The Role of Liquid Biopsy in Early Diagnosis of Lung Cancer

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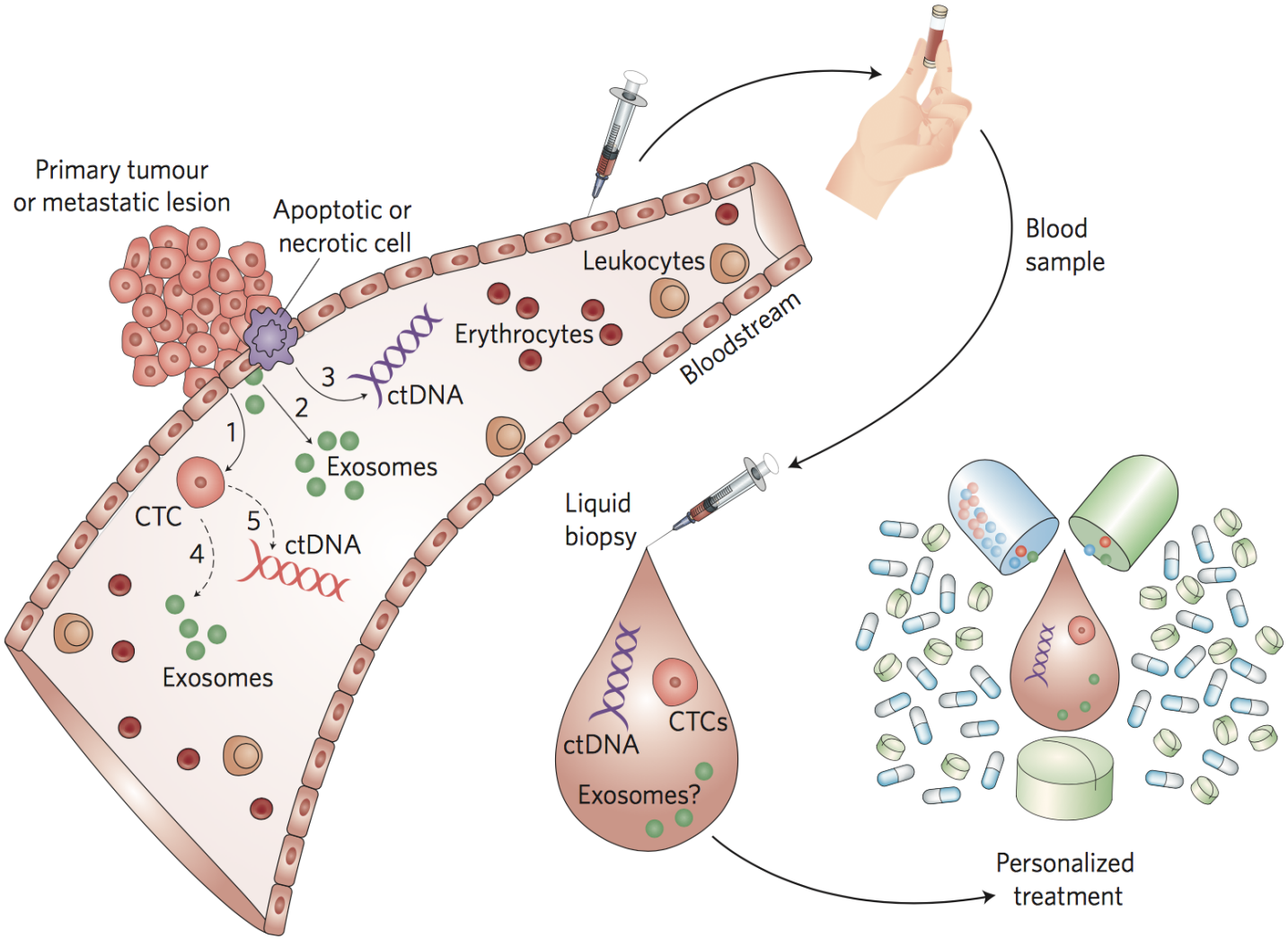
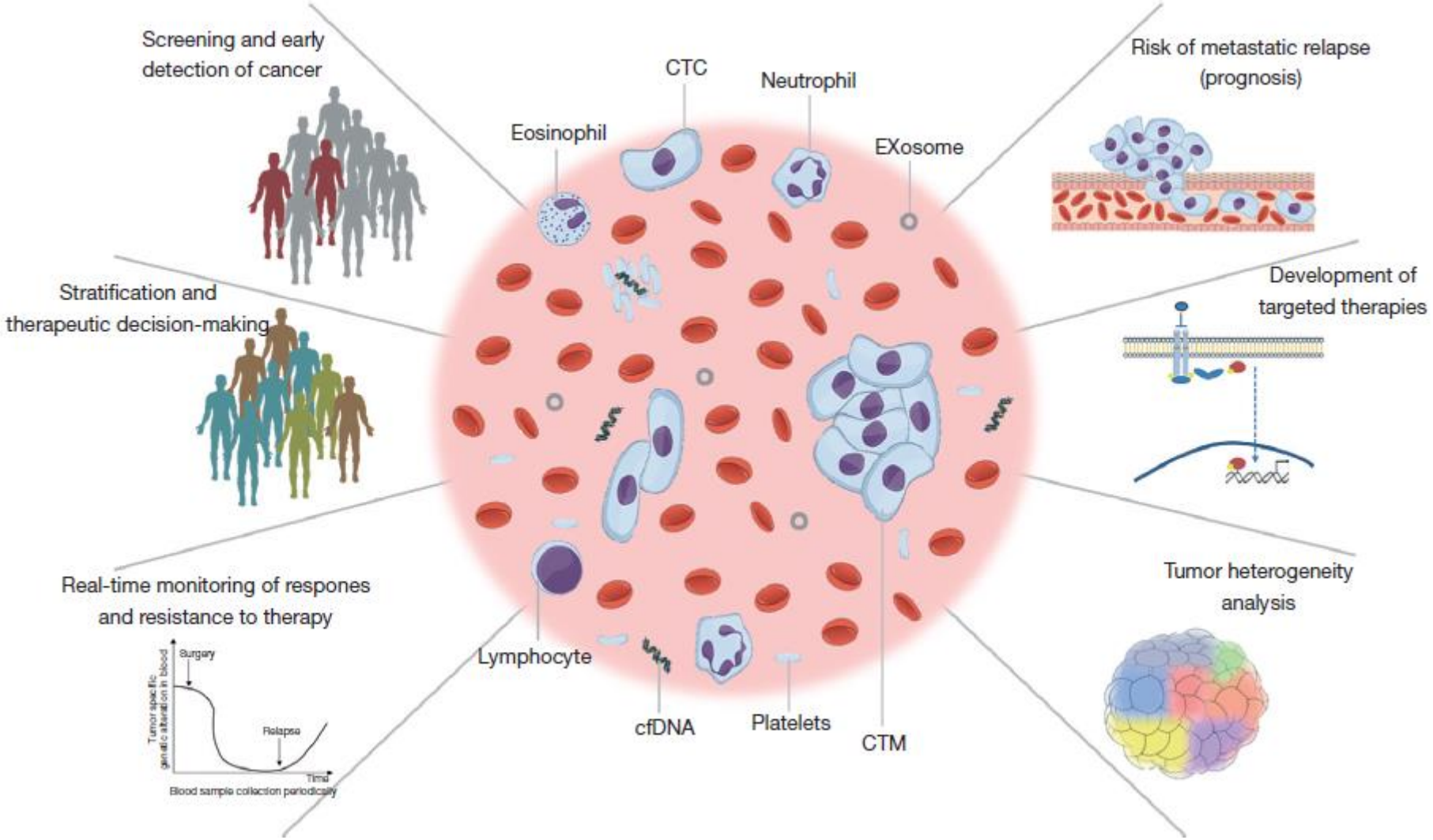
Obtaining Blood Samples



Alix-Panabie`res and Pantel, Nature Biomedical Engineering 2017

Clinical Utility of Liquid Biopsy



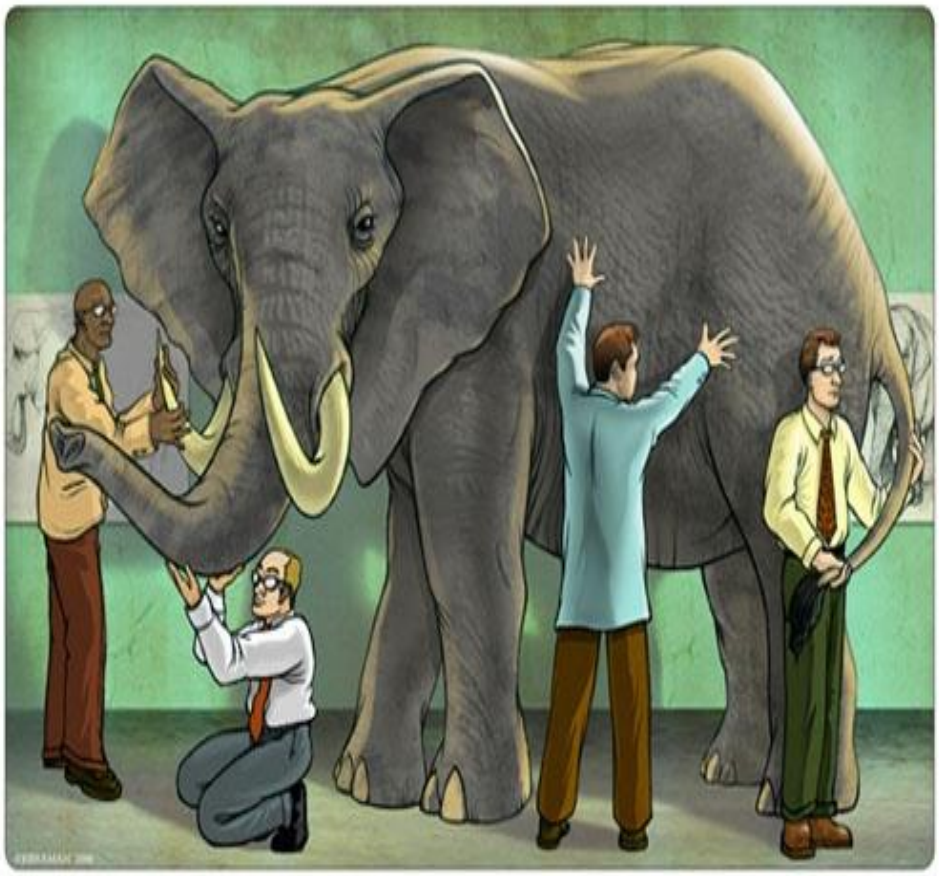
Transl Lung Cancer Res 2016;5(5):466-482

Table 1 A summary of advantages of liquid biopsy versus tissue biopsy

Tissue biopsy
Patient discomfort
Restricted or extremely risky access possibilities
Difficult to repeat (re-biopsies)
Tissue sample does not reflect the status of multiple sites
Tissues are usually collected months or years before treatment
A difficult tissue sample to run all the molecular tests
Impractical for periodic monitoring for progression/treatment resistance
Liquid biopsy
Non-invasive
Easily repeated
A blood sample can provide genetic information of the whole neoplastic process (primary and metastases)
Real-time monitoring: CTC persistence after treatment
Diagnostic: biomarker assessment for molecular profiling to choose the targeted agent
Prognosis: prediction of drug-response and resistance to treatment

Journal of Thoracic Disease 2017;9(Suppl 13):S1332-S1345

Will liquid biopsies replace tissue biopies?



