Liquid biopsy: an overview in Serbia

Miljana Tanić

Institute for Oncology and Radiology of Serbia

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Outline

1. Liquid biopsy in the clinics
   Molecular diagnostics for NSCLC, CRC, BRCA

2. Liquid biopsy research projects
   STEPUPIORS, TRACEPIGEN, EXPAND-EV

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IORS: Scientific and Research Service

dr sc. Radmila Janković

Molecular diagnostics:

- Non-small cell lung cancer
- Colorectal cancer
- Immuno-oncology diagnostics of hematological and solid malignanices

Clinical trials:

- A phase I-III, Multicenter Study Evaluating The Efficacy And Safety Of Multiple Therapies In Cohorts Of Patients Selected According To Biomarker Status, With Locally Advanced, Unresectable, Stage III Non-Small Cell Lung Cancer (PI: Davorin Radosavljević, Medical Oncology clinic, IORS; F. Hoffmann La Roche Ltd.)
- PIK3AC pilot

Research Projects:

- STEPUPIORS (PI:Milena Čavić, Experimental Oncology Department, IORS)
- TRACEPIGEN (PI: Miljana Tanić, Experimental Oncology Department, IORS)
- EXPAND-EV (PI: Milica Popović, Faculty of chemistry, UB)

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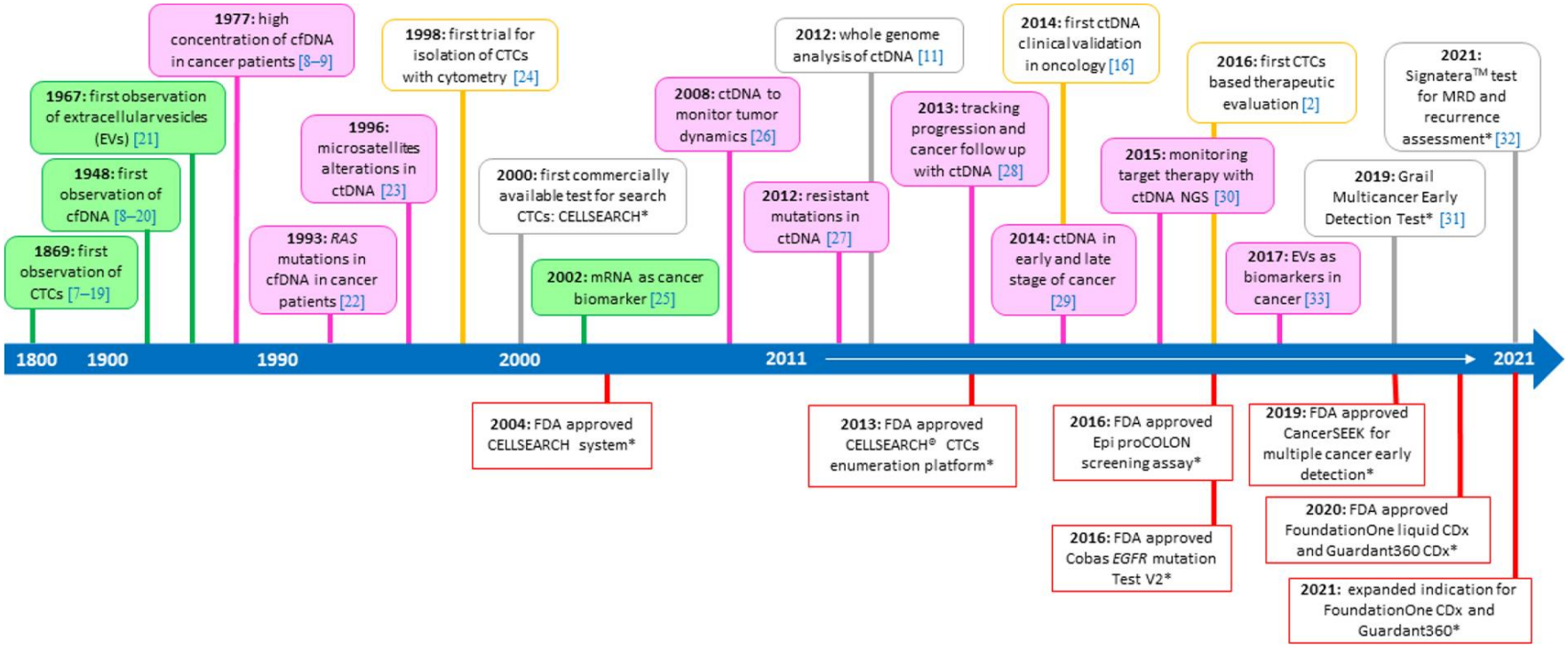
Liquid biopsy in the clinics

The timeline of liquid biopsy development



Explor Target AntitumorTher. 2023;4:102–38 https://doi.org/10.37349/etat.2023.00125

Credit: Radmila Jankovic

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LB Clinical testing

What?

Where?

How?

When?

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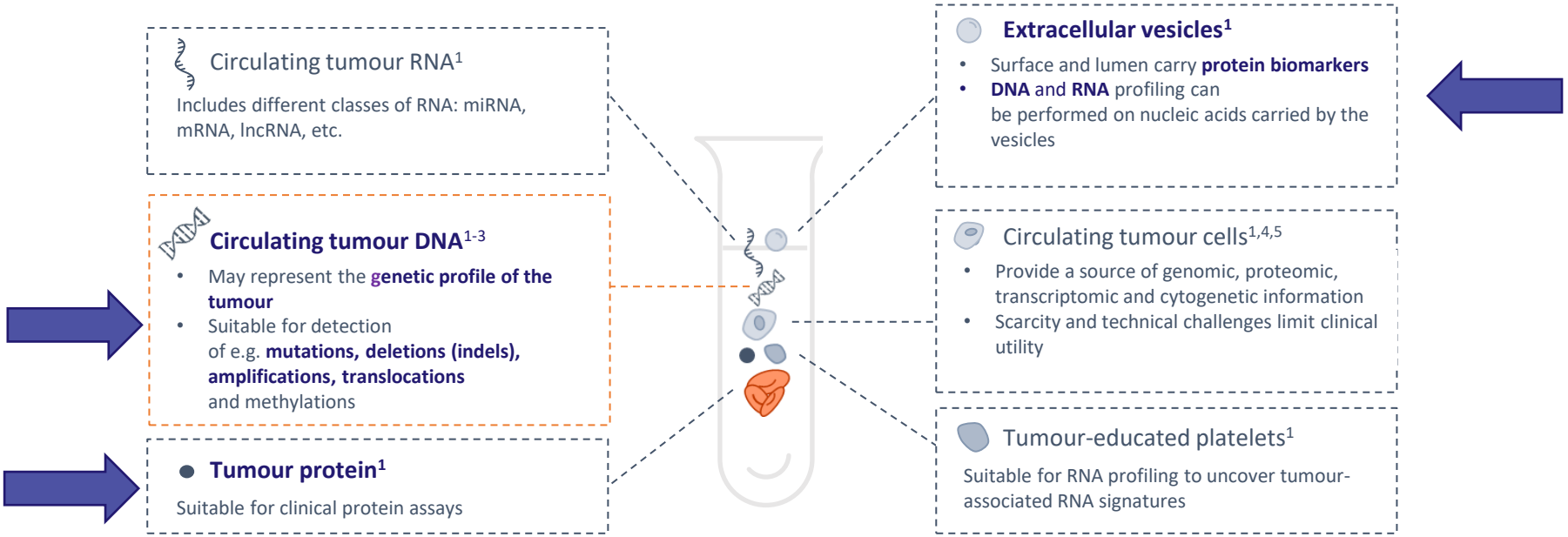
Liquid biopsies

Different LB components - different clinical and scientific applications



mRNA: messenger RNA; miRNA: micro RNA; lncRNA: long non-coding RNA.

