Pitfalls of liquid biopsy

Pitfalls of liquid biopsy

Atocha Romero
Hospital Universitario Puerta de Hierro

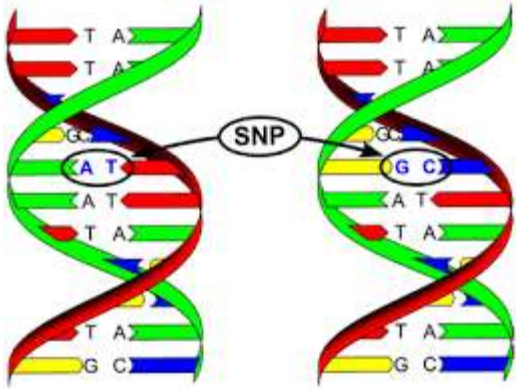
CONFLICTO DE INTERESES

- Grants from J&J and EMQN
- Consultant or advisory roles for Menarini and J&J
- Presentation for Illumina, Health in Code, and ThermoFisher Scientific, Menarini
- Support for attending meetings and/or travel from ThermoFisher Scientific, Bristol Myers Squibb, and Takeda

Pitfalls of liquid biopsy

1. Sensitivity and Detection Limits
2. Specificity and False Positives
3. Tumor Fraction estimation
4. Pre-analytical and Analytical Variability
5. Limited Clinical Validation and Utility:

Sensitivity and Detection Limits. Fusion detection



Indel examples

wild-type sequence

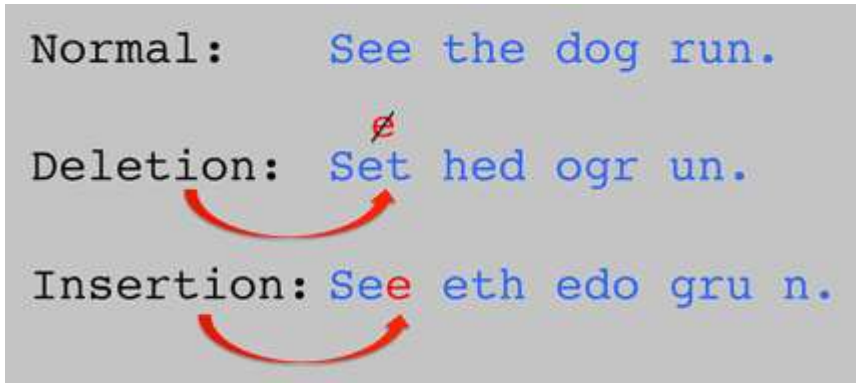
ATCTTCAGCCATAAAAGATGAAGTT

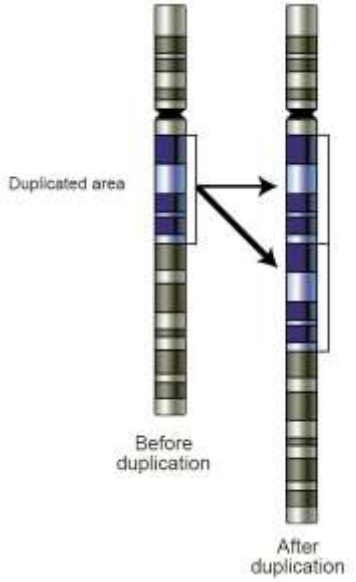
3 bp deletion

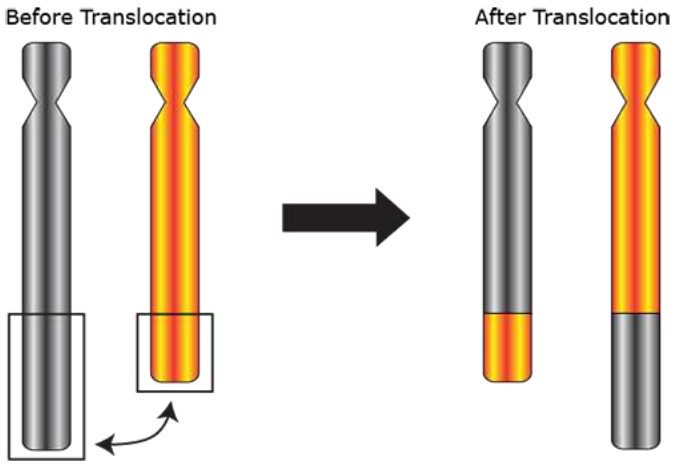
ATCTTCAGCCAAAGATGAAGTT

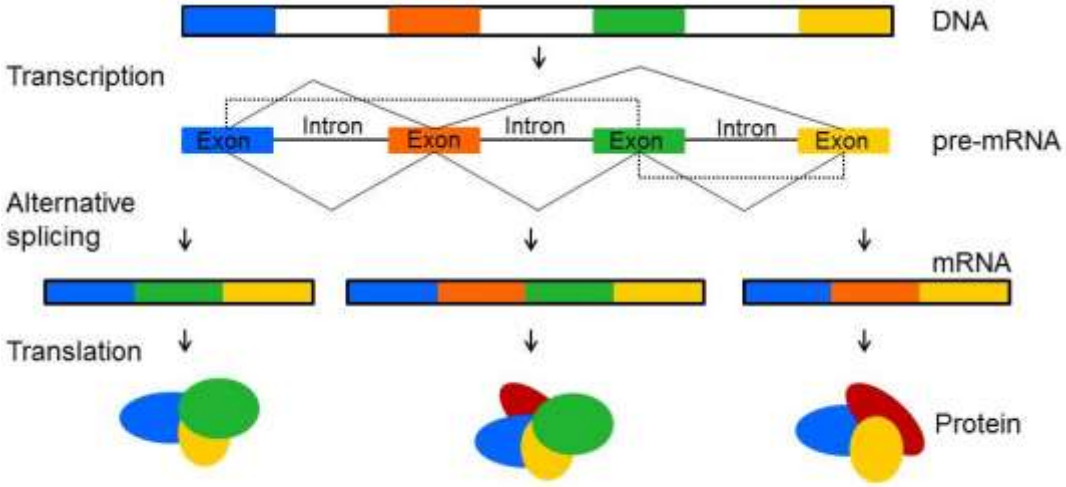
4 bp insertion (orange)

ATCTTCAGCCATATGTGAAAGATGAAGTT









Sensitivity and Detection Limits. Fusion detection

Sensitivity of next-generation sequencing assays detecting oncogenic fusions in plasma cell-free DNA

Julianna G. Supplee[a], Marina S.D. Milan[b], Lee P. Lim[c], Kristy T. Potts[c], Lynette M. Sholl[d], Geoffrey R. Oxnard[b], Cloud P. Paweletz[a,*]

[a] Belfer Center for Applied Cancer Science, Dana-Farber Cancer Institute, 360 Longwood Avenue, Boston, MA, 02215, USA
[b] Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA, 02215, USA
[c] Resolution Bioscience, Inc., 550 Kirkland Way, Kirkland, WA, 98033, USA
[d] Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA, 02115, USA

ARTICLE INFO

Keywords:
NSCLC
Cell-free DNA
Next-generation sequencing
Gene fusions

ABSTRACT

Objectives: Plasma genotyping represents an opportunity for convenient detection of clinically actionable mutations in advanced cancer patients, such has been well-documented in non-small cell lung cancer (NSCLC). Oncogenic gene fusions are complex variants that may be more challenging to detect by next-generation sequencing (NGS) of plasma cell-free DNA (cfDNA). Rigorous evaluation of plasma NGS assays in the detection of fusions is needed to maximize clinical utility.

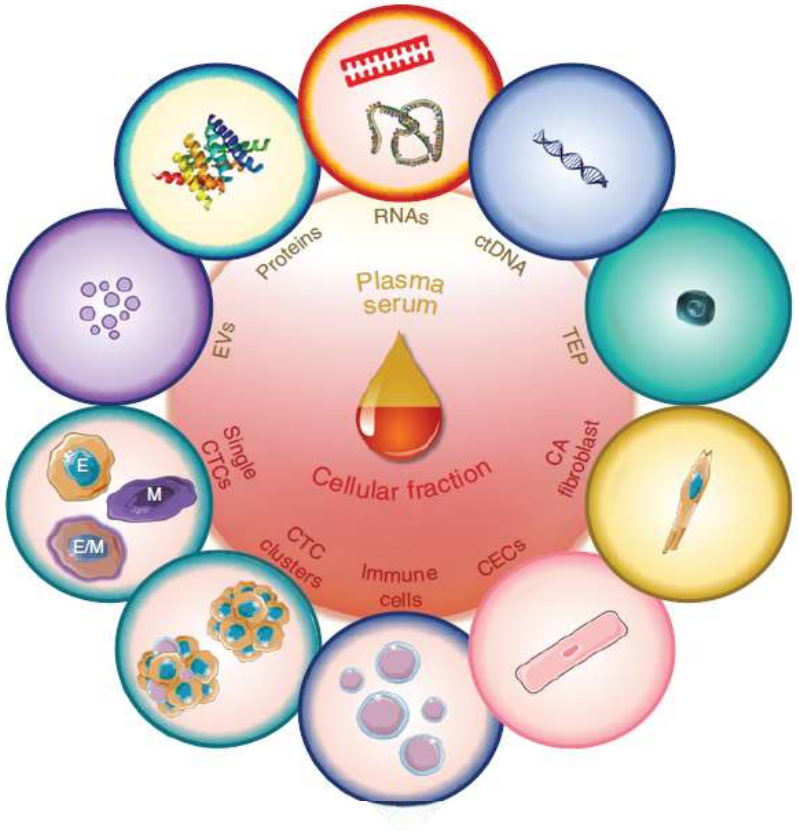
Materials and methods: : Additional plasma was collected from patients with advanced NSCLC and *ALK, ROS1,* or *RET* gene fusions in tissue who had undergone clinical plasma NGS using Guardant360™(G360, Guardant Health). We then sequenced extracted cfDNA with a plasma NGS kit focused on known driver mutations in NSCLC (ctDx-Lung, Resolution Bioscience) with cloud-based bioinformatic analysis and blinded variant calling.

Results: Of 16 patients assayed known to harbor an*ALK, ROS1,* or *RET* in tumor, G360 detected fusions in 7 cases, ctDx-Lung detected fusions in 13 cases, and 3 cases were detected by neither. Of the 7 fusions detected by both assays, G360 reported lower mutant allelic fractions (AF). In cases missed by G360, tumor derived *TP53* mutations were often detected confirming presence of tumor DNA. Raw sequencing data showed that inverted or out-of-frame variants were overrepresented in cases detected using ctDx-Lung but not by G360.

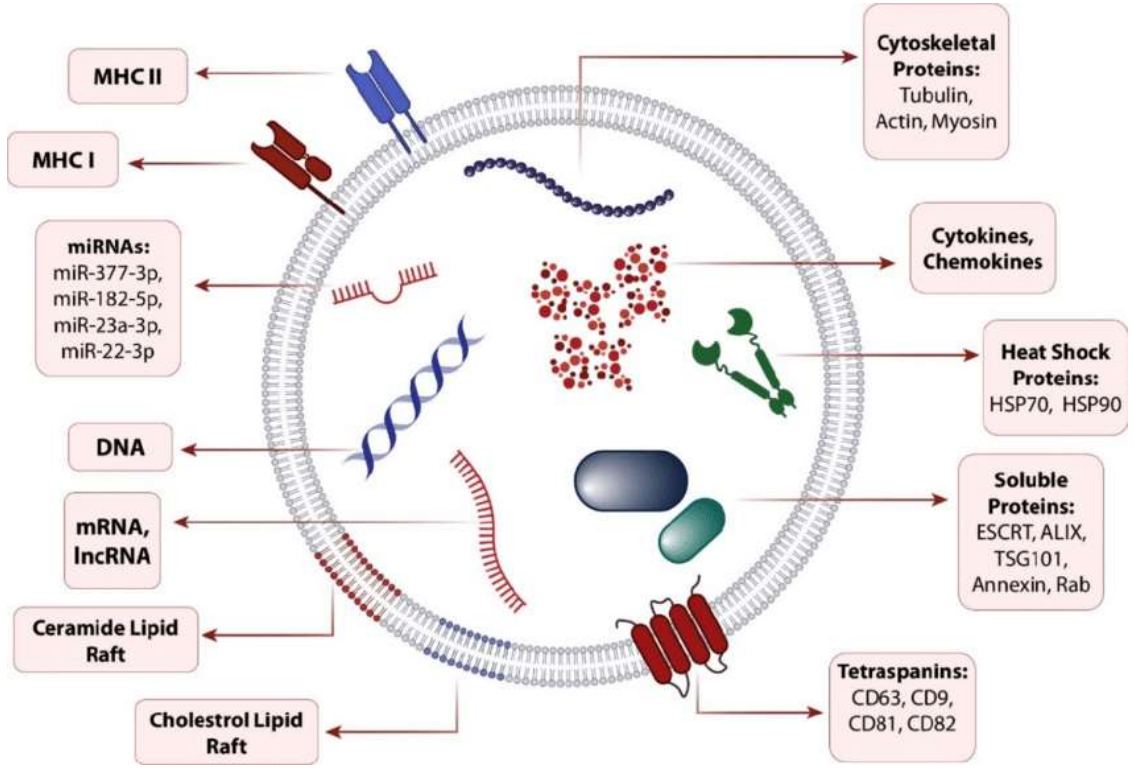
Conclusion: Focusing on complex, clinically actionable mutations using tumor as a reference standard allows for evaluation of technical differences in plasma NGS assays that may impact clinical performance. Noting the heterogeneity of fusion sequences observed in NSCLC, we hypothesize that differences in hybrid capture techniques and bioinformatic calling may be sources of variations in sensitivity among these assays.

