11th International Conference of Contemporary Oncology, 13-15.03.2019 Poznań

Genetic and epigenetic markers of non-small cell lung cancer in peripheral blood: an update

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National Institute of Tuberculosis and Lung Diseases, Warsaw

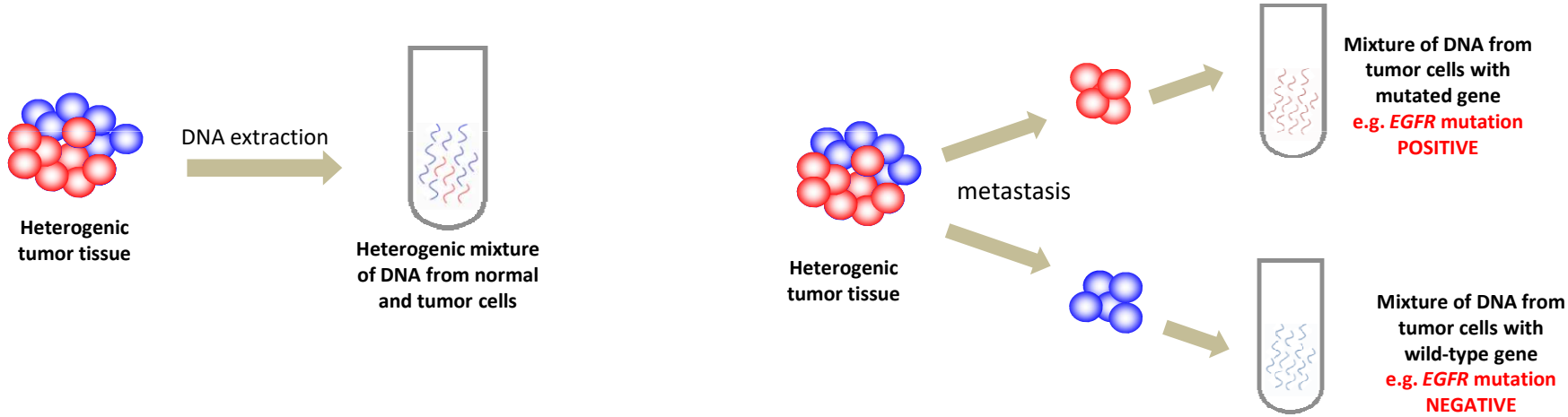
Current problems of tumor tissue-based molecular diagnostics in the era of precision medicine in NSCLC

1. Limited availability of good quality tumor tissue specimens in advanced NSCLC patients

- approx. 20% patients with advanced NSCLC cannot undergo tumor biopsy for clinical reasons
- approx. 15-25% tumor biopsies don't provide sufficient quality/quantity of genetic material for molecular analysis
- growing number of molecular and pathological markers to be evaluated in a scarce tumor tissue specimen

2. Heterogeneity of tumor tissue and cytology specimens affects the efficacy of molecular analysis

- **Cellular heterogeneity** (normal cells + tumor cells in tissue specimen)
- **Molecular heterogeneity** (distinct tumor cell clones presenting different profiles of molecular alterations)



3. Tumor biopsy is an invasive clinical procedure and may cause serious complications

- Most common complication: pneumothorax (half of the cases require interventional treatment)
- Other possible complications: pulmonary air embolism; plueral hemorrhages; hemoptysis; tumour cell dissemination in the biopsy site

4. The need of tumor re-biopsy upon progression on 1st line targeted treatment to check for resistance mechanism

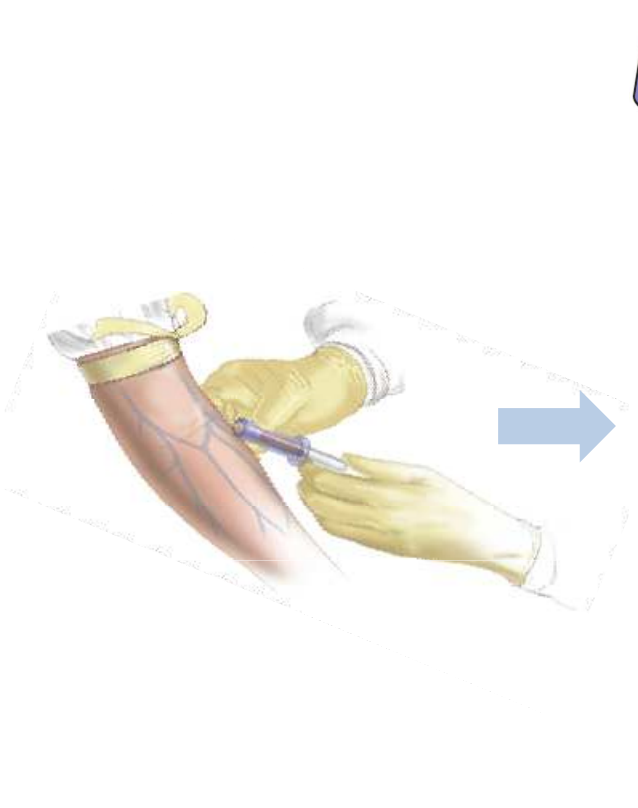
5. High economic costs of tumor biopsy procedure and tissue specimen evaluation by pathologists before molecular analysis and medical care for patient in a hospital before/after tumor biopsy

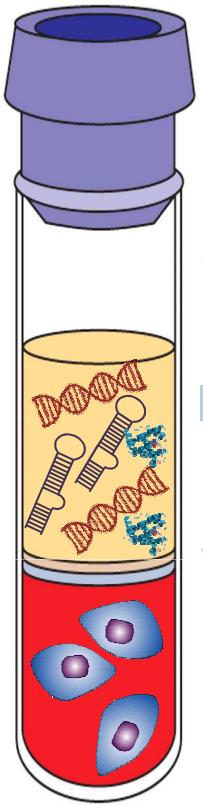
Development of non-invasive diagnostic approaches in NSCLC: what specimen to use?

Surrogate specimen	Invasiveness	Reproducibility	Genetic material shed by tumor	Molecular alterations (biomarker of interest)	Potential biomarker applications
Urine		++	▪ genomic DNA ▪ microRNA	▪ somatic mutations	▪ Screening and early detection ▪ Predictive biomarker: targeted therapy ▪ Treatment monitoring
Exhaled Breathe Condensate		+	▪ genomic DNA ▪ microRNA	▪ somatic mutations ▪ gene hypermethylation ▪ microsatellite instability ▪ microRNA expression	▪ Early detection
Induced sputum		++	▪ genomic DNA ▪ microRNA ▪ tumor cells	▪ somatic mutations ▪ gene hypermethylation ▪ microsatellite instability ▪ microRNA expression	▪ Early detection
Peripheral Blood		+++	▪ genomic DNA ▪ mitochondrial DNA ▪ nucleosomal DNA ▪ mRNA transcripts ▪ microRNA ▪ Circulating Tumor Cells	▪ cell-free DNA levels ▪ cell-free DNA integrity ▪ somatic mutations ▪ gene hypermethylation ▪ microsatellite instability ▪ microRNA expression ▪ gene mRNA expression	▪ Early detection ▪ Differential diagnosis of SPN ▪ Predictive biomarker: targeted therapy, radio-/chemotherapy ▪ Targeted therapy monitoring ▪ Prognostic biomarker
Bronchoalveolar lavage fluid		++	▪ genomic DNA ▪ microRNA ▪ tumor cells	▪ somatic mutations ▪ microsatellite instability ▪ microRNA expression	▪ Early detection
Pleural effusion fluid		+	▪ genomic DNA ▪ microRNA ▪ tumor cells	▪ somatic mutations ▪ gene hypermethylation ▪ microRNA expression	▪ Predictive biomarker: targeted therapy ▪ Prognostic biomarker
Cerebrospinal fluid		+	▪ genomic DNA ▪ microRNA	▪ somatic mutations	▪ Predictive biomarker: 2nd line EGFR TKI (osimertinib) in NSCLC pts with mets to CNS

1. **Liquid biopsy – definition and characteristics**
2. Detection of EGFR T790M mutation in liquid biopsy from NSCLC patients progressing on 1st line EGFR TKIs
3. Liquid biopsy in the monitoring of EGFR-TKI therapy effectiveness
4. Tumor Mutation Burden (TMB) in liquid biopsy as the predictive biomarker for immunotherapy in NSCLC
5. Future perspectives on the liquid biopsy –based applications in NSCLC diagnostics

The concept of 'liquid biopsy'

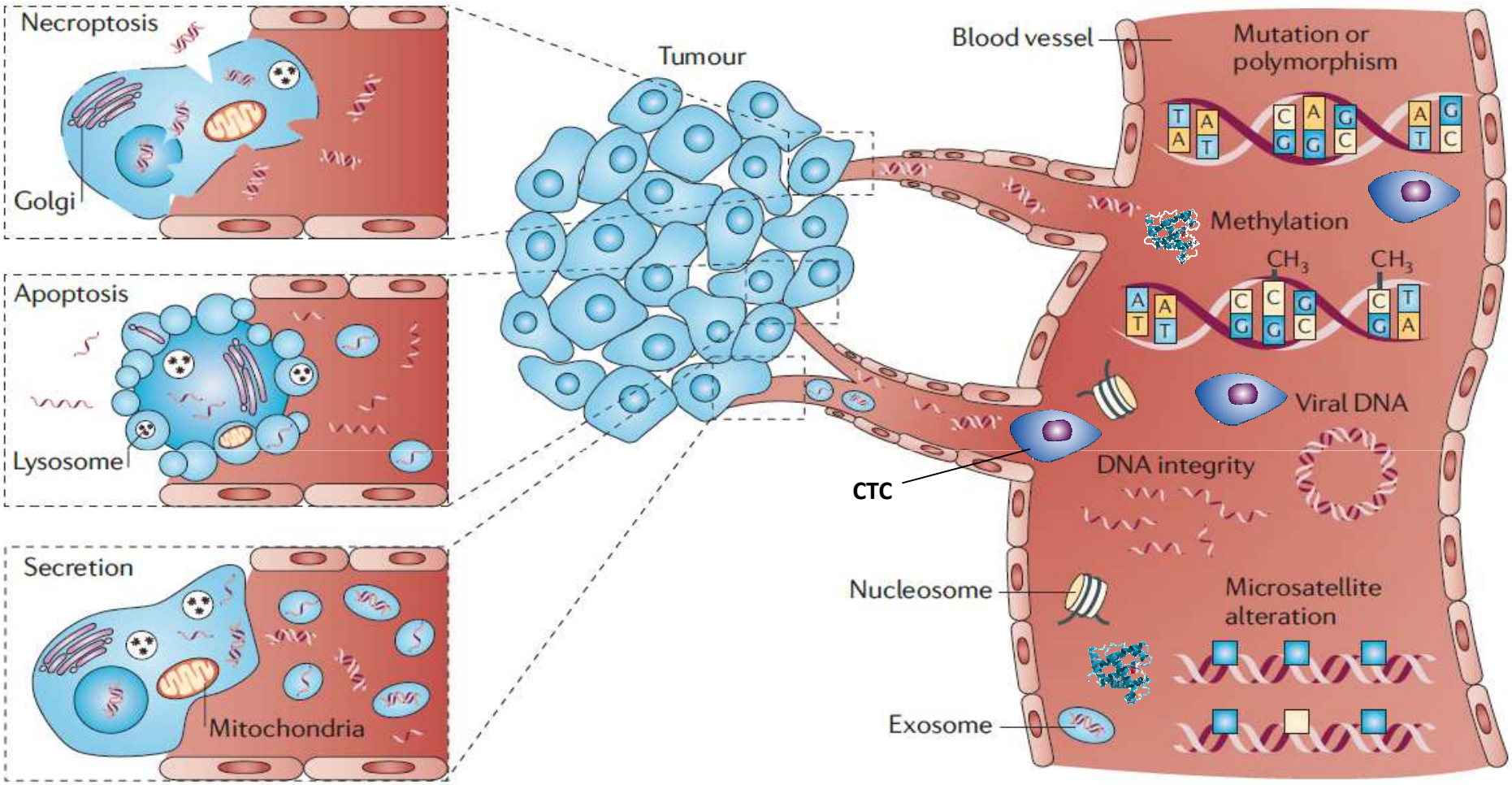




Tumor material	*Molecular biomarker*	*Detection method*
Cell-free DNA	• **cell-free DNA level** • **cell-free DNA integrity** • **somatic mutations** • **gene hypermethylation** • **gene fusions**	real-time PCR droplet-digital PCR BEAMing Next Generation Sequencing
Circulating RNA (microRNA)	• **microRNA expression** • **gene mRNA expression**	real-time PCR microarrays NanoString Next Generation Sequencing
Proteins	• **Plasma proteomic profiles**	Mass Spectrometry Protein arrays ELISA nephelometry
Circulating Tumor Cells	• **CTCs number** • **somatic mutations** • **gene fusions** • **gene mRNA expression** • **protein expression** • ***in vitro* culture**	real-time PCR droplet-digital PCR Next Generation Sequencing FISH immunofluorescence

Venous blood collection → **Plasma & cell fractions separation** → **Circulating biomarkers** → **Detection and analysis**

How tumor cells shed their genetic material into the blood circulation?



>> circulating blood captures and pools DNA/RNA released by all: primary and metastatic tumor sites within the body of cancer patient that overcomes to some extend the problem of tumor heterogeneity

>> DNA/RNA are relased by tumor cells constantly throughout the disease development

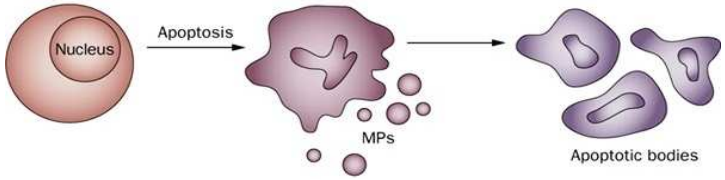
Schwarzenbach H et al. *Nat Rev Cancer. 2011 Jun;11(6):426-37.*

Molecular and biophysical forms of circulating DNA

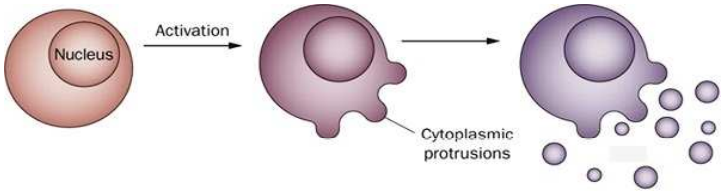
Liquid biopsy

Nucleosomal DNA

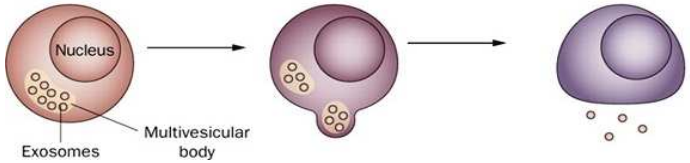
Apoptotic bodies



Microparticles



Exosomes



Virtosomes

- Complex of DNA-protein-lipid (no cell membrane)

DNA-protein complex

- Human serum albumins (HSA)
- Serum amyloid P component (SAP)
- Cellular receptors

How much circulating DNA of tumor origin in liquid biopsy?