1. De Rubis, G., et al. (2019) *Trends Pharmacol Sci* 40:172-86;
2. Francis, G. & Stein, S. (2015) *Int J Mol Sci* 16:14122-42; 3. Cheng, F., et al. (2016) *Oncotarget* 7:48832-41;
4. Kulkarni, R.P., (2019) *Sci Transl Med* 11:eaax1730;5. Ciurte, A., et al. (2018) *PLoS ONE* 13:e0208385;
6. FoundationOne Liquid CDx technical specifications (2020).

Credit: Radmila Jankovic

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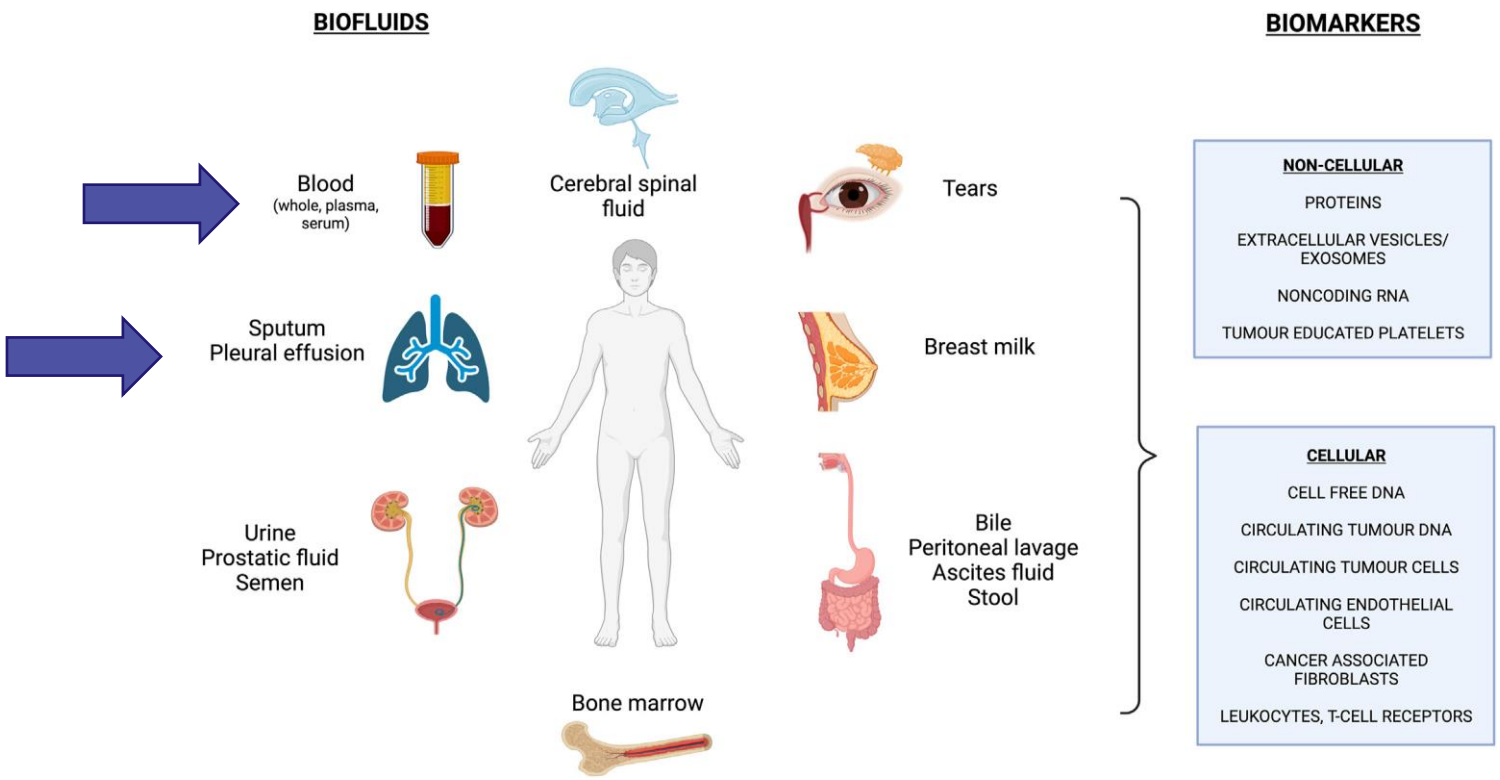
Liquid biopsy types in the clinics

The type of liquid biopsy sample drives the clinical sensitivity of the method



Mansur and Radovanovic, Frontiers in Genetics, 10.3389/fgene.2024.1348096

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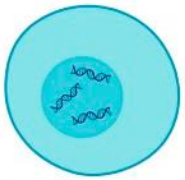
Challenges for ctDNA detection

cfDNA

- No tumor-specific
- Found in health subjects
- May carry germline mutations
- Source: non-cancerous cells

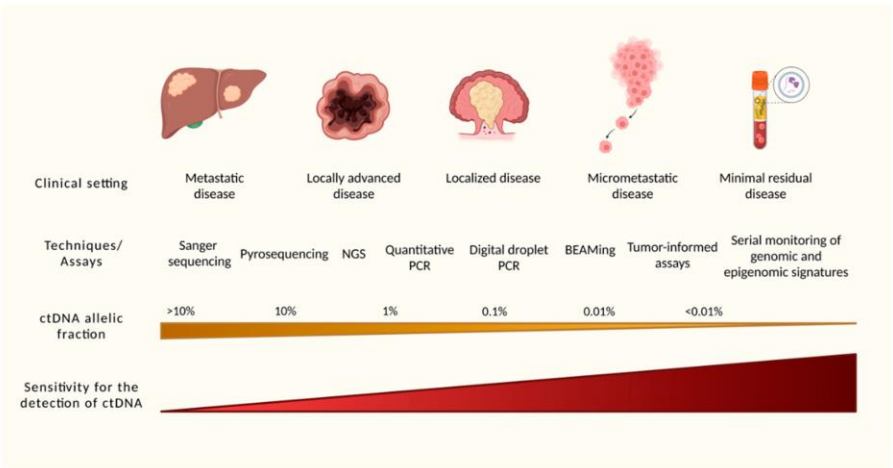


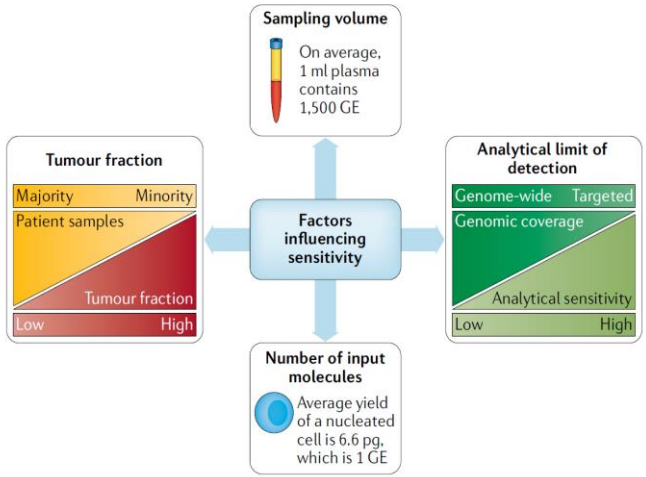
Similarities

- Low concentrations in bloodstream
- More abundant than CTCs
- Associated to disease progression

ctDNA

- Tumor-specific
- Not found in health subjects
- Harbors actionable mutations
- Mirrors tumor landscape
- Enable tracking tumor evolution
- Source: malignant cells