19%

Sensitivity and Detection Limits. Fusion detection. EVs



Alix-Panabières, C et al. Cancer Discov. 2021;11:858-873



Int J Mol Sci. 2022 Jun 3;23(11):6273. doi: 10.3390/ijms23116273.

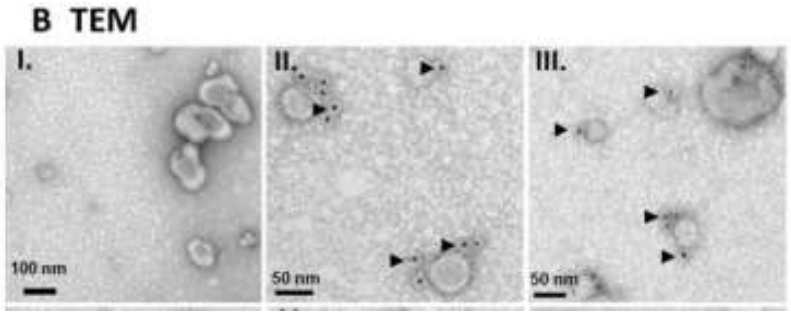
EVs

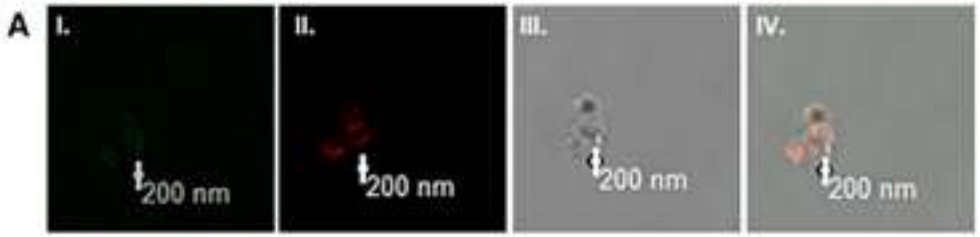
Clinical Chemistry 68:6
1–12 (2022)

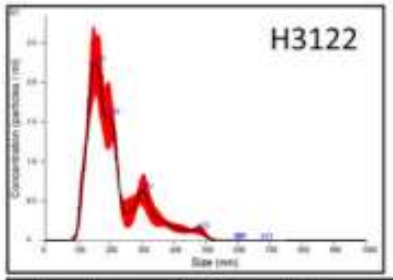
Molecular Diagnostics and Genetics

ALK-Fusion Transcripts Can Be Detected in Extracellular Vesicles (EVs) from Nonsmall Cell Lung Cancer Cell Lines and Patient Plasma: Toward EV-Based Noninvasive Testing

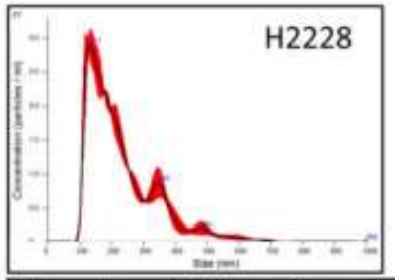
Estela Sánchez-Herrero,[a,b,†] Carmen Campos-Silva,[c,†] Yaiza Cáceres-Martell,[c] Lucía Robado de Lope,[a] Sandra Sanz-Moreno,[a] Roberto Serna-Blasco,[a] Alejandro Rodríguez-Festa,[a] Dunixe Ares Trotta,[a] Paloma Martín-Acosta,[d] Cristina Patiño,[e] María José Coronado,[f] Alexandra Beneitez,[g] Ricardo Jara,[g] Nerea Lago-Baameiro,[h] Tamara Camino,[h] Alberto Cruz-Bermúdez,[a] María Pardo,[h] Víctor González-Rumayor,[b] Mar Valés-Gómez ,[c,*] Mariano Provencio,[a,i] and Atocha Romero [a,i,*]







Mean ϕ	215.9 +/- 3.4 nm
Mode ϕ	154.7 +/- 6.3 nm
Particles/µL	$4.6 \cdot 10^8$ +/- $1.14 \cdot 10^8$



Mean ϕ	228.8 +/- 4.9 nm
Mode ϕ	126.6 +/- 4.8 nm
Particles/µL	$3.2 \cdot 10^8$ +/- $7.85 \cdot 10^7$

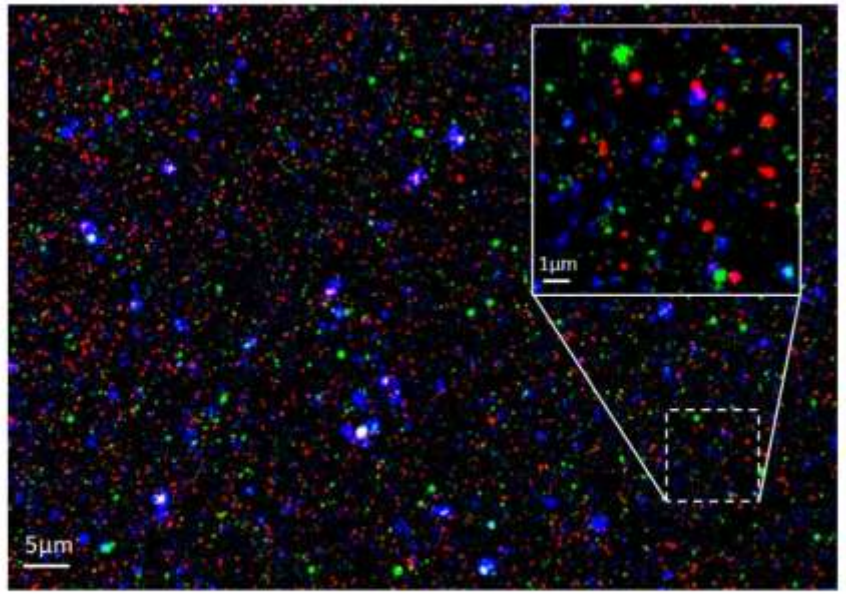
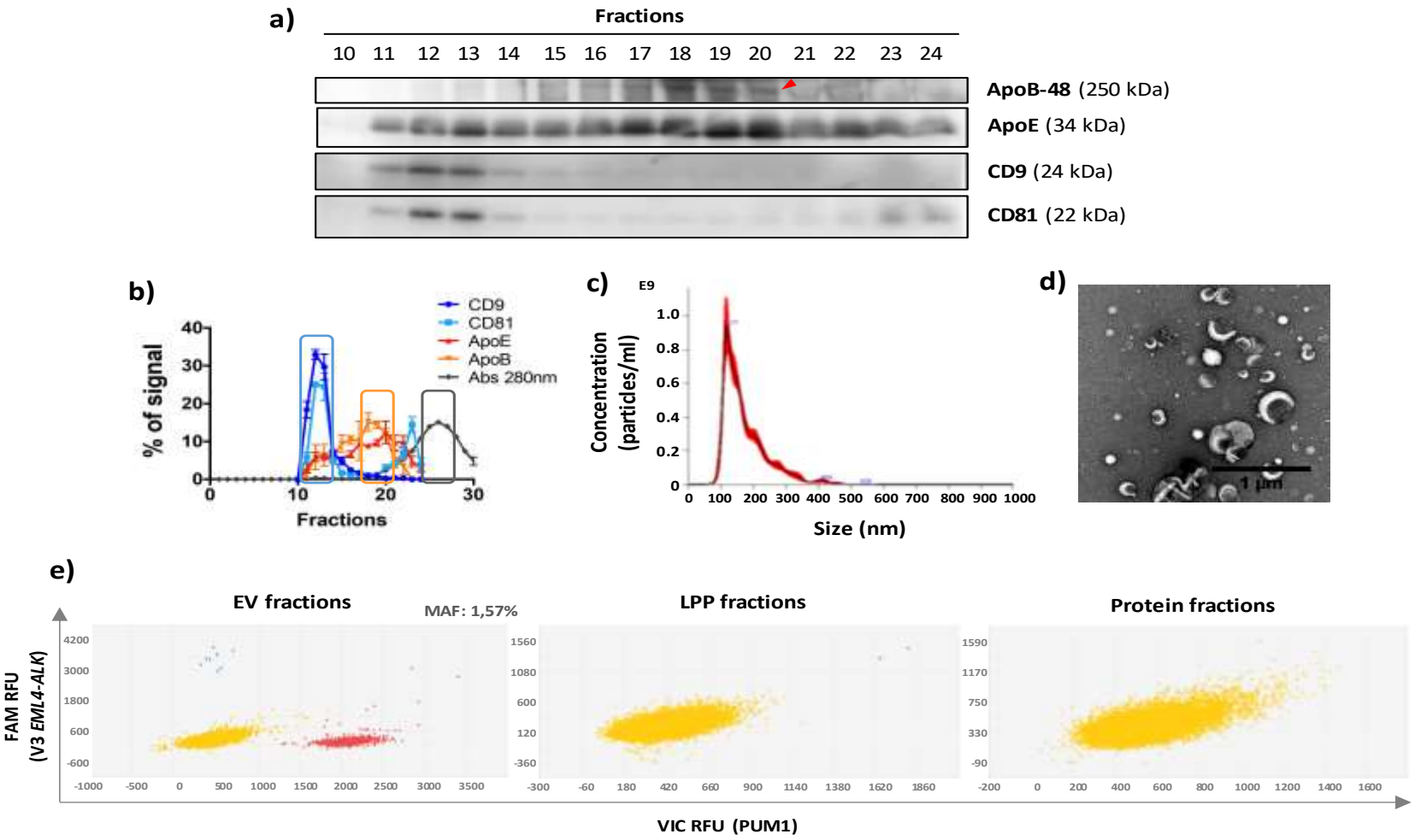


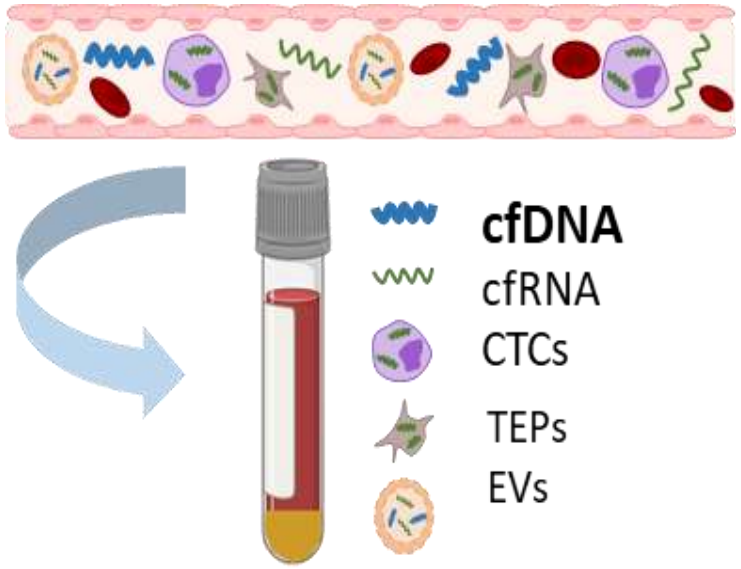
Figure S2. Characterization of EV-enriched preparations from NSCLC plasma samples by ExoView platform. CD9-captured EVs image in fluorescence mode: CD63 in red, CD9 in blue, *ALK** in green, CD63-*ALK** in yellow, CD63-CD9 in purple, *ALK**-CD9 in light blue, and CD63-CD9-*ALK** in white. *ALK**, *ALK* fusion protein.

EVs

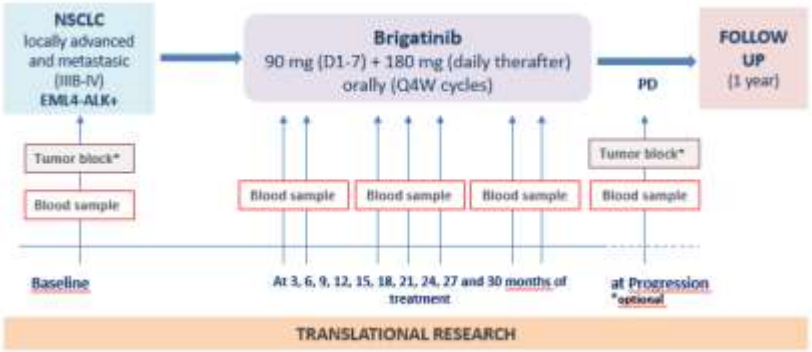


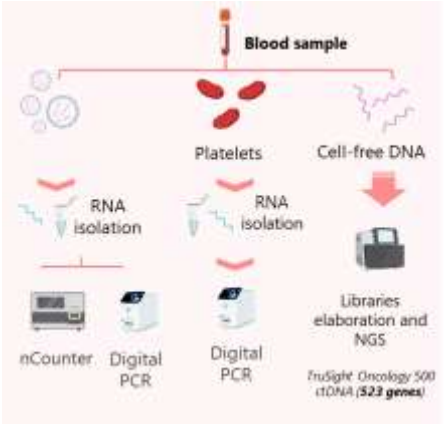
https://www.oaepublish.com/articles/evcna.2025.14