- **Circulating tumor DNA (ctDNA)** account for 0.01% – 10% of total cell-free DNA in liquid biopsy
- Dedicated molecular biology techniques presenting very high sensitivity need to be used for detection

Table 1. Allelic fractions for six FFPE and cfDNA matched samples from late-stage NSCLC samples. As expected, the allelic fraction in an FFPE tumor samples is greater than that measured in plasma when the variant is derived from cancer cells.[1]

Samples	Variant	FFPE samples	cfDNA
1	*EGFR*-L858R	**71.42%**	2.62%
2	*TP53*-R158L	**51.89%**	4.32%
3	*MET*-T1010I	43.87%	51.75%
	KRAS-G12C	**34.62%**	0.28%
4	NA	No detection	No detection
5	*EGFR*-L858R	**58.44%**	7.28%
	MET-T1010I	41.93%	48.72%
	TP53-Y220C	**35.54%**	1.93%
6	*TP53*-R158L	**10.19%**	1.26%

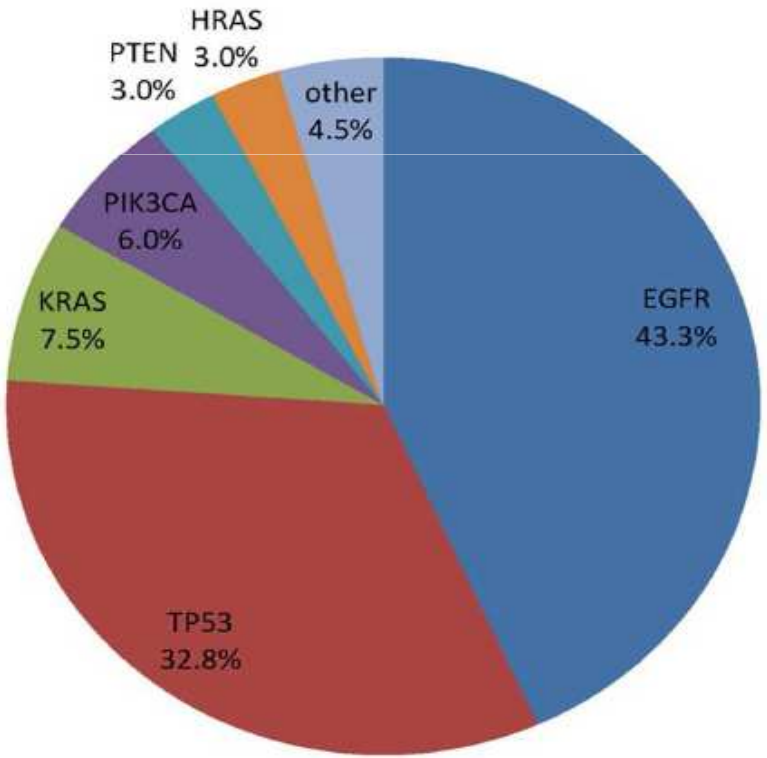
[1] Bold: somatic mutations; normal: germline mutations.

thermofisher.com/cfdna-assays

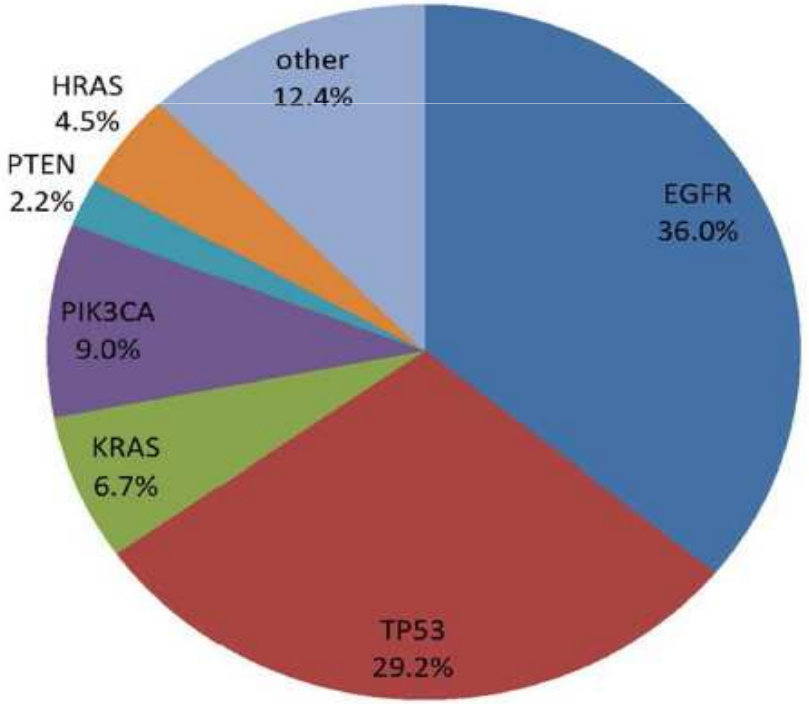
Liquid biopsy and tumor tissue present very similar patterns of molecular alterations in NSCLC

n=76 (I-III)

Frequency (%) of somatic mutations in tumor tissue

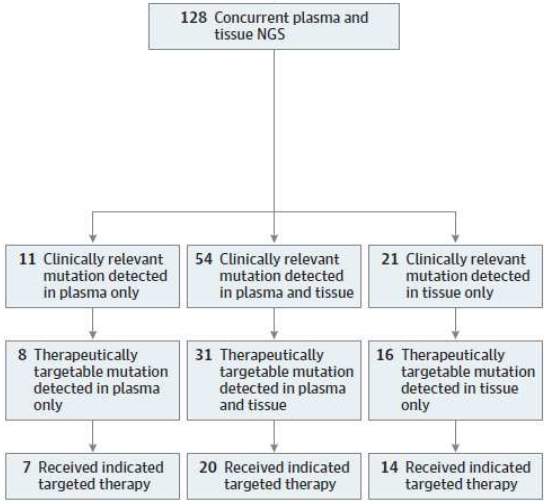


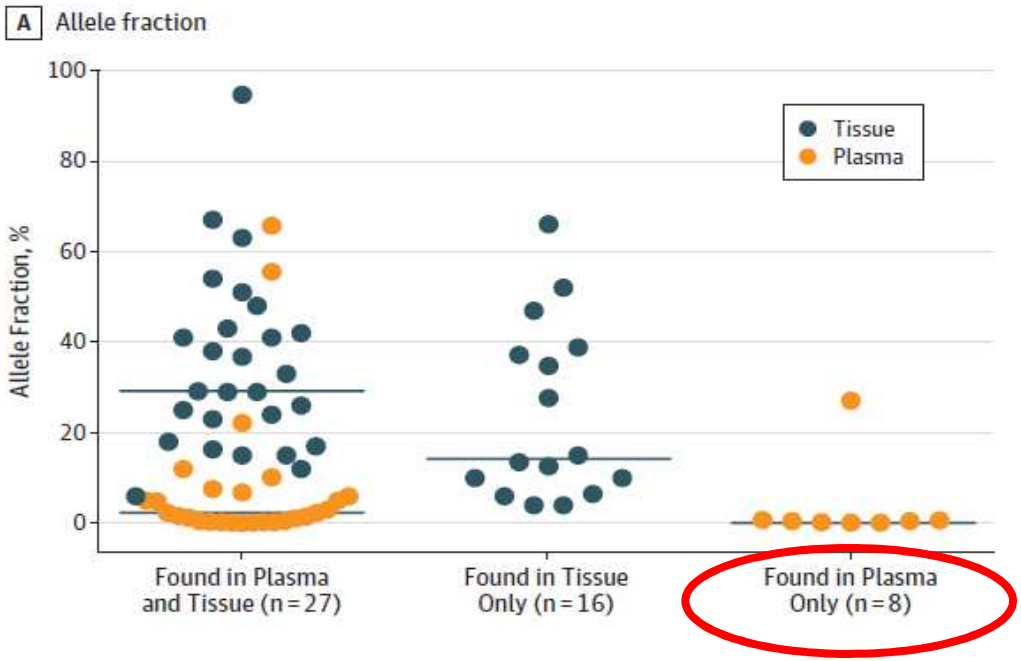
Frequency (%) of somatic mutations in liquid biopsy

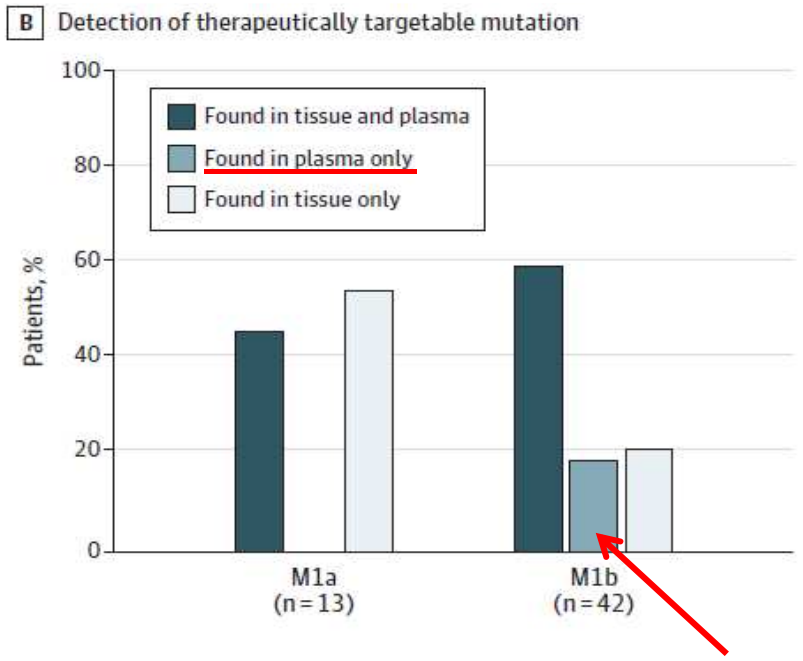


Kezhong Chen i wsp. The Journal of Thoracic and Cardiovascular Surgery 2017

Molecular profiling of liquid biopsy may supplement the data from tumor tissue analysis in metastatic NSCLC







Aggarwal C et al. JAMA Oncol. 2018 Oct 11.

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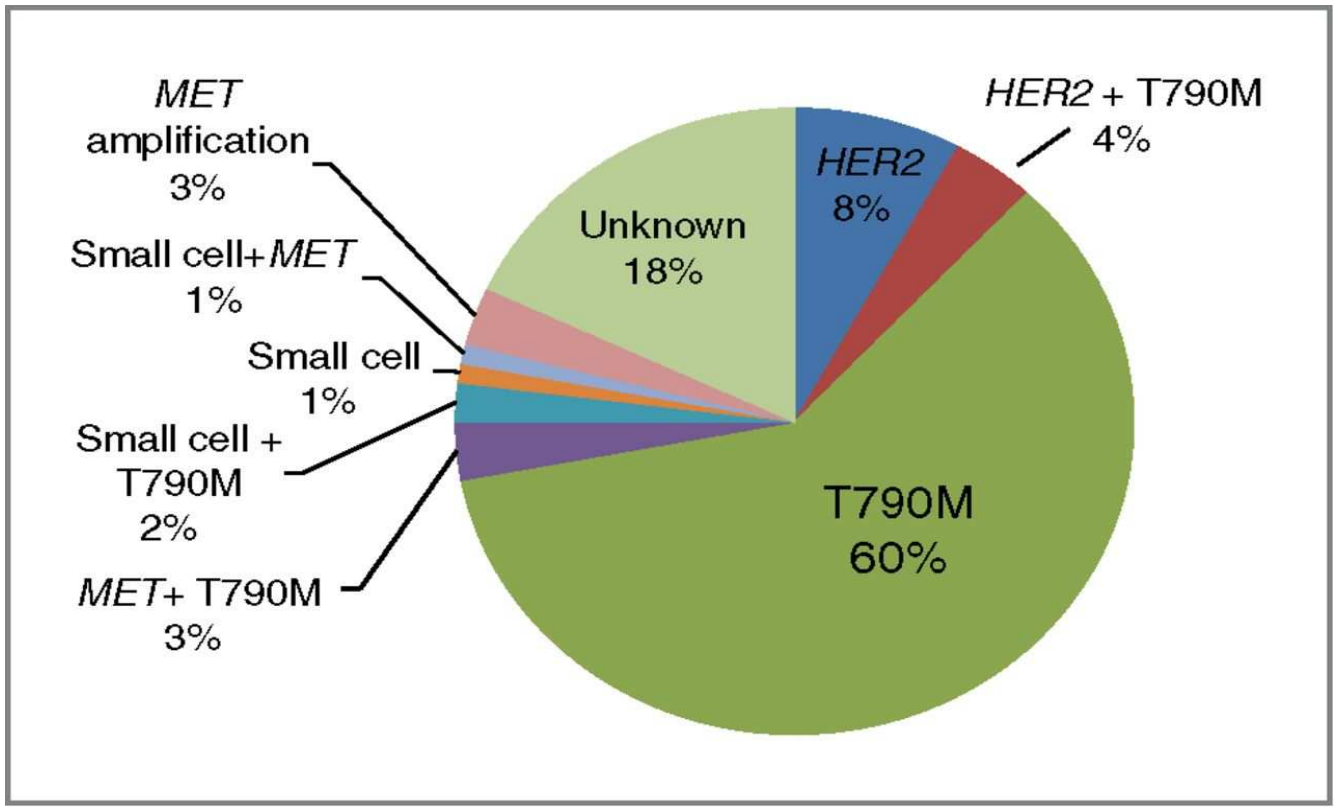
The efficacy of EGFR TKIs in 1st line treatment of advanced NSCLC