Jácome, A.A.; Johnson, B. *Cells* (**2023**), *12*(7), 1068; https://doi.org/10.3390/cells12071068

Heitzer et al. NatRev Genet 2019

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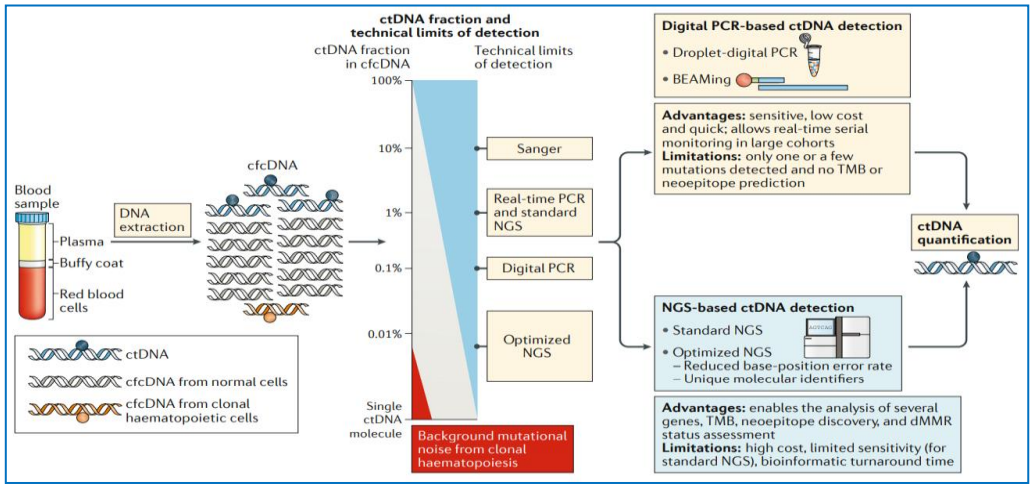
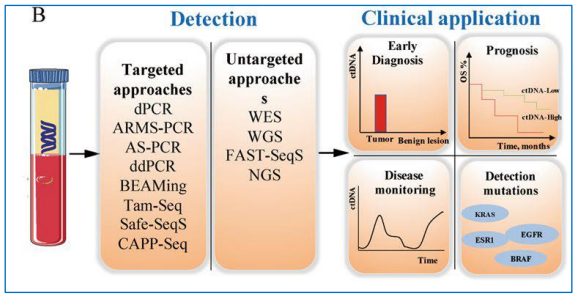
Challenges for ctDNA detection

Technical considerations: depending on the ctDNA content and further clinical application, different ctDNA plasma assays may be indicated

- **Digital PCR** methods enable highly sensitive targeted assays but only on very few loci
- **NGS** allows query of many more genes, enables analysis of TMB, MSI





Cabel L, et al. Nat Rev Clin Oncol. (2018) Oct;15(10):639-650. doi: 10.1038/s41571-018-0074-3.
Zhou *et al.* Molecular Cancer (2022) *21:86,* https://doi.org/10.1186/s12943-022-01556-2
Jácome, A.A.; Johnson, B. *Cells* (**2023**), *12*(7), 1068; https://doi.org/10.3390/cells12071068

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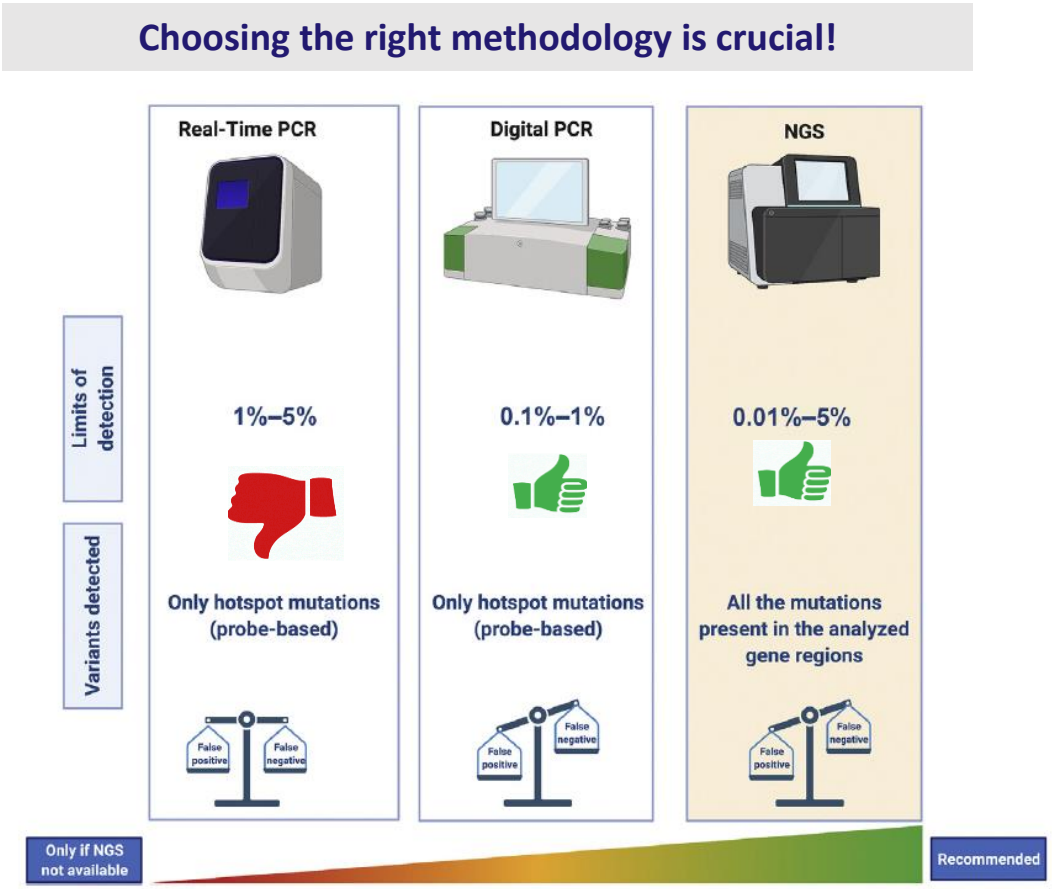
cfDNA detection in the clinics

Mutation positive
NOT able to detect



Rolfo et al., J Thorac Oncol, 16, 1647-1662,2021

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Clinical guidelines for ctDNA

ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

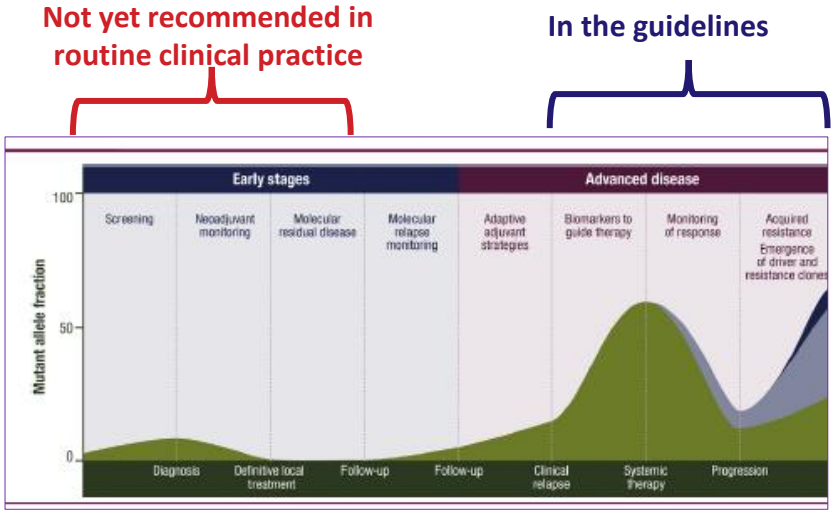
ANNALS OF ONCOLOGY driving innovation in oncology

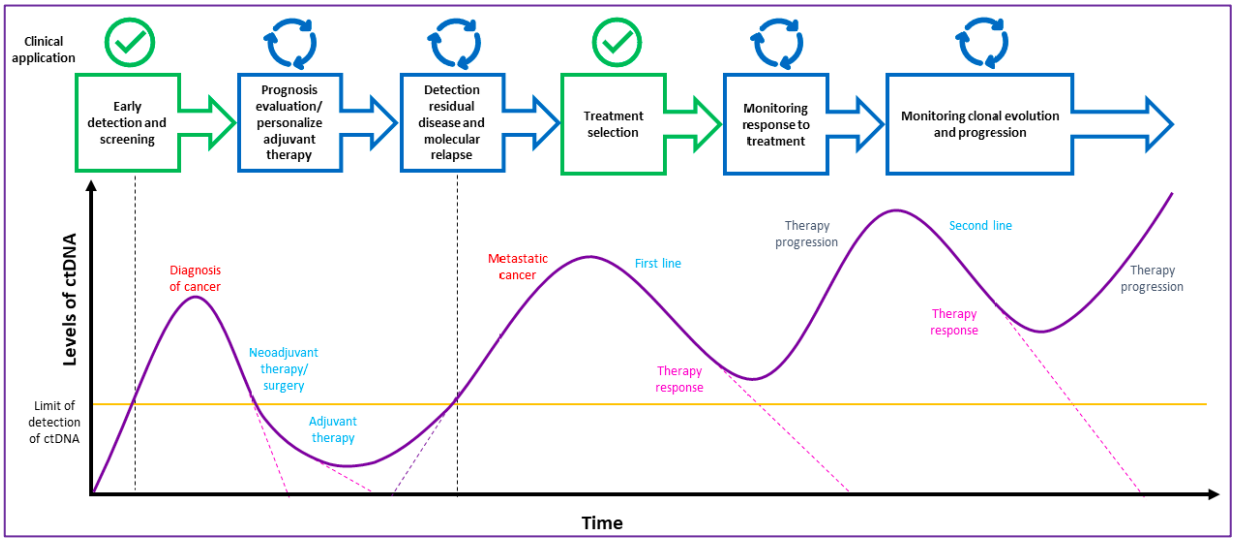
2022

SPECIAL ARTICLE

ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group

J. Pascual[1], G. Attard[2], F.-C. Bidard[3,4], G. Curigliano[5,6], L. De Mattos-Arruda[7,8], M. Diehn[9], A. Italiano[10,11,12], J. Lindberg[13], J. D. Merker[14], C. Montagut[15], N. Normanno[16], K. Pantel[17], G. Pentheroudakis[18], S. Popat[19,20], J. S. Reis-Filho[21], J. Tie[22,23], J. Seoane[24,25], N. Tarazona[26,27], T. Yoshino[28] & N. C. Turner[19,20*]





Clinical application largely depends on the amount of ctDNA that changes during the course of the disease!

Explor Target AntitumorTher. 2023;4:102–38 | https://doi.org/10.37349/etat.2023.00125

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Non-small cell lung cancer

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With NSCLC, the tissue is still the issue!

Table 2. Tumour-specific table for advanced cancer genotyping

Tumour type	Indications	ESCAT tier and level of evidence	Recommendation
Non-small-cell lung cancer	*EGFR* (for common, uncommon, exon 20 insertions, T790M and other resistance mutations e.g. C797X).	IA[120]	ctDNA genotyping recommended in treatment-naive cancer patients and resistance upon prior TKIs. Caution should be kept as ctDNA assays will miss histological trans-differentiation.
	ALK (for fusions and acquired resistance kinase domain mutations).	IA[121-125]	
	MET (for exon 14 splice site mutations, and acquired resistance mutations)	IB[126,127]	
	KRAS (for G12C and non-tier 1 other *KRAS* mutations)	IB[128]	
	BRAF (for V600E)	IB[129,130]	
	RET (for fusions and acquired resistance kinase domain mutations)	IB[131]	
	ROS1 (for fusions and acquired resistance kinase domain mutations)	IB[132,133]	
	NTRK 1/2/3 (for fusions and acquired resistance mutations)	IC[134]	
	MET (for high-level copy number gain/amplification)	IIA[135]	
	ERBB2 (for exon 20 insertions and transmembrane mutations, and amplification)	IIB[136-138]	
	BRAF (for non-V600E class I-III mutations)	IIB[139]	

✓LB assays for genotyping should be collected when cancer is progressing, either treatment naive or after prior lines of therapy. Samples collected when a tumour is responding to therapy will have decreased sensitivity.

✓ For genotyping of advanced cancer, the choice between RT-PCR, digital PCR and NGS assays in a clinical practice setting should be defined by availability, reimbursement status and the number of tier I actionable genetic aberrations in a tumour-specific context.

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Non-small cell lung cancer

Diagnostic algorithm for liquid biopsy use in treatment-naive advanced/metastatic NSCLC

2021

STATE OF THE ART: CONCISE REVIEW

IASLC

Liquid Biopsy for Advanced NSCLC: A Consensus Statement From the International Association for the Study of Lung Cancer

Christian Rolfo, MD, PhD, MBA, Dr.hc.,[a] Philip Mack, PhD,[a]

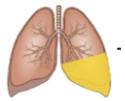
Advanced NSCLC with unknown genotype

Tissue sample available for tumor genotyping

Tissue sample unavailable for tumor genotyping

"Plasma first approach"

Plasma cfDNA genotyping



Re-biopsy for tumor tissue genotyping in case of absence of targetable drivers in plasma

Tumor tissue adequate for genotyping

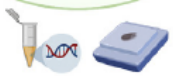
Tumor tissue scant/of uncertain adequacy for genotyping

"Sequential approach"

"Complementary approach"

Tumor tissue genotyping

Concurrent tumor tissue and cfDNA genotyping



cfDNA analysis in case of incomplete tumor genotyping

Rebiopsy is recommended when no targetable alterations are detected

Even when tissue is available, LB is recommended as a "sequential" or "complimentary" approach