Liquid Biopsy

- Circulating tumor cells (CTCs)
- Circulating cell-free DNAs (cfDNAs)
- Circulating microRNAs (miRNAs)
- Exosomes
- Circulating proteins

- Samples: blood(serum/plasma), urine, CSF, saliva, BALF, effusion

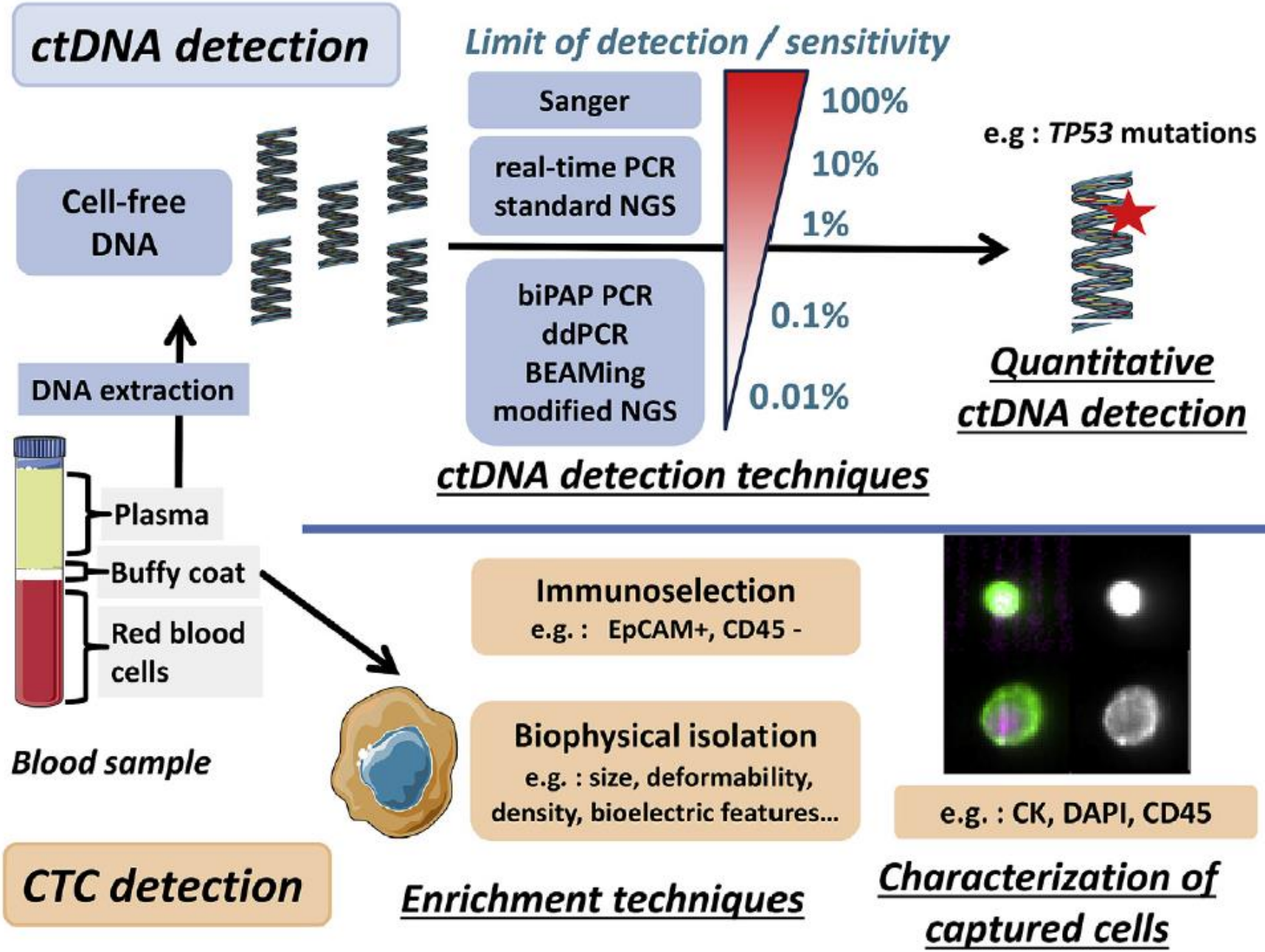


Figure 1. Overview of circulating tumor cell (CTC) and circulating tumor DNA (ctDNA) detection and characterization. Abbreviations: ddPCR = Dropplet digital polymerase chain reaction, BEAM = beads, emulsions, amplification and magnetics, NGS = Next-generation sequencing, PAP = pyrophosphorolysis-activated polymerization, CK = cytokeratin, EpCAM = Epithelial cell adhesion molecule.

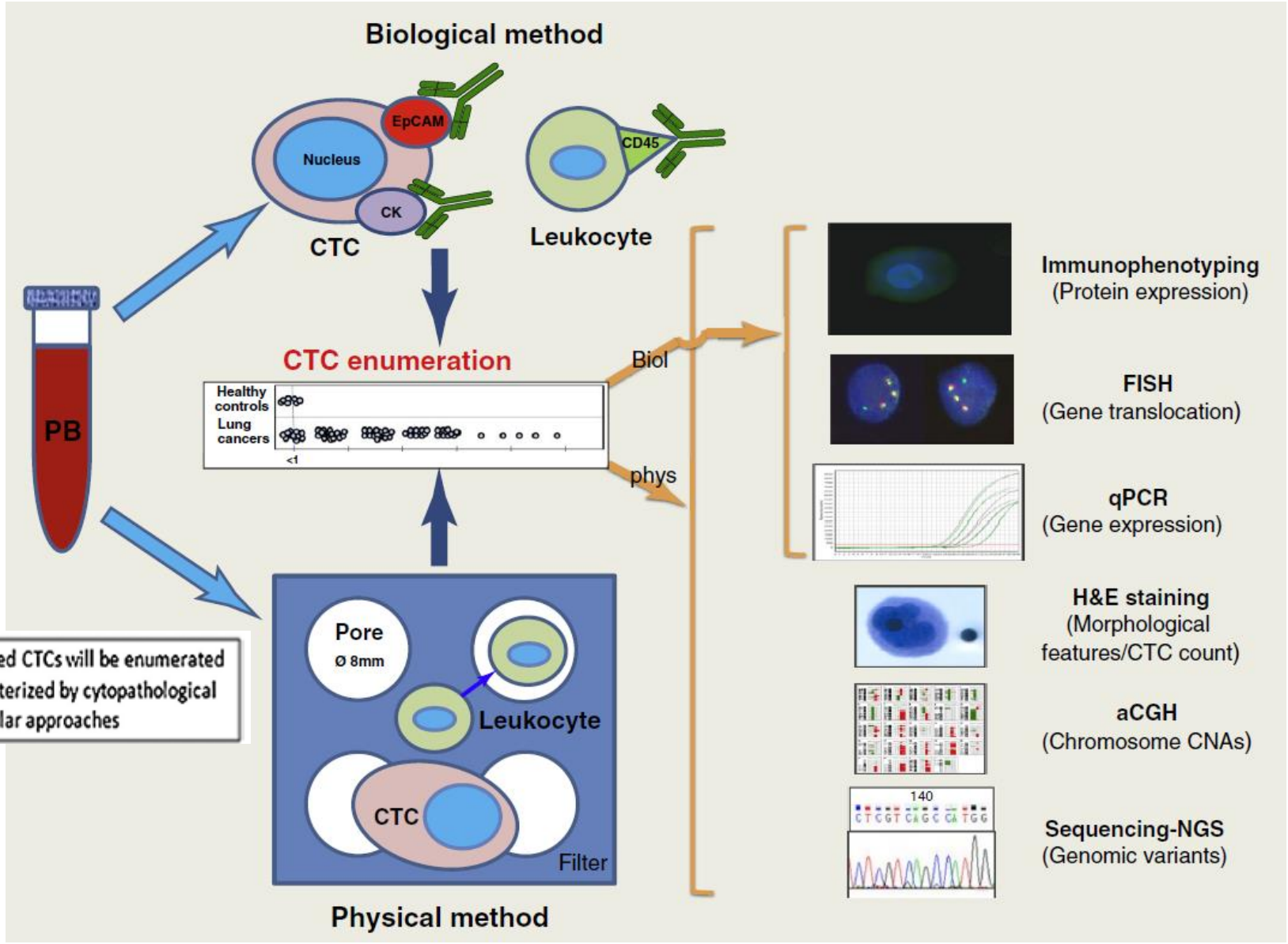
Clinical Relevance of CTCs

Applications

- Prognosis
- Monitor Metastasis
- Drug Activity
- Cell culture
- Xenograft Model

Approaches

- Enumeration
- Molecular characterization
- DNA/RNA NGS Sequencing



Schematic representation of the most commonly used techniques for circulating tumor cell (CTC) detection in non-small-cell lung cancer (NSCLC). Whole peripheral blood (PB) of a cancer patient is collected and processed for CTC enrichment according to either biological (ISET; isolation by size of epithelial tumor cells) or physical (CS; CellSearch®) methods. The identified CTCs and circulating tumor clusters are enumerated and characterized by cytopathological and molecular approaches. *Abbreviations*: aCGH, array-based comparative genomic hybridization; CNA, copy number aberration; EpCAM, epithelial cell adhesion molecule; CK, cytokeratins; FISH, fluorescent *in situ* hybridization; H&E, hematoxylin–eosin; NGS, next-generation sequencing.