Table 1. Benefit of first-line EGFR TKIs: nine randomized phase III studies						
Study	TKI	CTx	N	PFS (months)	HR (95% CI)	OS (months)
IPASS[1]	Gefitinib	Cb/Pac	261	9.5 vs. 6.3	0.48 (0.36–0.64)	21.6 vs. 21.9
First-signal[2]	Gefitinib	Cis/Gem	42	8.0 vs. 6.3	0.54 (0.26–1.10)	27.2 vs. 25.6
NEJ002[3]	Gefitinib	Cb/Pac	194	10.8 vs. 5.4	0.35 (0.22–0.41)	30.5 vs. 23.6
WJTOG 3405[4]	Gefitinib	Cis/Doc	172	9.2 vs. 6.3	0.49 (0.33–0.71)	30.9 vs. NR
OPTIMAL[5]	Erlotinib	Cis/Gem	164	13.1 vs. 4.6	0.16 (0.10–0.26)	Not mature
EURTAC[6]	Erlotinib	Cis/Doc or Gem	174	10.4 vs. 5.1	0.34 (0.23–0.29)	19.3 vs. 19.5
ENSURE[7]	Erlotinib	Cis/Gem	217	11.0 vs. 5.6	0.42 (0.27–0.66)	26.3 vs. 25.5
LUX-Lung 3[8]	Afatinib	Cis/Pem	308	11.1 vs. 6.9	0.47 (0.34–0.65)	31.5 vs. 28.3
LUX-Lung 6[9]	Afatinib	Cis/Gem	364	11.0 vs. 5.6	0.28 (0.20–0.39)	23.6 vs. 23.5

Most NSCLC patients treated with 1- and 2-generation EGFR TKIs acquire drug resistance between 9 and 13 month

Dr. Filippo de Marinis at the Canadian Lung Cancer Conference (CLCCO) 2016 Annual Meeting

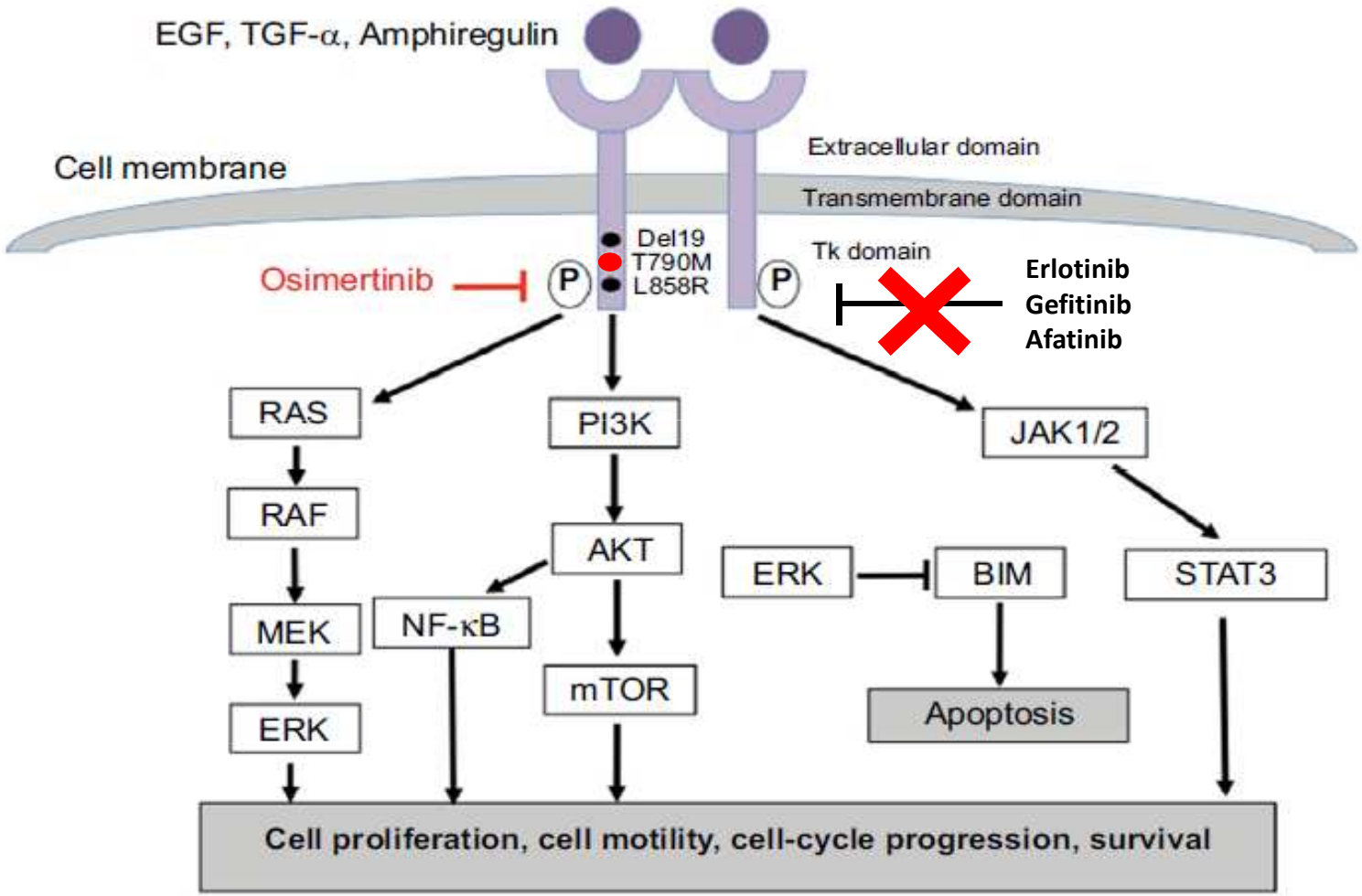
Major molecular mechanisms of acquired resistance to 1- and 2-generation EGFR TKIs



N=155

Helena A. Yu et al. Clin Cancer Res 2013 (19) (8) 2240-2247.

EGFR signaling pathway and mechanism of action of osimertinib



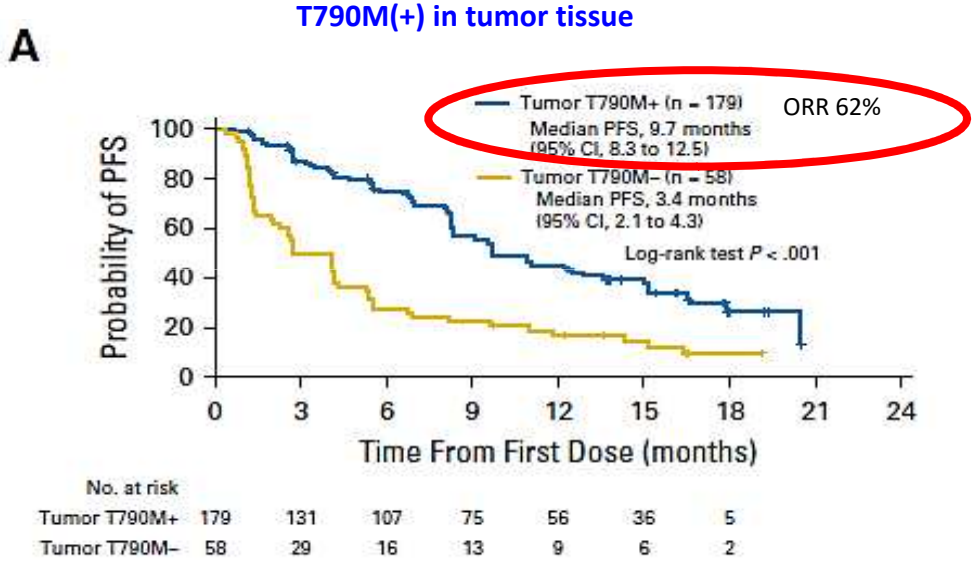
Santarpia M et al. Lung Cancer: Targets and Therapy 2017;8:109-125

The EGFR T790M mutation in liquid biopsy shows predictive value for osimertinib treatment outcome

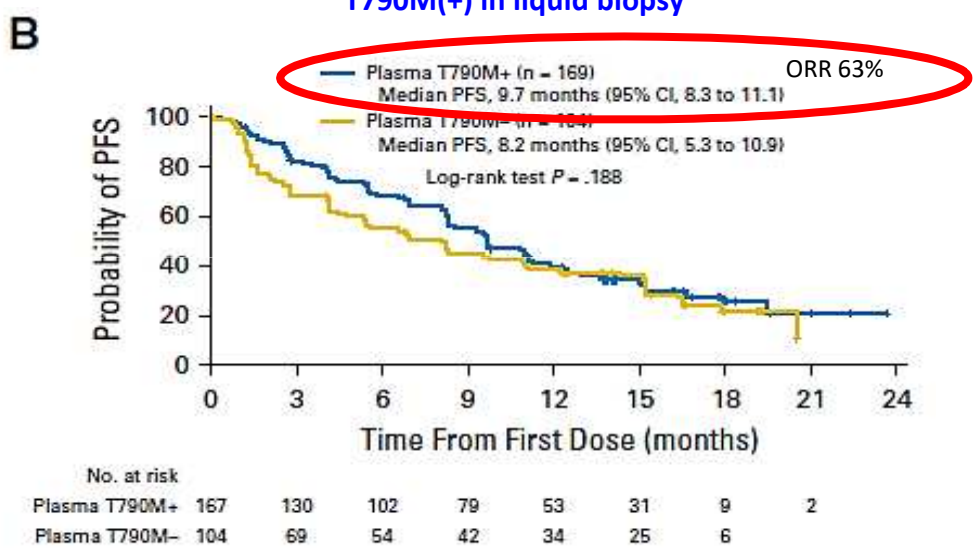
AURA Clinical Trial

Progression Free Survival (PFS)

T790M(+) in tumor tissue



T790M(+) in liquid biopsy



Oxnard GR i wsp. *J Clin Oncol. 2016, 34:3375-3382*

The sensitivity/specificity of EGFR T790M detection in ctDNA differ between techniques

Table 1. Comparison of Selected ctDNA Assays for Detection of *EGFR* Mutations

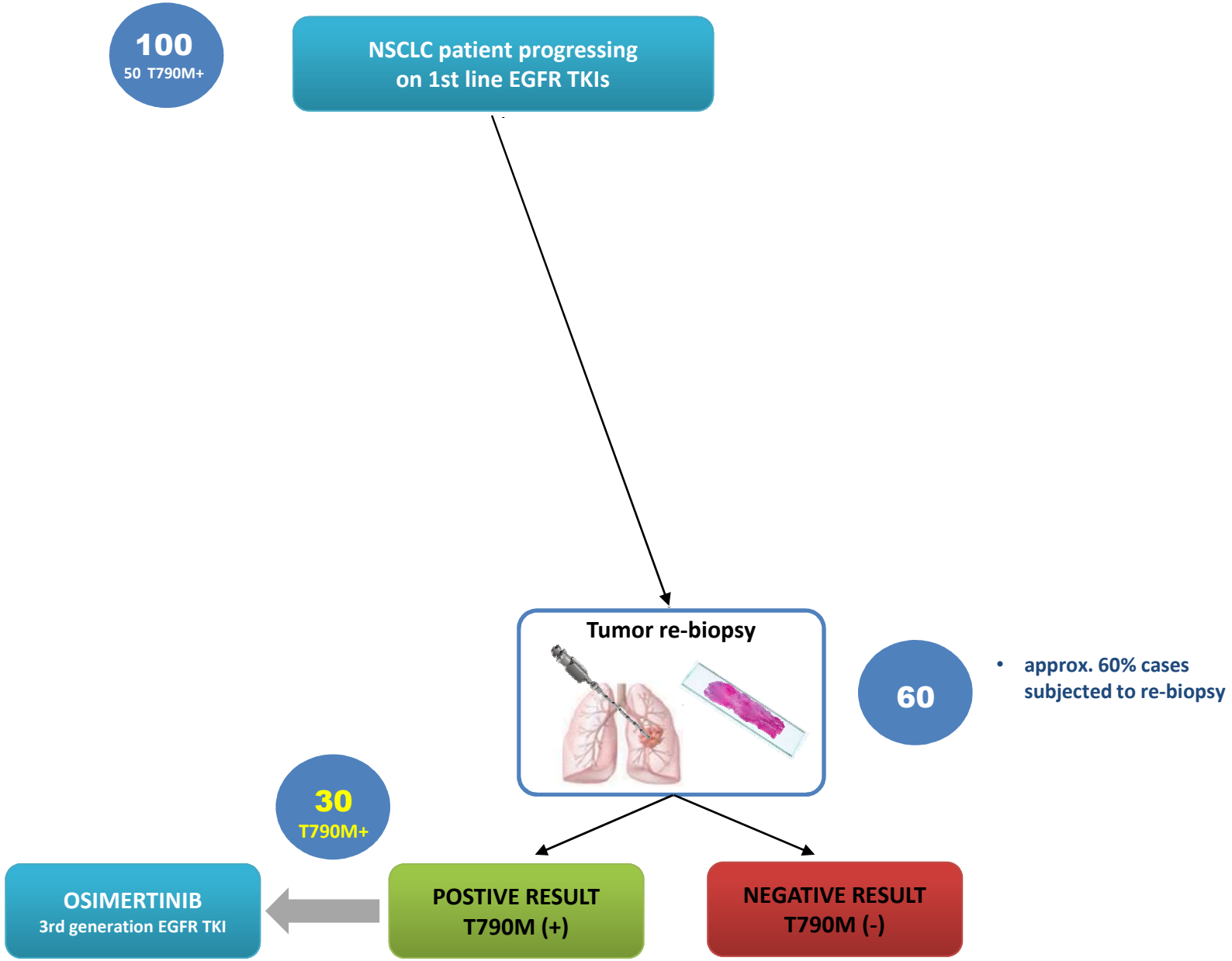
Platform	Key Test Characteristics	Validation Study (Prospective vs Retrospective)	Sensitivity (for *EGFR* Mutations)	Specificity (for *EGFR* Mutations)	Reference(s)
cobas	The only ctDNA assay that is currently approved by the FDA for detection of *EGFR* mutations	Retrospective	76.7% (*EGFR* exon 19 del and L858R)	98.2% (*EGFR* exon 19 del and L858R)	[14]
	Detects known SNV mutations Semi-quantitative	Retrospective	61.4% (*EGFR* T790M)	78.6% (*EGFR* T790M)	[15,16]
Scorpion-ARMS	Detects known SNV mutations Semi-quantitative	Retrospective	61.8%–85.7% (*EGFR* exon 19 del and L858R)	94.3%–100% (*EGFR* exon 19 del and L858R)	[2,17]
ddPCR	Has rapid turnaround time Detects known SNV mutations Quantitative	Prospective	74%–82% (*EGFR* exon 19 del and L858R)	100% (*EGFR* exon 19 del and L858R)	[13]
		Retrospective	77% (*EGFR* T790M)	63% (*EGFR* T790M)	[13]
BEAMing	Detects more complex alterations, such as copy number changes and translocations Quantitative	Retrospective	70% (*EGFR* T790M)	69% (*EGFR* T790M)	[20]
NGS	Profiles large gene panels Does not require prior knowledge of the molecular alteration of interest Detects more complex alterations, such as copy number changes and translocations Quantitative	Retrospective	79% (*EGFR* and *KRAS*)	100% (*EGFR* and *KRAS*)	[23]
		Retrospective	87%–100% (*EGFR* exon 19 del and L858R)	96%–100% (*EGFR* exon 19 del and L858R)	[41]
		Retrospective	93% (*EGFR* T790M)	94% (*EGFR* T790M)	[41]