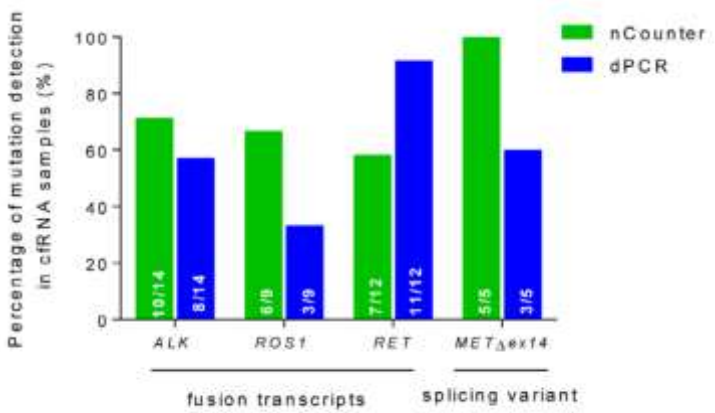
cfRNA, Evs and platelets



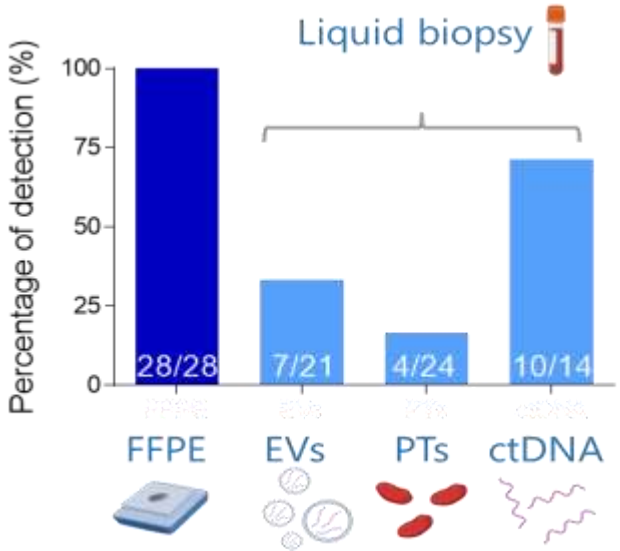
CUBIK TRIAL

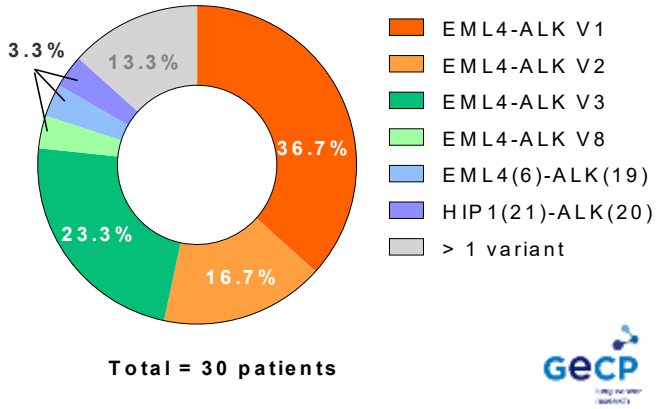






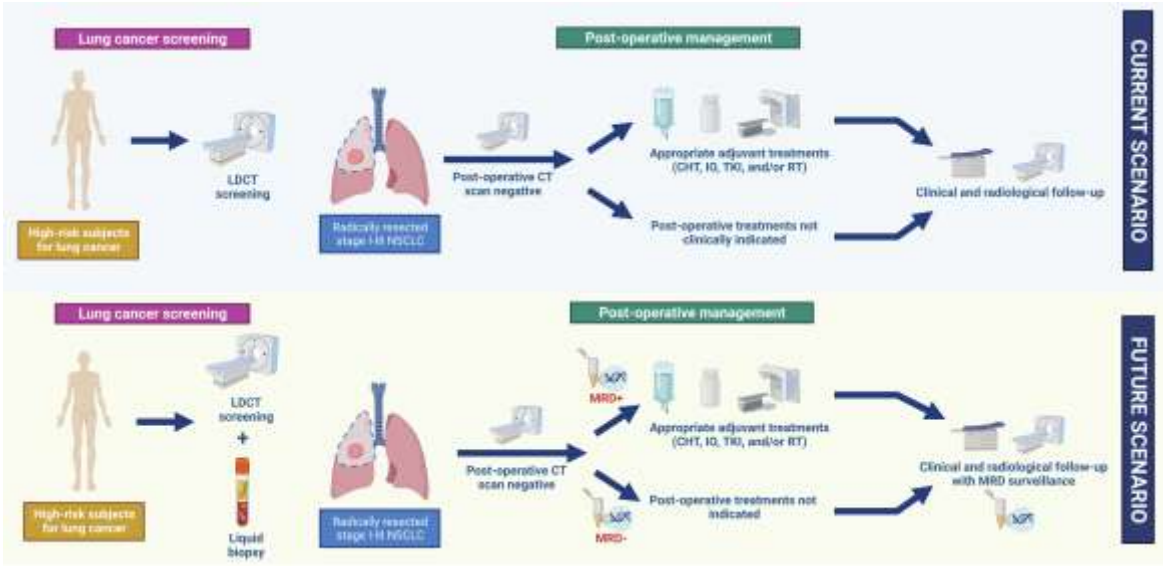
https://pubmed.ncbi.nlm.nih.gov/37243883/





Total = 30 patients

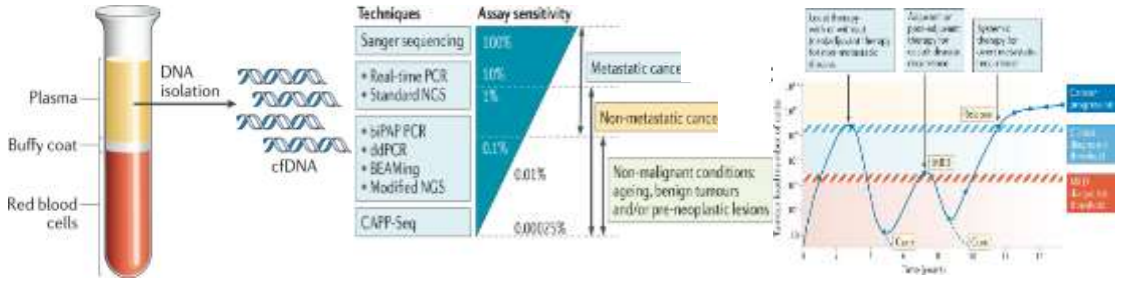
Sensitivity and Detection Limits MRD in NSCLC



https://www.thelancet.com/journals/ebiom/article/PIIS2352-3964%2823%2900170-6/fulltext

NADIM II (NCT03838159)

GUARDANTREVEAL



Pantel, K et al. Nat Rev Clin Oncol. 2019;16:409-424.

10% detection rate

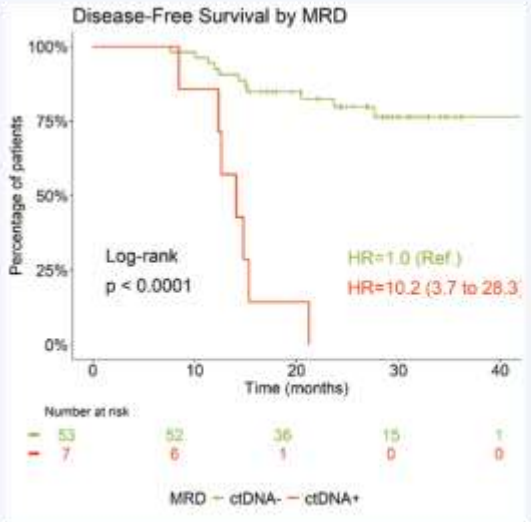
MRD in NADIMII

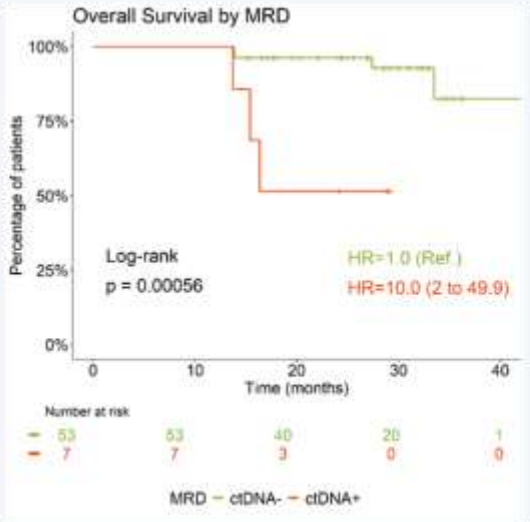
MRD positive vs. **MRD negative**

ANY vs. ANY

MRD	pCR	Non-pCR	Total
Negative	19 (36%)	34 (64%)	53
Positive	0 (0%)	7 (100%)	7
Total	19 (32%)	41 (68%)	60

P = 0.085



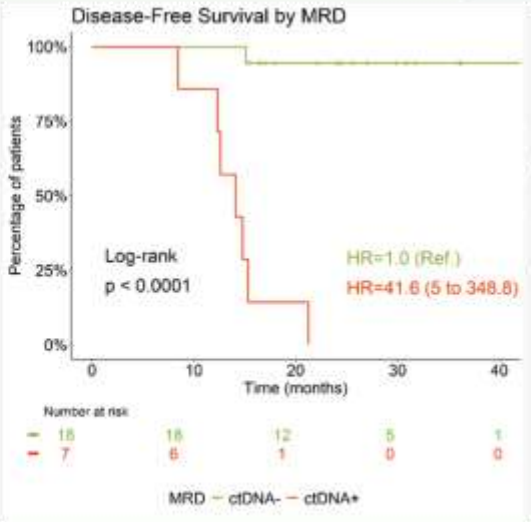


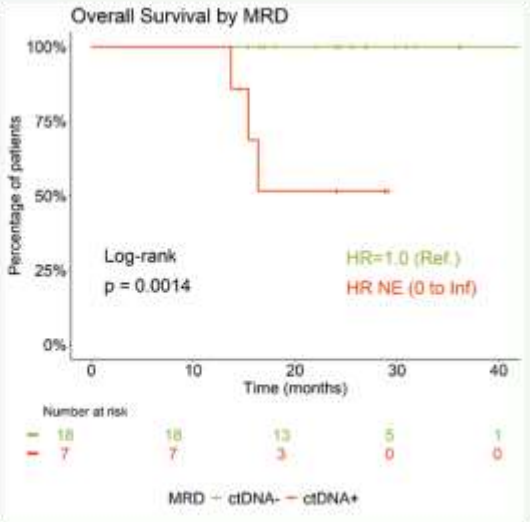
MRD positivo vs. **MRD negativo**

ANY vs. ALL SAMPLES

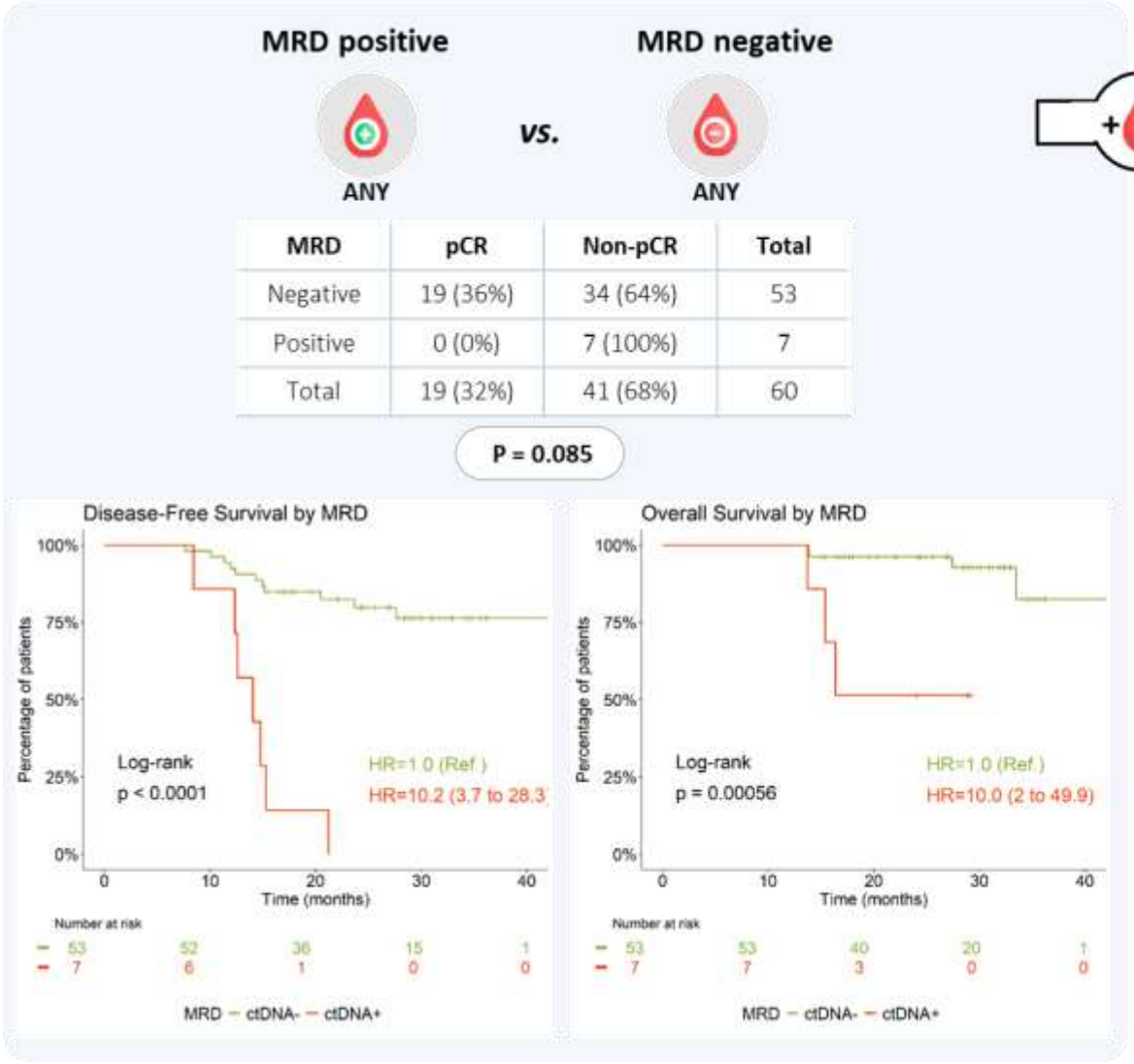
MRD	pCR	Non-pCR	Total
Negative	9 (50%)	9 (50%)	18
Positive	0 (0%)	7 (100%)	7
Total	9 (36%)	16 (64%)	25

