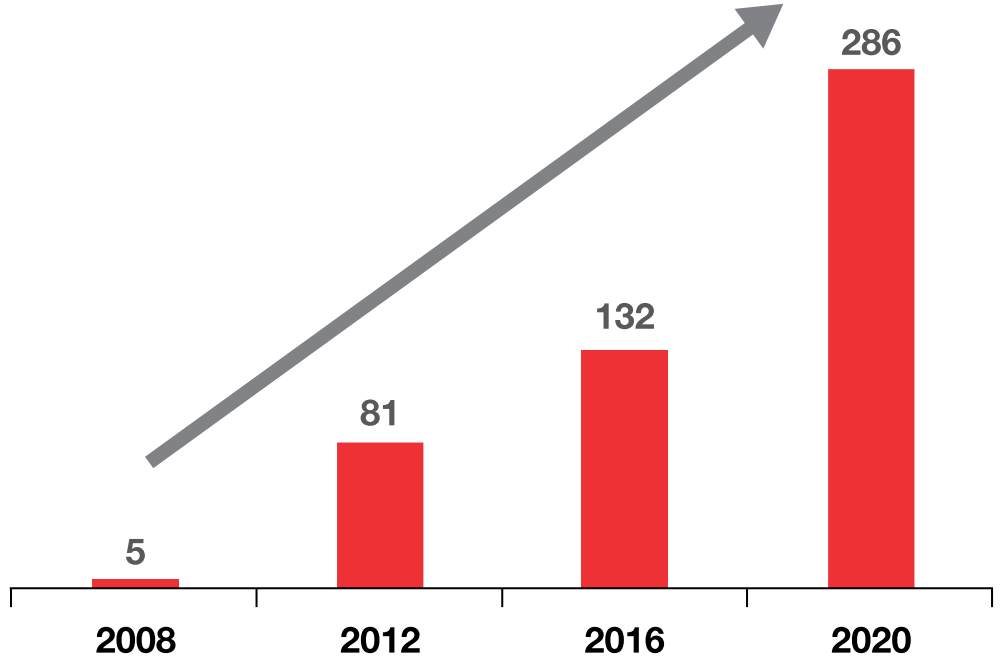
Biomarkers identified by sequencing can be important diagnostic and therapeutic targets

Percentage of oncology trials incorporating biomarkers [1]



Source: LEK Consulting

Personalized medicines on the market [2]*



1. Food and Drug Administration, updated December 2019, fda.gov.
2. Modified from https://www.personalizedmedicinecoalition.org/Userfiles/PMC_Corporate/file/PMC_The_Personalized_Medicine_Report_Opportunity_Challenges_and_the_Future.pdf.

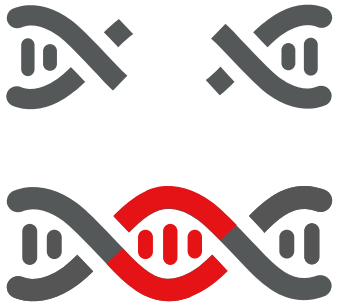
* Methodological notes: The number of personalized medicines was calculated by combining information from the Personalized Medicine Coalition's Case for Personalized Medicine (2008–2014); Personalized Medicine Report (2017); Personalized Medicine at FDA: An Annual Research Report (2014–2019); data from the U.S. FDA Table of Pharmacogenomic Biomarkers in Drug Labeling, accessed June 5, 2020 (https://www.fda.gov/drugs/science-and-research-drugs/table-pharmacogenomic-biomarkers-drug-labeling tables); and the Clinical Pharmacogenetic Implementation Consortium's Genes-Drugs Table, accessed June 5, 2020 (https://cpicpgx.org/genes-drugs). See Appendix B for a complete list of the 286 medicines counted in 2020.

Types of genomic alterations relevant to oncology

Four primary genetic variant classes detectable by NGS:

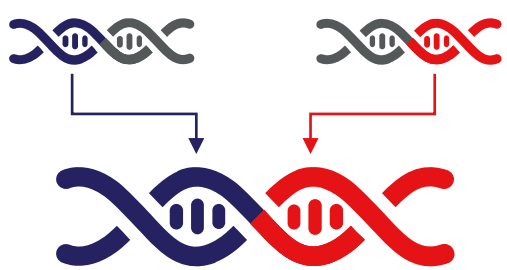
SNV
Single nucleotide variant

- Single nucleotide variants, substitutions, or point mutations



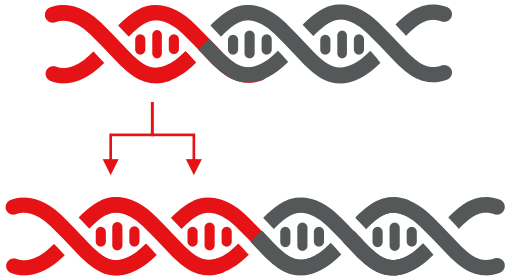
Indel
Insertion or deletion

- **Insertions and deletions (indels):** frameshift mutations resulting in changes in amino acid sequence that lead to truncated protein expression



Fusion

- **Translocation:** segment of chromosome transferred to different part
- **Fusion:** portion of different genes join

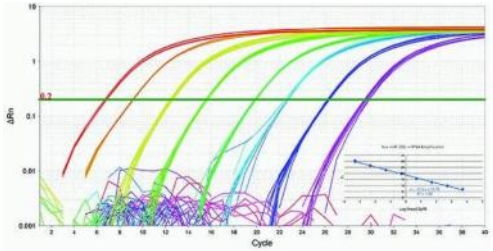


CNV
Copy number variant

- **Copy number variants:** change in copy number leading to either gain or loss of function.

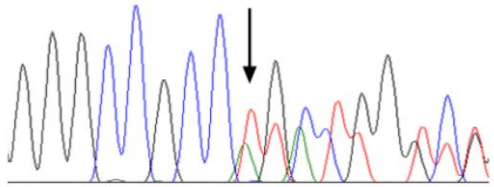
Molecular tests for oncology genomic biomarkers

qPCR



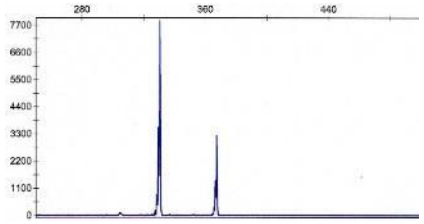
Determines presence or absence of certain variants in specific genes (ex: fusions)

Sanger sequencing



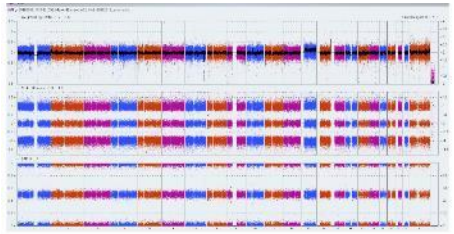
Determines sequence of specific genes to identify certain mutations (ex: indels)

Fragment analysis



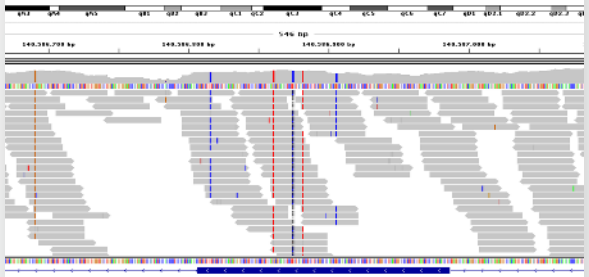
Determines presence or absence of certain variants in specific genes (ex: SNVs)

Microarray



Determines representation of many genes across genome (ex: CNVs)