Techniques used for CTCs

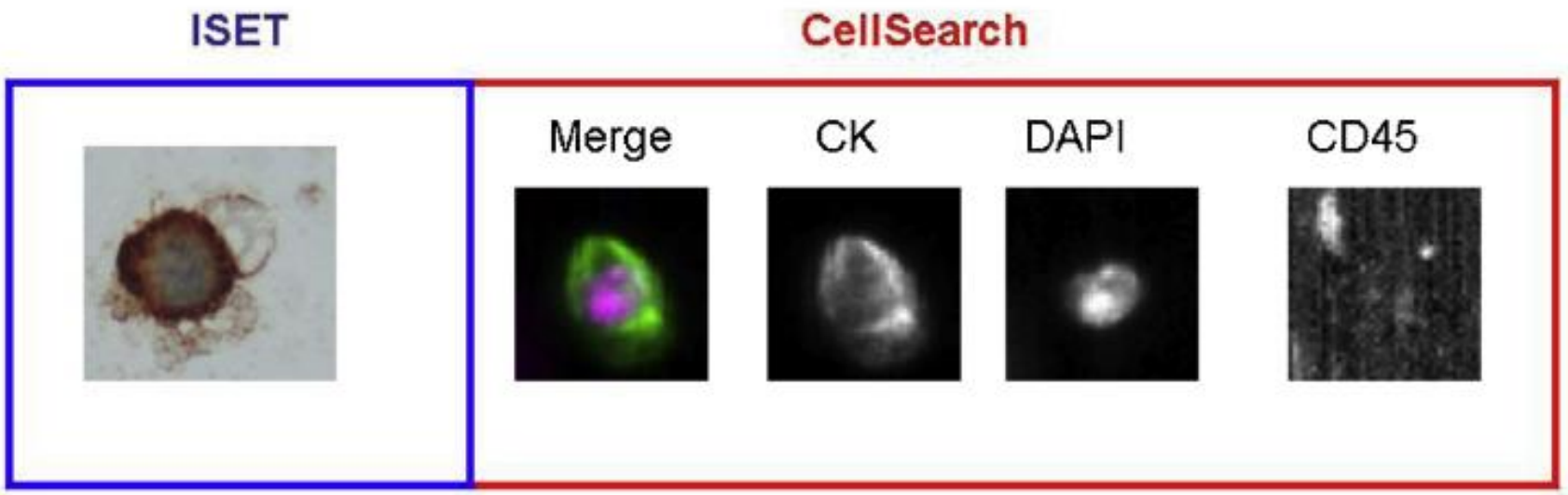


Fig. 3. Analysis of CTCs in a blood sample of a representative patient.
ISET: the picture shows a tumor cell isolated by size and evaluated for Cytokeratin19 expression by IHC.
CellSearch: the photos show the same cell stained for the combination (Merge) of CK (green) and DAPI (purple); CK FITC only; DAPI only; CD45 APC only.

Comparison of Techniques used for CTCs

Table 1 Main advantages and disadvantages of techniques used for CTCs enrichment and detection in lung cancer

Technique	Advantages	Disadvantages
Enrichment		
Immunomagnetic separation (CellSearch)	Semi-automatic magnetic separation method—easy to use. Highly reproducibility. High specificity. Fully validated. FDA-approved method	EpCAM antibody dependent. Difficult to extract pure CTC for further analysis. Low sensitivity. Expensive
Density gradient centrifugation (Ficoll–Hypaque or OncoQuick)	Simple method. No EpCAM antibody dependent. Isolates mononuclear cells based on different density gradient. Non-expensive	Poor enrichment. Low specificity and sensitivity. May lost large number of CTCs in processing
Size-based filtration (ISET)	Isolates cells based on different size (CTC larger than WBC). No EpCAM antibody dependent. Effective method. Tumor cells could be used for the subsequently analysis. Isolates CTM	Small tumor cells may be omitted. Low sensitivity and specificity
Detection		
Cytometric	Able to assess cell enumeration, morphology and characteristics. Cells also can be isolated for the subsequently molecular characterization	Detection depends on epithelial or tumor biomarker expression. No specific CTC marker exists. False-positive cells may be assessed. Low sensitivity
Nucleic acid based (RT-PCR)	Antibody independent. High sensitivity and specificity, particular in multimarker assay. Enrichment step is not necessary. Quantitative analysis	Difficult to produce. Unable to assess cell enumeration, morphology and characteristics. The false-positive is high

CTC circulating tumor cell, *EpCAM* epithelial cell adhesion molecule, *CTM* circulating tumor microemboli, *ISET* isolation by size of epithelial tumor cells

CTCs as Prognostic marker in Lung Cancer

Study	Technology	Sensitivity (% of patients positive for CTCs)	Prognostic significance
Hofman et al. (37) 208 NSCLC patients (stages I–IV)	ISET	50%	>50 CTCs corresponded with shorter OS and PFS
Tanaka et al. (35) 125 lung cancer patients (stages I–IV) 25 patients with non-malignant diseases	CellSearch	30% in all patients 71% in metastatic patients	CTC count was higher in lung cancer than non-malignant patients. CTC count was higher in patient with distant metastasis
Kreb et al. (19) 101 NSCLC patients (Stages III–IV)	CellSearch	21% at baseline (32% at stage IV, 7% at stage IIIB)	>5 CTCs/7.5 ml blood predicted shorter PFS and OS. A reduction in CTC count after chemotherapy predicted improved survival
Dorsey et al. (72) 30 NSCLC patients received radiation therapy	Telomerase-based assay	65% before RT	CTC count decreased in patients responding to RT
Juan et al. (73) 37 NSCLC patients [Advanced stage (IIIB–IV)]	CellSearch	24% at baseline	No significant prognostic conclusion was made
Muinelo-Romay et al. (34) 43 NSCLC patients (stages IIIB and IV)	CellSearch	42% at baseline	>5 CTCs/7.5 ml blood at baseline predicted shorter PFS and OS. CTC count increase during chemotherapy correlated with worse PFS and OS
Punnoose et al. (33) 41 NSCLC patients (Advanced stage)	CellSearch	76% at baseline	Reduction in CTC count after chemotherapy predicted longer PFS
Sienel et al. (74) 62 NSCLC patients (stages I–III)	Ficoll-Hypaque Centrifugation	18% in pulmonary venous (PV) blood	Presence of CTCs in PV blood was associated with shorter survival especially in patients with lymph node involvement
Hashimoto et al. (75) 30 NSCLC patients (stages I–IV)	CellSearch	73% in PV blood before surgery	CTC count in PV blood significantly increased after surgery, which predicted lymphatic tumor invasion
Funaki et al. (76) 130 NSCLC patients (stages I–IV)	RosetteSep kit	74% in PV blood after tumor resection Circulating tumor microemboli (CTM) in 33%	The presence of CTM in PV blood predicted worse PFS
Chudasama et al.(77) 20 NSCLC patients (stages III–IV)	ScreenCell	25% at baseline, 75% after endobronchial cryotherapy (EC)	CTC count increased after EC
Carlsson et al. (70) 104 NSCLC patients (stages I–IV) 25 patients with benign diseases	HD-CTC assay	50% positive to CTM	CTM along with clinical and imaging data can serve as predictor of malignant vs benign diseases
Pirozzi et al. (114) 45 NSCLC patients (stages I–III)	Ficoll-Hypaque Centrifugation	24% in PV blood	No association found between presence of CTCs and prognosis
Nel et al. (79) 43 NSCLC patients (stages IIB–IV)	Ficoll-Paque CD45 magnetic depletion	100%	Presence of mesenchymal markers CD133 and N-cadherin in CTCs predicated shorter PFS
Hou et al. (81) 97 SCLC patients	CellSearch and ISET	CTCs in 85% CTM in 32%	More than 50 CTCs/7.5 ml blood predicated shorter OS
Huang et al. (82) 26 SCLC patients	CellSearch	Not reported Median CTC count at baseline is 75 (0–3430)	CTC count decreased after chemotherapy CTC count at baseline and change of CTC numbers after treatment not associated with survival

18%~85%

Front ONcol 2015 Sep 30;5:209

Summary of CTCs Study

Summary of relevant circulating tumor cell (CTC) detection techniques and relative clinical indications

Refs	Sample number	Technique	CTC cut-off per (/) ml blood	Clinical indication
[36]	9	CS immunocytology	n.d.	Prognosis
[37]	116	CS	≥1/7.5	Diagnosis and prognosis
[38]	250	ISET, cytology	n.d.	Diagnosis
[39]	208	ISET	50/10	Diagnosis and prognosis
[40]	210	CS, ISET	≥2/7.0	Prognosis
[41]	101	CS	≥5/7.5	Prognosis and prediction
[45]	34	CS, CTC-chip, IF	≥3/7.5	*EGFR* expression
[46]	41	CS, IF, qPCR	≥1/7.5	*EGFR, KRAS, BRAF, NRAS, AKT1, PIK3CA* mutations[a]
[47]	5	LCM, PCR	n.d.	*EGFR* mutation
[48]	18	CS, ISET, FISH, rt-PCR	≥4/10	*ALK* rearrangement, monitoring treatment
[51]	33	CS	≥1/7.5	Prediction
[46]	41	CS	≥5/7.5	Prediction
[52]	37	CS	≥2/7.5	Prediction, prognosis
[53]	43	CS	≥5/7.5	Prognosis, prediction

[a] Mutational status was assessed in CTCs and ctDNA. *Abbreviations*: ALK, anaplastic lymphoma kinase; CS, CellSearch®; ctDNA, circulating DNA; EGFR, epidermal growth factor receptor; FISH, fluorescent *in situ* hybridization; IF, immunofluorescence; ISET, isolation by size of epithelial tumor cells; LCM, laser cell microdissection; n.d., not defined; SARMS, Scorpion Amplification Refractory Mutation System.

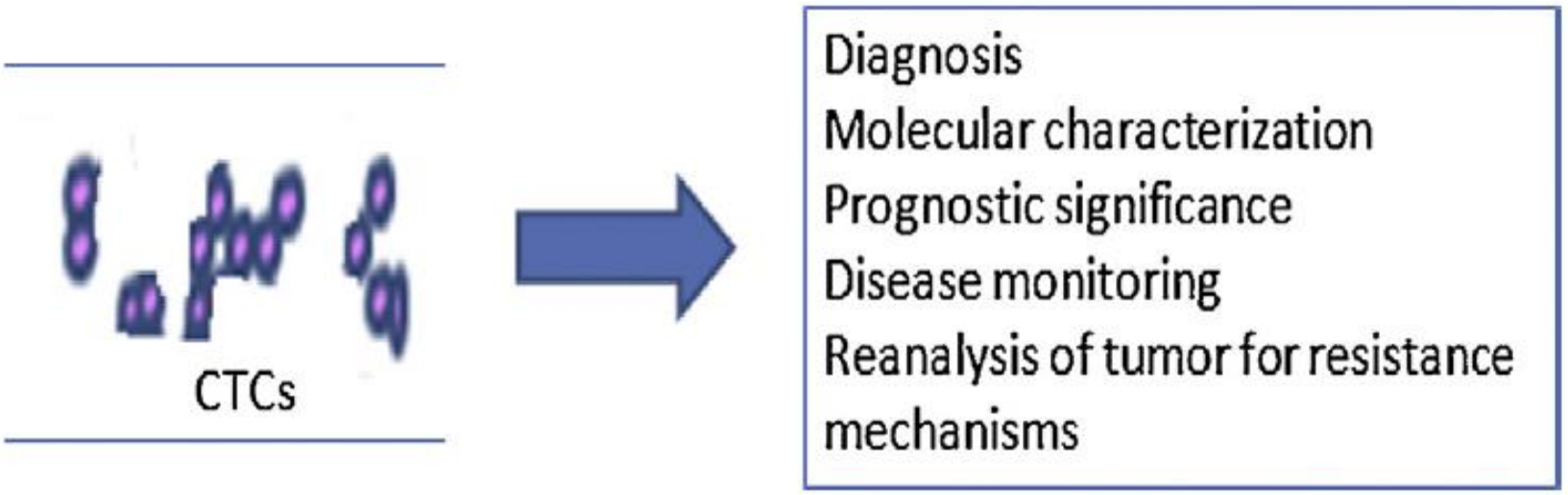


Fig. 4. Possible applications of CTCs in patients with NSCLC.