P = 0.027



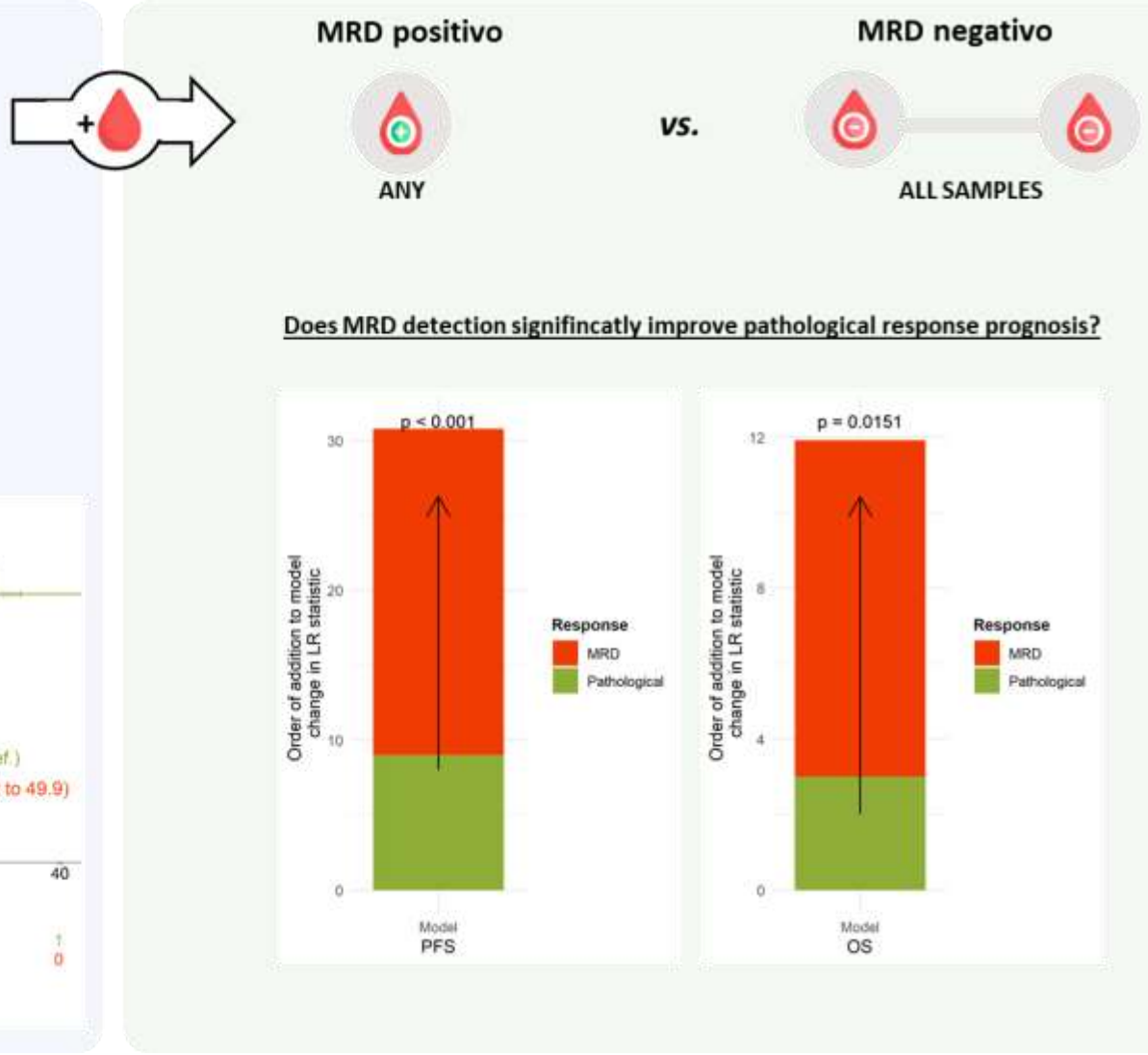


MRD in NADIMII



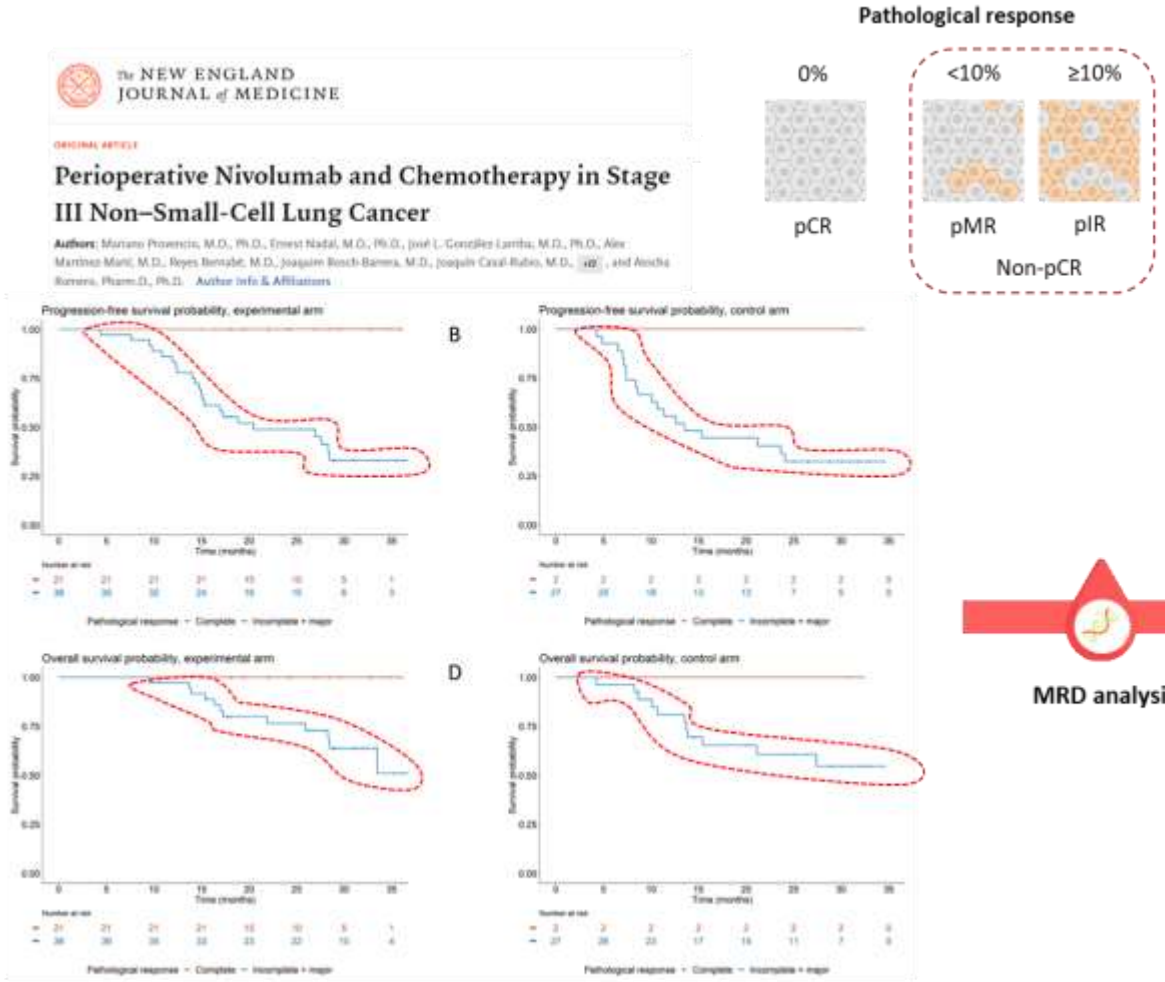
MRD	pCR	Non-pCR	Total
Negative	19 (36%)	34 (64%)	53
Positive	0 (0%)	7 (100%)	7
Total	19 (32%)	41 (68%)	60

P = 0.085

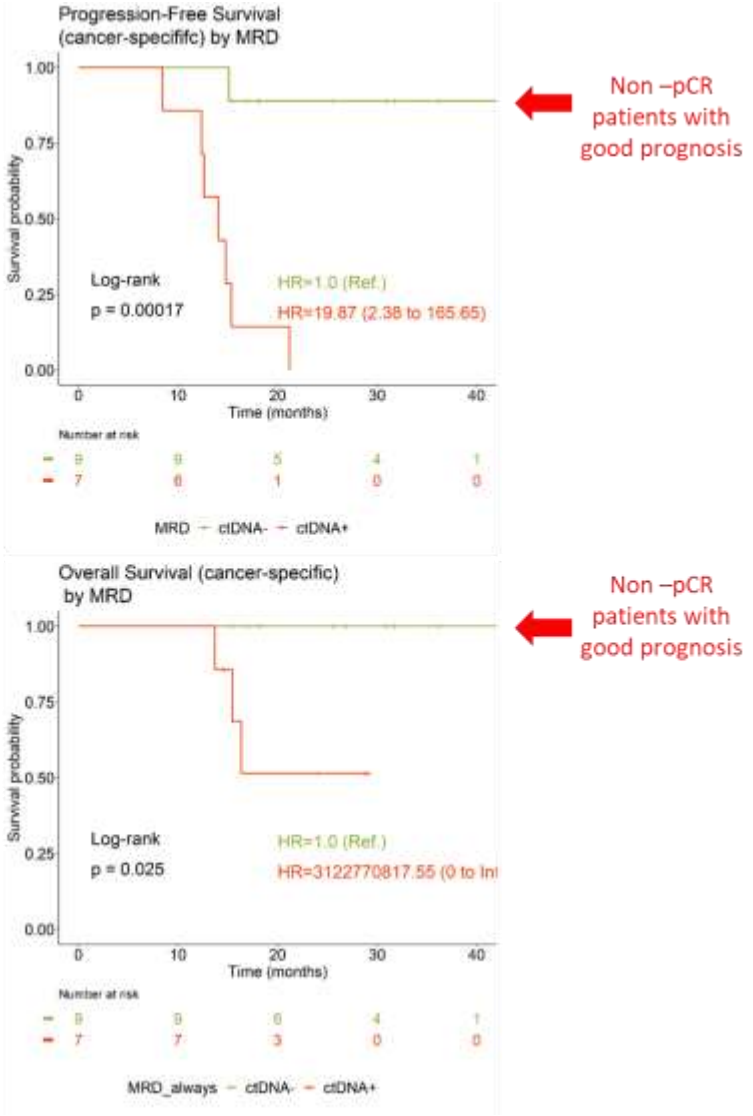


Does MRD detection signifincatly improve pathological response prognosis?

MRD in NADIMII



MRD analysis



Non –pCR patients with good prognosis

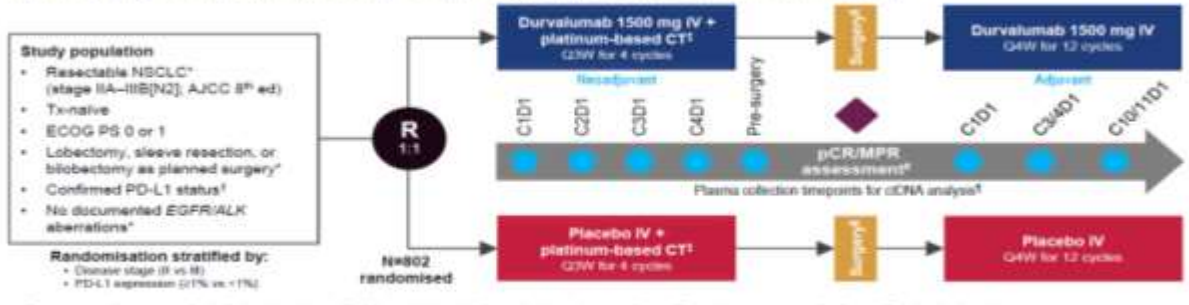
Non –pCR patients with good prognosis

M Provencio et al., NEJM 2023 (PMID: 37379158)

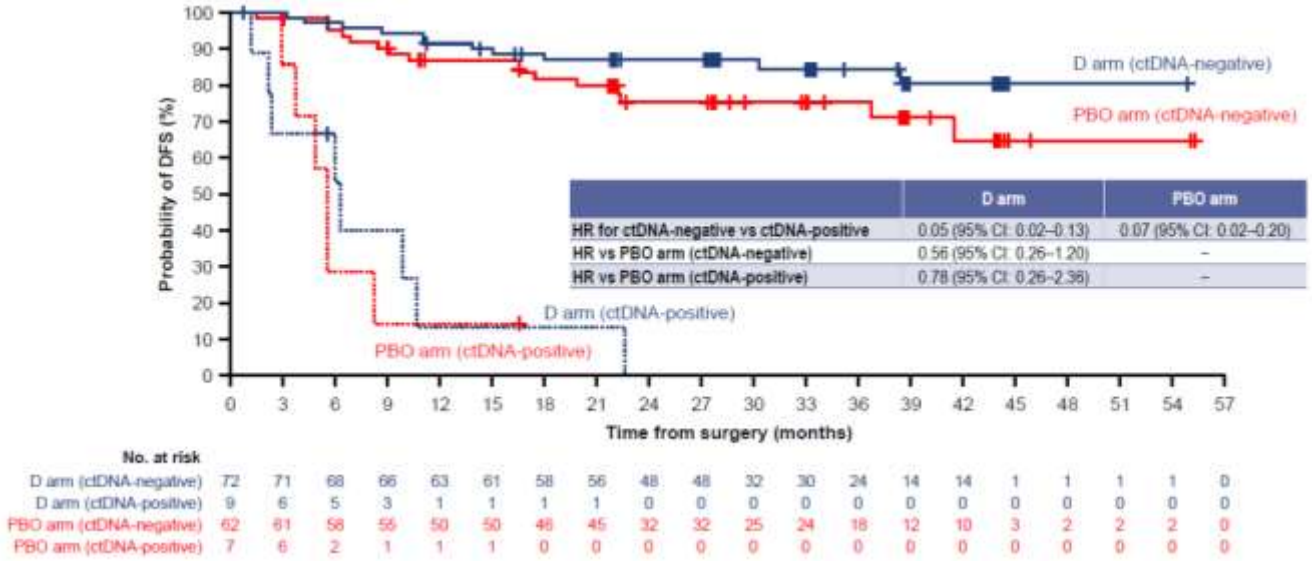
MRD in NSCLC

AEGEAN Study Design

Phase 3, global, randomised, double-blind, placebo-controlled study



- Plasma samples were collected at protocol-specified timepoints, including prior to each neoadjuvant Tx cycle, surgery, and adjuvant Tx at select cycles
- ctDNA analysis was performed using Invitae Personalized Cancer Monitoring,™ a tumour-informed MRD assay,[11] with the exploratory analyses reported here based on data from