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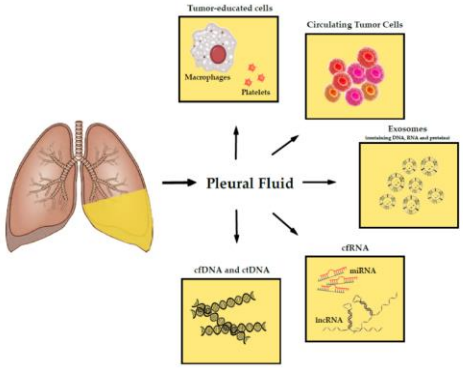
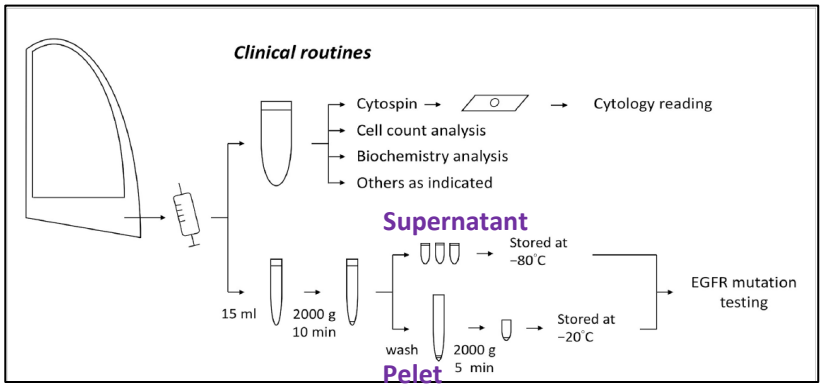
Workflow

Significance of molecular profiling from pleural effusions





Year	Genomic Approach	Patients and Samples	Mutations Detected	Detection Rate	Concordance
2020	NGS	21 cancer patients (7 lung adenocarcinomas): 15 PFs, 5 peritoneal fluids, 1 pericardial fluid, 8 cell blocks, and 3 tissues	Mutations on 130 gene-panel of cancer related genes	71.4% global mutation rate	72.3% PF-tissue
2020	NGS	108 lung cancer patients: PF cell blocks	Mutations on 17 gene-panel containing lung cancer associated genes	86% *EGFR*, 41.7% *TP53*, 9% BCL2, 21.3% *BRAF*, 19.4% *PIK3CA*, 21.3% *PTEN*, 18.5% FGFR1, 25% *MET*, 27.8% *RET*, 5.6% *KRAS*, and 5.6% *ALK*	Not evaluated
2018	NGS and ARMS-PCR	30 NSCLC patients: PF cell blocks and tissues	Mutations on 9 gene-panel containing lung cancer associated genes	Global mutation rate: 83.33% in both tissue and PF cell blocks	86.7% PF cell blocks-tissu

Pleural punctate represents a substantial source of ctDNA for molecular profiling

Sorolla MA et al. , cancers, 13, 2798,2021
Chiang CL et al., Frontiers in Oncology, 12, 8101,2022

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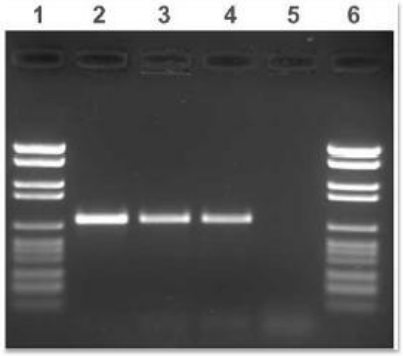
Hotspot detection

Molecular diagnostics based on PCR methods

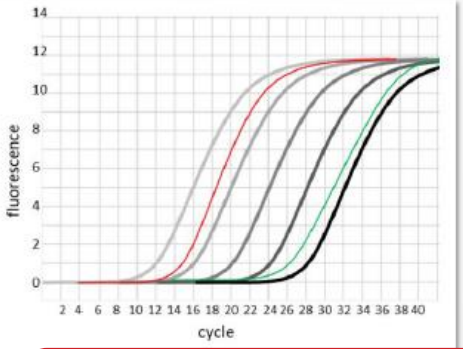
Classical PCR



Endpoint

Semi-quantitative

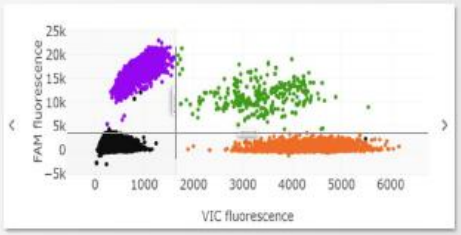
Quantitative PCR (qPCR)



Real-time analysis

Relative quantification

Digital PCR (dPCR)



Endpoint

Absolute quantification

Source: ThermoFisher

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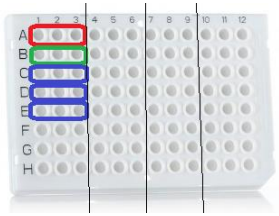
Hotspot detection

cobas® EGFR Mutation Test

The **cobas**® EGFR Mutation Test is a real-time PCR test for the qualitative detection and identification of mutations in exons 18, 19, 20 and 21 of the epidermal growth factor receptor (EGFR) gene in DNA derived from formalin-fixed paraffin-embedded (FFPET) human non-small cell lung cancer (NSCLC) tumor tissue.

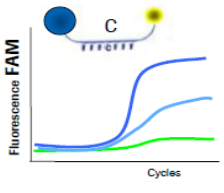


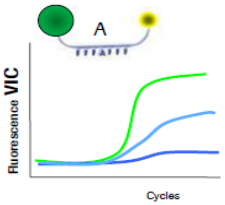
3 MMIXes

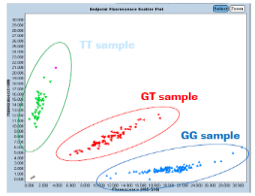
1 Positive Control

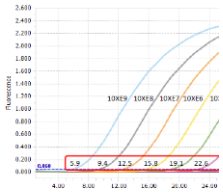
1 Negative control

Up to 30 samples per plate/per run









MUTATIONS DETECTED:

Exon 18 – Mutation Call G719X (3 mutations)

Exon 19 – Mutation Call Exon 19 Deletion (29 different deletions)

Exon 20 – Mutation Calls T790M, S768I, Exon 20 Insertion (7 mutations)

Exon 21 –Mutation Call L858R (2 mutations)

IN TOTAL: 41 MUTATIONS, 3 reactions per pt, up to 30 pts in one run, hands on ~ 8hours