Table 2
CTCs: future perspectives.

1. Development of standardized methods for the CTCs detection
2. Establishment of recognized cut-off values
3. More powerful and less expensive technologies
4. Correlation with radiological imaging
5. Comparison with other liquid biomarkers (ctDNA, exosomes, secretome)
6. Large well-designed controlled trials

BMJ Open

Circulating tumour cells as a potential screening tool for lung cancer (the AIR study): protocol of a prospective multicentre cohort study in France

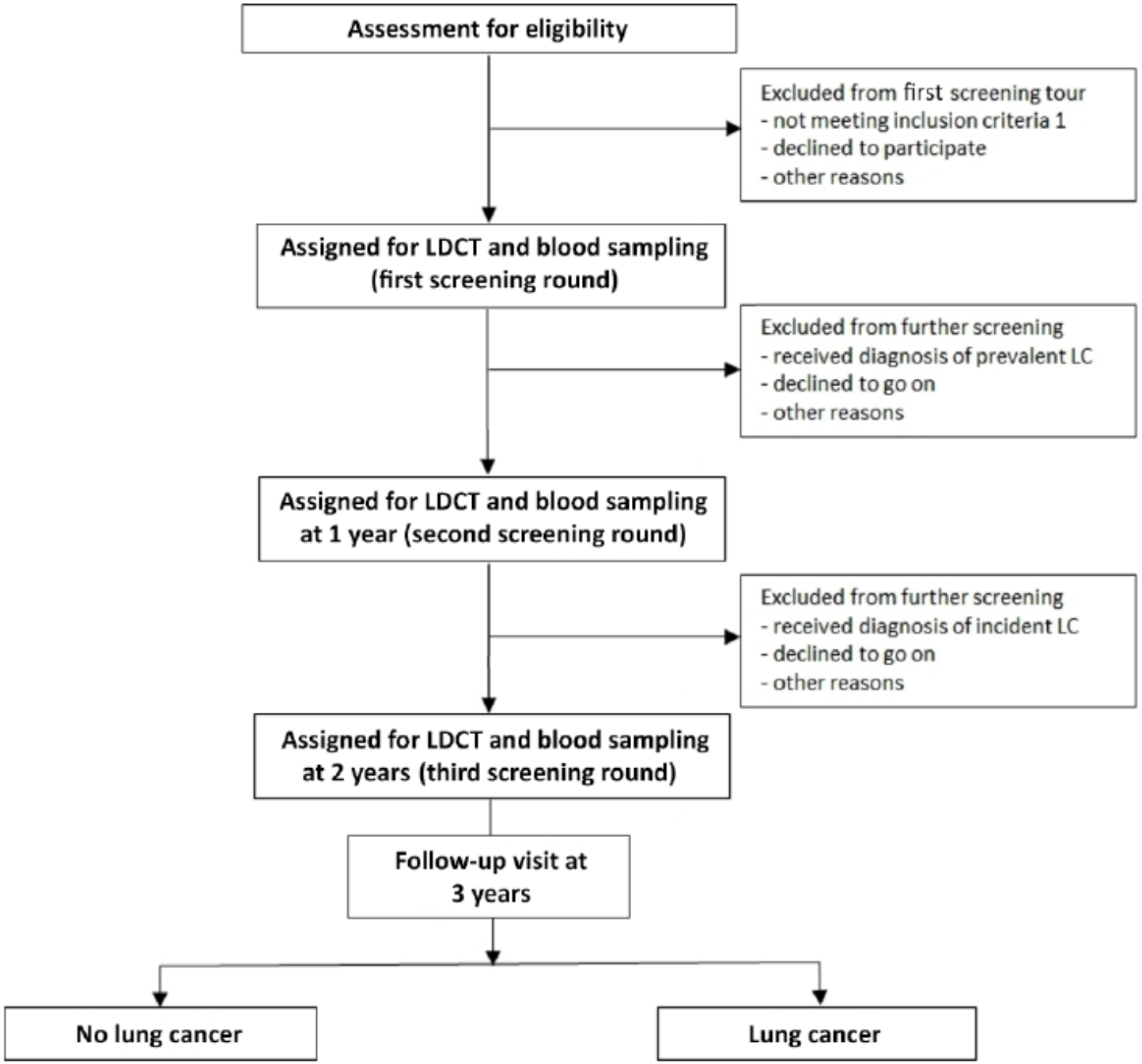
Inclusion criteria	Exclusion criteria
► Age ≥55 years ► Tobacco pack-years ≥30 ► If former smoker, quit within 15 years ► Presence of COPD* ► Affiliation to the French social security system	► Chest CT examination in the 12 months prior to eligibility assessment, except chest CT made in the prior 6 weeks ► Known pulmonary nodule or abnormality warranting radiological follow-up or further diagnostic work-up ► Treatment for, or evidence of, any cancer other than skin basocellular carcinoma in the 5 previous years ► Acute respiratory tract infection treated with antibiotics in the previous 12 weeks ► Unexplained weight loss ≥10% in the previous 12 months ► Recent haemoptysis ► History of lung volume reduction with coils, glue or valves ► Thoracic metallic implants or devices such as Harrington fixation rods ► Participation in another cancer screening trial ► Participation in a cancer prevention study, other than a smoking cessation study ► Vulnerable persons: adults under guardianship, adults under trusteeship or persons deprived of their liberty, participants under 18 years old, medical and/or psychiatric problems

*Compatible medical history and fixed airflow limitation as defined by postbronchodilator FEV1/FVC <0.7.[39]
COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

BMJ Open

Circulating tumour cells as a potential screening tool for lung cancer (the AIR study): protocol of a prospective multicentre cohort study in France

Methods and analysis The AIR Project is a prospective, multicentre, double-blinded, cohort study conducted by a consortium of 21 French university centres. The primary objective is to determine the operational values of CTCs for the early detection of LC in a cohort of asymptomatic participants at high risk for LC, that is, smokers and ex-smokers (≥30 pack-years, quitted ≤15 years), aged ≥55 years, with chronic obstructive pulmonary disease (COPD). The study participants will undergo yearly screening rounds for 3 years plus a 1-year follow-up. Each round will include LDCT plus peripheral blood sampling for CTC detection. Assuming 5% prevalence of LC in the studied population and a 10% dropout rate, a total of at least 600 volunteers will be enrolled.



Flow chart of the AIR Project. LC, lung cancer; LDCT, low-dose chest CT.

BMJ Open 2017;7:e018884

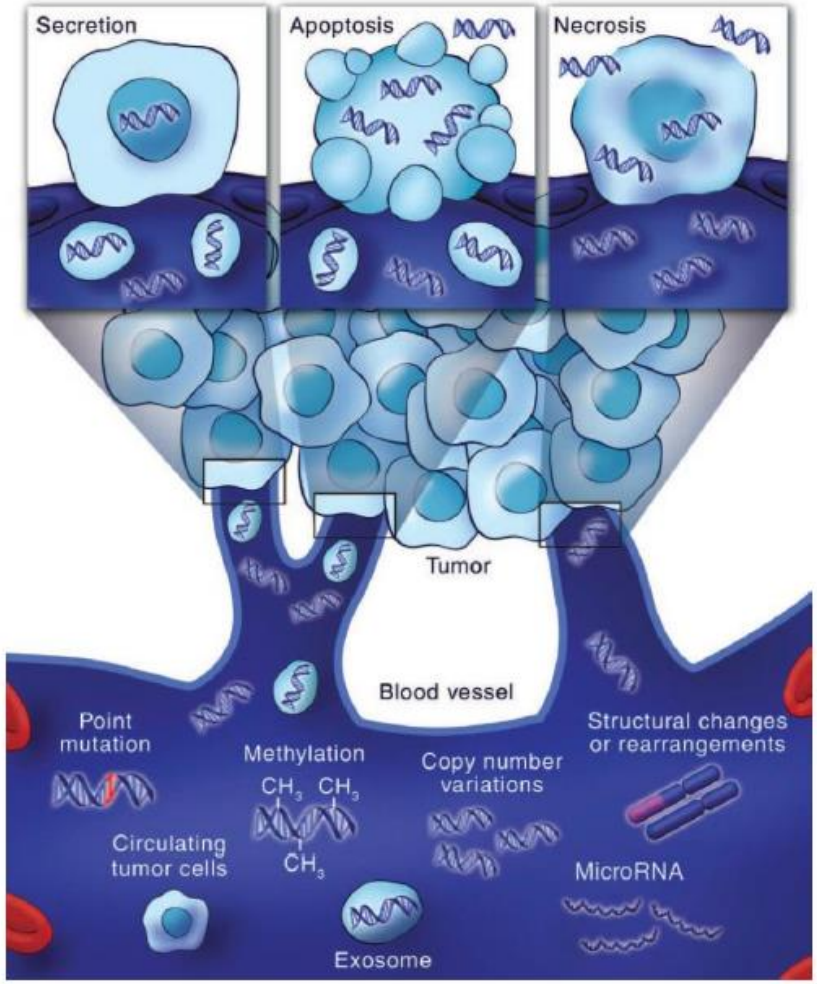
Strengths and limitations of this study

- This study focuses on an unmet need, that is, identification of persons at high risk for lung cancer (LC).
- Previous results from our group suggest that circulating tumour cells (CTCs) is an early event in LC and therefore could be used as a screening tool for LC.
- The main limitation is the insufficient power of this study to show a clinical benefit (ie, reduction of LC mortality).
- Another limitation may be the detection of CTCs from a cancer developing in another organ (such as the bladder) in this high-risk population, although ancillary methods are under development to better characterise the origin of the detected CTCs.