NGS



Determines sequence of many genes to identify multiple types of variants (ex: fusion, SNV, indel, CNV)

Advantages of Next-Generation Sequencing

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One test

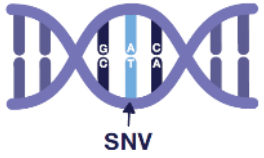
for

All key biomarkers

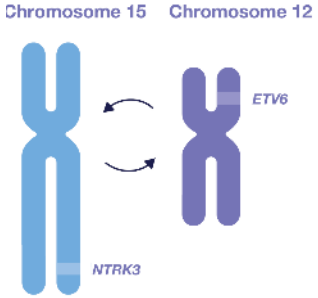
PML-RARA	*RUNX1*
FLT3-ITD	*BCR-ABL*
CALR	*JAK2*
KIT	*IDH1*
TP53	*IDH2*
NPM1	*MPL*
ASXL1	*+ more*

Multiple Variant Types

SNVs, InDels



Gene Fusions



across

Multiple samples

- Rapid
- Efficient
- High sensitivity
- High specificity
- Novel variants

Simultaneous evaluation of multiple genomic biomarkers across multiple samples

Genomic profiling-directed therapy may improve patient outcomes

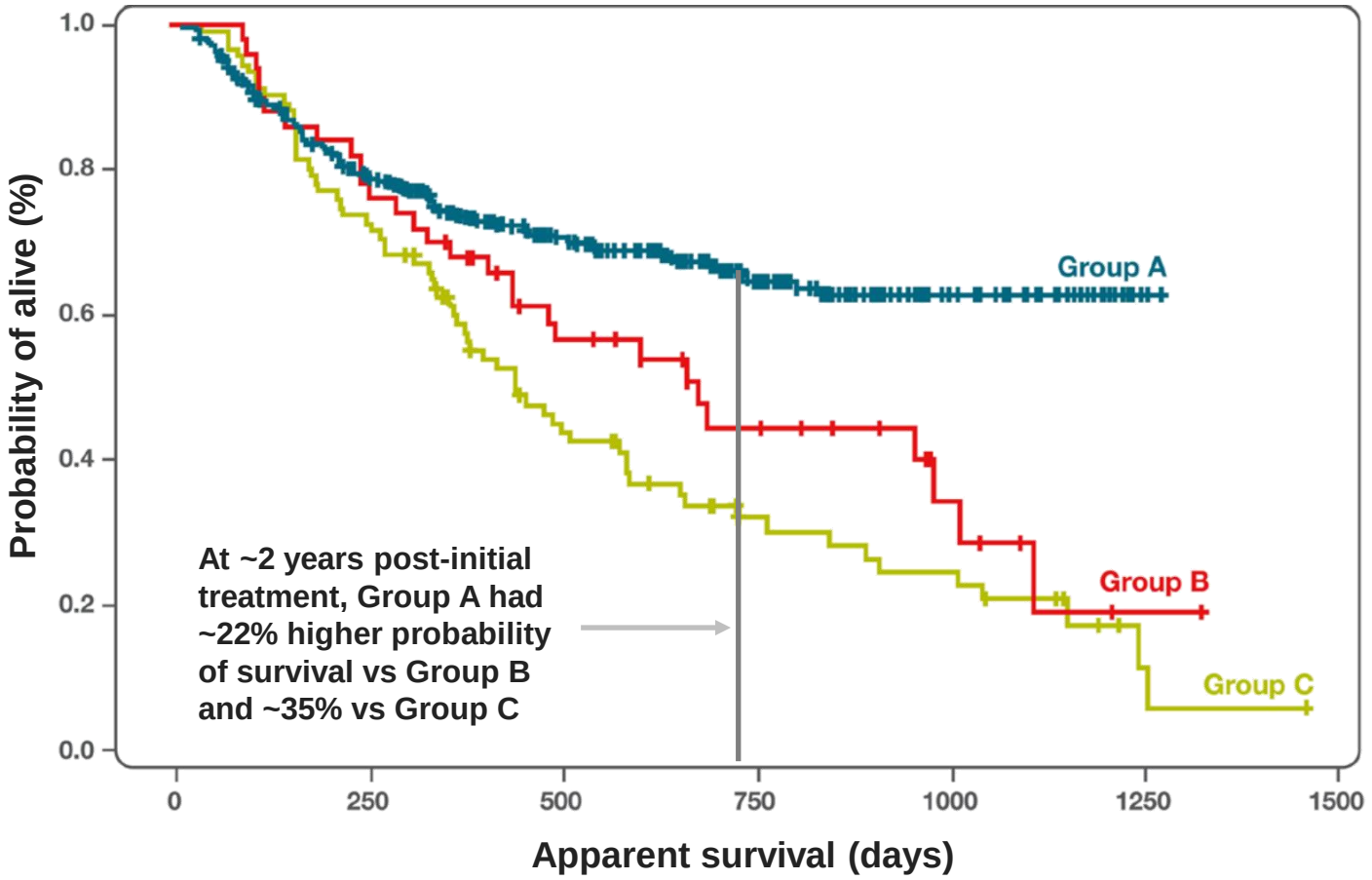
Retrospective study of 525 newly diagnosed stage IV NSCLC patients harboring actionable oncogenic drivers[1]

Group A
Treated with TKI after genomic profiling results (n=384)

Patients treated prior to genomic profiling results (Chemo, IO, or Chemo+IO) (n=141)

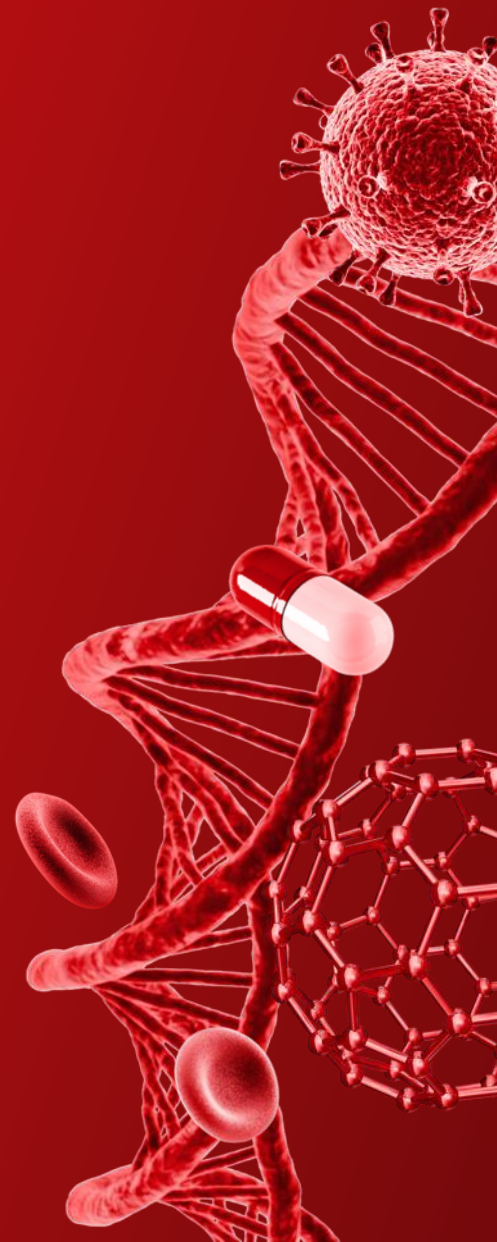
Group B
Started treatment before genomic profiling results and **switched** to TKI within 35 days (n=51)