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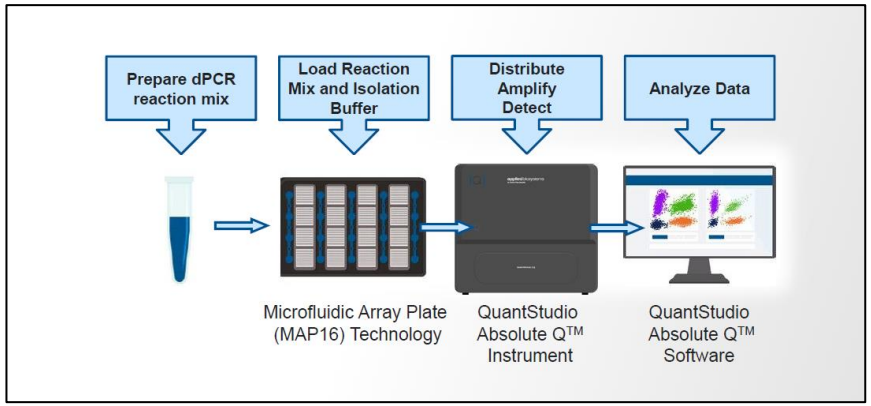
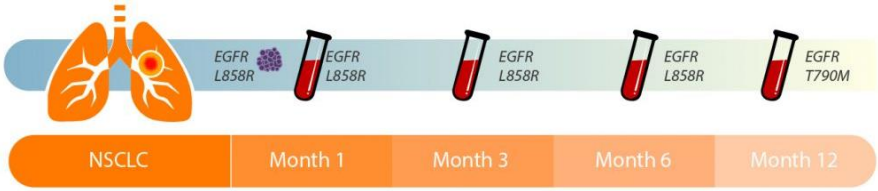
Hotspot detection

Step by step workflow

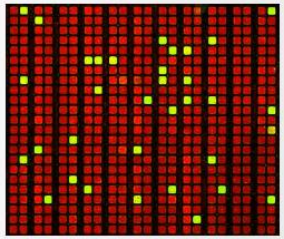


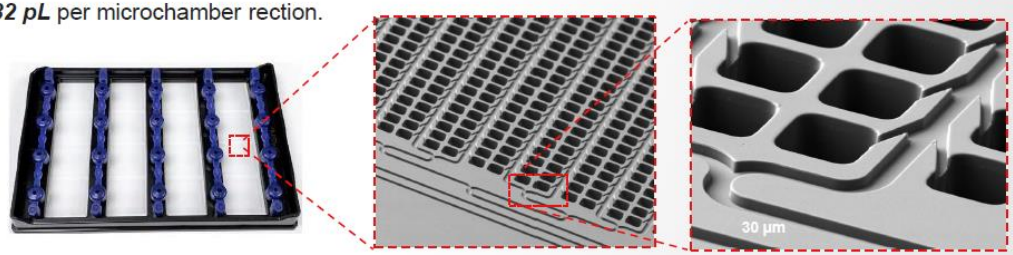
T790M and L858R assays



Microfluidic Array Plate

- ***16 reaction arrays*** (opaque squares) per plate for dPCR reactions
  - Run in intervals of 4
  - Unused reaction arrays can be run later.
- Each reaction array is made up of ***20,480 fixed microchambers***
  - Microchambers are connected by a distribution network of channels that is used to deliver reaction mix and Isolation Buffer.
- ***432 pL*** per microchamber rection.





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ctDNA from pleural effusions

Translational Oncology 37 (2023) 101772

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

EGFR mutation testing from pleural effusions of non-small cell lung cancer patients at the institute for oncology and radiology of Serbia

	Baseline testing Liquid biopsy (blood) (*n*=217) n (%)	Testing at progression on *EGFR* TKIs Liquid biopsy with rebiopsy (blood) (*n*=407) n (%)	Liquid biopsy (pleural effusion) (*n*=20) n (%)
EGFR status			
EGFR mut	22 (10.14)	242 (59.46)	16 (80)
EGFR wt	189 (87.10)	164 (40.29)	4 (20)
NA	6 (2.76)	1 (0.25)	0 (0)
EGFR mutation type			
Ex19del	14 (63.64)	92 (38.02)	5 (31.25)
L858R	3 (13.64)	36 (14.88)	1 (6.25)
L861Q	1 (4.55)	3 (1.24)	0 (0)
G719X	0 (0)	5 (2.07)	0 (0)
Ex20Ins	1 (4.55)	14 (5.78)	0 (0)
S768I	1 (4.55)	0 (0)	0 (0)
Double mutants	1 (4.55)	91 (37.60)	10 (62.50)
Triple mutants	0 (0)	1 (0.41)	0 (0)
T790M	1 (4.55)	89** (36.77)	10*** (62.50)

M. Vukovic et al. Translational Oncology 37 (2023) 101772

Distribution of *EGFR* mutation types in patients' liquid biopsy samples at progression on *EGFR* TKIs (analyzes were done by qPCR and dPCR).

	Cobas® 4800		QuantStudio Absolute Q Digital PCR	
	Liquid biopsy with rebiopsy (blood) (*n*=25) n (%)	Liquid biopsy (pleural effusion) (*n*=5) n (%)	Liquid biopsy with rebiopsy (blood) (*n*=25) n (%)	Liquid biopsy (pleural effusion) (*n*=5) n (%)
EGFR status*				
EGFR mut	11 (44)	3 (60)	7 (28)	4 (80)
EGFR wt	14 (56)	2 (40)	18 (72)	1 (20)
NA	0 (0)	0 (0)	0 (0)	0 (0)
EGFR mut type				
Ex19del	6 (54.54)	1 (33.33)	/	/
L858R	1 (9.10)	0 (0)	1 (14.29)	0 (0)
Double mut**	4 (36.36)	2 (66.67)	2 (28.57)	1 (25)
T790M***	4 (36.36)	2 (66.67)	4 (57.14)	3 (75)

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ctDNA from pleural effusions

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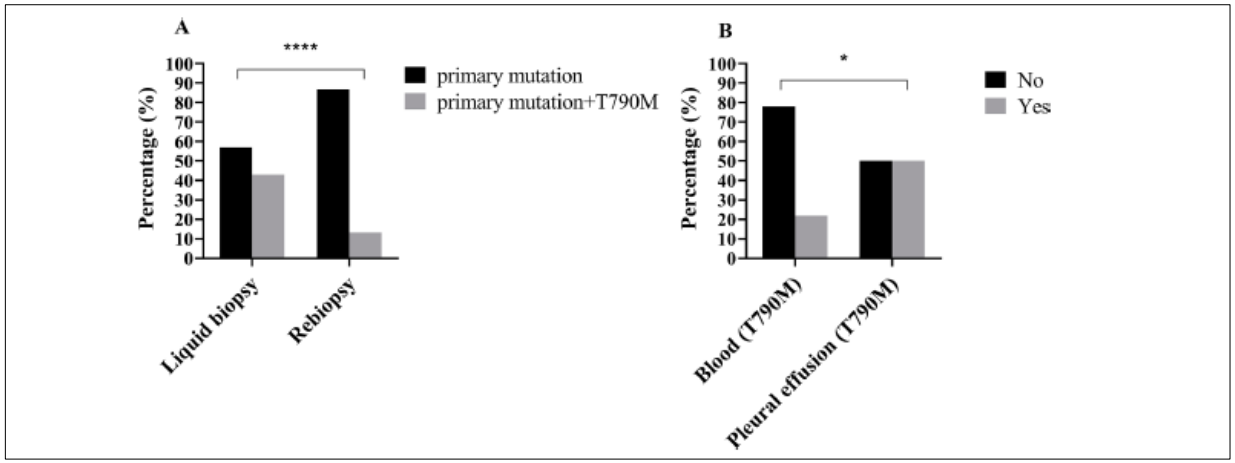
journal homepage: www.elsevier.com/locate/tranon

EGFR mutation testing from pleural effusions of non-small cell lun
patients at the institute for oncology and radiology of Serbia

Detection of the resistant T790M mutation in paired liquid biopsy samples at progression on *EGFR* TKIs (blood and pleural effusion) on qPCR and dPCR.