ARMS = amplification refractory mutation system; BEAMing = beads, emulsion, amplification, and magnetics; ctDNA = circulating tumor DNA; ddPCR = digital droplet polymerase chain reaction; del = deletion; FDA = US Food and Drug Administration; NGS = next-generation sequencing; SNV = single nucleotide variant.

approx. 60-70% sensitivity of T790M detection in liquid biopsy for most common diagnostic tests

Komatsubara KM et al. Oncology (Williston Park). 31(8):618–627

Current diagnostic algorithm to qualify NSCLC patients for 2nd line EGFR TKI



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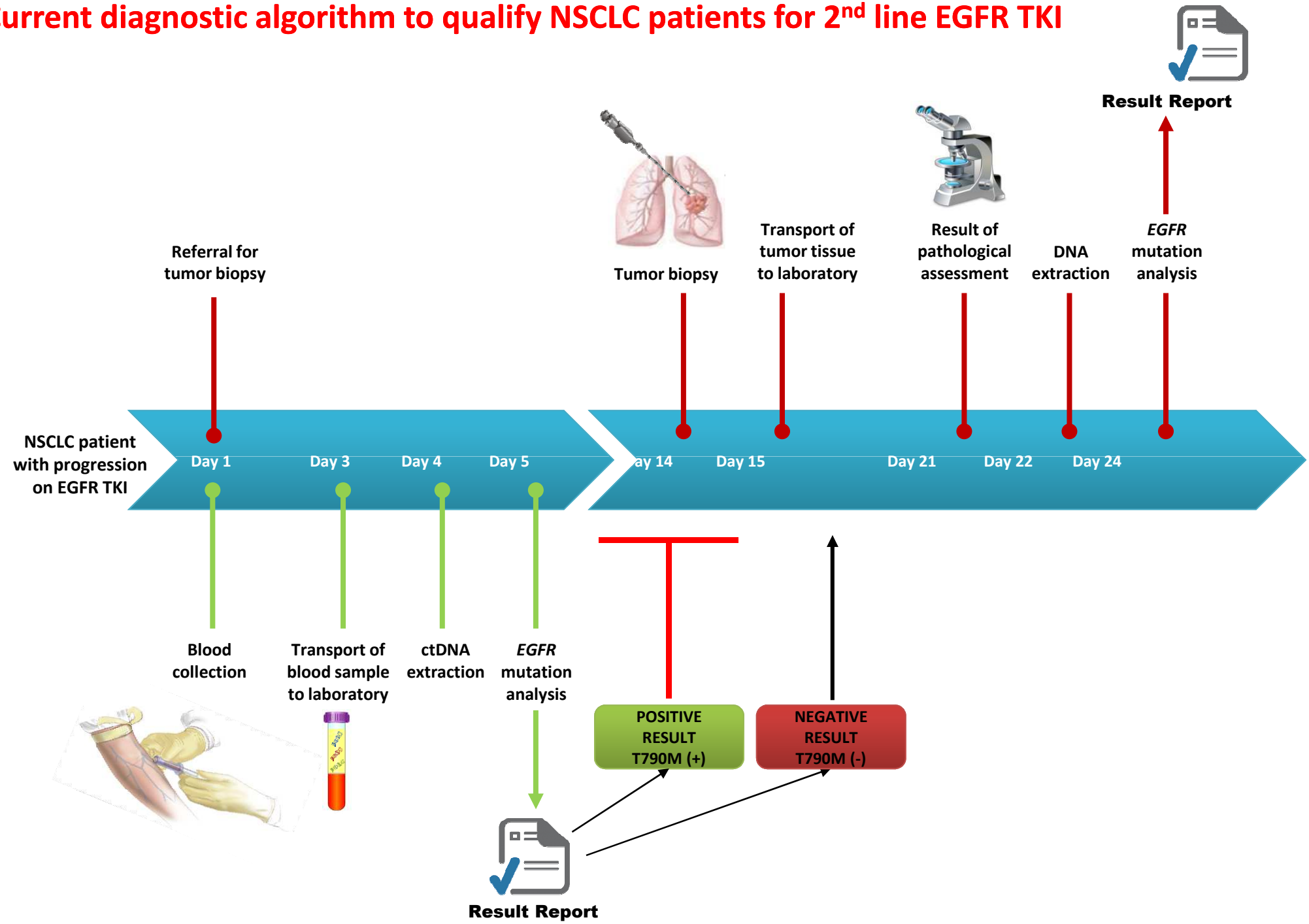
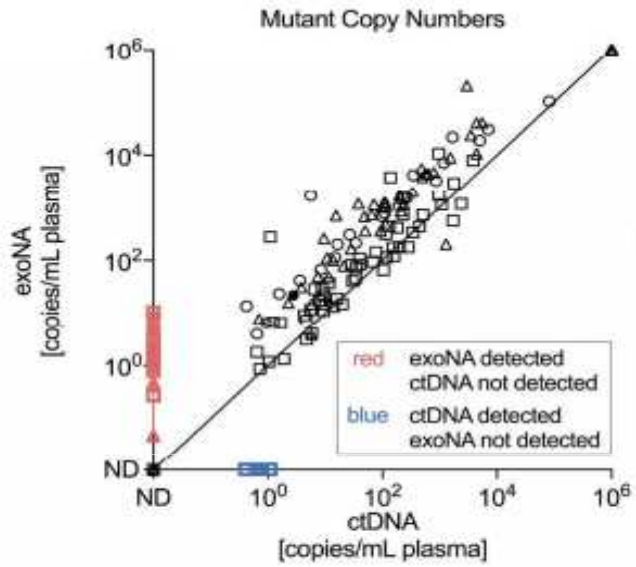


Cell-free DNA fractionation may enhance the sensitivity of mutation detection - exosomal DNA/RNA

N=84

A



B

Tumor mutation-positives	Activating	T790M
exoNA detected ctDNA not detected	11	5
ctDNA detected exoNA not detected	1	2

C

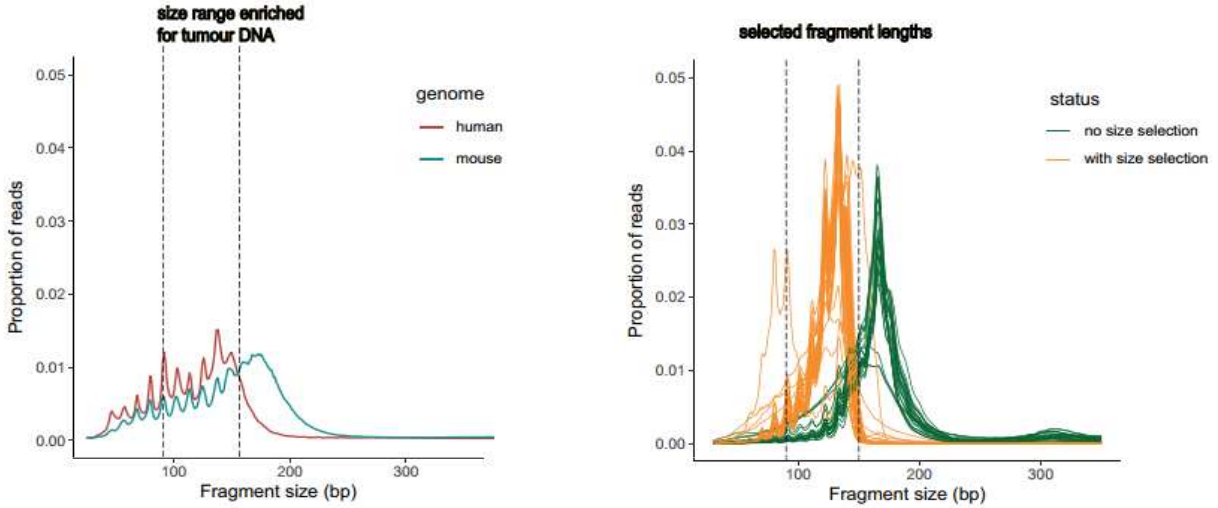
Complete cohort		Mutant cps/mL Median	Range	*p*-value
Activating *EGFR* MUT	exoNA	234	0-202092	<0.0001
	ctDNA	24	0-82406	
T790M *EGFR* MUT	exoNA	12	0-10642	<0.0001
	ctDNA	6	0-3867	

		Tissue Biopsy Result Activating[a]	T790M		Activating	T790M
exoNA (EXO1000)[b] NGS	+	53	44	Sensitivity	98%	90%
	-	1	5			
ctDNA (BEAMing)[b]	+	44	41	Sensitivity	82%	84%
	-	10	8			

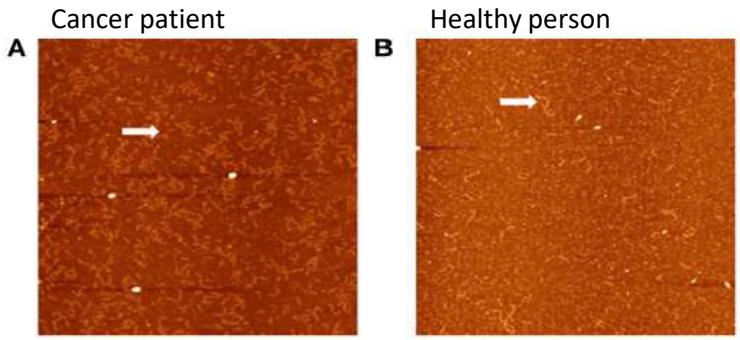
Krug AK et al. Ann Oncol. 2018 Mar 1;29(3):700-706.

Cell-free DNA fractionation may enhance the sensitivity of mutation detection - short DNA fragment analysis

cfDNA fragment size distribution

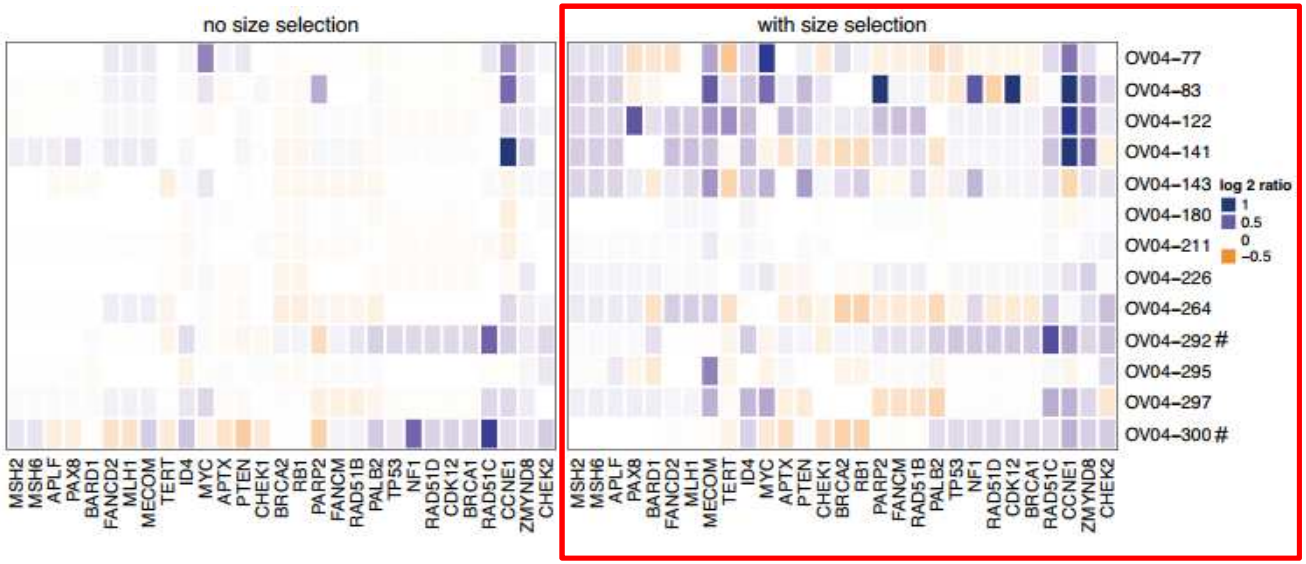


Electron microscopy image of cfDNA fragments



Dalong Pang et al. Front Mol Biosci. 2015; 2: 1.