BMJ Open 2017;7:e018884

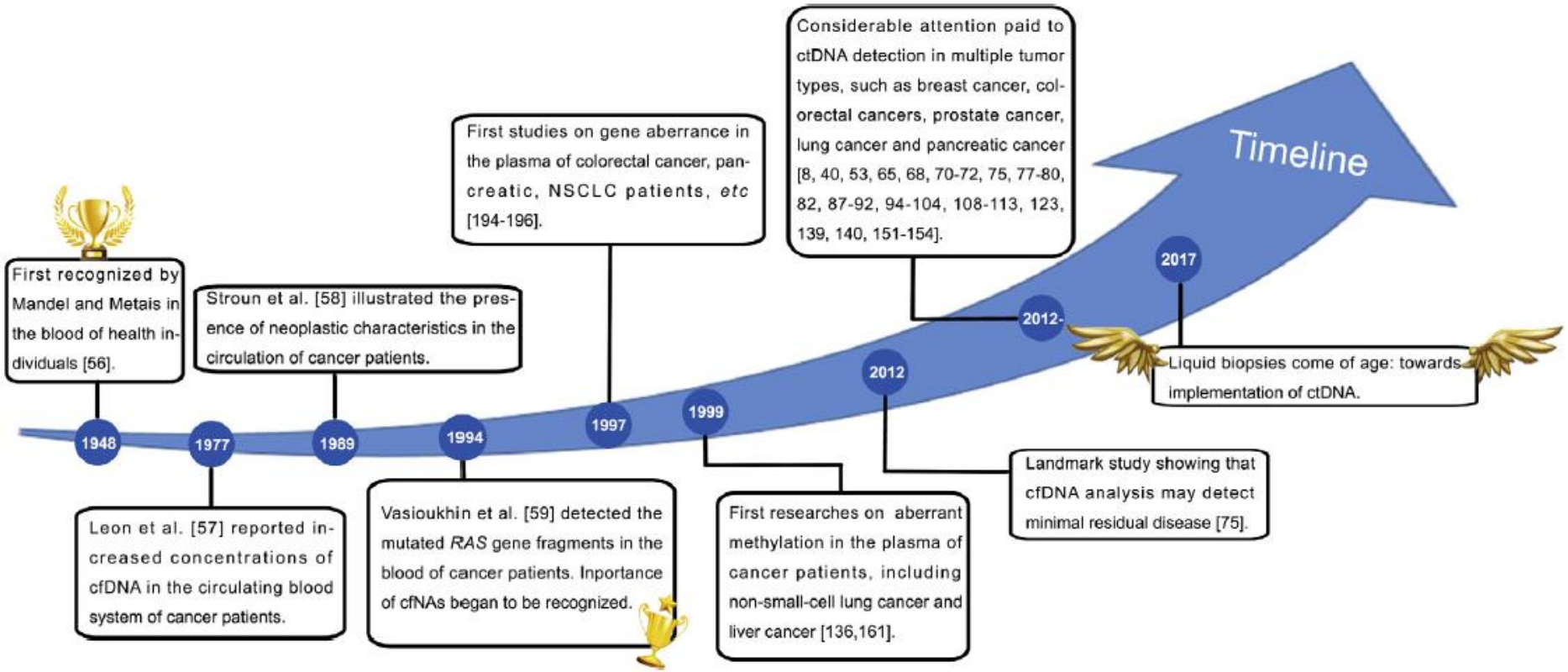
Circulating Tumor DNA (ctDNA)

- ctDNA is tumour DNA that has been shed into the bloodstream
- ctDNA can be present in 0.01% - >90% of the total Cell Free DNA (cfDNA)
- The amount of ctDNA is related to the tumour burden and varies between patients with different clinical presentations



Journal of Clinical Oncology 2014:32

Landmarks in the detection of ctDNA in patients



*Genomics Proteomics Bioinformatics 15(*2017) 59-72

Clinical Relevance of ctDNA

Applications

- Tumor genotyping
- Monitoring tumor burden, therapeutic response and treatment
- Minimal residual disease monitoring and early detection

PCR-based approaches

- Nested real-time PCR
- PARE
- ARMS-Scorpion PCR
- PAP-A amplification
- BEAMing
- Microfluidic digital PCR
- Droplet-based digital PCR

NGS-based approaches

- Targeted deep sequencing
- Tam-Seq
- Safe-Seq
- CAPP-Seq
- Ion-AmpliSeqTM
- Whole exome sequencing
- Whole genome Sequencing

Increasing breadth of analysis

PCR-based Methods

Cobas v2.0
(Cobas)

Therascreen
(Qiagen)

Digital Droplet PCR
(Biorad)

Next generation sequencing

Ion Proton
(Thermo Fisher)

Next-Seq
(Illumina)

Review

Cell-free DNA and next-generation sequencing in the service of personalized medicine for lung cancer

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Keywords: lung cancer, cell-free DNA, next-generation sequencing, personalized medicine, biomarkers

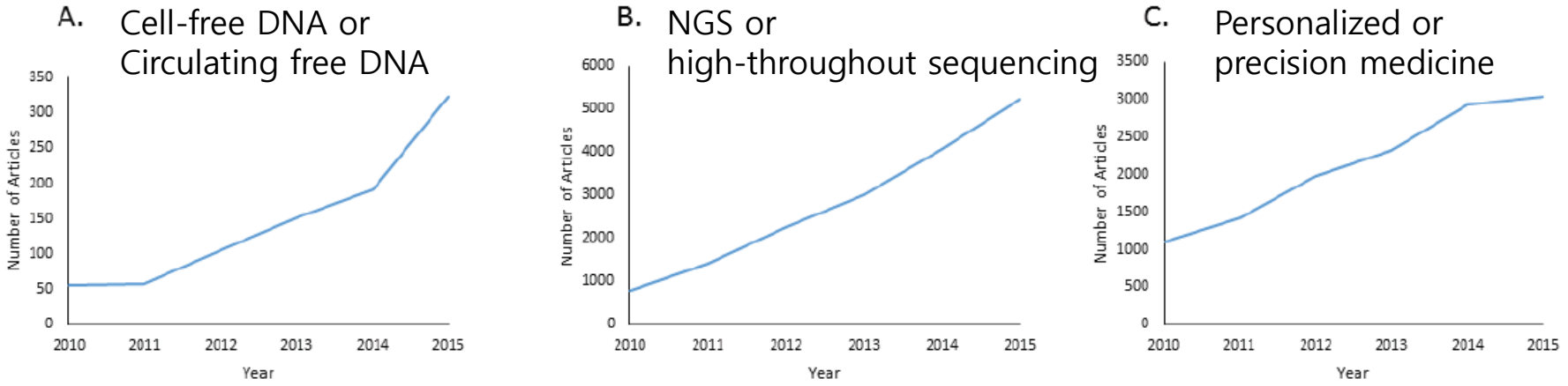


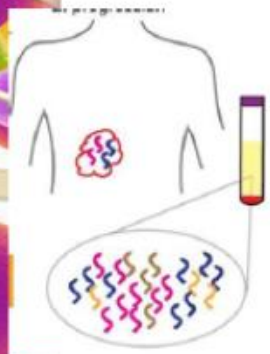
Figure 1: Rise of publications in cell-free DNA, next-generation sequencing, and personalized medicine. A. Increase in publications regarding cell-free DNA from 2010 until 2015. Number of articles determined by Pubmed search of "cell-free DNA" OR "circulating free DNA." **B.** Increase in publications regarding next-generation sequencing from 2010 until 2015. Number of articles determined by Pubmed search of "next-generation sequencing" OR "high-throughput sequencing." **C.** Increase in publications regarding personalized medicine from 2010 until 2015. Number of articles determined by Pubmed search of "personalized medicine" OR "precision medicine."

Reference	Cancer Type	Tissue Type	Methods	Genes/ Mutations	Results
[89]	NSCLC	Plasma cfDNA and tumor tissue	Therascreen® Real-Time PCR and peptide nucleic acid (PNA) clamp Real-Time PCR	EGFR exon 19 del, L858R	Therascreen® RT-PCR: 65.4% sensitivity (sens.), 100% specificity (spec.); 55% detection for exon 19 del. in plasma cfDNA compared to tissue, 100% detection for L858R in plasma cfDNA compared to tissue PNA clamp: 61.5% sens., 100% spec.; 50% detection for exon 19 del in plasma cfDNA compared to tissue, 100% detection for L858R in plasma cfDNA compared to tissue
[90]	NSCLC	Plasma and serum cfDNA and tumor tissue	PNA clamp Real-Time PCR (Taqman)	*EGFR* exon 19 del, L858R	PNA clamp (Taqman): 78% overall concordance rate between plasma/serum cfDNA and tissue, 83.9% exon 19 del concordance rate, 70.7% L858R concordance rate Overall survival (OS): Median OS was shorter in patients with L858R than exon 19 del in cfDNA (13.7 months, 95% CI 7.1-17.7, versus 30.0 months, 95% CI 19.3-37.7)
[91]	NSCLC	Serum cfDNA and tumor tissue	Amplification Refractory Mutation System (ARMS) and Scorpion real-time PCR	*EGFR*	NGS: 23.7% detection rate in cfDNA, 61.5% detection rate in tumor tissue; 56.9% false negative rate for cfDNA Progression Free Survival (PFS): For patients positive for EGFR mutations, those who received gefitinib had longer PFS than those who received carboplatin-paclitaxel.
[93]	NSCLC	Plasma cfDNA and tumor tissue	ARMS Real-Time PCR	*EGFR*	Overall sensitivity in cfDNA: 17.2% Higher sensitivity in later-stage patients: 1.6% in stage IA, 7.9% in stage IB, 11.1% in stage IIA, 20.0% in stage IIB, and 33.3% in stage IIIA Higher sensitivity with lower levels of tumor differentiation: 0%, 15.7% and 36.8% in highly, moderately and poorly differentiated tumors, respectively. Positive ratios of plasma cfDNA compared to tumor tissue: Exon 19 del: 22.5%, L858R: 7.0%, L861Q: 75.0%, exon 20 insertions: 14.3%; T790M, G719X, S768I: 0%
[94]	NSCLC	Plasma cfDNA and tumor tissue	Droplet digital PCR (ddPCR) and ARMS	*EGFR* exon 19 del, L858R	ddPCR of cfDNA compared to ARMS tumor analysis: Exon 19 del: 81.8% sens., 98.4% spec., 94.2% concordance L858R: 80.0% sens., 95.8% spec., 93.0% concordance
[95]	NSCLC	Plasma cfDNA and tumor tissue	ddPCR	*EGFR* exon 19 del, L858R, T790M	Exon 19 del: 76.5% sens., 100% spec., 86.2% concordance with tumor tissue before treatment L858R: 70.8% sens., 100% spec., 87.9% concordance with tumor tissue before treatment Response to TKI: 40 patients with either L858R or exon 19 del in cfDNA at baseline showed a decrease in mutant levels after treatment. The T790M mut. was detected in 8 patients 2-12 months before progression was detected radiographically and in 6 patients at progression.
[96]	NSCLC	Plasma cfDNA and tumor tissue	ARMS and combination of mutant enriched PCR (me-PCR) and denaturing high performance liquid chromatography (DHPLC)	*EGFR* exon 19 del, L858R	Me-PCR and DHPLC: 77.3% sens., 89.6% spec., 85.1% concordance between cfDNA (me-PCR and DHPLC) and tissue (ARMS) Response to TKI: In tumor tissue: Objective response rate (ORR) of 69.4% for patients with *EGFR* mutations; ORR of 13.0% for patients without EGFR mutations In plasma cfDNA: ORR of 64.5% for patients with *EGFR* mutations; ORR of 28.6% for patients without *EGFR* mut.
[97]	NSCLC	Plasma cfDNA and tumor tissue	ddPCR and NGS	*EGFR*	ddPCR: 74% concordance rate between cfDNA and tissue Survival: Longer PFS and OS for patients with *EGFR* mutations in cfDNA and tumor samples versus *EGFR* mutations only in tumor (Median: PFS: 12.6 months versus 6.7 months; OS: 35.6 months versus 23.8 months) ddPCR and NGS: Limit of quantification: 0.04% for ddPCR, 5% for NGS; NGS had 89% sens. and 100% spec.
[98]	NSCLC	Plasma cfDNA and tumor tissue	Digital PCR	*EGFR* T790M	Activating Tumor Mutations: 88.2% of tumor samples had *EGFR* mutations; 58.8% of plasma cfDNA samples had *EGFR* mutations. Resistance Mutations: T790M mutation in plasma cfDNA detected in patients after receiving EGFR-TKIs; 81.8% sensitivity, 85.7% specificity, 83.3% concordance between plasma cfDNA and tumor tissue