	D arm	PBO arm
HR for ctDNA-negative vs ctDNA-positive	0.05 (95% CI: 0.02–0.13)	0.07 (95% CI: 0.02–0.20)
HR vs PBO arm (ctDNA-negative)	0.56 (95% CI: 0.26–1.20)	–
HR vs PBO arm (ctDNA-positive)	0.78 (95% CI: 0.26–2.36)	–

nature medicine

Article

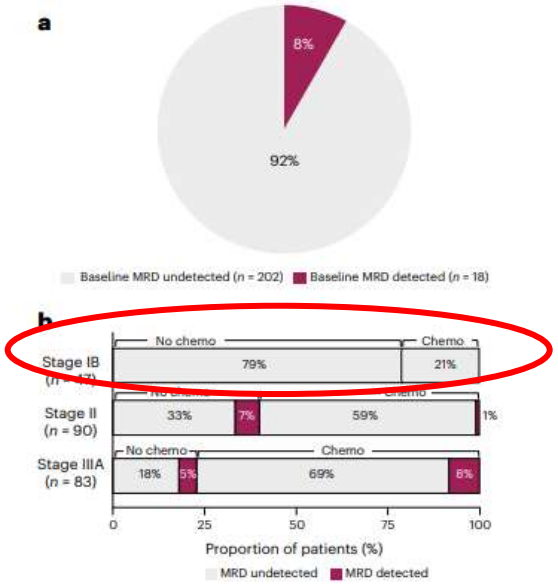
https://doi.org/10.1038/s41591-025-03577-y

Molecular residual disease analysis of adjuvant osimertinib in resected *EGFR*-mutated stage IB–IIIA non-small-cell lung cancer

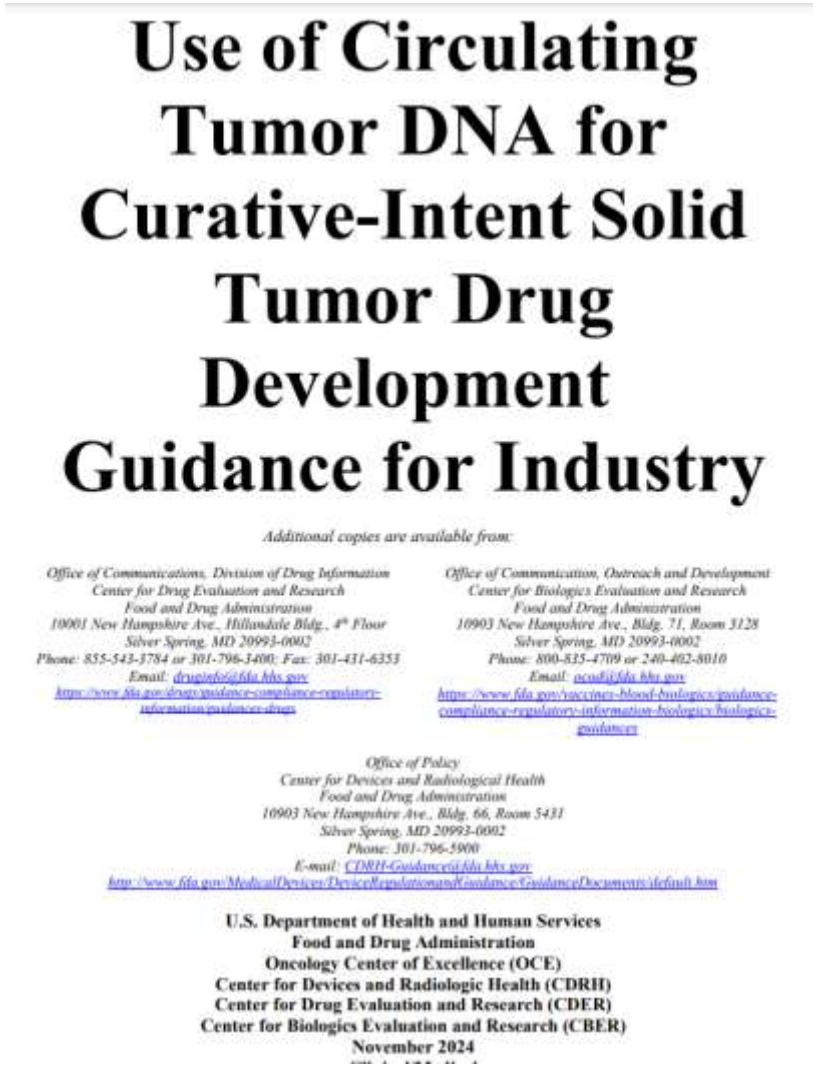
MRD status at randomization:

- Detection rate: 8% (18/220)
- 4% receiving Osimertinib (5/12)
- 12% receiving placebo (13/108)

		MRD status,†n	
		Detected (n=68)	Undetected (n=152)
DFS status,* n	Event (n=96)	62	34
	Censor (n=124)	6	118
Concordance of MRD with DFS,* % (95% CI)			
PPA (sensitivity)			65 (55–74)
NPA (specificity)			95 (91–99)
PPV			[illegible]
NPV			78 (71–84)
OPA			[illegible]



Associations of ctDNA Clearance during Neoadjuvant Treatment with Pathological Response and Event-free Survival in Patients with Resectable NSCLC. Martin Reck. ESMO 2024



Use of Circulating Tumor DNA for Curative-Intent Solid Tumor Drug Development Guidance for Industry

Additional copies are available from:

Office of Communications, Division of Drug Information
Center for Drug Evaluation and Research
Food and Drug Administration
10001 New Hampshire Ave., Hillandale Bldg., 4th Floor
Silver Spring, MD 20993-0002
Phone: 855-543-3784 or 301-796-3400; Fax: 301-431-6353
Email: druginfo@fda.hhs.gov
https://www.fda.gov/drugs/guidance-compliance-regulatory-information/guidances-drugs

Office of Communication, Outreach and Development
Center for Biologics Evaluation and Research
Food and Drug Administration
10903 New Hampshire Ave., Bldg. 71, Room 3128
Silver Spring, MD 20993-0002
Phone: 800-835-4709 or 240-402-8010
Email: ocod@fda.hhs.gov
https://www.fda.gov/vaccines-blood-biologics/guidance-compliance-regulatory-information-biologics/biologics-guidances

Office of Policy
Center for Devices and Radiological Health
Food and Drug Administration
10903 New Hampshire Ave., Bldg. 66, Room 5431
Silver Spring, MD 20993-0002
Phone: 301-796-5900
E-mail: CDRH-Guidance@fda.hhs.gov
http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/default.htm

U.S. Department of Health and Human Services
Food and Drug Administration
Oncology Center of Excellence (OCE)
Center for Devices and Radiologic Health (CDRH)
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)
November 2024

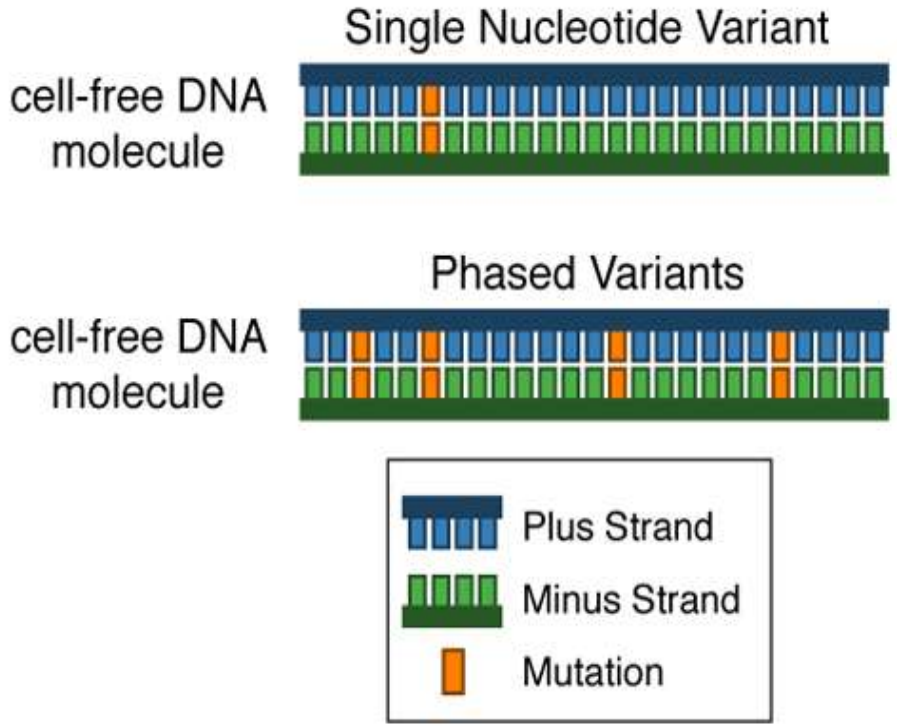
ctDNA MRD could be used for treatment optimization, to add on therapy for patients who are at higher risk of disease recurrence (i.e., ctDNA MRD positive) or to de-escalate therapy for patients with lower risk of disease recurrence (i.e., ctDNA MRD negative)

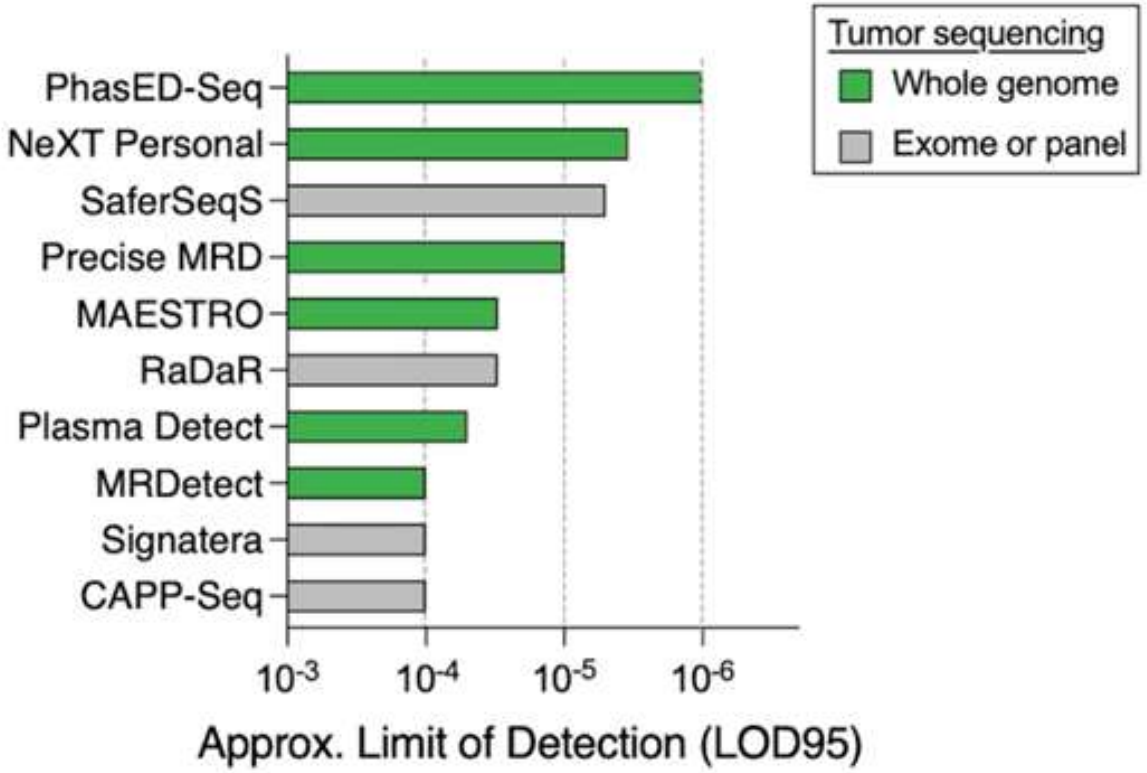
The MRD assay should have high sensitivity and negative predictive value (NPV) for supporting de-escalation of treatment and high specificity and positive predictive value (PPV) for supporting escalation of treatment.