Group C
Started treatment before genomic profiling results and did **not switch** to TKI within 35 days (n=90)



1. Smith RE, et al. *Journal of Clinical Oncology* (2022), doi: 10.1200/JCO.2022.40.16_suppl.1530

Thank you



Use of Automated Next-Generation Sequencing for Rapid Profiling of Myeloid Malignancies

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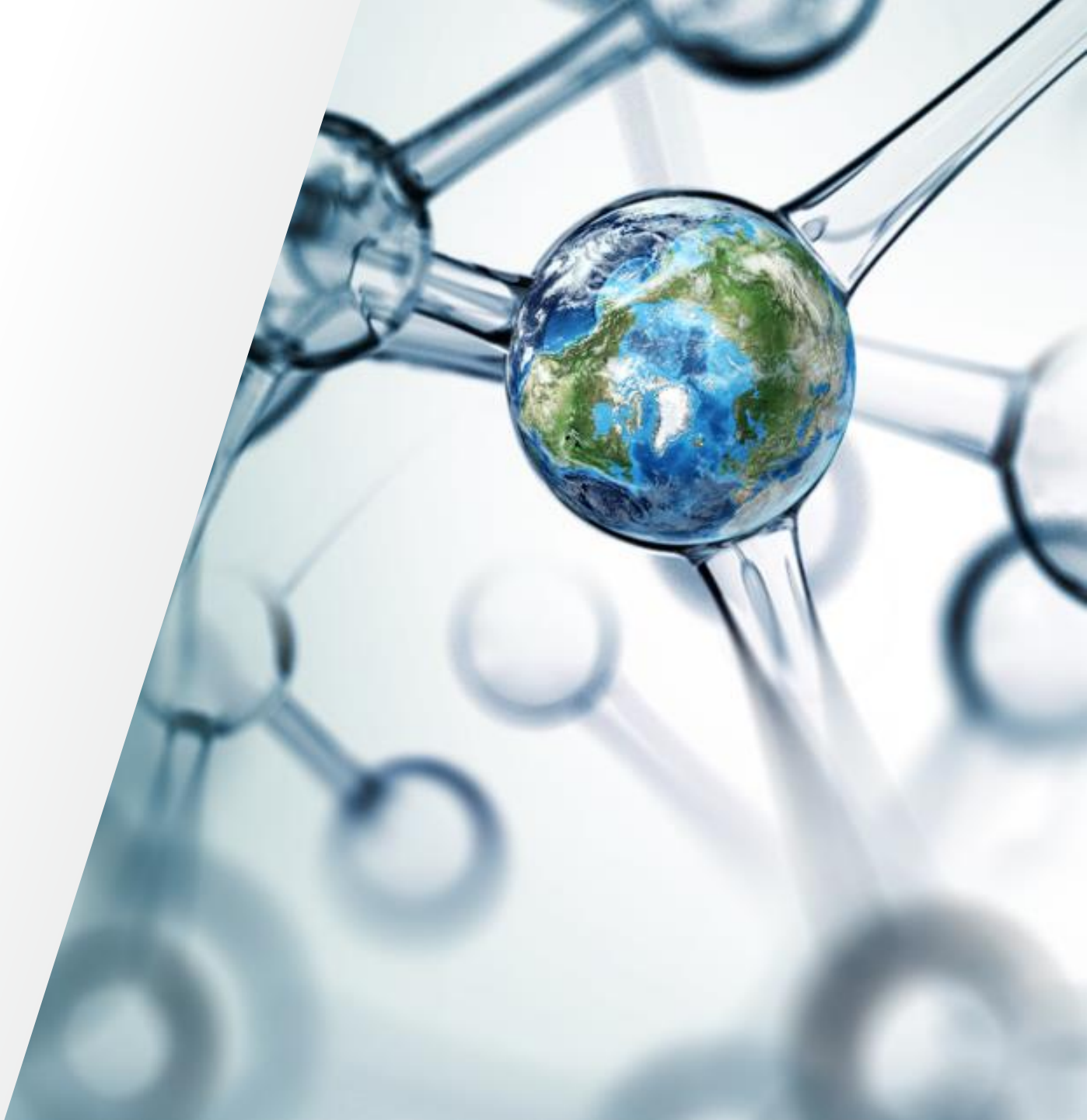
Assistant Professor of Pathology
Medical Director Molecular Oncology Laboratory
Atrium Health Wake Forest Baptist



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Outline

- Clinical cases
- Myeloid Neoplasms Generalities
- The Atrium Wake Forest Experience
- Summary

Clinical Case # 1

24-year-old male

Diagnosed with acute myeloid leukemia 07/2021

Flow cytometry:

- Monocytic differentiation

Conventional cytogenetics:

- Normal karyotype

FISH AML panel:

- Normal

***FLT3* = Positive *FLT3* ITD mutation**

NGS AML profile = *WT1* mutation

Clinical Case # 1

- Patient has had a poor response to conventional chemotherapy
- Have not achieved morphologic remission
- Persistent infections and cytopenias
- **In-house NGS panel ordered on 7/2022**

DNA Alterations Identified

Gene	AA Change	Mutant Allele Frequency (%)
WT1	p. (A387Vfs*4)	24.7

Gene Fusions Identified

Fusion Genes	Variant ID	Locus
NUP98::NSD1	NUP98-NSD1.N12N6	chr11:3765739 - chr5:176662822
NUP98::NSD1	NUP98-NSD1.N11N6	chr11:3774546 - chr5:176662822

Clinical Case # 2

42-year-old male

Presented in June 2022 to outside hospital with cytopenias

Bone marrow:

- Myeloid predominance with maturational arrest (myelocyte predominance)

Flow cytometry:

- Abnormal myeloid population (CD34-, CD117+)

Conventional cytogenetics:

- Normal karyotype

FISH AML panel = Normal

Clinical Case # 2

- Patient went into DIC on second day of admission
- **In-house NGS panel ordered**

Fusions Identified

Fusion Genes	Variant ID	Locus
STAT5B-RARA	STAT5B-RARA.S15R3	chr17:40362189 - chr17:28504568

Myeloid Neoplasms – General Concepts

Diverse spectrum of hematopoietic malignancies

Derived from common progenitor myeloid stem cell

Complex underlying molecular mechanisms

Can be divided as acute and chronic

Classification based on morphologic, immunophenotypic, cytogenetic and molecular features

Myeloid Neoplasms - Classification

WHO 2022

Precursor lesions

Clonal Hematopoiesis (Including age-Related)

Clonal hematopoietic cells without diagnosed hematologic disorder or cytopenia

Clonal Hematopoiesis of Indeterminate Potential (CHIP)

CH with somatic mutations of **myeloid malignancy-associated genes** detected in the blood or bone marrow present at a **variant allele fraction of ≥ 2%** in individuals without a diagnosed hematologic disorder or unexplained cytopenia.

Khoury, Joseph D et al. “The 5th edition of the World Health Organization Classification of Hematolymphoid Tumors: Myeloid and Histiocytic/Dendritic Neoplasms.” Leukemia vol. 36,7 (2022): 1703-1719

Myeloid Neoplasms – Classification (con’t.)

Update Formally Defines CH Entities

Clonal Cytopenia(s) of Undetermined Significance (CCUS)

CH detected in the presence of one or more persistent cytopenias that are otherwise unexplained by hematologic or non-hematologic conditions and that do not meet diagnostic criteria for defined myeloid neoplasms.