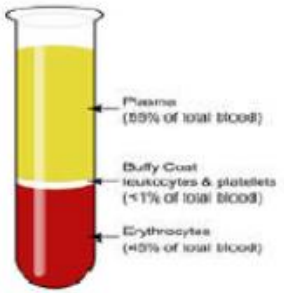
Reference	Cancer Type	Tissue Type	Methods	Genes/ Mutations	Results
[99]	NSCLC	Plasma cfDNA and tumor tissue	Plasma ddPCR and tumor genotyping	*EGFR* exon 19 del, L858R, T790M and KRAS mutations	ddPCR of cfDNA compared to tumor genotyping: *EGFR* Exon 19 deletion: 100% positive predictive value (PPV) and 82% sensitivity *EGFR* L858R: 100% PPV and 74% sensitivity *EGFR* T790M: 79% PPV and 77% sensitivity *KRAS* G12X: 100% PPV and 64% sensitivity
[100]	NSCLC	Plasma cfDNA and tumor tissue	Cobas® EGFR Mutation Test, Therascreen® EGFR Mutation Test, ddPCR and BEAMing dPCR	*EGFR* exon 19 del, L858R and T790M	In a first set of 38 plasma samples: Cobas® Test: 86% and 90% sens., 100% spec., 89% and 97% concordance with tumor tissue for *EGFR* exon 19 del and L858R, resp. Therascreen® Test: 82% and 78% sens., 100% spec., 87% and 95% concordance with tumor tissue for *EGFR* exon 19 del and L858R, resp. ddPCR: 90% sens., 100% spec., 97% concordance with tumor tissue for *EGFR* L858R. BEAMing dPCR: 93% and 100% sens., 100% and 93% spec., 95% concordance with tumor tissue for *EGFR* exon 19 del and L858R, resp. In a second set of 72 plasma samples: Cobas® Test: 73% sens., 67% spec. for *EGFR* T790M BEAMing dPCR: 81% sens., 58% spec. for *EGFR* T790M
[101]	NSCLC with *EGFR* exon 19 del or L858R and acquired *EGFR*-TKI resistance, selected for AZD9291 treatment	Plasma cfDNA	BEAMing dPCR	*EGFR* T790M	Outcomes on AZD9291: If cfDNA *EGFR* T790M-positive, ORR of 63% and median PFS of 9.7 months If tumor *EGFR* T790M-positive, ORR of 62% and median PFS of 9.7 months Conclusions: If cfDNA is T790M-positive, no need for tumor genotyping. However, if cfDNA is T790M-negative, tumor genotyping is warranted
[102]	NSCLC with acquired resistance to AZD9291 treatment	Plasma cfDNA	NGS and ddPCR	*EGFR* exon 19 del, L858R, T790M and C797S	Upon AZD9291 treatment, different resistance phenotypes can emerge from *EGFR* T790M-positive patients : Acquisition of *EGFR* C797S, maintenance of T790M positivity without C797S or loss of *EGFR* T790M. Conclusions: Several mechanisms result in the emergence of resistance to AZD9291. Therapies that overcome resistance due to *EGFR* C797S mutation are needed.
[103]	NSCLC	Tumor tissue	Targeted NGS and Real-Time PCR	*EGFR* T790M and other EGFR and non-EGFR mutations	NGS: T790M detected in 60.0% of patients (all patients previously treated with EGFR-TKIs); Other mutations detected: *TP53* P72R (86.7%), *KDR* Q472H (33.3%), and *KIT* M541L (13.3%); NGS is able to detect T790M mutation better than real-time PCR
[104]	NSCLC	Tumor tissue	NGS deep sequencing	*EGFR*	NGS: 24.6% of samples had compound mutations; 66.7% of compound mutations had an atypical mutation with *EGFR*-TKI sensitizing mutation Survival: Shorter OS for patients with compound mutations (72.8 months) versus patients without compound mutations (83.7 months) Co-occurring mutations: Patients with compound mutations are more likely to have co-occurring mutations in other genes than patients with simple mutations
[105]	NSCLC	Plasma cfDNA, malignant pleural effusion (MPE), and tumor tissue	ARMS for all samples, Sanger sequencing and immunohistochemistry (IHC) for MPE cell block and tumor tissue samples	*EGFR*	ARMS: In MPE cell block samples compared to tissue: 81.8% sens., 80.0% spec., 81% concordance In MPE supernatant compared to tissue: 63.6% sens., 100% spec., 81% concordance In plasma cfDNA compared to tissue: 67.5% sens., 100% spec., 84.9% concordance In MPE supernatant compared to MPE cell block: 69.2% sens., 100% spec., 85.2% concordance Sanger sequencing compared to ARMS: In tumor: 81.8% sens., 100% spec., 91.3% concordance In MPE cell blocks: 40% sens., 100% spec., 72.7% concordance IHC compared to ARMS: In tumor: 54.8% sens., 97.1% spec., 77.3% concordance In MPE cell blocks: 50% sens., 100% spec., 76.9% concordance

Reference	Cancer Type	Tissue Type	Methods	Genes/ Mutations	Results
[106]	NSCLC	Plasma cfDNA and tumor tissue	NGS deep sequencing	*EGFR* exon 19 del, T790M, L858R	NGS of plasma cfDNA compared to tissue samples: For exon 19 deletions: 50.9% sens., 98.0% spec. For L858R: 51.9% sens., 94.1% spec. For T790M in patients after EGFR-TKI: 94.2% spec.
[107]	NSCLC	Plasma cfDNA, tumor tissue, whole blood circulating tumor cells (CTC)	Targeted NGS with PCR amplification and Cobas® EGFR PCR	*EGFR* T790M	Targeted NGS of CTC samples: T790M detected in 50% of samples; 57% concordance between CTC samples and concurrent tissue samples (74% concordance between CTC samples and all tissue samples) Cobas® EGFR PCR for cfDNA samples: T790M detected in 50% of cfDNA samples; 60% concordance between cfDNA and concurrent tissue samples (61% concordance between cfDNA and all tissue samples) cfDNA samples compared to CTC samples: 65% concordance
[110]	NSCLC	Plasma cfDNA and tumor tissue	Cobas® EGFR PCR and ultra deep NGS	*EGFR* exon 19 del, T790M, L858R	Cobas® PCR for cfDNA: 72% sens., 100% spec.; 71% baseline concordance rate between plasma cfDNA and tissue, 73% progression concordance rate between plasma cfDNA and tissue Ultra Deep NGS for cfDNA: 74% sens., 100% spec.; 74% baseline concordance rate between plasma cfDNA and tissue, 73% progression concordance rate between plasma cfDNA and tissue
[121]	Several tumor types, including NSCLC	Tumor tissue	NGS, IHC, and qualitative Real-Time PCR (qRT-PCR)	*MET*	NGS: *MET* exon 14 mutations detected in 28/933 NSCLC patients (3.0%); 61% deletions, 39% point mutations; 29% of patients also had *EGFR* copy gain; 71% of patients had at least 1 mutation in *TP53* or *MDM2* IHC: c-MET expressed more in stage IV samples with *MET* exon 14 mutations than stage I-III samples with *MET* exon 14 mutations qRT-PCR: *MET* exon 14 skipping occurred in 96% of samples tested
[122, 123]	Several tumor types, including NSCLC	Tumor tissue	Targeted NGS	*MET*	NGS: *MET* mutations detected in 221/38,028 specimens, 3% of which (131) were lung adenocarcinomas; *MDM2* and *CDK4* amplification often occurred with *MET* exon 14 splicing mutations but not with *MET* amplification; Patients with *MET* exon 14 alterations, including c.2888-5_2944del62, c.3028G>C, and c.3028+1G>T, showed partial responses to MET inhibitors
[124] case report	NSCLC-Sarcomatoid	Metastasis of primary lung tumor	NGS	*MET*	Mutations detected: c.2888-5_2890TTAAGATC>A and c.3028+2T>G (both thought to contribute to exon 14 skipping), p.H1094Y (c.3280C>T) Crizotinib response: partial response, decrease in lung mass
[125] case report	NSCLC-Adenocarcinoma	Adrenal lesion-metastasis of primary lung tumor	NGS	*MET*	Mutation detected: Intronic deletion c. 2887-18_2887-7del12 Crizotinib response: lung mass improvement and decrease in size of adrenal lesion after 5 weeks
[126] case report	NSCLC-Adenocarcinoma	Metastasis of primary lung tumor	NGS	*MET*	Mutations Detected: *MET* c.2888-19>2888-2delCTTTCTCTCTGTTTTAA, c.3028G>C, c.3028+1G>A, c.3024_3028delAGAAGGTATATT, p.V1001_F1007del (c.3001_3021delGTAGACTACCGA GCTACTTTT), c.3028+1G>T, c.3028G>T, and c.3017_3028delCTTTTCCAGAAGGT *MET* TKI response: 3/4 patients who received TKIs exhibited complete or partial responses

Isolation and processing of ctDNA



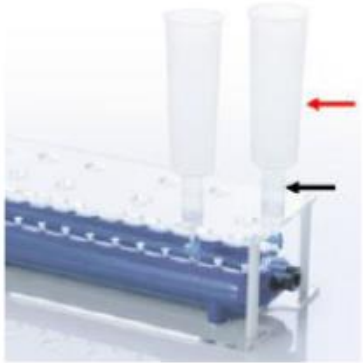
Blood sample taken



Sample spun down to isolate the plasma

Plasma is stored at -80°c

ctDNA is extracted from the plasma using the QIAamp Circulating Nucleic Acid or some other commercial kit



Applications::
Next-generation sequencing
Quantative PCR
BEAMing
Digital PCR

Major techical advantage compared to CTC

Technologies for Detection of ctDNA

Selected technologies for detection of ctDNA.