- MRD detection with 1st generation assays has a high positive predictive value.
- Sensitivity remains a challenge

Approaches to improve LOD

Phased variants in ctDNA

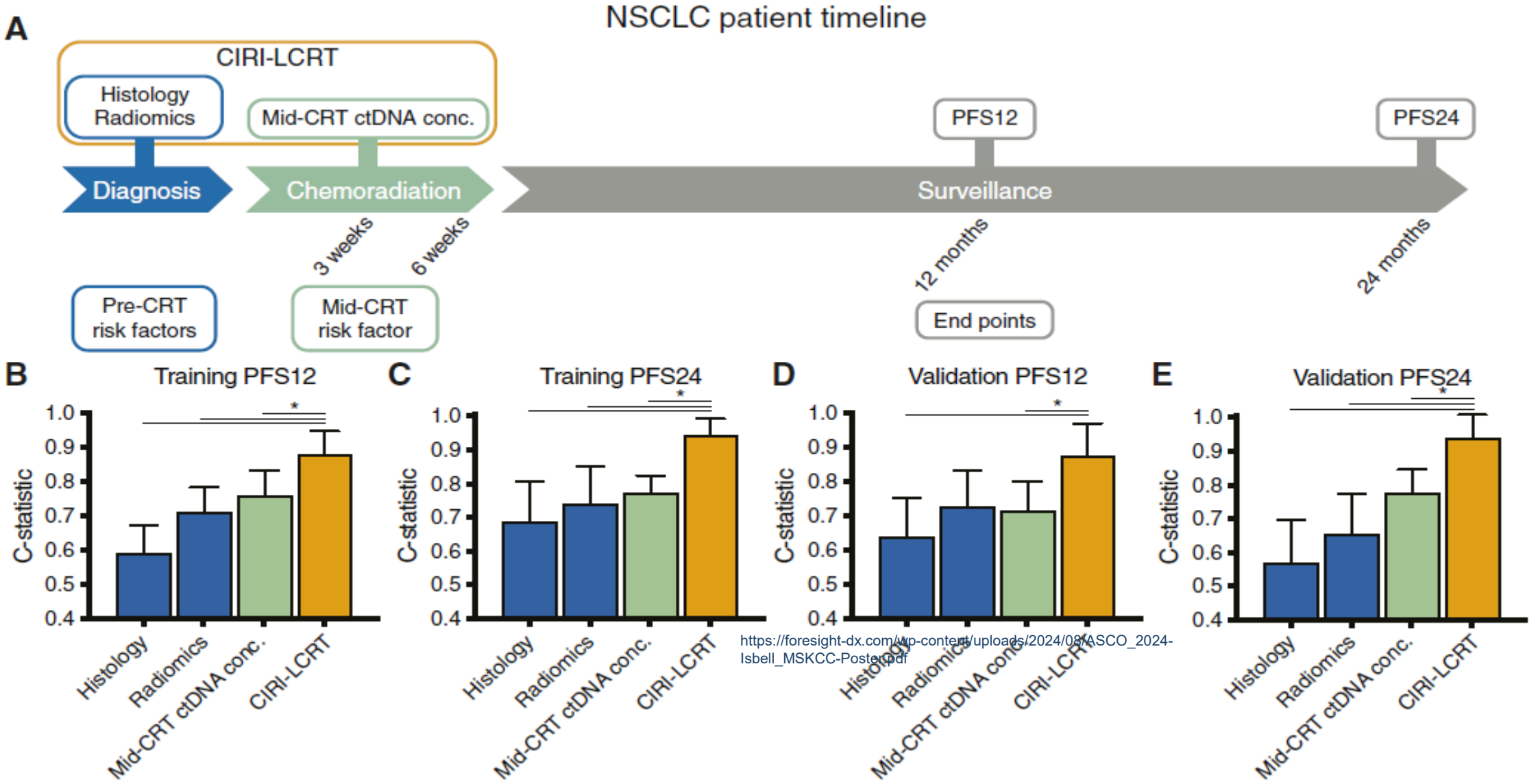




Nat Biotechnol. 2021 Dec;39(12):1537-1547

https://foresight-dx.com/wp-content/uploads/2024/08/ASCO_2024-Isbell_MSKCC-Poster.pdf

Approaches to improve LOD



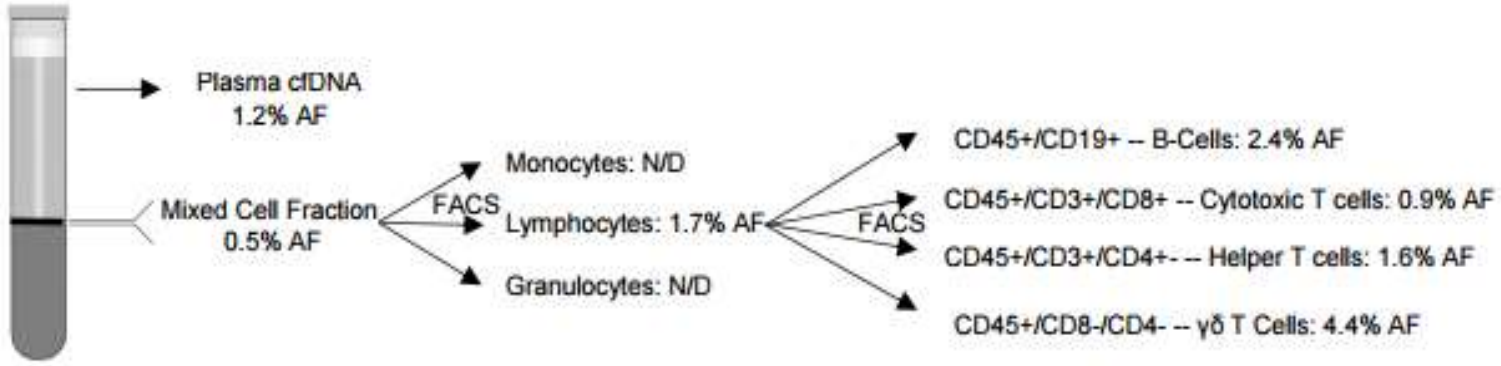
https://foresight-dx.com/wp-content/uploads/2024/08/ASCO_2024-Isbell_MSKCC-Poster.pdf

Cancer Discov 2025;15:1609–29 doi: 10.1158/2159-8290.CD-24-1704

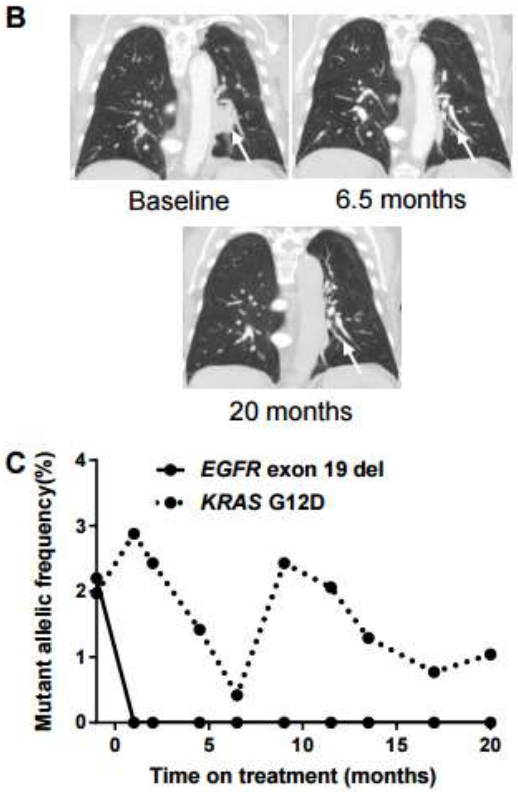
Specificity and False Positives
Clonal haematopoiesis

- CHIP is defined by the presence of somatic mutation in blood or BM but without other diagnostic criteria for a haematological malignancy.
- More frequent in aged patients, patients with solid tumours
- More likely to be detected with deeper sequencing approaches.
- May account for false positive ctDNA sequencing results.

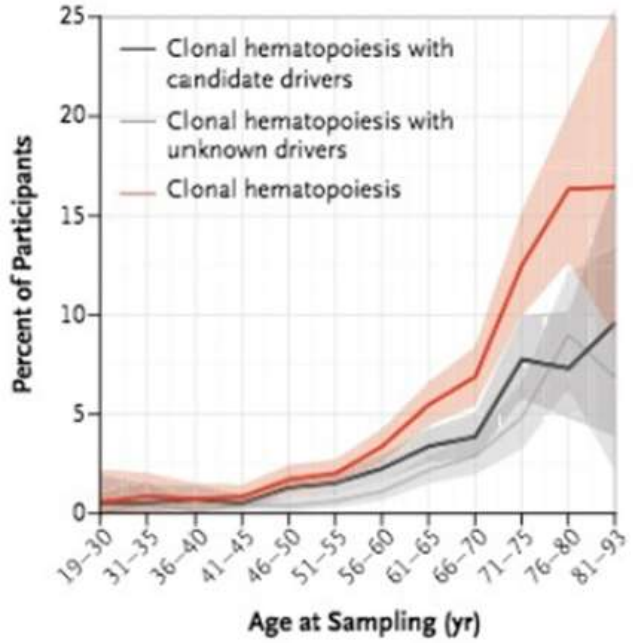
KRAS G12D



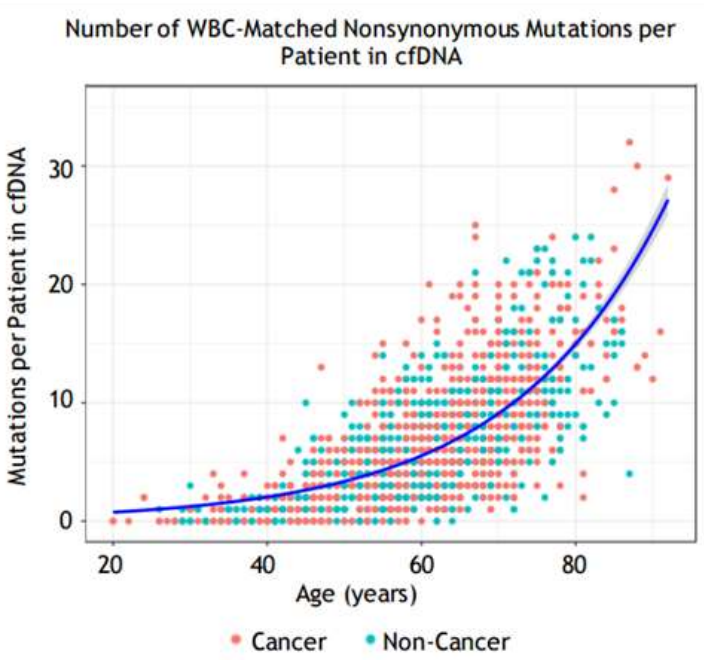
Hu Y et al. False-Positive Plasma Genotyping Due to Clonal Hematopoiesis.Clin Cancer Res. 2018 Mar 22.



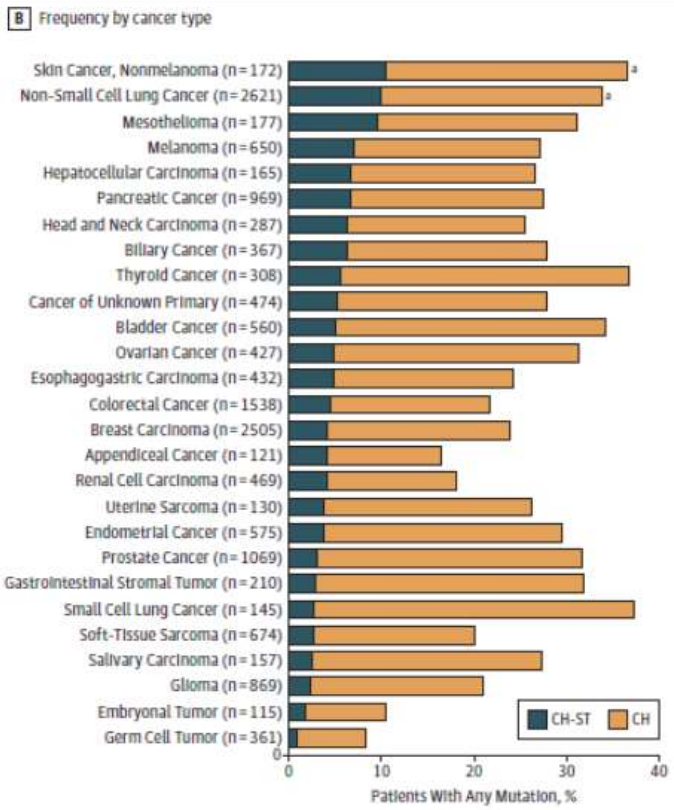
Specificity and False Positives



Genovese G., *et al.* 2014 NEJM

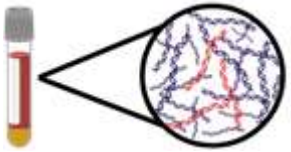


Charles Swanton et al. Journal of Clinical Oncology 36, no. 15_suppl (May 20 2018) 12003-12003.