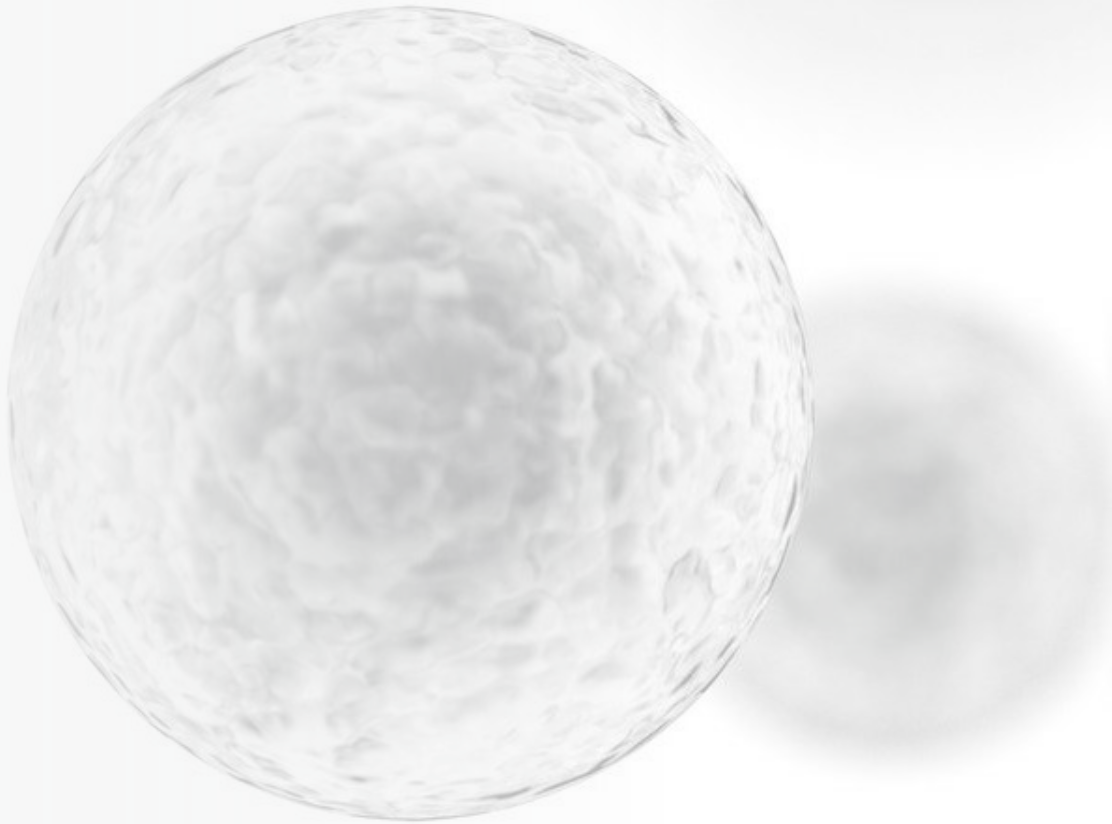
Idiopathic cytopenia(s) of unknown significance (ICUS)

Sustained and unexplained cytopenia without evidence of blood cancer-associated driver mutations.

Myeloid Neoplasms - Classification

Myeloproliferative Neoplasms

- Chronic Myeloid Leukemia (CML)
- Polycythemia Vera (PV)
- Essential Thrombocythemia (ET)
- Primary Myelofibrosis (PMF)
- Chronic Neutrophilic Leukemia (CNL)
- Chronic Eosinophilic Leukemia (CEL)
- Juvenile Myelomonocytic Leukemia (JMML)
- MPN, NOS



Myeloid Neoplasms - Classification

Myelodysplastic Neoplasms

MDN with defining genetic abnormalities:

MDN with low-blasts and 5q deletion

MDN with low-blasts and *SF3B1* mutation

MDN with low-blasts with bi-allelic *TP53* inactivation

MDN, morphologically defined:

MDN with low-blasts

MDN with increased blasts

MDN, hypoplastic

MDN of childhood

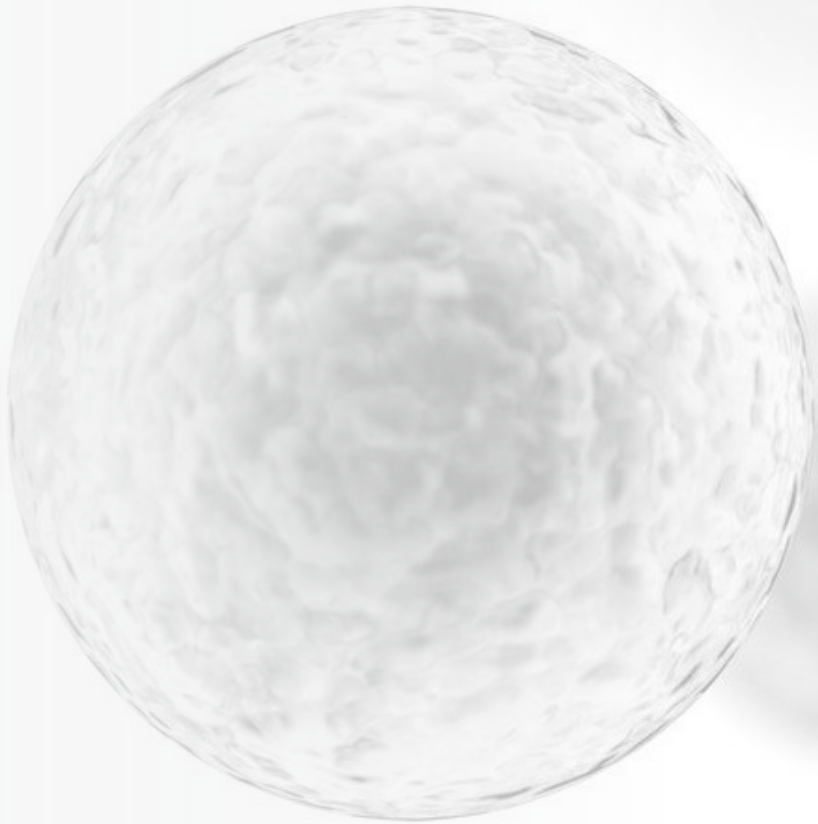
Myeloid Neoplasms - Classification

Myelodysplastic/Myeloproliferative Neoplasms

- Chronic Myelomonocytic Leukemia (CMML)
- Myelodysplastic/myeloproliferative Neoplasm with Neutrophilia
- Myelodysplastic/myeloproliferative Neoplasm with *SF3B1* mutation and thrombocytosis
- Myelodysplastic/myeloproliferative Neoplasm, NOS