Technology	Method	Advantage	Limitations	Examples
PCR based	Quantitative PCR	Cost-effective	Detection limited to specified sequence	ARMS allele-specific PCR for detection of EGFR mutations in NSCLC
		High sensitivity and specificity for specific sequence		Ice-cold PCR for detection of BRAF and KRAS mutations in advanced cancers
	Digital PCR	Improved sensitivity and specificity compared to quantitative PCR	Detection limited to specified sequence	Digital droplet PCR for detection of PI3K mutation and HER2 amplification in breast cancer
		Relatively low cost		BEAMing for detection of PI3KCA mutations in breast cancer
NGS based	Targeted sequencing	Allows simultaneous detection of fairly large number of sequences	Higher cost	Tam-Seq panel to detect multiple potential mutations in patient sample (eg, EGFR, PI3KCA, BRAF, and MET)
	Whole-exome sequencing	Allows assessment of copy number changes	Need for bioinformatics support	Assessment of copy number changes, longitudinal tracking of aberrations detected
	Whole-genome sequencing	Allow for detection of genomic rearrangements	Longer readout time required	Assessment of potential mutations or genomic rearrangements, longitudinal tracking of aberrations detected

ARMS, amplification refractory mutation system; BEAMing, beads, emulsions, amplification, and magnetics.

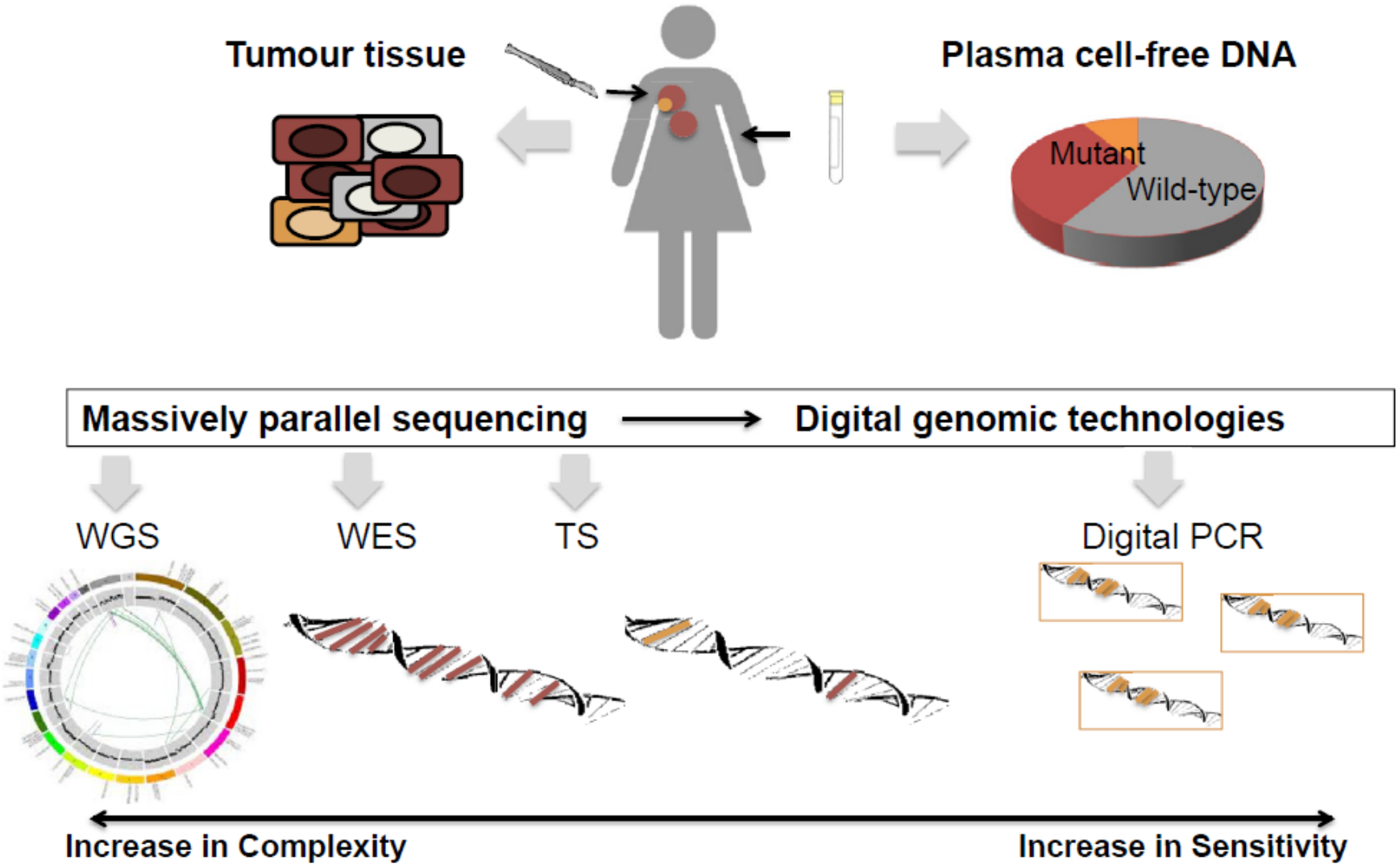


Figure 1 – Schematic for assessing plasma cell-free circulating tumour DNA (ctDNA) and tumour tissue biopsies using massively parallel sequencing and digital genomic technologies. Note that for digital PCR it is essential to know *a priori* the genomic alteration to be targeted. WGS, whole genome sequencing, WES, whole exome sequencing, TS, targeted sequencing.

Limitation of Screening Test

Challenge: Low fraction of tumor-derived DNA

Mutant allele fraction: $\frac{\text{Tumor-derived DNA}}{\text{[Normal+tumor derived DNA]}}$

Metastatic
>5%, average 8-10%
e.g. **Stage IV metastatic patients ~26% have >10% MAF**

Early stage
<1%

Zehir, Berger et al 2017 Nat Med

Possible problems?

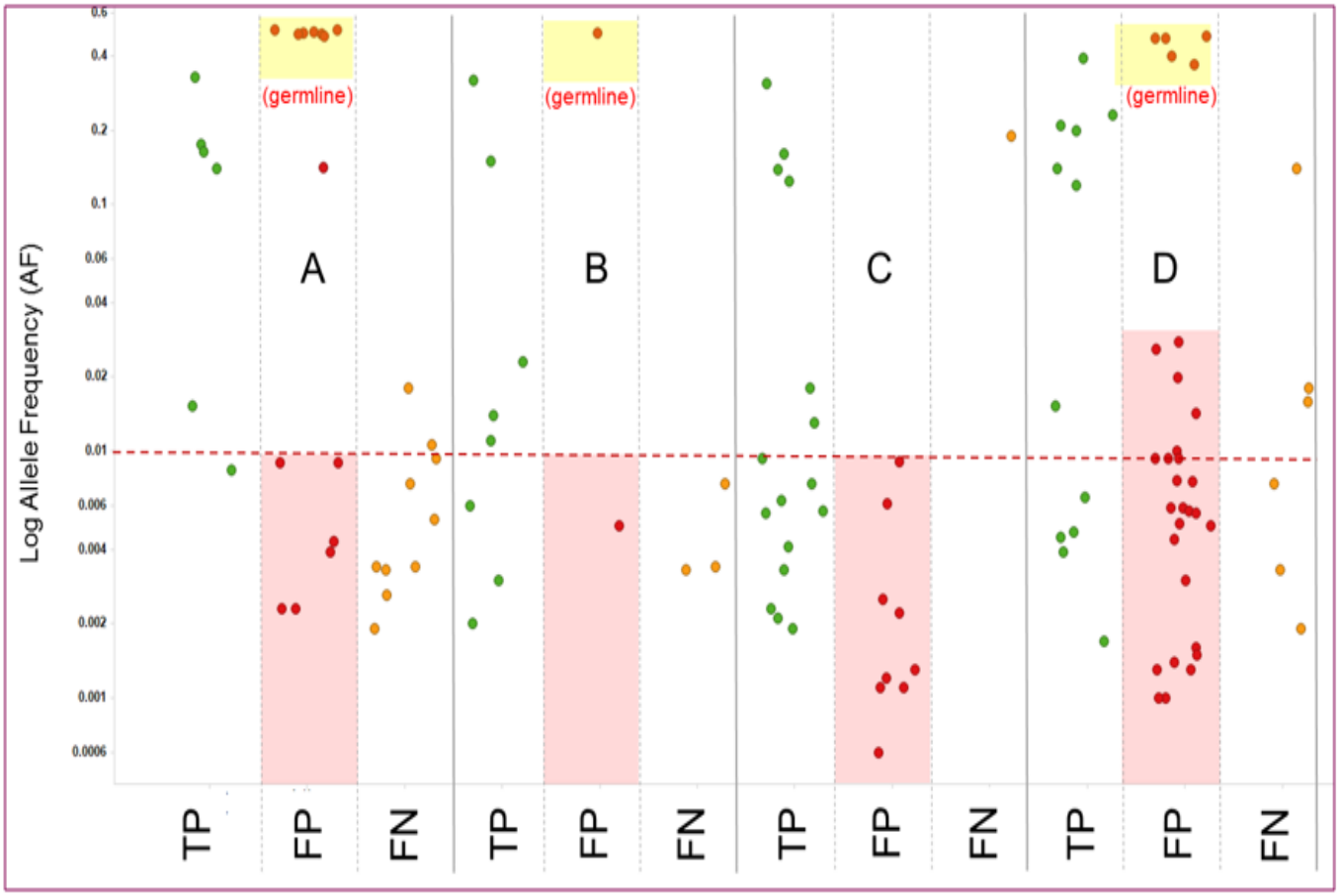
- ctDNA is unstable: requires fast processing
- Getting only 30ng of ctDNA per 5ml plasma extraction
- The technique used must be sensitive enough to pick up the low level variants

Technique	Sensitivity	Optimal Application
Sanger sequencing	> 10%	Tumor tissue
Pyrosequencing	10%	Tumor tissue
Next-generation seqeuncing	2%	Tumor tissue
Quantative PCR	1%	Tumor tissue
ARMS	0.10%	Tumor tissue
BEAMing, PAP, Digital PCR, TAM-Seq	0.01% or lower	ctDNA, rare variants in tumor tissue