Molecular profiling of cfDNA using NGS



Florent Mouliere, et al. bioRxiv 2017, 134437

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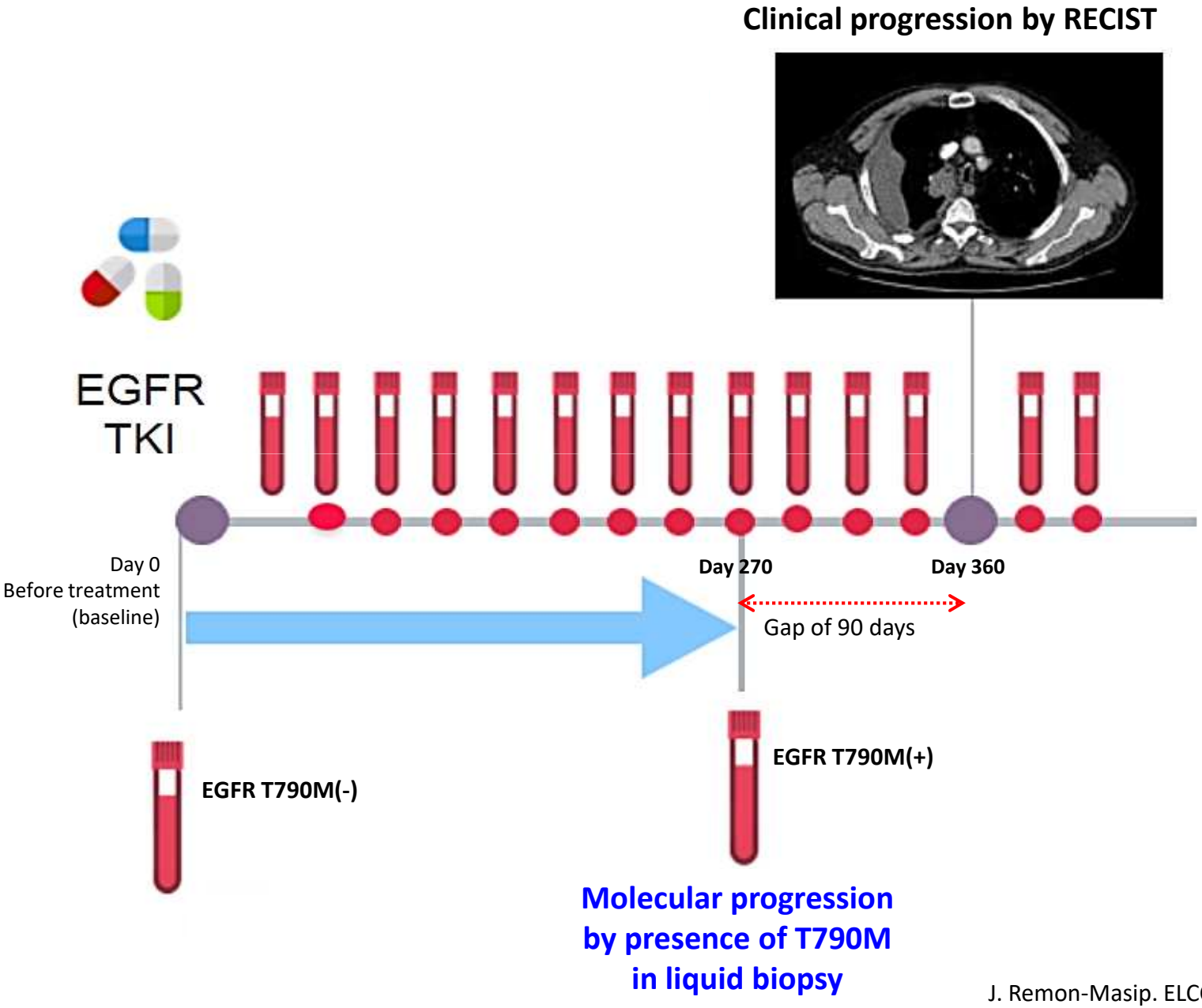
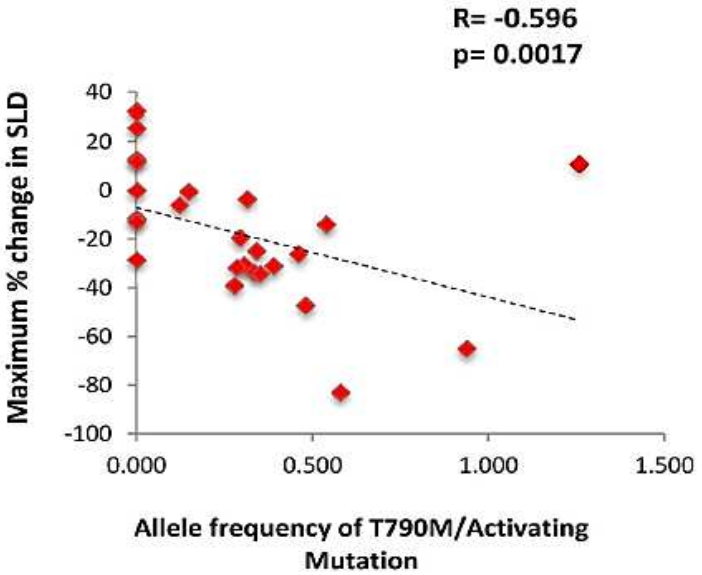
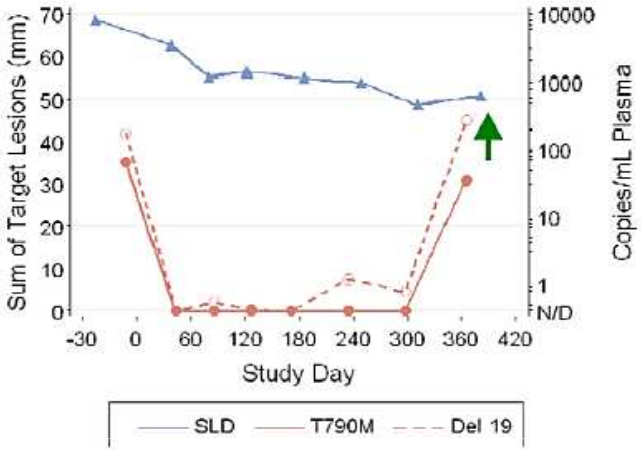
The concept to monitor EGFR TKI treatment efficacy using serial blood sampling

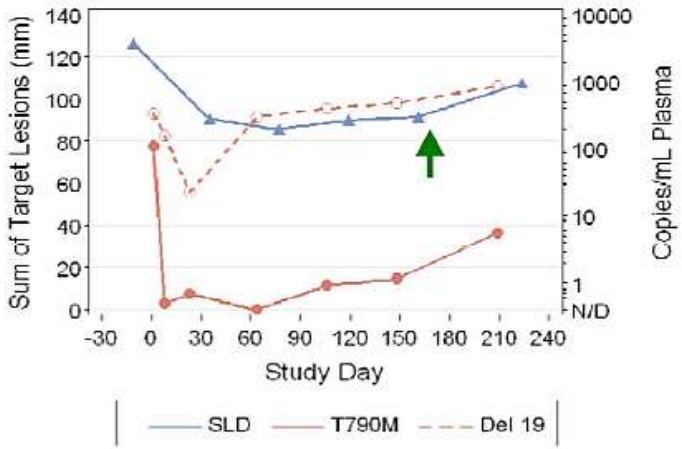


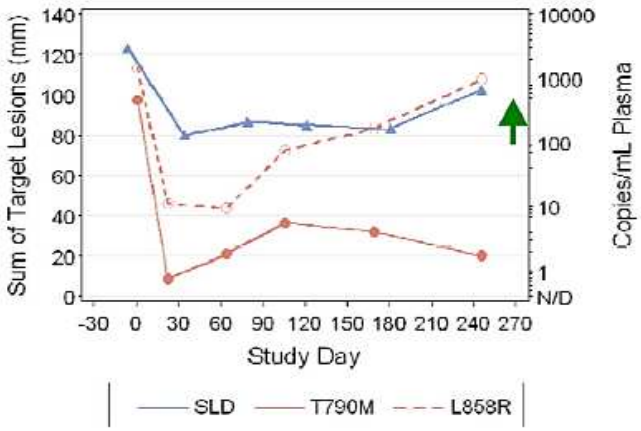
J. Remon-Masip. ELCC 2018, Geneva

The real-time monitoring of EGFR TKI treatment efficacy using serial blood sampling - proof of concept





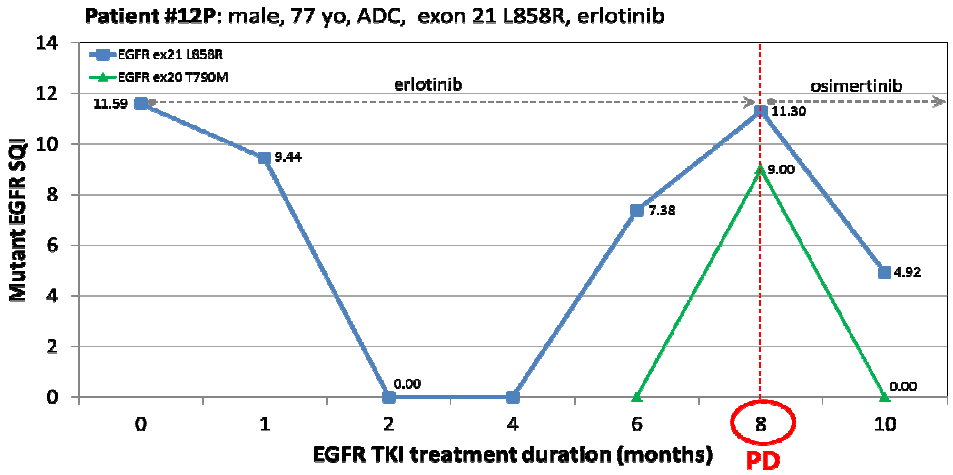


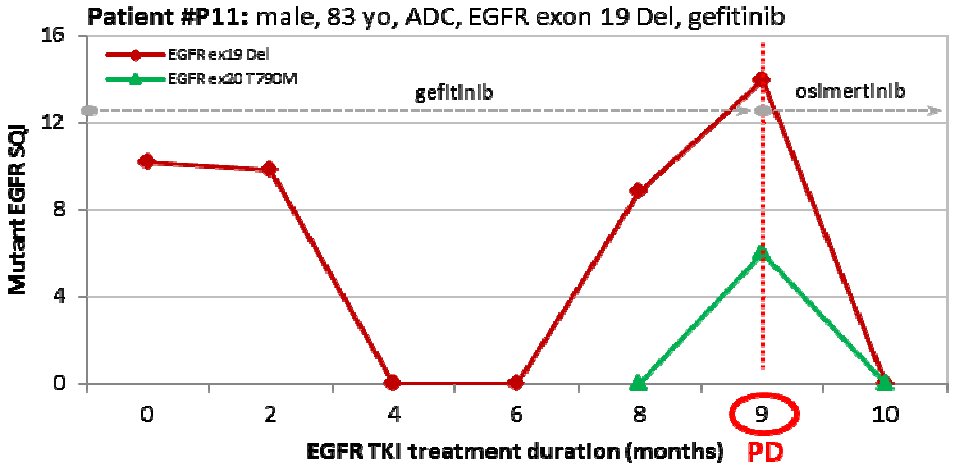


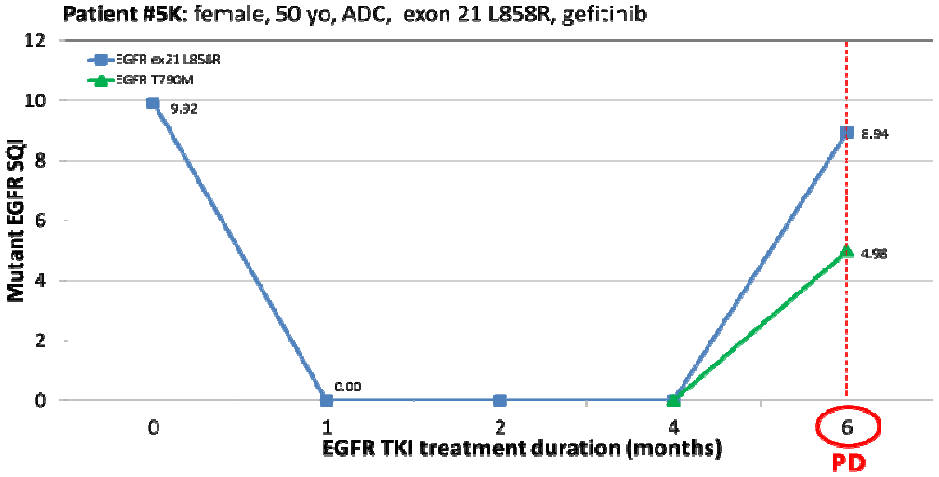
SLD = sum of the longest diameter (RECIST)

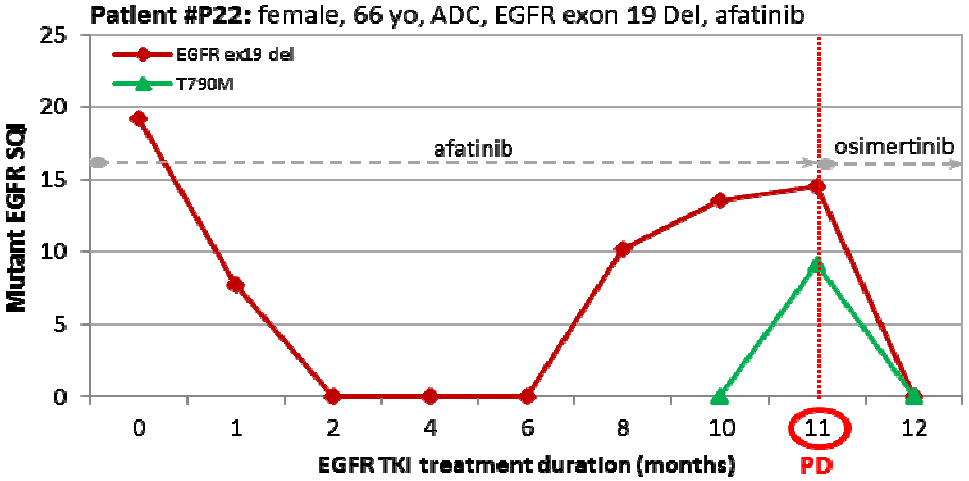
Piotrowska Z i wsp. *Cancer Discov. 2015;5(7):713-22*

The real-time monitoring of EGFR TKI treatment efficacy using serial blood sampling - proof of concept





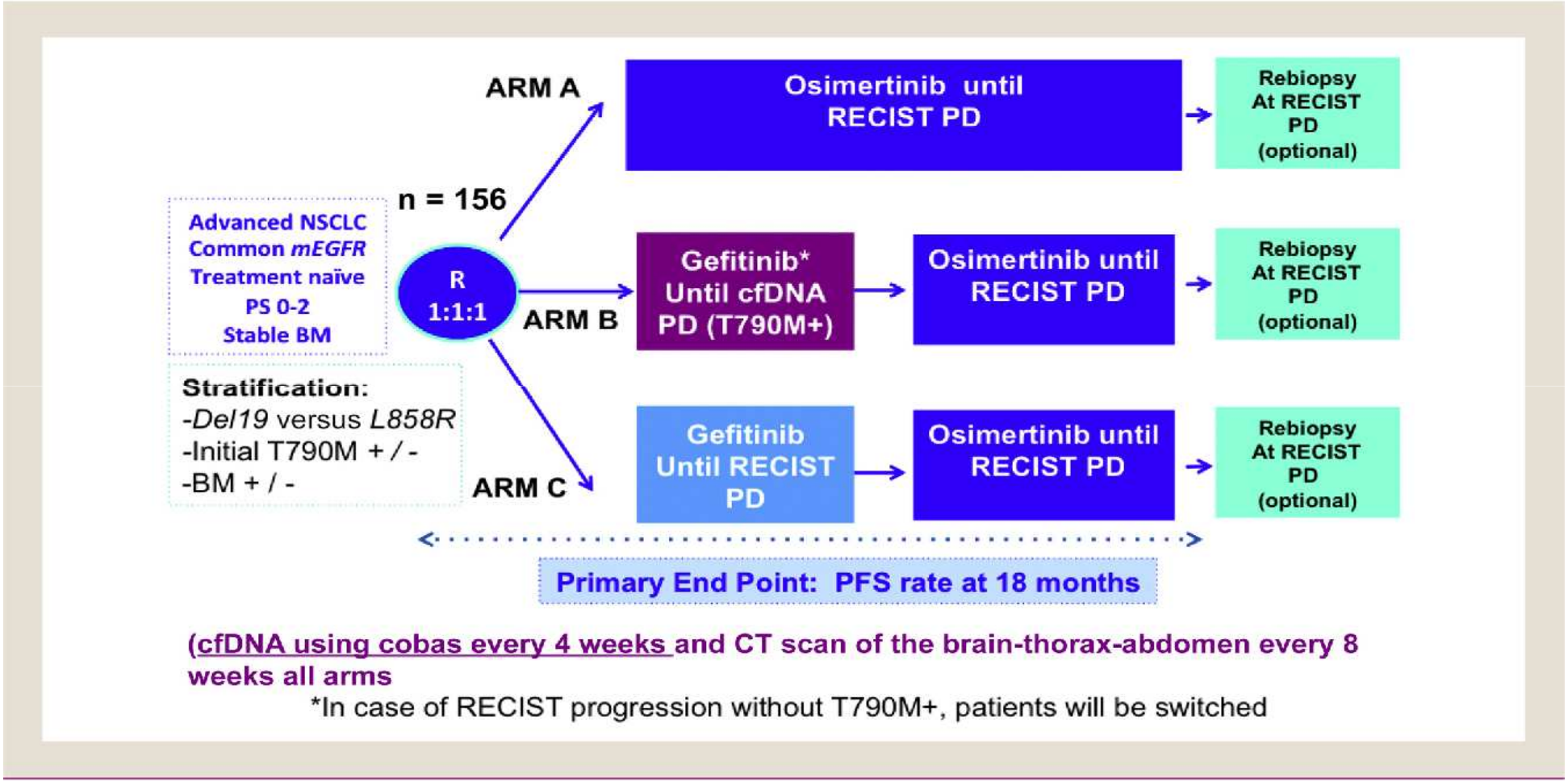




In our study, the rise of T790M mutation level in plasma preceded clinical progression in time by 4-28 weeks