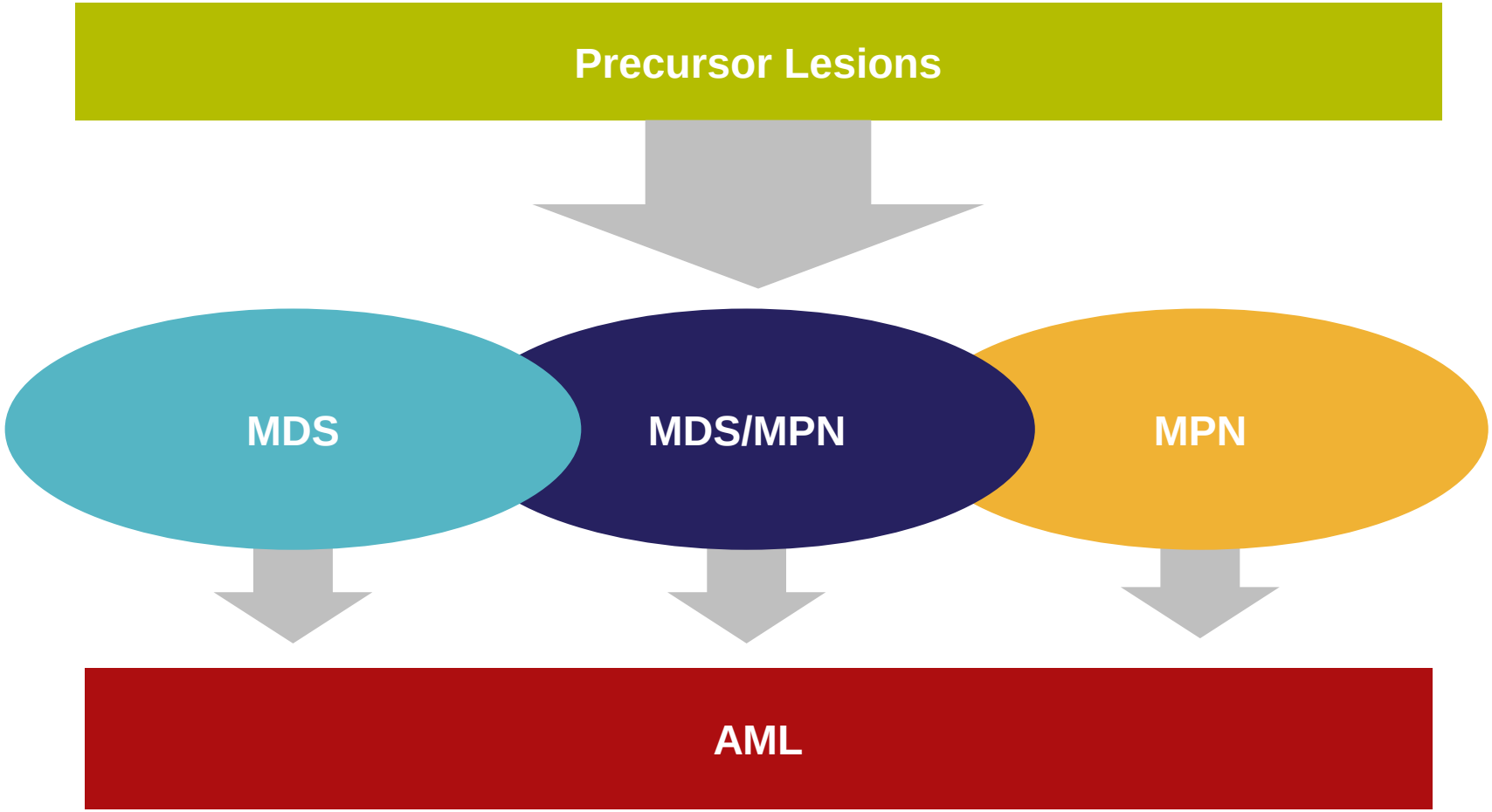
Myeloid Neoplasms - Classification

Acute Myeloid Leukemia (AML)



- AML with defining genetic abnormalities
- AML, defined by differentiation
- Myeloid Sarcoma