QuantStudio Absolute Q Digital PCR (T790M)	Cobas® 4800 (T790M)	
	Yes	No
Yes	6	4
No	0	20

* Cohen's kappa test; kappa=0.667 – "Good agreement".



EGFR mutation detection of primary and T790M mutation at progression on *EGFR* TKIs: in liquid biopsy and rebiopsy blood plasma samples **(A)** and in blood plasma overall and pleural effusion samples **(B)**. **p*=0.01, *****p*<0.0001.

Our results showed that the detection rate of the primary mutation was lower in the first testing from the liquid biopsy compared to the rebiopsy, while the situation with the detection of the primary T790M mutation was reversed.

When comparing the detection success rate of the resistant T790M mutation in blood and PE, a statistically significant difference was obtained in favour of PE (21.87% and 50%, respectively, p=0.01, Fig. 2B).

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qPCR vs ddPCR

IORS; cobas® EGFR Mutation Test v2 vs. dPCR

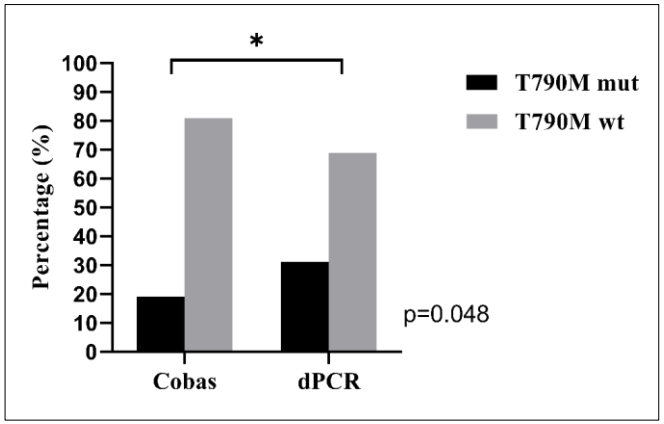
Cobas EGFR v2 (2022-2023); 110 analiza (75 pacijenata)

- **Bez mutacija** – **48/110** = **43.64%**
- **T790M** – **21/110** = **19.1%** (**1/21** T790M only; **12/21** T790M + ex19del; **8/21** T790M + L858R)
- **L858R** – **15/110** = **65%** (**7/15** L858R only; **8/15** T790M + L858R)
- **Ex19del** – **45/110** = **40.91%** (**33/45** ex19del only; **12/45** T790M + ex19del)
- **G719X** – **1/110** = **0.91%**

dPCR (2023-2024); 124 analize (78 pacijenata)

- **Bez mutacija** – **78/119** = **65.55%**
- **T790M** – **37/119** = **31.1%** (**28/37** T790M only; **9/37** T790M + L858R)
- **L858R** – **13/20** = **65%** (**4/13** L858R only; **9/13** T790M + L858R)

**Napomena; korišćeni samo T790M i L858R eseji*



Fisher's exact test

Laboratorija za molekularnu genetiku: unpublished data

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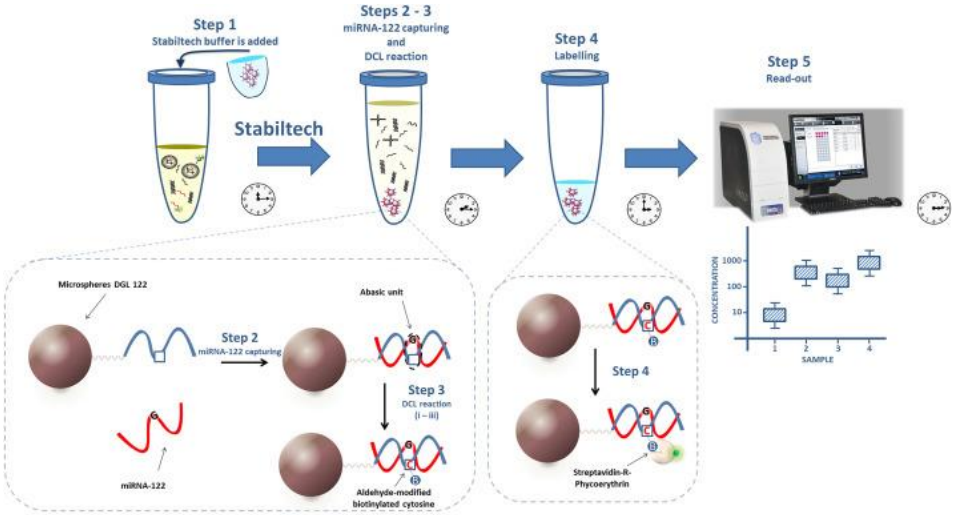
Research projects

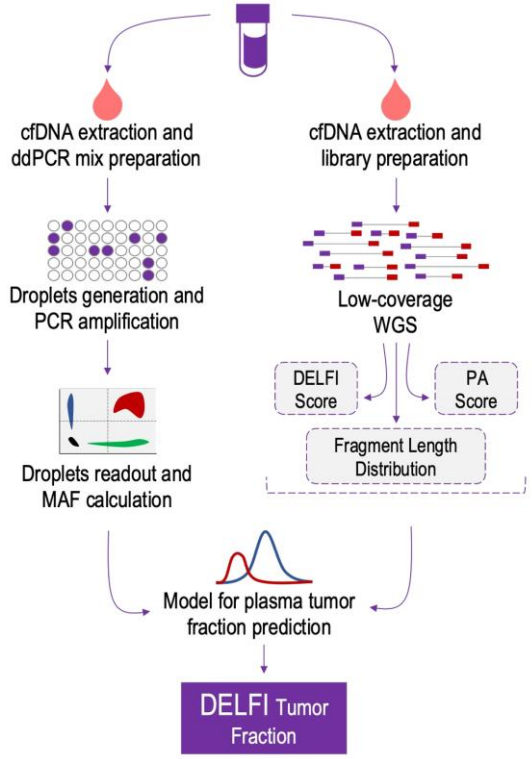
STEPUPIORS
Horizon Twinning

Determine the molecular profile associated with response to neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer

Milena Čavić, PI

transcriptomics
proteomics
radiomics
fragmentomics





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Research projects

TRACEPIGEN

PROMIS 2019 Serbian Science Fund

Tracking systemic therapy resistance of lung and colorectal cancer through targeted NGS analysis of genetic and epigenetic variants in liquid biopsies