Szpechcinski A. et al. P2.13-14 Abstract. WCLC2018, Toronto, Canada

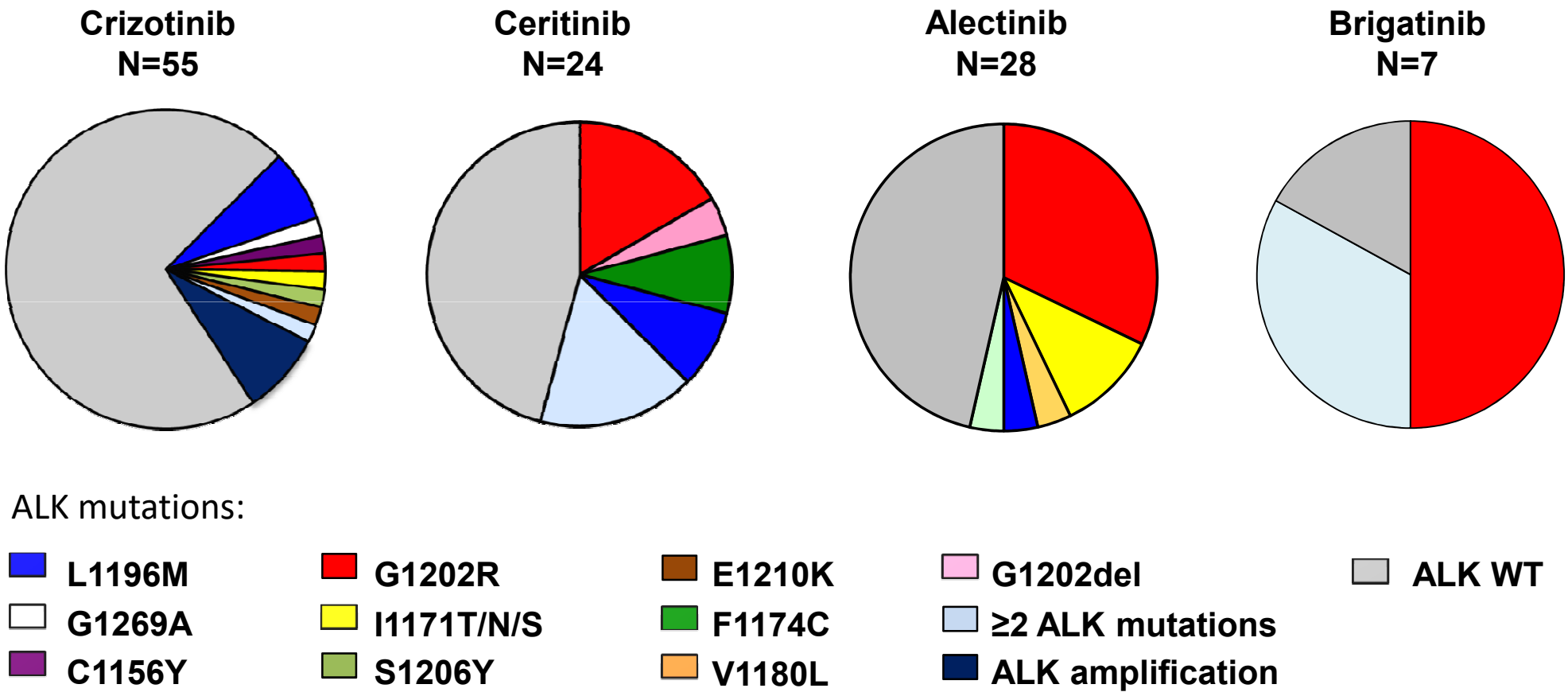
Clinical evaluation of EGFR TKI treatment outcome in patients with T790M in liquid biopsy - prospective study (APPLE Trial 2017-2027)



Abbreviations: BM = brain metastases; cfDNA = cell-free tumor DNA; COBAS = cobas EGFR Mutation Test from Roche Molecular Diagnostics (Pleasanton, CA); CT = computed tomography; NSCLC = non–small-cell lung cancer; PFS = progression-free survival; PS = performance status; RECIST = Response Evaluation Criteria In Solid Tumors.

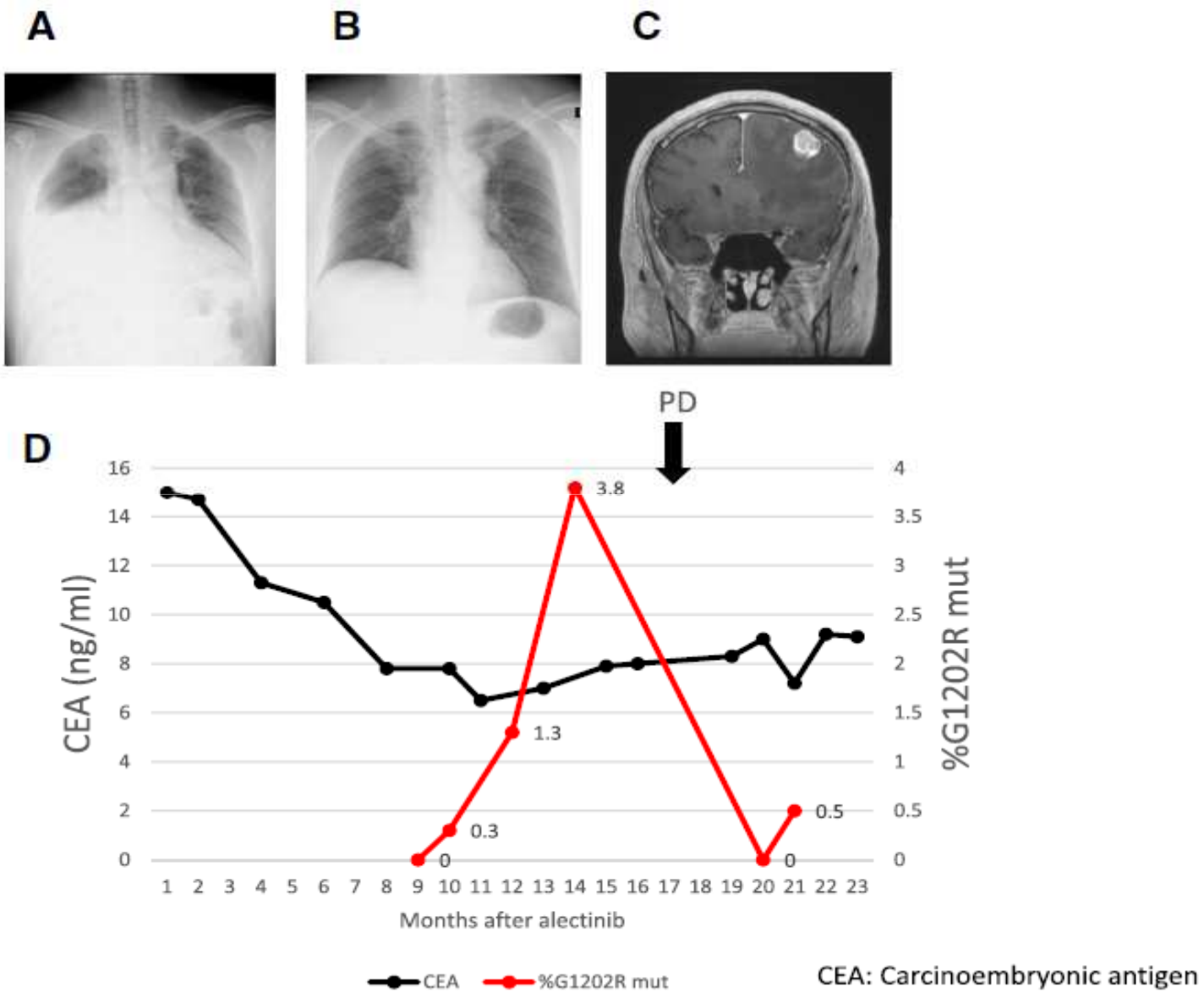
J. Remon et al. Clinical Lung Cancer, 2017; 18(5):583-8

Detection of ALK mutations associated with acquired resistance to ALK TKI therapy



Gainor et al., Cancer Discov 6: 1118-33, 2016
J. Chorostowska-Wynimko, 2018

Detection of ALK mutations associated with acquired resistance to ALK TKI therapy



Yoshida R et al. BMC Cancer. 2018;18(1):1136.

1. Liquid biopsy – the characteristics
2. Detection of EGFR T790M mutation in liquid biopsy from NSCLC patients progressing on 1st line EGFR TKIs
3. Liquid biopsy in the real-time monitoring of EGFR-TKI therapy effectiveness
4. **Tumor Mutation Burden (TMB) in liquid biopsy as the promising predictive biomarker for immunotherapy in NSCLC**
5. Future perspectives on the liquid biopsy –based applications in NSCLC diagnostics

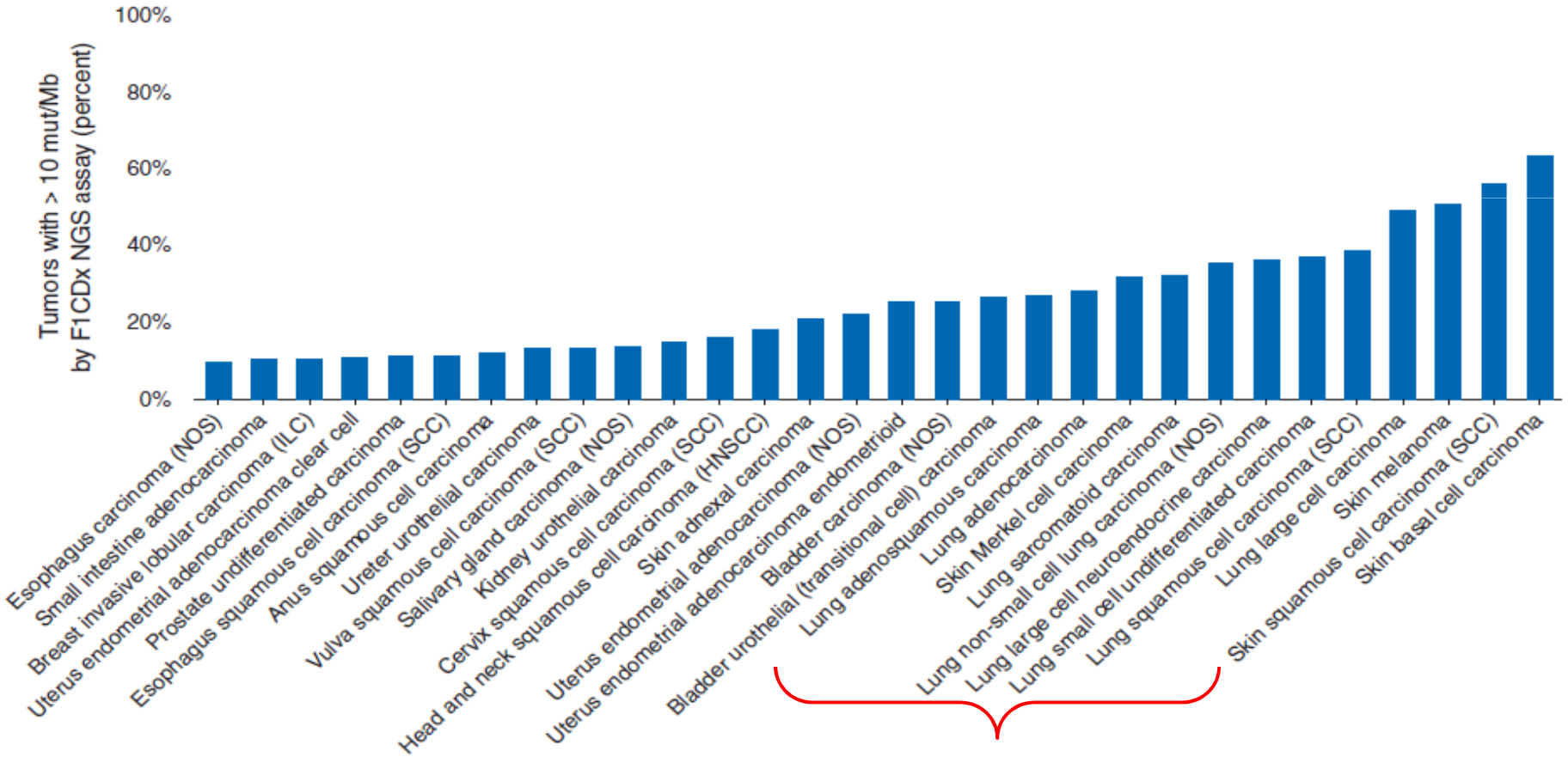
Tumor Mutation Burden (TMB) as potential predicitve biomarker for immunotherapy

Tumor Mutation Burden (TMB) = the mean number of somatic mutations per each 1 mln base pairs in genome (number of mutations/Mb genome)