Progression to AML



Genetic Pathways Impacted by Mutations in Myeloid Neoplasms

Epigenetics:
ASXL1
BCOR
BCORL1
DNMT3A
EZH2
IDH1/2
TET2

Signaling:
CALR
CBL
CSF3R
FLT3
JAK2
KIT
KRAS
MPL
NF1
NRAS
PTPN11

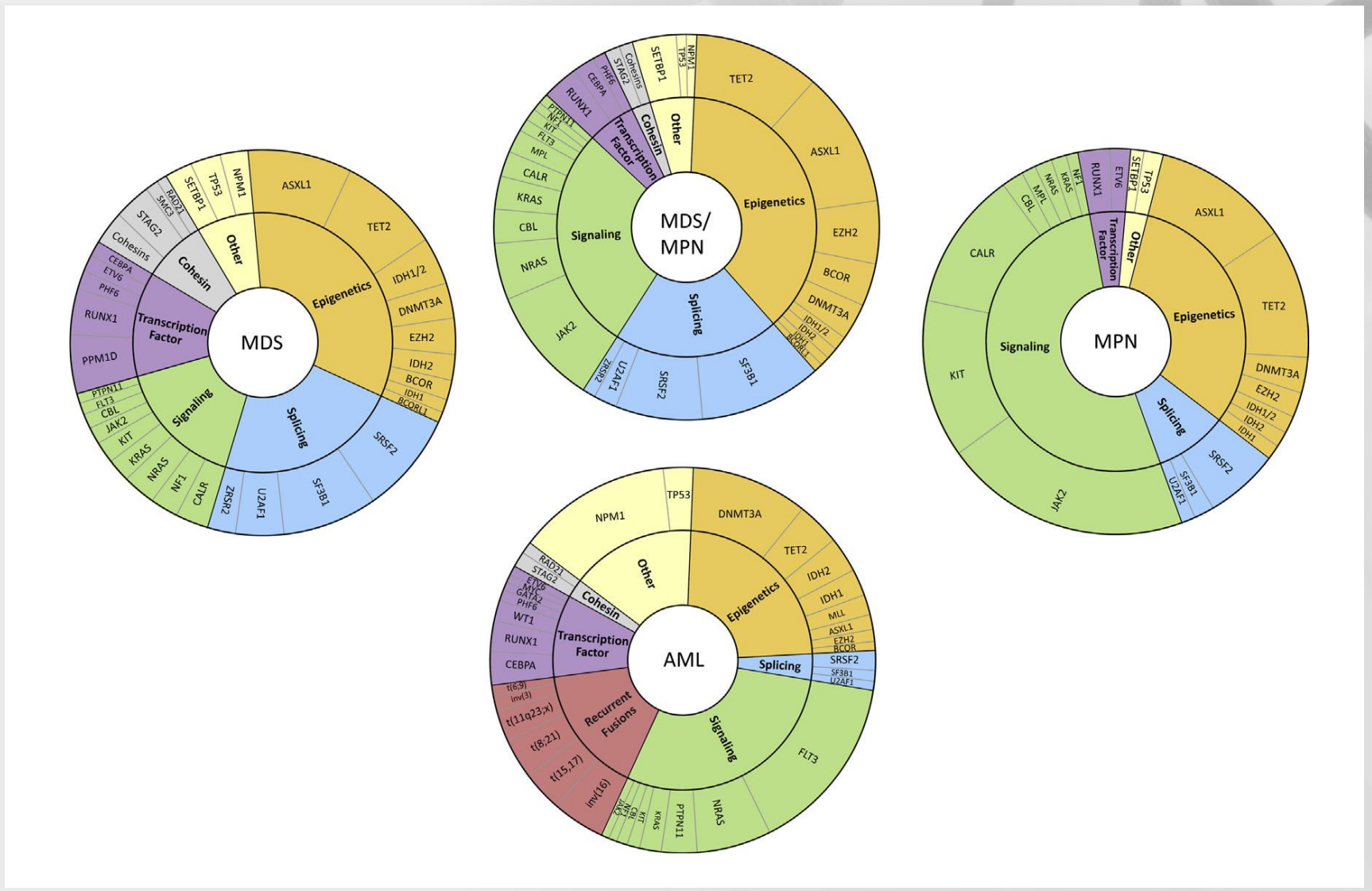
Spliceosome:
SF3B1
SRSF2
U2AF1
ZRSR2

Transcription Factors:
CEBPA
ETV6
PHF6
PPM1D
RUNX1

Cohesins:
RAD21
SMC31
STAG2

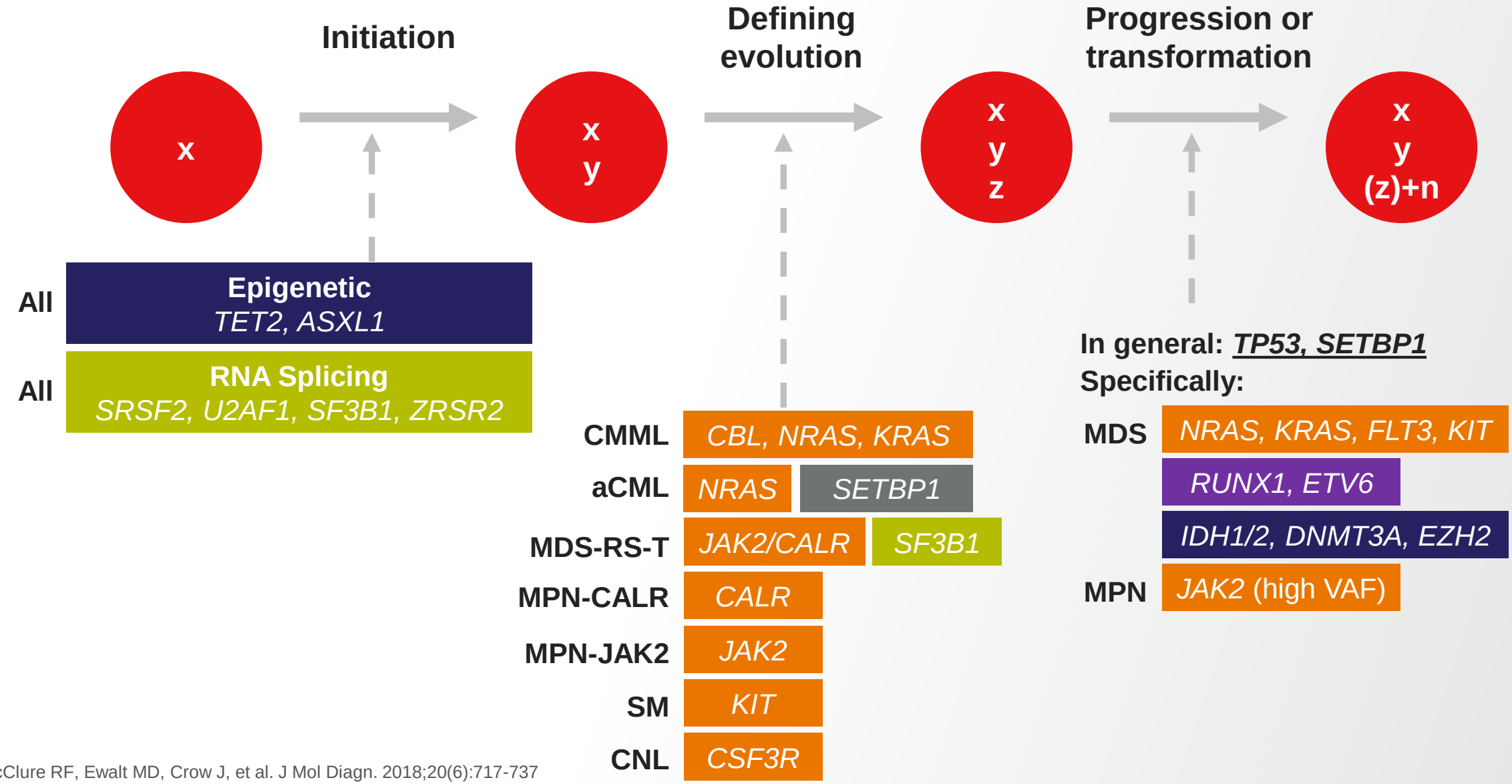
Other:
TP53
NPM1
SETBP1

Impacted pathways differ between myeloid neoplasms



Sadigh S, Kim AS.. Surg Pathol Clin. 2021 Sep;14(3):517-528.

Common changes acquired in the differentiation and progression of myeloid neoplasms



McClure RF, Ewalt MD, Crow J, et al. J Mol Diagn. 2018;20(6):717-737

Myeloid Neoplasms – Genomics Data

Useful for:

Classification

Prognosis

- ELN (European Leukemia Net)
- NCCN (National Comprehensive Cancer Network)
- GIPSS (Genetically Inspired Prognostic Scoring System)

Potential therapies

- Inhibitors of *FLT3* (midostaurin), *IDH1* (ivosidenib), *IDH2* (enasidenib), and splicing factor pathways (H3B-8800)

The Atrium Health Wake Forest Experience

Before June 2022 all NGS-based testing for myeloid neoplasms was sent-out to a reference laboratory

Limitations for implementation

- Small laboratory (3.5 FTE technologists)
- Absence of bioinformatics support
- Limited physical space
- Need for automation

Send out testing has a number of disadvantages

Send out testing offers advantages and disadvantages

Advantages

- Billing (pre-authorizations)
- No additional personnel needed

Disadvantages

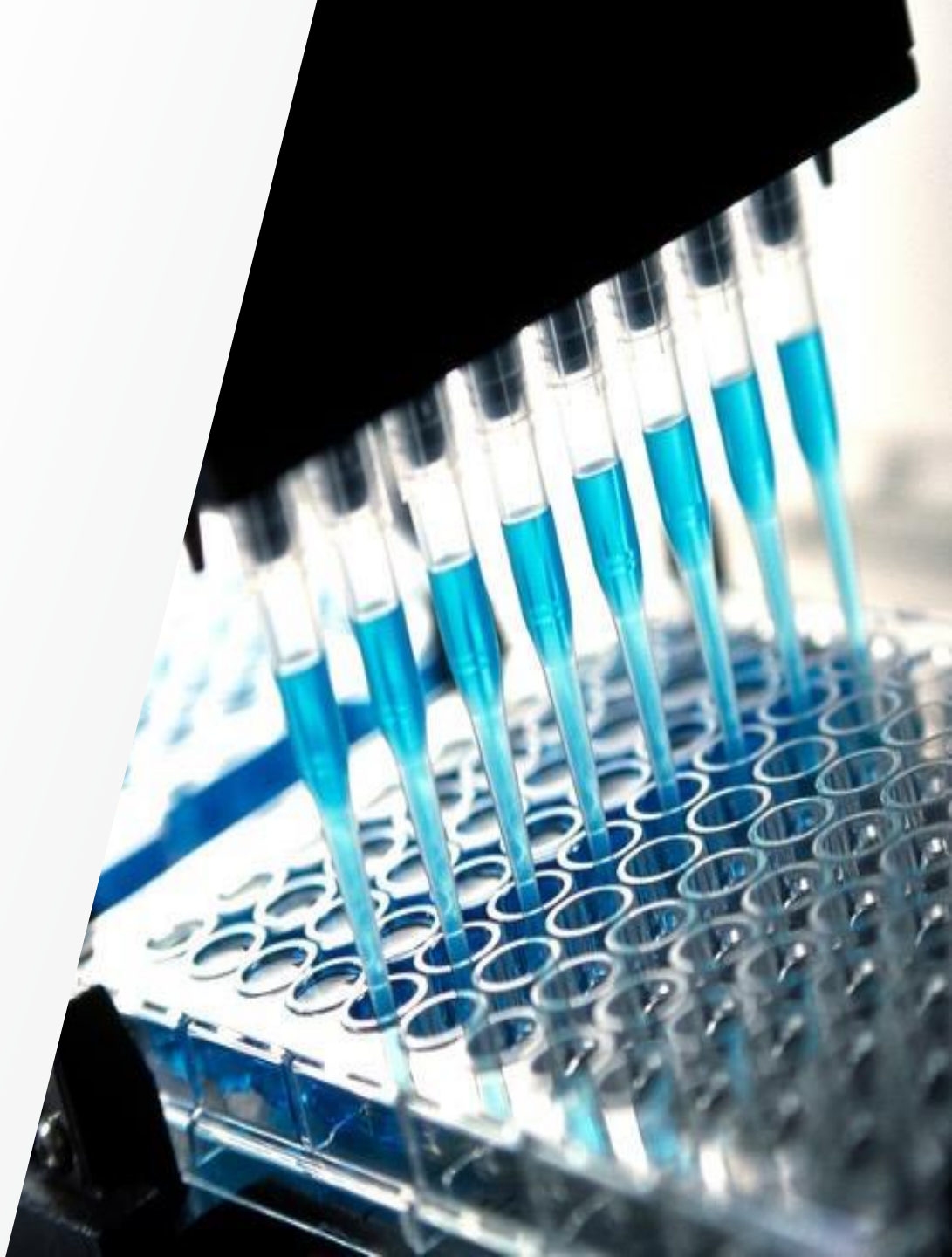
- Long turn-around time (2-3 weeks)
- Expensive
- No access to sequencing data
- Decreased accessibility by pathologists and trainees