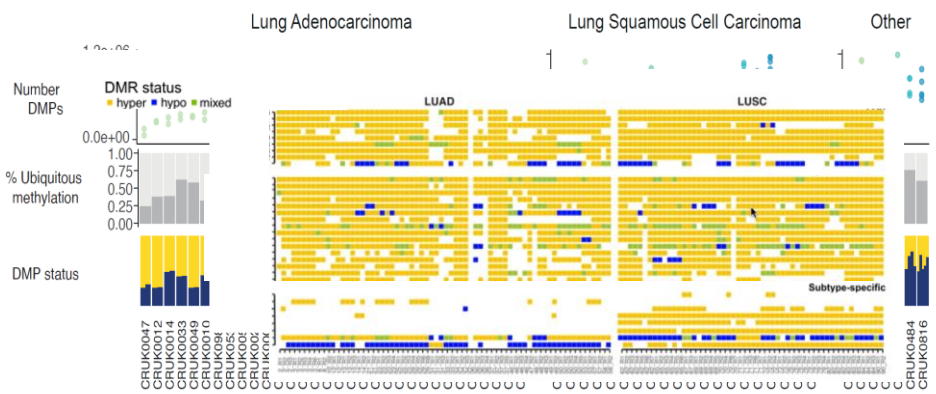
Miljana Tanić, PI

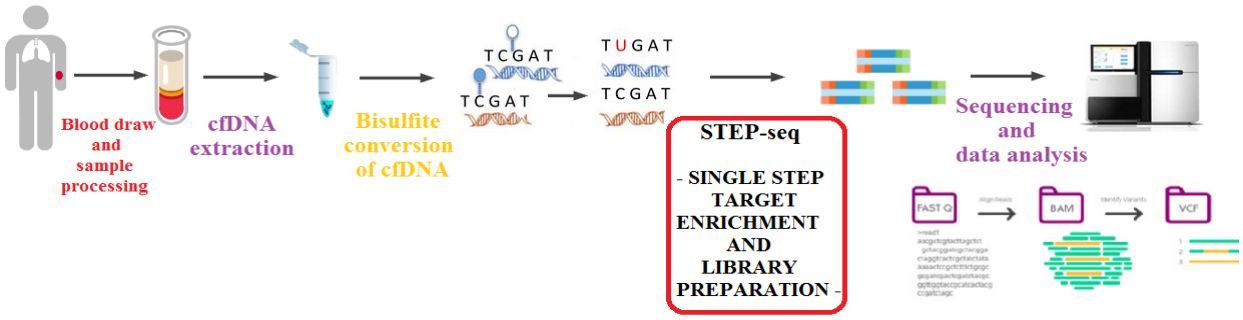
Chemo/targeted therapy for advanced stage

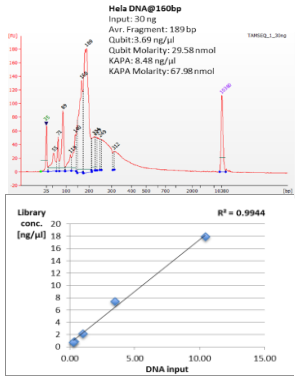
3-10K € /month

Therapy resistance

TRACERx







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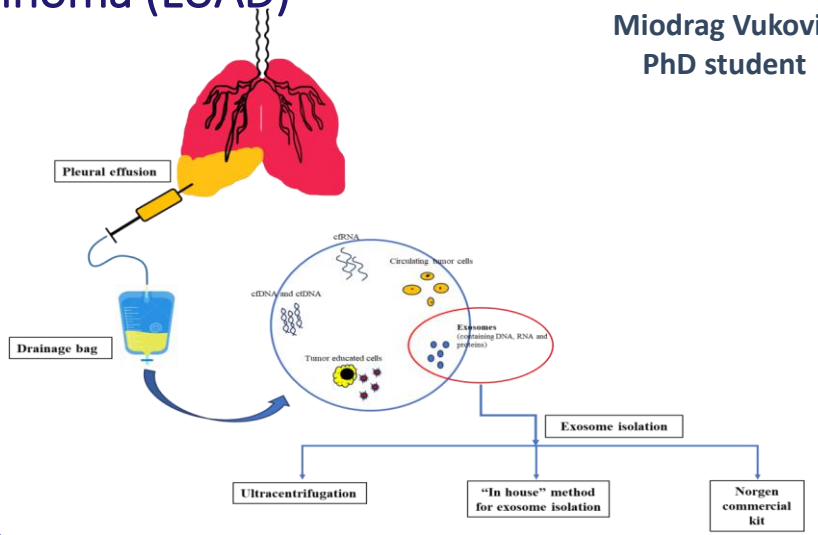
Research projects

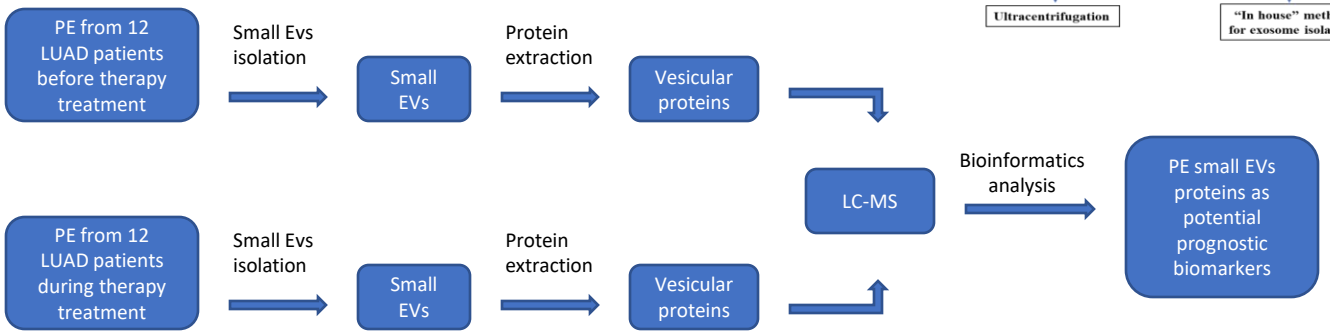
EXPAND–EV
Horizon MSCA Staff Exchanges

Miodrag Vuković
PhD student

Small extracellular vesicles (EVs) from pleural effusion of patients with advanced lung adenocarcinoma (LUAD)

- First goal – isolation and characterization of small EVs with three different methods from pleural effusion (PE) of patients with advanced LUAD (PE pool from 5 patients)
- Final goal – choosing the best isolation method, isolation of small EVs from PE of 12 patients with advanced LUAD before and during therapy treatment, extraction of vesicular proteins and their proteomic analysis for seeking potential biomarkers of therapy response





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Laboratory for immunology

Diagnostics

Analysis of immunophenotypic characteristics of various subsets of leukocytes in peripheral blood (PB), bone marrow aspirate, liquor, effusion in patients with hematological malignancies (leukemia, lymphoma, multiple myeloma), immunodeficiency, solid tumors by flow cytometry

Research

Analysis in PB of cancer patients (melanoma, breast cancer, multiple myeloma) before and during therapy (immunotherapy, targeted therapy):

- the prevalence, the expression of activating (NKG2D, NKp46, DNAM-1, CD28) and inhibitory (PD-1, CTLA-4, TIM-3, LAG-3, TIGIT) receptors and coreceptors, the functional characteristics of antitumor (NK, NKT, Th, CTL cells) and immunossupressive (Treg lymphocytes, MDSC, TAM) immune cells by flow cytometry
- the level of cytokines (IFN-γ, IL-2, TGF-β, TNF-α, IL-6, IL-8, IL-10, VEGF) and soluble ligands (PD-L1, MICA) in sera and plasma by ELISA method
- the expression of genes for cytokines and enzyme IDO in mRNA isolated from PB mononuclear cells by qPCR

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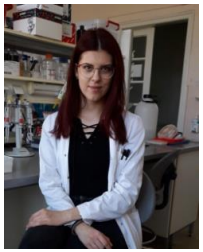

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Republic of Serbia
MINISTRY OF SCIENCE, TECHNOLOGICAL DEVELOPMENT AND INNOVATION

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