Whole exsome sequencing (WES) by next generation sequencing (NGS)

Targeted sequencing of hot-spots in genome (usually 70-300 genes covered) by NGS

Human exome = ca. 180,000 exons of 30 mln (30 Mb) base pairs of total lenght

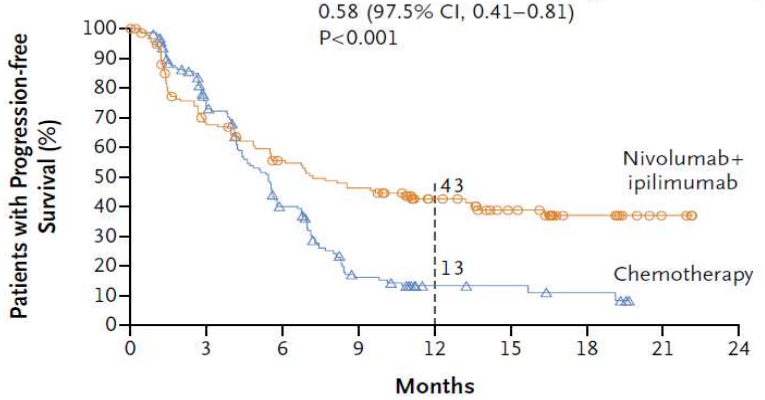


T. A. Chan et al. Annals of Oncology 30: 44–56, 2019

Tumor Mutation Burden (TMB) as potential predicitve biomarker for immunotherapy

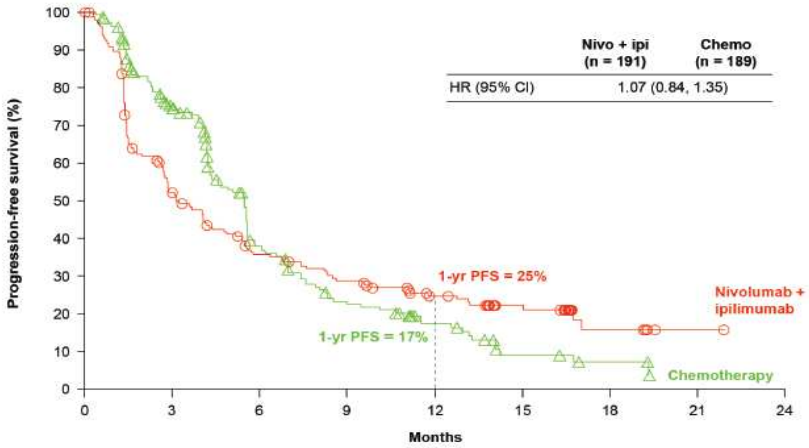
High TMB ≥10 Mutations/Mb

A Progression-free Survival



No. at Risk									
Nivolumab + ipilimumab	139	85	66	55	36	24	11	3	0
Chemotherapy	160	103	51	17	7	6	4	0	0

Low TMB <10 Mutations/Mb

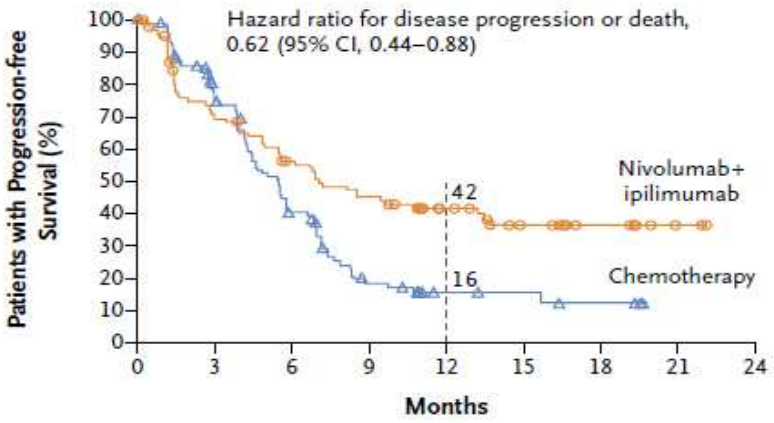


No. at risk									
Nivo + ipi	191	92	58	46	31	18	6	1	0
Chemo	189	122	53	30	17	6	3	0	0

High TMB ≥10 Mutations/Mb

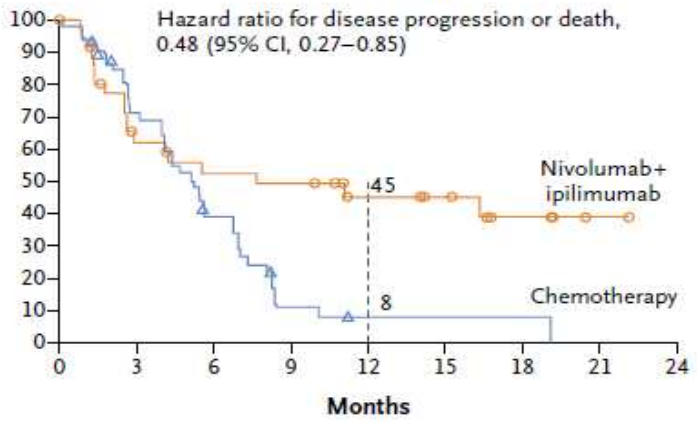
A Tumor PD-L1 Expression

PD-L1 Expression of ≥1%



No. at Risk									
Nivolumab + ipilimumab	101	65	50	40	26	16	7	2	0
Chemotherapy	112	73	35	13	6	5	3	0	0

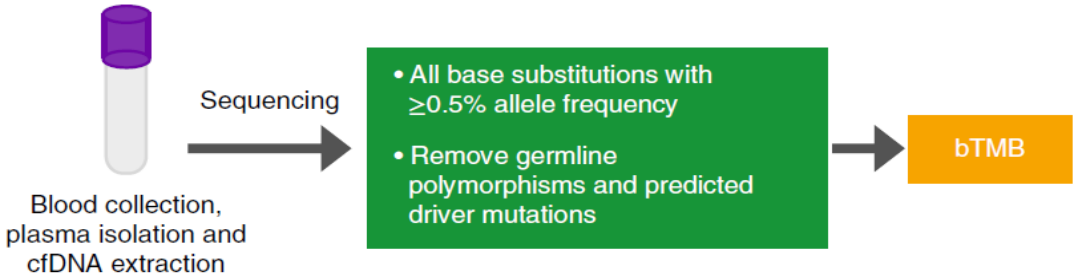
PD-L1 Expression of <1%



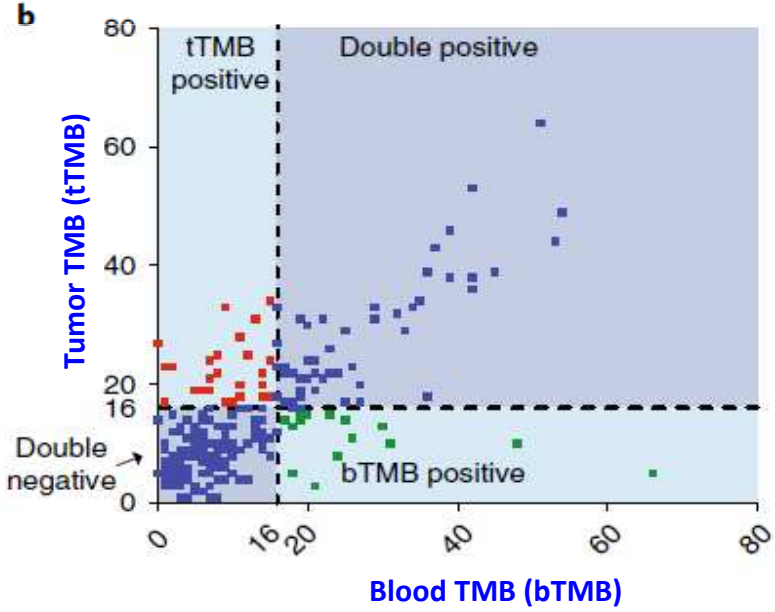
	38	20	16	15	10	8	4	1	0
	48	30	16	4	1	1	1	0	0

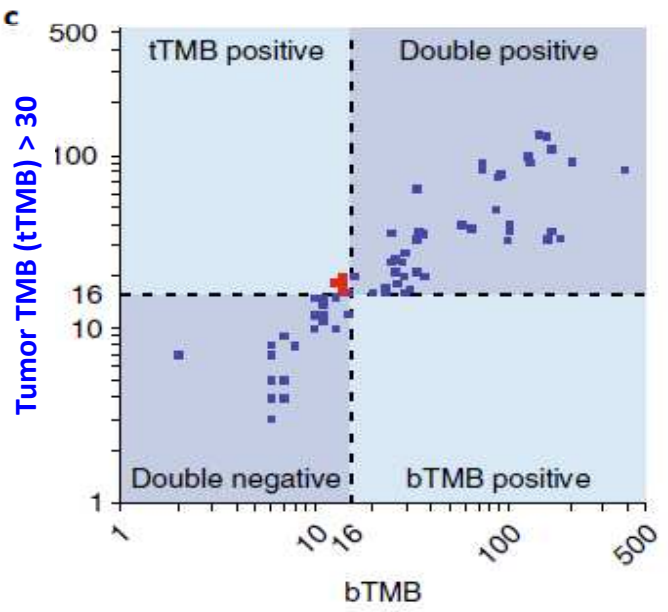
M.D. Hellmann, et al. N Engl J Med 2018;378:2093-104.

Development of Tumor Mutation Burden in blood (bTMB)



Correlation between tumor TMB and blood TMB (N = 259 patient samples)

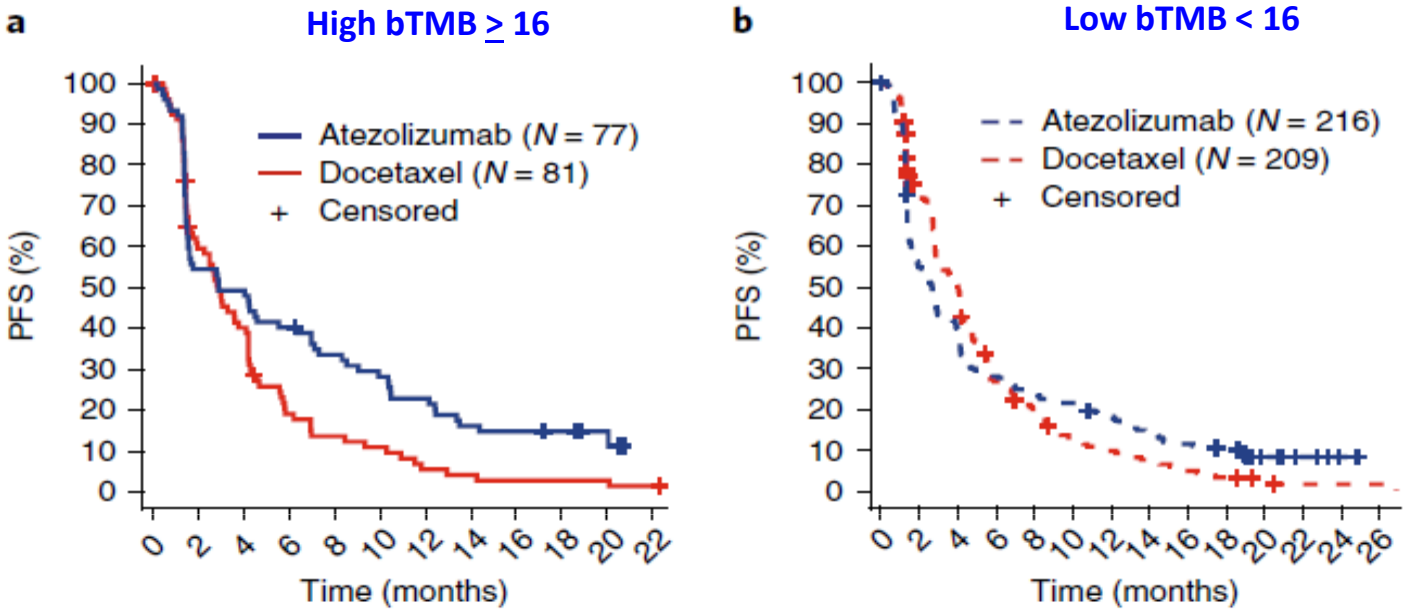




bTMB	PPA (%)	NPA (%)
≥10	100	100
≥11	94.6	92.3
≥12	89.1	92.9
≥13	94	84.2
≥14	90	89.5
≥15	85.7	90
≥16	89.1	100
≥17	90.2	89.3
≥18	92.1	83.9
≥19	94.4	81.8
≥20	97.1	82.4

David R. Gandara et al. Nat Med. 2018 Sep;24(9):1441-1448.

Development of Tumor Mutation Burden in blood (bTMB)
- Retrospective clinical validation (OAK study)



Correlation of PD-L1 expression and bTMB counts

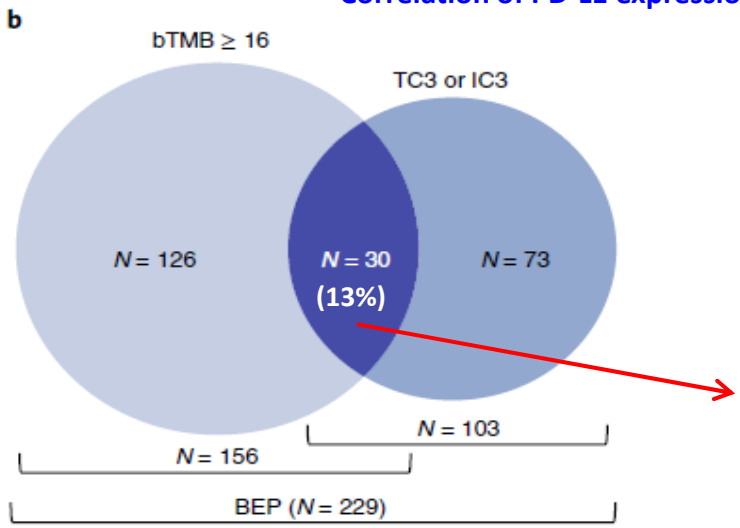


Table 1 | OS and PFS HRs in the OAK BEP with valid bTMB and PD-L1 IHC results

	N	PFS HR (95% CI)	OS HR (95% CI)
bTMB ≥ 16	156	0.64 (0.46–0.91)	0.64 (0.44–0.93)
TC3 or IC3	103	0.62 (0.41–0.93)	0.44 (0.27–0.71)
bTMB ≥ 16 and TC3 or IC3	30	0.38 (0.17–0.85)	0.23 (0.09–0.58)

N represents the number of patients in each subgroup. TC3 or IC3, ≥50% of tumor cells or ≥10% of tumor-infiltrating immune cells expressing PD-L1.