Bringing testing “in-house”

Integrated Semiconductor Sequencer – Myeloid NGS assay

Validation process

- Assisted by sequencing vendor’s analytical validation team
- 60 unique samples used (most from our archival library)
- 8-week process
- One instrument failure (required a camera replacement) during this period



In-house testing has a number of advantages

Since June 2022 in-house testing

Advantages

- Fast turn-around time (2-3 days in average)
- Low hands-on technologist time (<2 hours)
- No need for a bioinformatics specialist
- Complete access to sequencing data
- High accessibility by pathologists and trainees

Disadvantages

- Billing (pre-authorizations)
- Pipeline closed system (allows for minimal modifications)
- Relatively rigid reporting system

Myeloid NGS Assay

DNA/RNA-based amplicon sequencing assay

Advantages

- Rapid
- Relatively low input
  - A minimum of 27.75 ng purified DNA (at 1.11ng/uL)
  - A minimum of 14.25 ng of RNA (at 0.95 ng/uL)
- Panel composition highly applicable to myeloid neoplasms

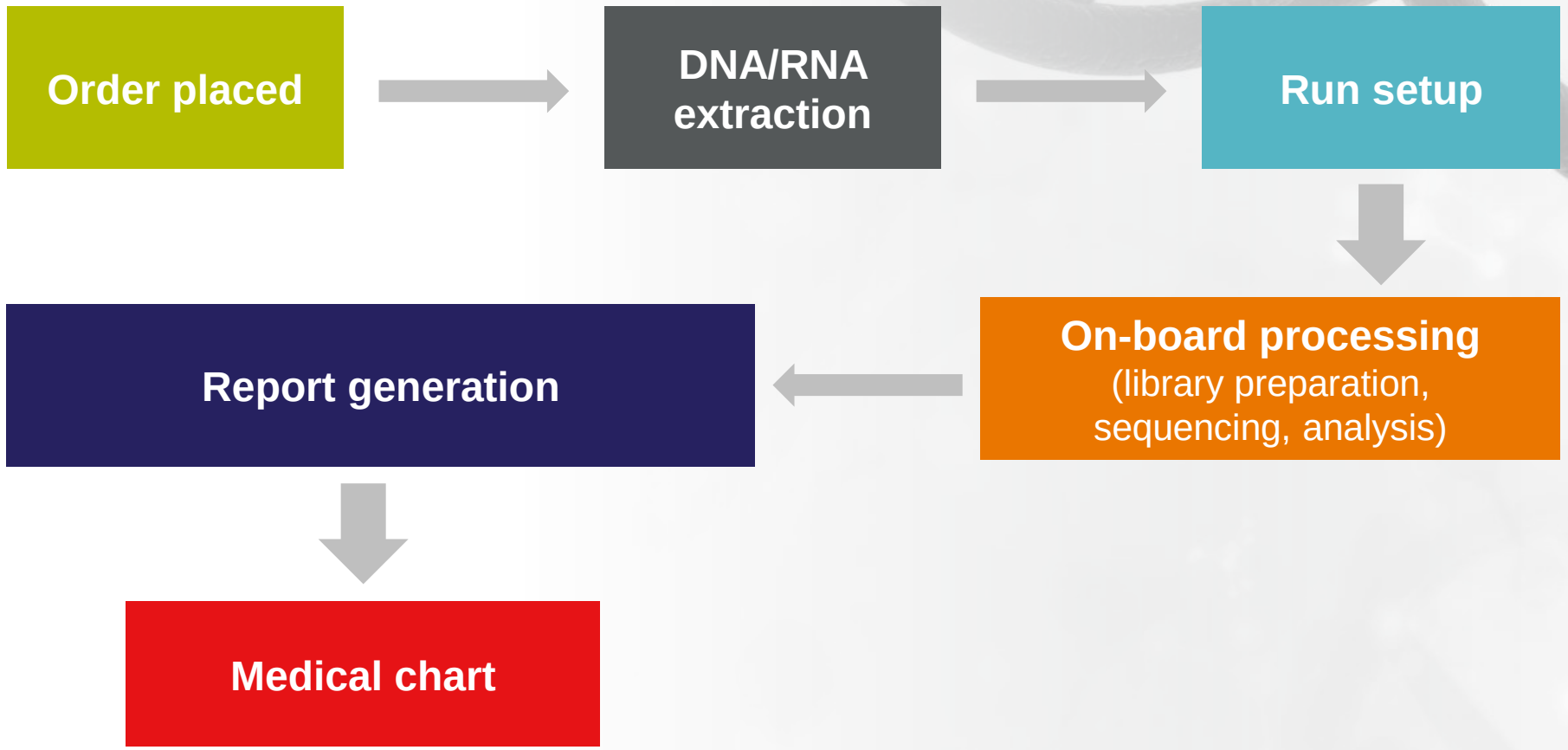
Disadvantages

- Inconsistent calling of certain variants [homopolymers such as ASXL1 NM_015338:c.1934dupG (p.G646fs)]
- Pipeline with relatively conservative filters