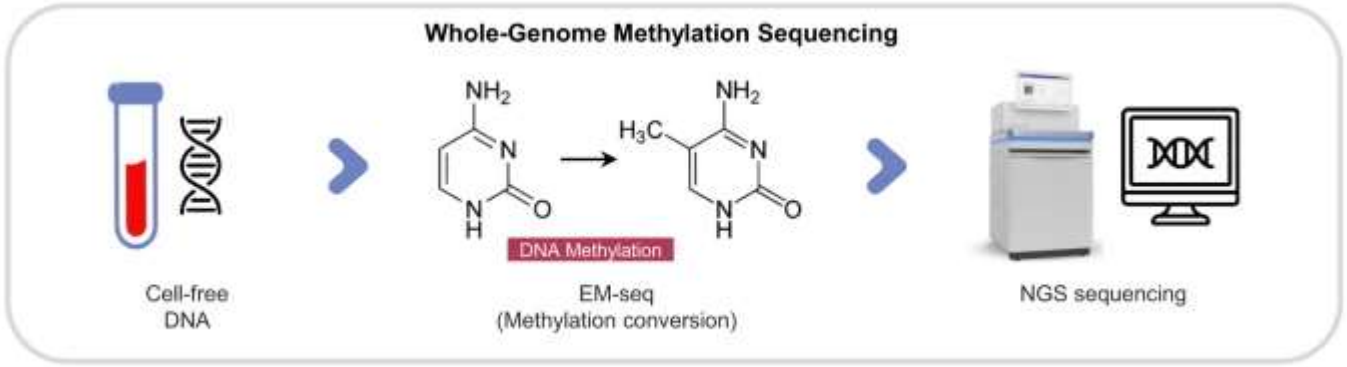
Tumor Fraction estimation

Methods for estimating tumor fraction in circulating tumor DNA (ctDNA) using liquid biopsy

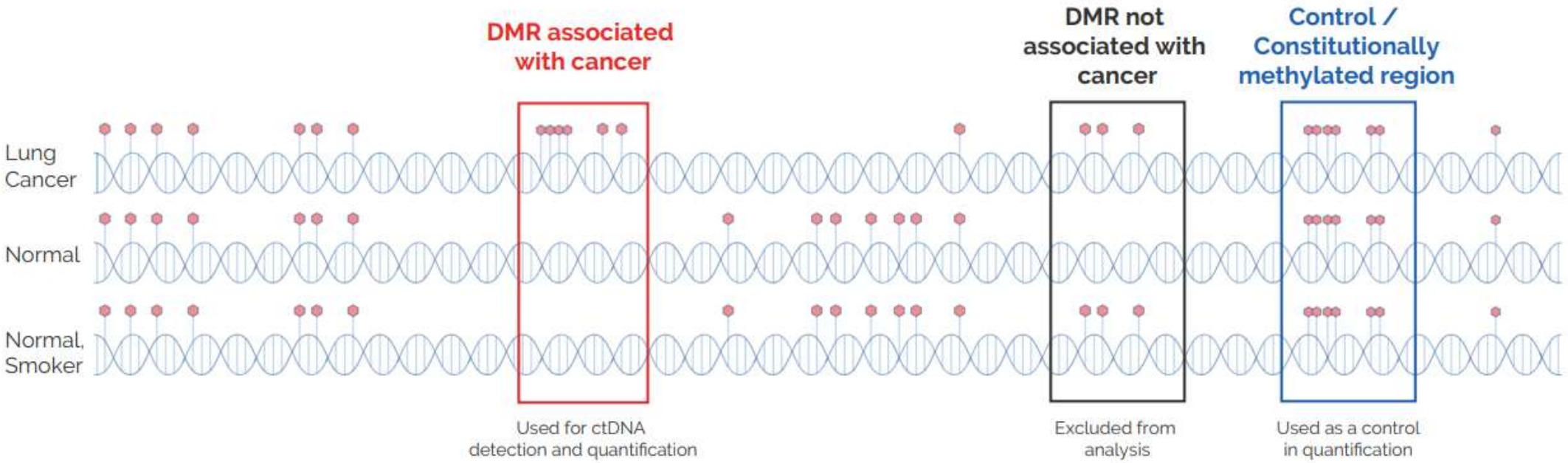


1. **Variant Allele Frequency (VAF) of Tumor-Specific Mutations**
2. **Genome-Wide Copy Number Alterations (CNAs):** The amplitude of these alterations in cfDNA is used to infer the fraction of ctDNA, especially in tumors with high chromosomal instability
3. **Methylation Profiling**
4. **Fragment Size Analysis**
5. **Bioinformatic Deconvolution**

TUMOR FRACTION. METILATION

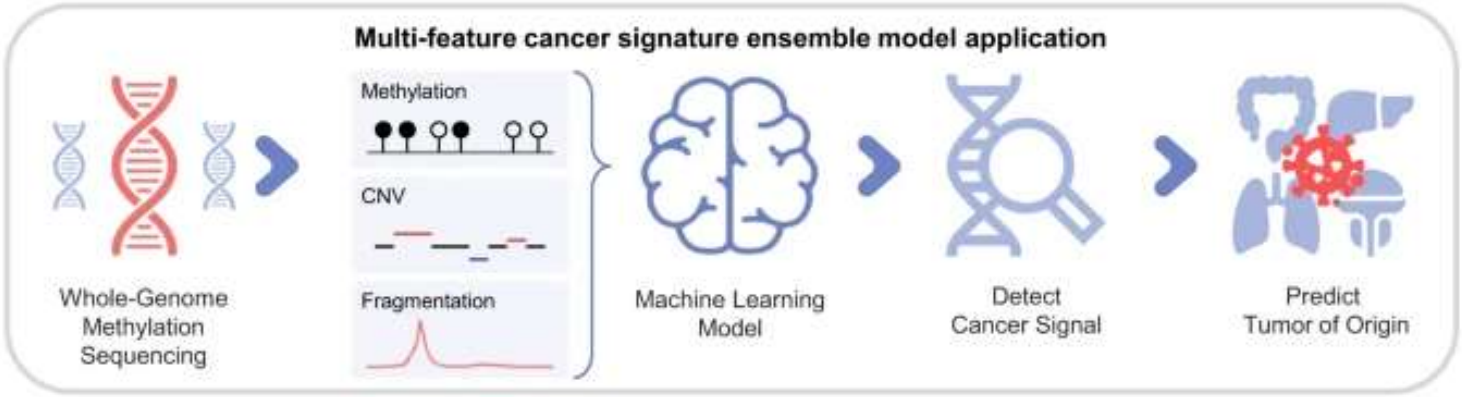


https://www.nature.com/articles/s12276-023-01119-5

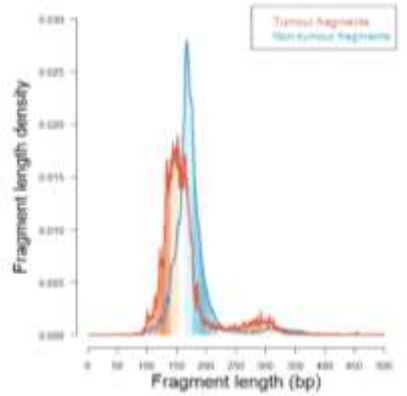


Quantifying ctDNA Using a Tissue-Free Test for Minimal Residual Disease (MRD) Detection. Guardant Health

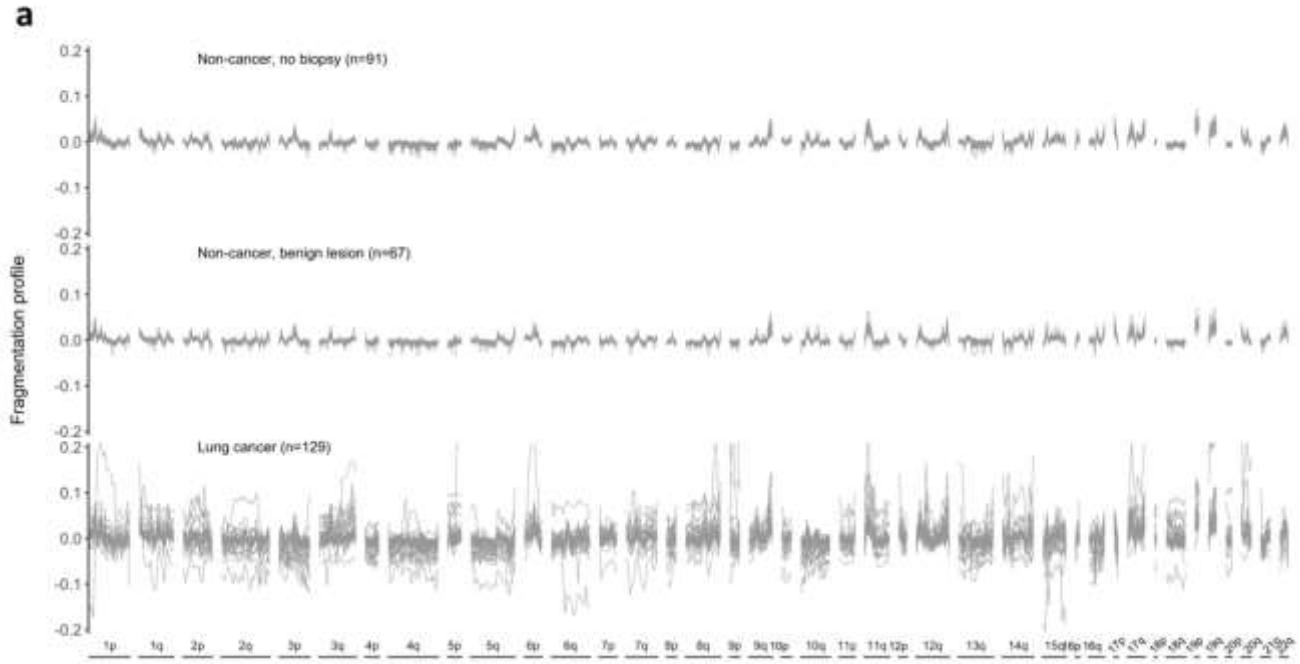
FRAGMENTOMICS



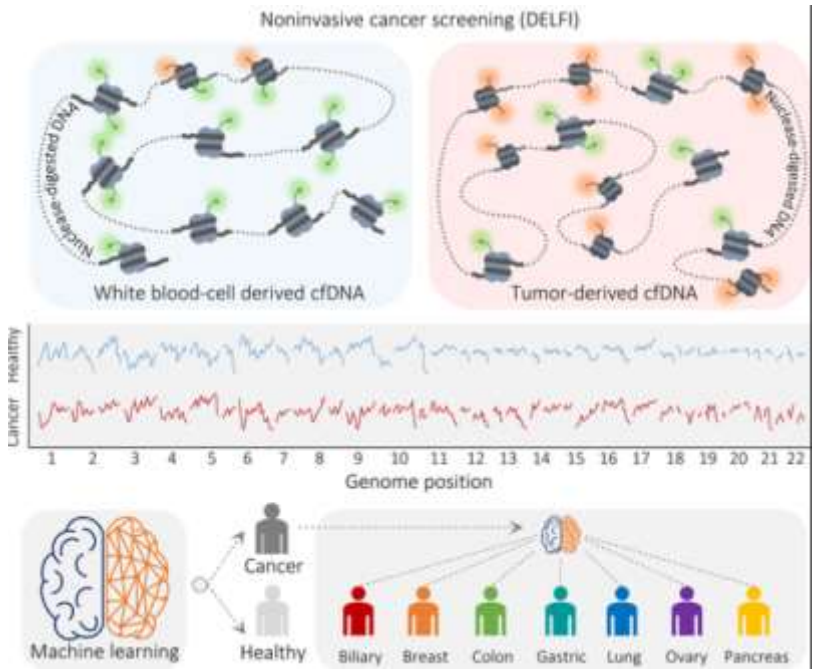
https://www.nature.com/articles/s12276-023-01119-5



Vessies et al., Molecular Oncology 16:2719–2732 (2022)