David R. Gandara et al. Nat Med. 2018 Sep;24(9):1441-1448.

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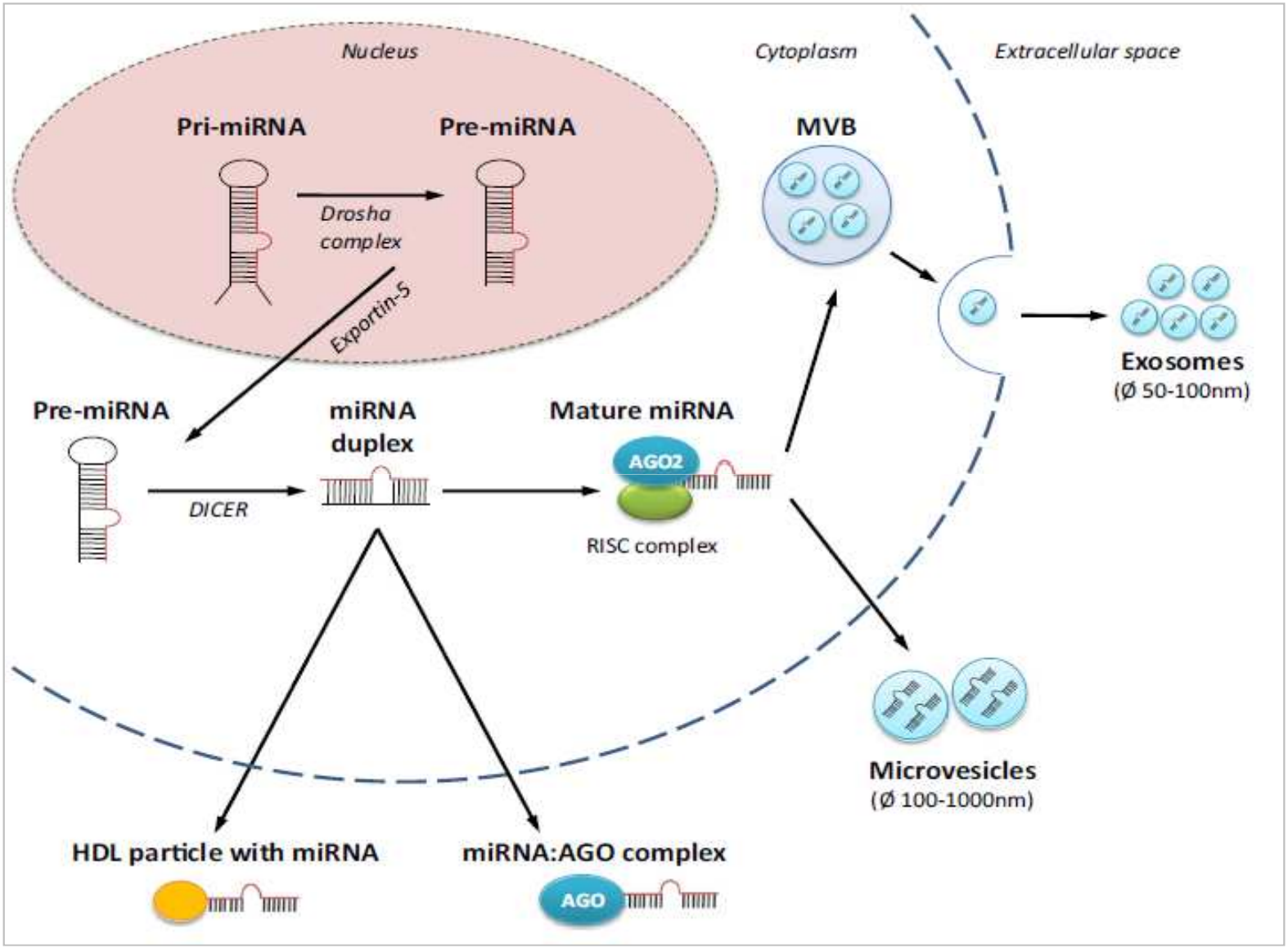
Biogenesis of circulating microRNA



Florczuk M, Szpechcinski A. Targeted Oncology 2017; Target Oncol. 2017;12(2):179-200

Circulating microRNA as the potential biomarker for NSCLC screening

Clinical Utility of a Plasma-Based miRNA Signature Classifier Within Computed Tomography Lung Cancer Screening: A Correlative MILD Trial Study

Gabriella Sozzi, Mattia Boeri, Marta Rossi, Carla Verri, Paola Suatoni, Francesca Bravi, Luca Roz, Davide Conte, Michela Grassi, Nicola Sverzellati, Alfonso Marchiano, Eva Negri, Carlo La Vecchia, and Ugo Pastorino

J Clin Oncol 32:768-773. © 2014

- Screening for lung cancer in high-risk population using chest CT scanning combined with analysis of circulating microRNA expression
- 939 participants: 870 participants with high-risk of lung cancer (age, smoking)

  69 patients with lung cancer

- **Diagnostic performance of microRNA/CT approach:**

- only CT scan: **79% sensitivity, 81% specificity**, **19.4% false-positive results**
- only circulating microRNA: **87% sensitivity, 81% specificity**
- Combined circ-microRNA + CT scans: **88% sensitivity, 80% specificity**, **3,7% false-positive results in CT**

Circulating microRNA as the potential biomarker for NSCLC screening

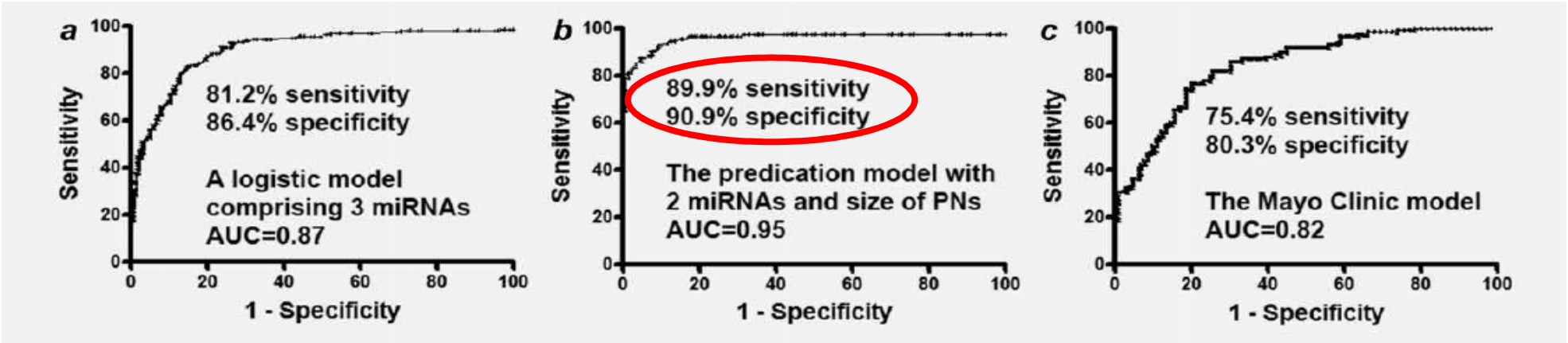
A classifier integrating plasma biomarkers and radiological characteristics for distinguishing malignant from benign pulmonary nodules

Yanli Lin[1], Qixin Leng[1], Zhengran Jiang[1,2], Maria A. Guarnera[1], Yun Zhou[3], Xueqi Chen[4], Heping Wang[5], Wenxian Zhou[5], Ling Cai[5], HongBin Fang[5], Jie Li[6], Hairong Ji, Yun Su[9] and Feng Jiang[1,8]

Int. J. Cancer: 141, 1240–1248 (2017) © 2017 UICC

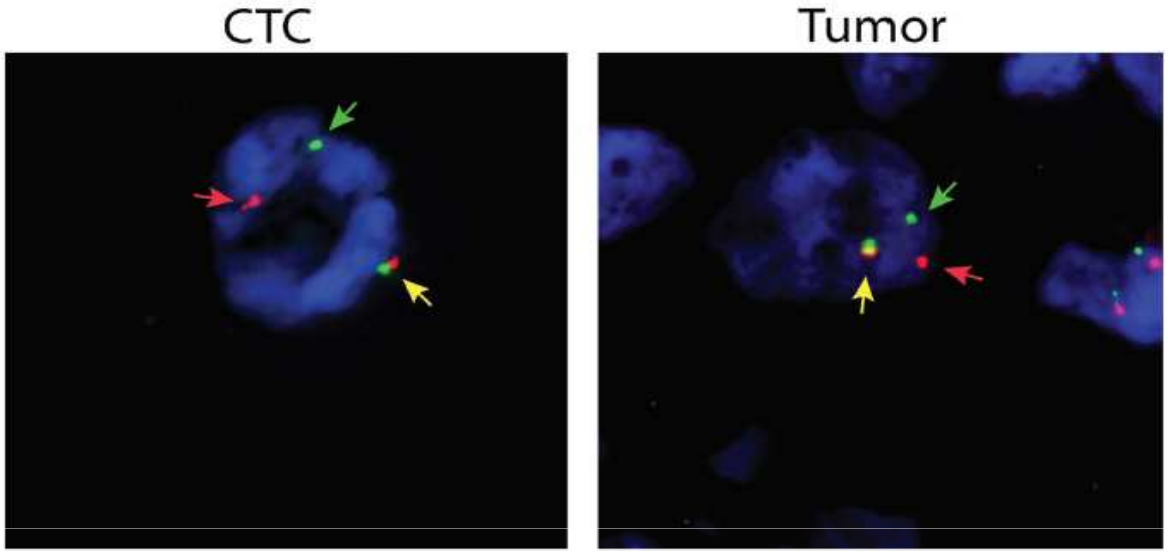
- N= 135 former and current smokers
- SPN detected in CT scan
- 69 patients had malignant SPNs

circulating microRNA expression | **circ microRNA + CT scan** | CT scan



ALK rearrangement detection in Circulating Tumor Cells using FISH technique is feasible

ALK rearrangement detection by FISH technique



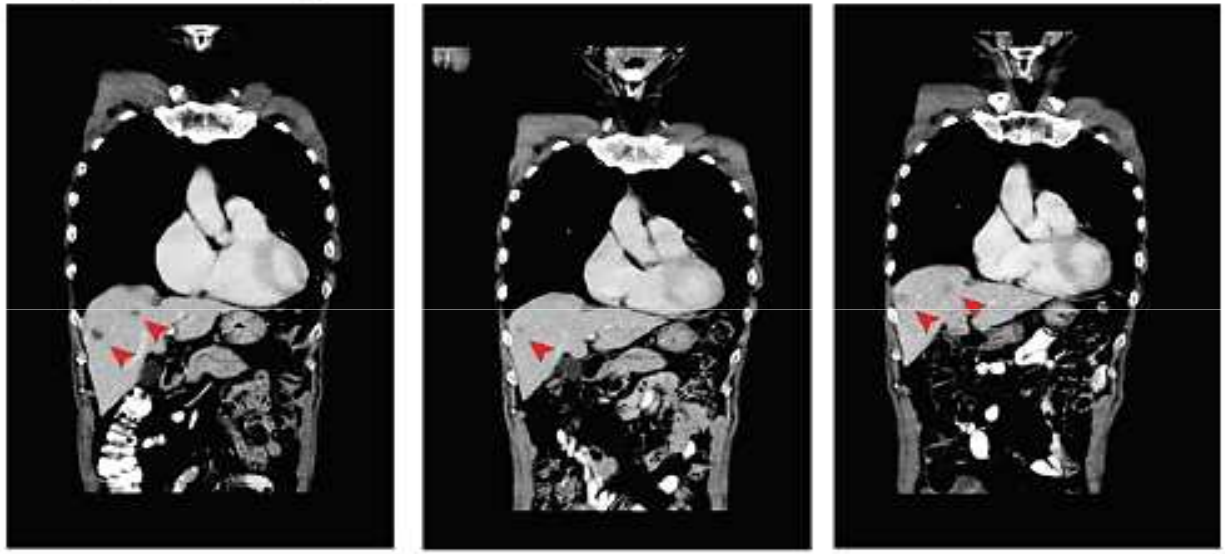
Faugeroux V et al. ***Front Oncol. 2014;4:281***
ALK+ patients: 30-100% CTCs with rearranged ALK
ALK- patients: 0-10% CTCs with rearranged ALK (false postives?)

Pailler E et al. ***J Clin Oncol. 2013;31(18):2273-81.***
99.99% concordance between CTCs and tumor biopsy for ALK rearrangement detection

Tan CL et al. ***Oncotarget. 2016.***
90% concordance between CTCs and tumor biopsy for ALK rearrangement detection

Kim YH et al. ***Oncol Lett. 2018; 15(6): 8959–8964*****.**
CTC positive for ALK rearrangement were observed in 16/22 patients (72.7%) with ALK+ tumors

Monitoring of ALK TKI treatment efficacy using Circulating Tumor Cells



	Baseline (0 months)	Partial response (3 months)	Progression (5 months)
CTC count (CTCs/1.88 ml)	6	3	11
ALK rearrangment patterns	1F1R1G	1F1R1G	1F1R1G,2R2G,1F1R

Tan CL i wsp. *Oncotarget. 2016.*

Summary

1. Tumor tissue specimens are the pillars of pathological and molecular diagnosis of NSCLC and currently they cannot be permamently replaced by any surrogate material.

2. Liquid biopsy and other surrogate specimens as additional sources of tumor genetic material may greatly supplement the current algorithm of molecular diagnosis in NSCLC.

3. Currently, the only well-established diagnostic application of liquid biopsy in NSCLC is the molecular evaluation of *EGFR* gene mutation status as the predictive biomarker for targeted therapy.

4. The greatest limitations of liquid biopsy: - ultrasensitive techniques are required, some of them are still not validated for in vitro diagnostics (IVD); - high risk of false-negative results (level of mutated variant below detection limit, some tumors do not shed much DNA into blood).

5. It is expected that other molecular alterations, e.g. *ALK* fusions, *ALK* mutations, *BRAF* mutations will be evaluated in liquid biopsy in the close future.

6. Comprehensive molecular analysis of liquid biopsy using Next Generation Sequencing is feasible and reliable, however, it is still technically challenging. The evaluation of Tumor Mutation Burden in blood shows promise to become a predictive biomarker for immunotherapy.

7. Diagnostic and clinical value of circulating microRNA, Circulating Tumor Cells and plasma proteomic profiling in NSCLC has not been confirmed yet. Some promising data need further validation in the independent studies.