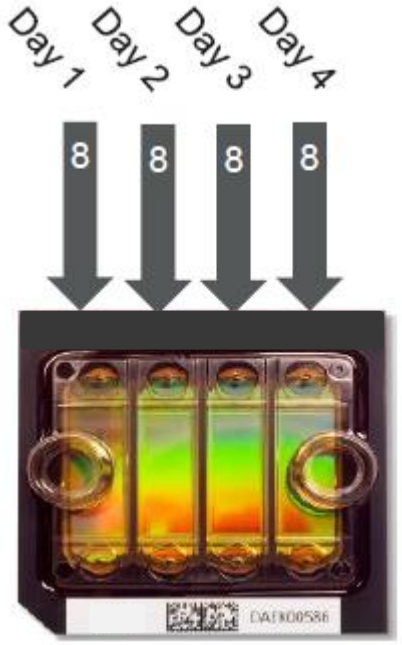
Myeloid NGS Assay Content

DNA panel: hotspot genes (28)		DNA panel: full genes (17)		RNA panel: fusion driver genes (35)			RNA panel: expression genes (5)	RNA panel: expression control genes (5)
ANKRD26	*KRAS*	*ASXL1*	*PRPF8*	*ABL1*	*HMGA2*	*NUP98*	*BAALC*	*EIF2B1*
ABL1	*MPL*	*BCOR*	*RB1*	*ABL2*	*JAK2*	*NUP214*	*MECOM*	*FBXW2*
BRAF	*MYD88*	*CALR*	*RUNX1*	*BCL2*	*KAT6A (MOZ)*	*PAX5*	*MYC*	*PSMB2*
CBL	*NPM1*	*CEBPA*	*SH2B3*	*BRAF*	*KAT6B*	*PDGFRA*	*SMC1A*	*PUM1*
CSF3R	*NRAS*	*ETV6*	*STAG2*	*CCND1*	*KMT2A*	*PDGFRB*	*WT1*	*TRIM27*
DDX41	*PPM1D*	*EZH2*	*TET2*	*CREBBP*	*KMT2A PTDs*	*RARA*		
DNMT3A	*PTPN11*	*IKZF1*	*TP53*	*EGFR*	*MECOM*	*RUNX1*		
FLT3 (ITD,	*SMC1A*	*NF1*	*ZRSR2*	*ETV6*	*MET*	*TCF3*		
TKD)	*SMC3*	*PHF6*		*FGFR1*	*MLLT10*	*TFE3*		
GATA2	*SETBP1*			*FGFR2*	*MRTFA (MKL1)*	*ZNF384*		
HRAS	*SF3B1*			*FUS*	*MYBL1*			
IDH1	*SRSF2*				*MYH11*			
IDH2	*U2AF1*				*NTRK2*			
JAK2	*WT1*				*NTRK3*			
KIT								

From sample to result



Multiplexing allows multiple samples to be processed together



1 chip → **4 lanes**

1 lane → **8 samples = 6 patients + 2 controls**

1 lane → **Processing time (DNA+RNA extraction, library prep, sequencing, analysis) = ~26 hours**

After 6 patient samples (PBL or BM) are received, estimated TAT = 2-3 days

Chip stability = 2 weeks (ideally minimum of 24 patients/2 weeks)

Clinical Cases – Follow up

Case 1: NGS fusion detection leads to clinical trial eligibility

Patient #1

- Because of the fusion detected by NGS, patient was eligible for a clinical trial

J Oncol. 2019; 2019: 1609128. PMCID: PMC6699323
Published online 2019 Jul 30. doi: 10.1155/2019/1609128 PMID: 31467532

Mutated *WT1*, *FLT3-ITD*, and *NUP98-NSD1* Fusion in Various Combinations Define a Poor Prognostic Group in Pediatric Acute Myeloid Leukemia

Naghmeh Niktoreh, [1] Christiane Walter, [1] Martin Zimmermann, [2] Christine von Neuhoff, [3] Nils von Neuhoff, [1] Mareike Rasche, [1] Katharina Waack, [4] Ursula Creutzig, [2] Helmut Hanenberg,[✉ 1, 5] and Dirk Reinhardt[✉ 1]

Clinical Cases – Follow up

Case 2: NGS identifies rare AML subtype, guiding therapy options

Patient #2

- Patient failed initial chemotherapy, now on an alternative salvage regimen
- Looking for potential trials

Case Reports > Leuk Res. 2019 Mar;78:21-23. doi: 10.1016/j.leukres.2019.01.004.
Epub 2019 Jan 15.

STAT5b-RARa-positive acute myeloid leukemia: Diagnostic and therapeutic challenges of a rare AML subtype

G Ciangola [1], C Gurnari [1], G Paterno [1], M Mirabile [2], M Angelini [3], S Lavorgna [1], T Ottone [1], S Travaglini [1], L Cicconi [1], F LoCoco [4]

Summary

- Myeloid neoplasms are highly heterogeneous molecular entities
- Molecular data plays a critical role in classification, prognosis and selection of targeted therapies
- Automated NGS platforms are great options for a significant number of laboratories
  - “Democratization of NGS”
- Improvements and evolutions on available panels and pipelines is necessary

Selected References

1. Carbonell, D., et al., *Next-Generation Sequencing Improves Diagnosis, Prognosis and Clinical Management of Myeloid Neoplasms.* Cancers (Basel), 2019. **11**(9).

2. Ferrone, C.K., et al., *Validation, Implementation, and Clinical Impact of the Oncomine Myeloid Targeted-Amplicon DNA and RNA Ion Semiconductor Sequencing Assay.* J Mol Diagn, 2021. **23**(10): p. 1292-1305.

3. Izevbaye, I., et al., *Clinical Validation of a Myeloid Next-Generation Sequencing Panel for Single-Nucleotide Variants, Insertions/Deletions, and Fusion Genes.* J Mol Diagn, 2020. **22**(2): p. 208-219.

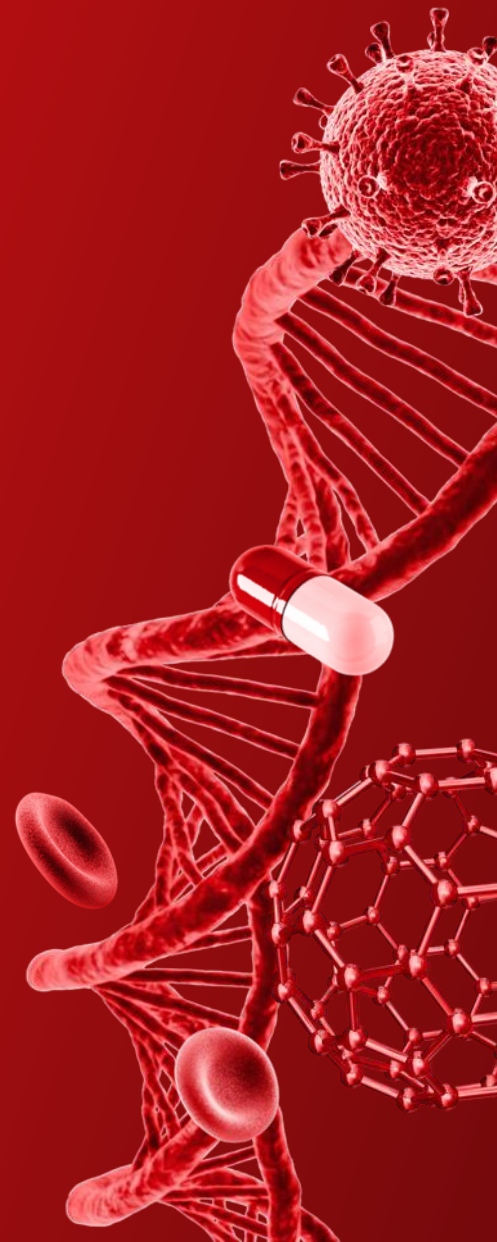
4. McClure, R.F., et al., *Clinical Significance of DNA Variants in Chronic Myeloid Neoplasms: A Report of the Association for Molecular Pathology.* J Mol Diagn, 2018. **20**(6): p. 717-737.

5. Papaemmanuil, E., et al., *Genomic Classification and Prognosis in Acute Myeloid Leukemia.* N Engl J Med, 2016. **374**(23): p. 2209-2221.

6. Park, J., et al., *Analytical and Potential Clinical Performance of Oncomine Myeloid Research Assay for Myeloid Neoplasms.* Mol Diagn Ther, 2020. **24**(5): p. 579-592.

7. Sadigh, S. and A.S. Kim, *Molecular Pathology of Myeloid Neoplasms: Molecular Pattern Recognition.* Surg Pathol Clin, 2021. **14**(3): p. 517-528.

Thank You



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