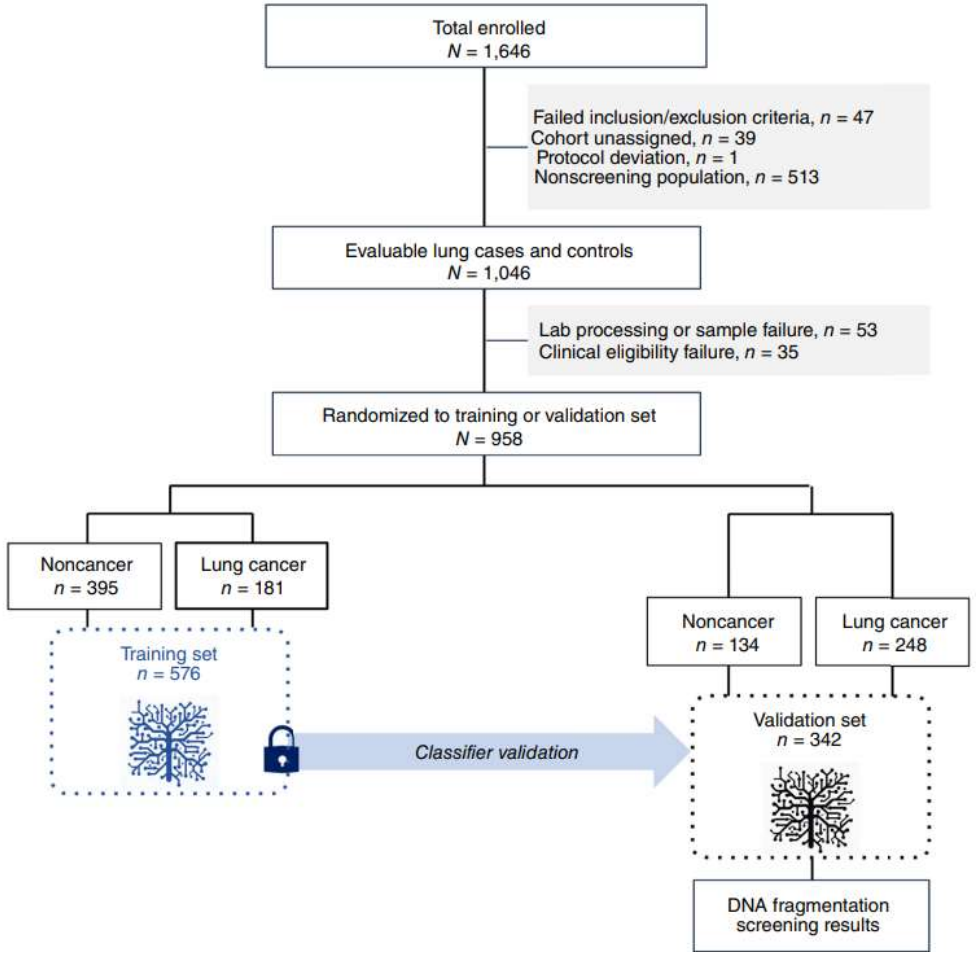


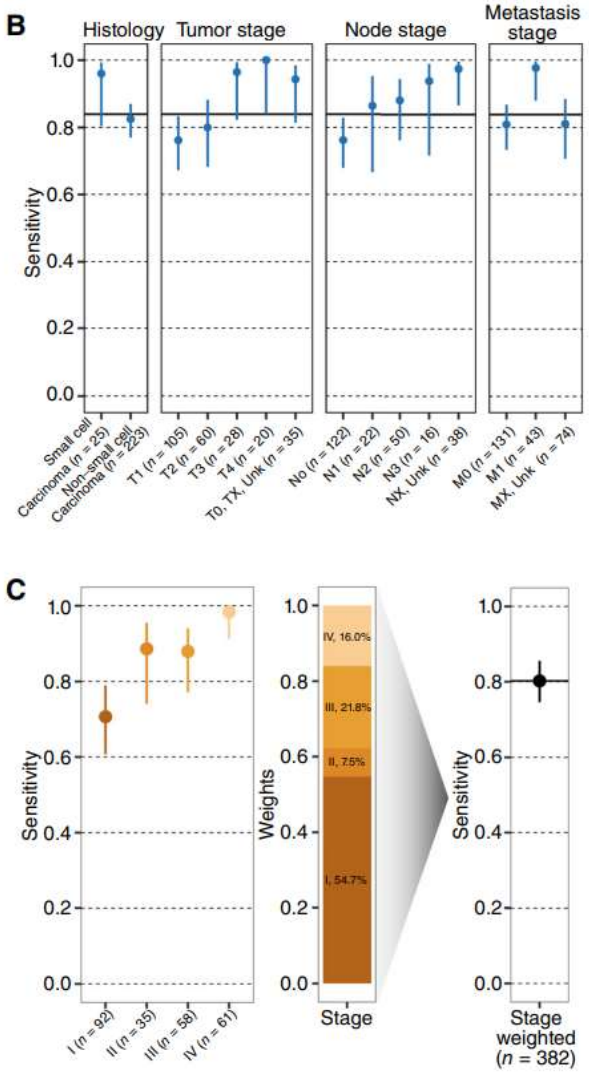
Mathios D, et al. Nature Communications, 2021;12(1):5060.



Nature. 2019 Jun;570(7761):385-389.

FRAGMENTOMICS

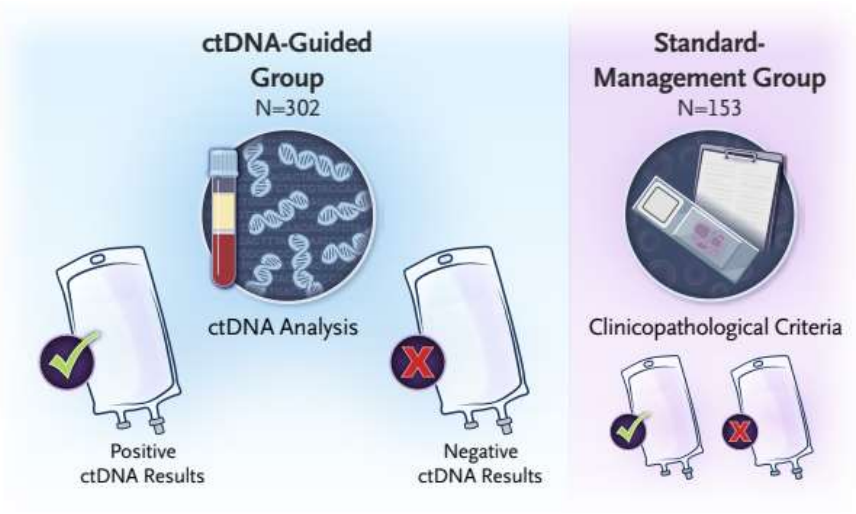




https://aacrjournals.org/cancerdiscovery/article/14/11/2224/749197/Clinical-Validation-of-a-Cell-Free-DNA-Fragmentome

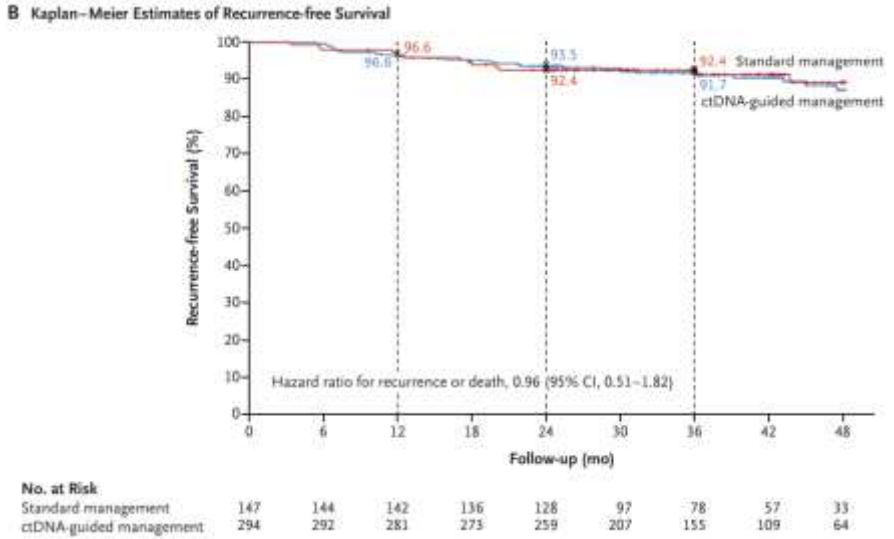
Clinical Validation

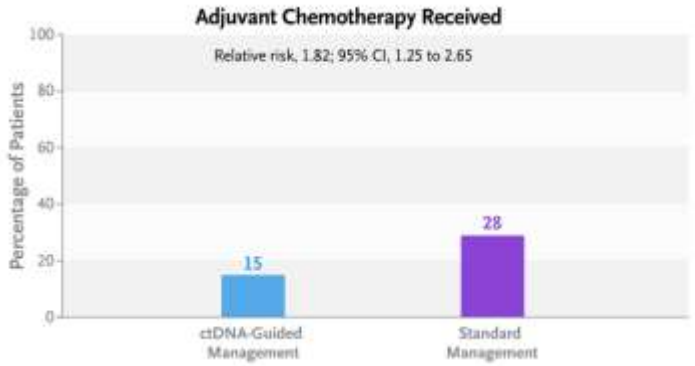
https://www.nejm.org/doi/full/10.1056/NEJMoa2200075#ap2

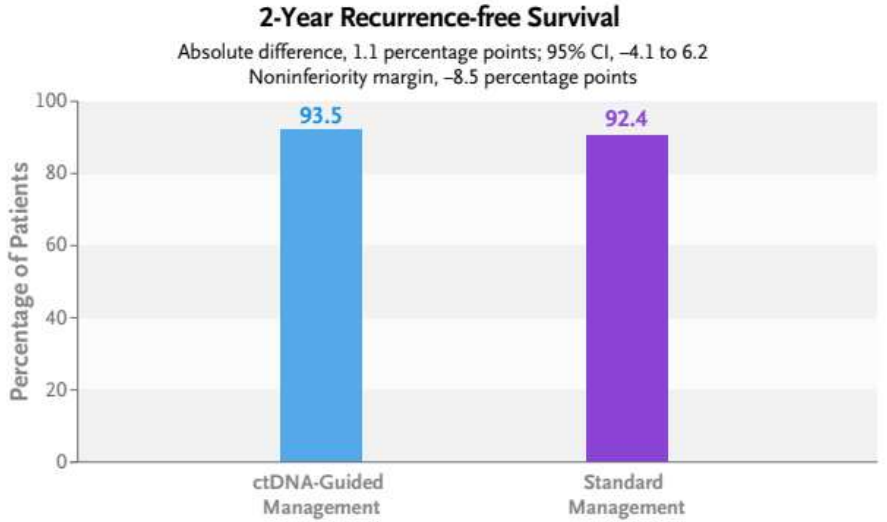


Stage II Colon Cancer

- R0 resection
- ECOG 0 – 2
- Staging CT within 8 weeks
- Provision of adequate tumor tissue within 4 weeks post-op
- No synchronous colorectal cancer

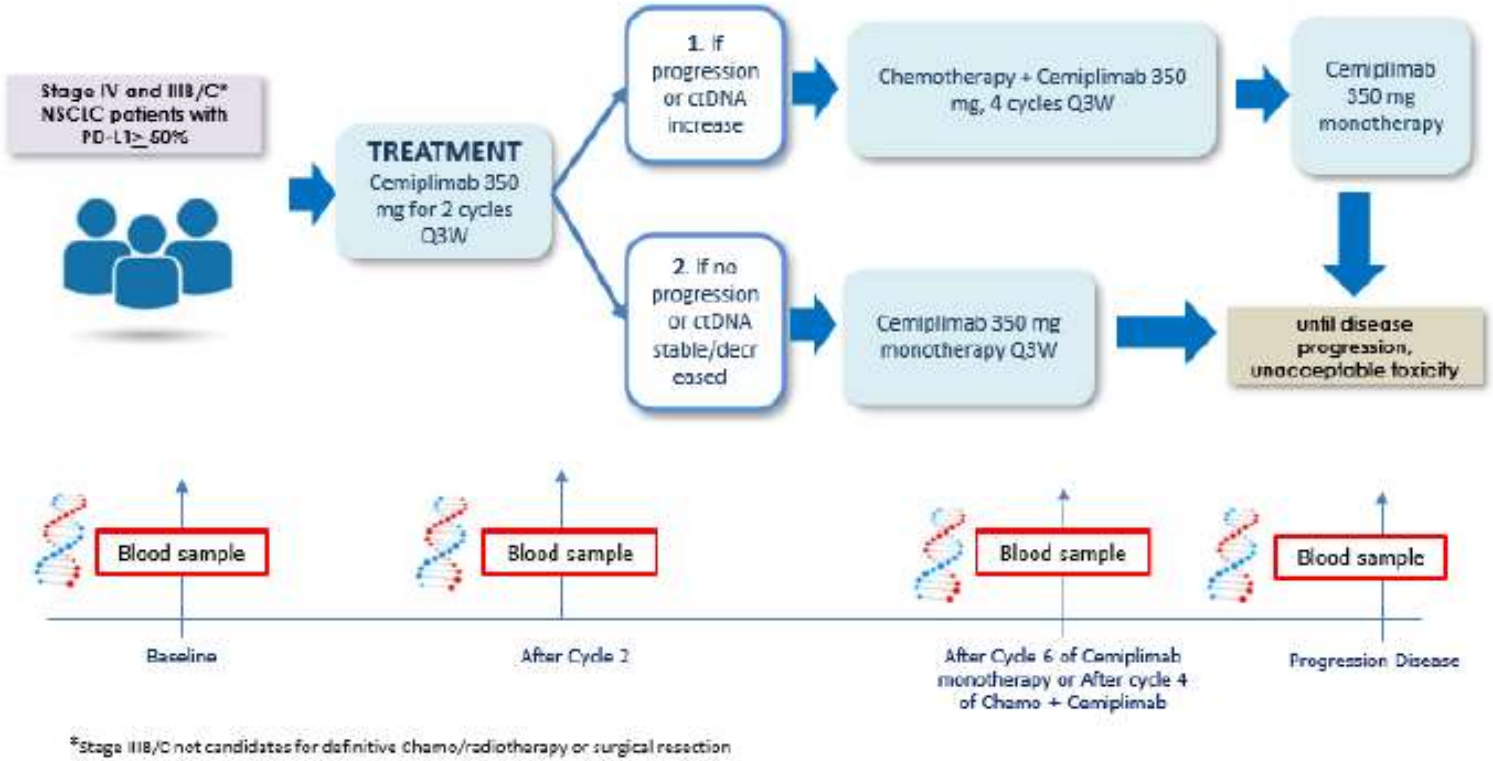






Clinical Validation

Palace



Primary endpoint: OS at 24 months in the ITT population

26 pts

Pre-analytical and Analytical Variability

External Quality Assessment (EQA) for Circulating free DNA (cfDNA) testing in Lung cancer 2023

Table 5: Number of participating laboratories

Number of registrations	357
Number of withdrawals	22
Number of laboratories that did not submit results	19*
Total number participating laboratories	316

* Participation in this EQA is sponsored and has a limited number of available places; we request laboratories to only register if they are planning to submit results.

Sixteen laboratories using either the Oncomine™ Lung Cell-Free Total Nucleic Acid (cfTNA) Research Assay or the Oncomine™ Pan-Cancer Cell-Free Assay did not report the presence of the insertion in exon 20 of *EGFR*. There were other laboratories using these assays which did report the presence of the variant. Further investigation of this demonstrated that although this region of *EGFR* was included in the regions sequenced it was not in the "hotspot" file required to call variants using the kit bioinformatics. Many of these sixteen laboratories did not provide sufficient information in their reports to enable readers of the reports to determine that the assay used would not detect all variants in exon 20 of *EGFR* and therefore received deductions from their interpretation scores.

Take home message

- Liquid biopsy sensitivity remains limited for MRD analysis.
- Specificity issues arise due to clonal hematopoiesis (CHIP).
- Tumor fraction estimation is critical and method-dependent.
- High pre-analytical and analytical variability; standardization is essential.
- Clinical validation is still limited.

16th CONGRESS Lung ON CANCER

BARCELONA 27 / 28 NOVEMBER 2025

THANK YOU

aromero@idiphim.org

Plan de Recuperación, Transformación y Resiliencia

Unión Europea
Fondo Europeo de Desarrollo Regional

INSTITUTO DE INVESTIGACIÓN SANITARIA PUERTA DE HIERRO - SEGOVIA DE ARANA
SaludMadrid Hospital Universitario Puerta de Hierro Majadahonda

GECP lung cancer research