Diaz and Bardelli, 2014 Journal of Clincial Oncology 32

Tumor / plasma discordance

- 24 cfDNA samples sent for plasma NGS, compared to tumor:



Stetson et al, AACR, 2017

Tumor / plasma discordance

- 24 cfDNA samples sent for plasma NGS, compared to tumor: **lots of discordance!**
- Let's think some causes…
  1. Variable shed of tumor DNA into cfDNA
  2. Genomic heterogeneity
  3. Complexities of cfDNA content
  4. Problems with assay performance

Stetson et al, AACR, 2017

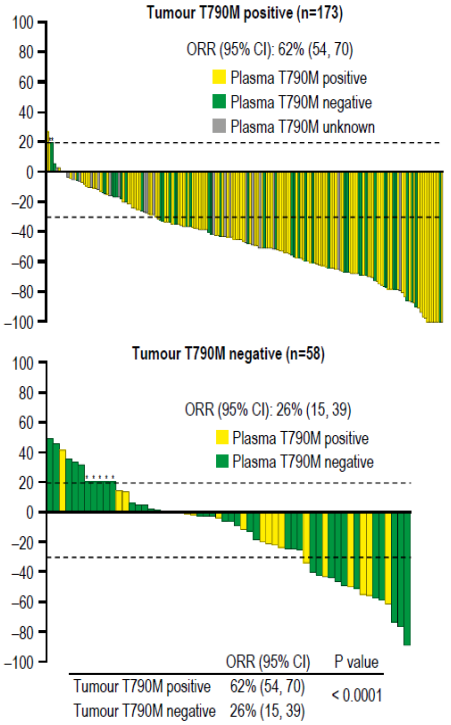
Plasma *EGFR* T790M

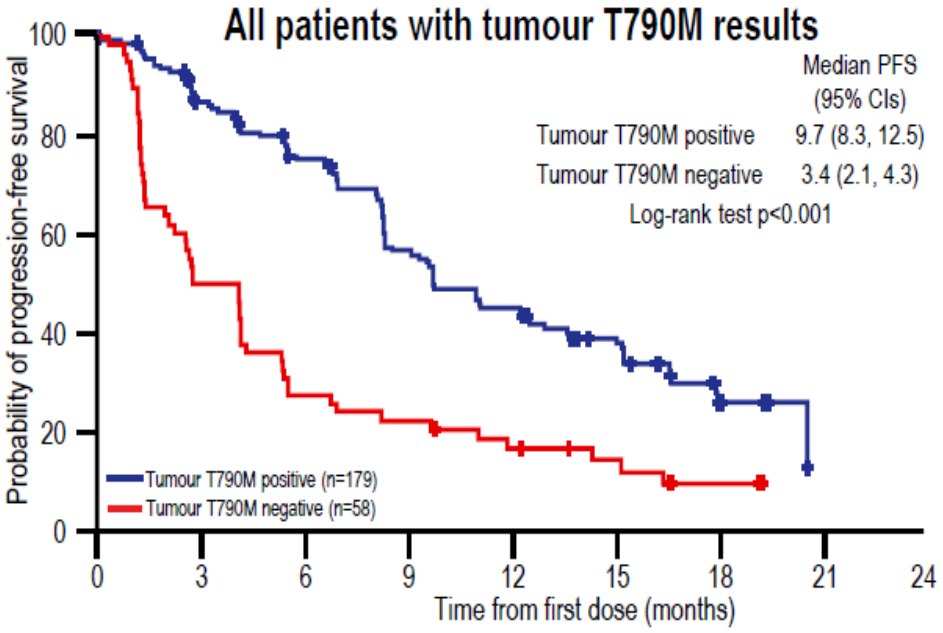
- Osimertinib (AZD9291) :

  T790M status associated with outcome, <u>requires a biopsy</u>

T790M+ in tumor: 62% RR

T790M- in tumor 26% RR

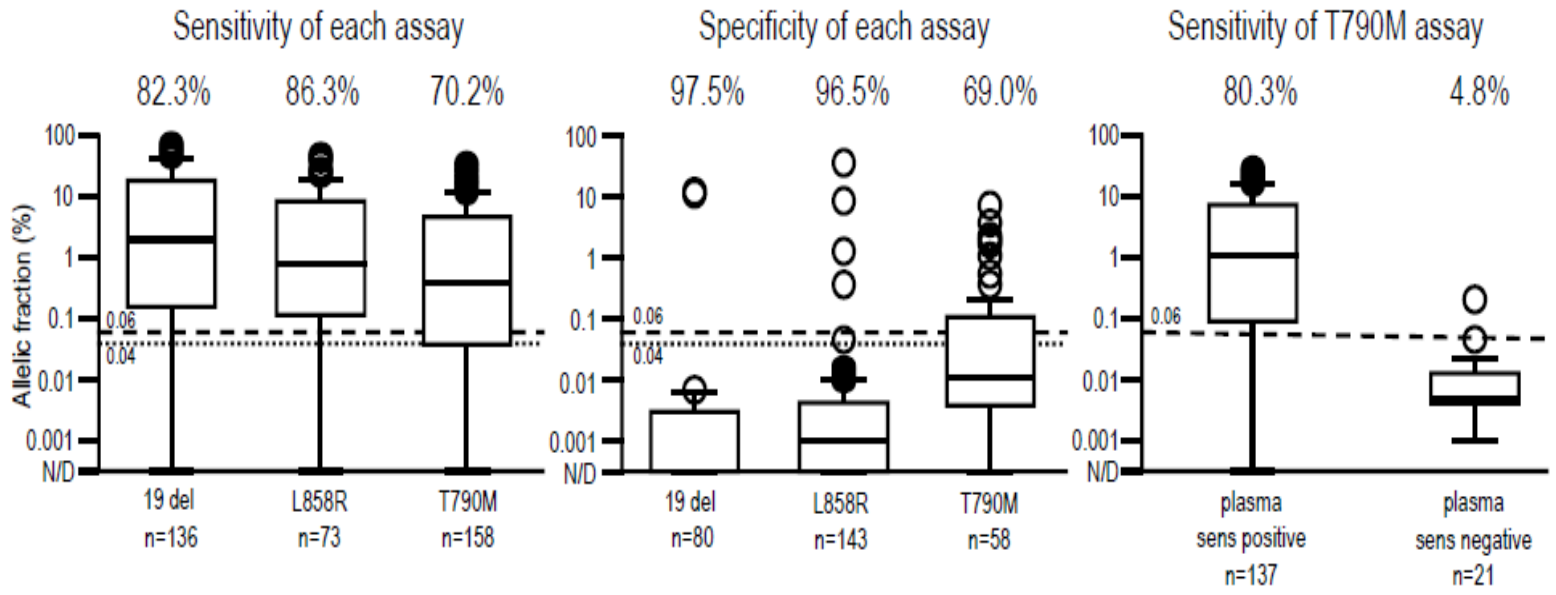




Oxnard et al, JCO, 2016

Plasma *EGFR* T790M

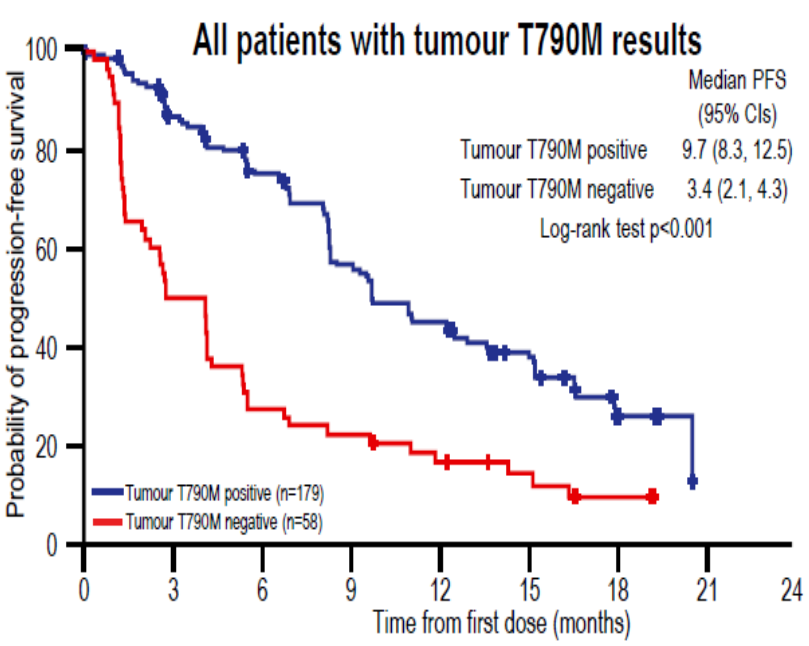
- Plasma from AURA trial sent for BEAMing
  - Paired tumor and plasma available for 216 patients
- Sensitivity compared to tumor genotype was 70%-86%
- High specificity (>95%) for driver EGFR mutations but only 69% specificity for T790M

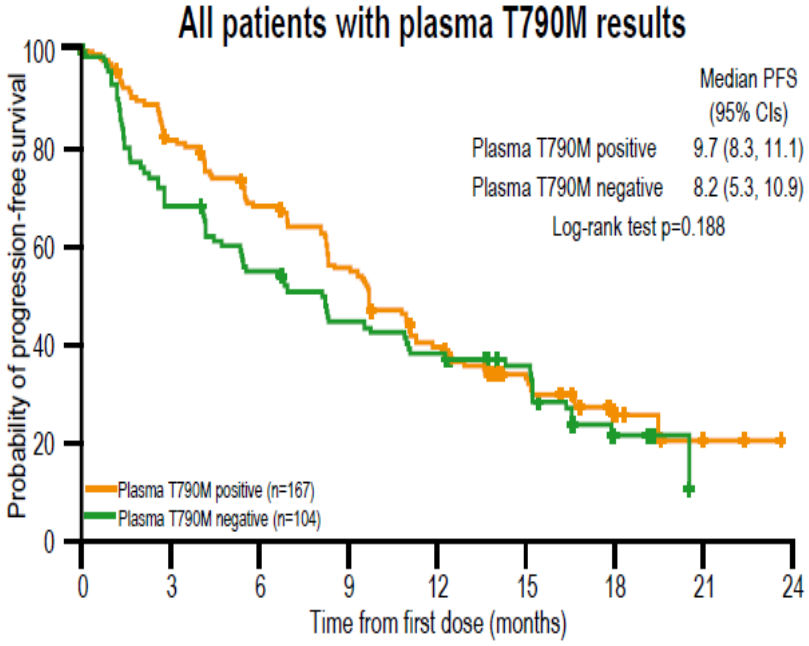


Oxnard et al, JCO, 2016

Plasma *EGFR* T790M

- Plasma from AURA trial sent for BEAMing
  - Paired tumor and plasma available for 216 patients
- Plasma T790M testing not as predictive as tumor T790 M testing

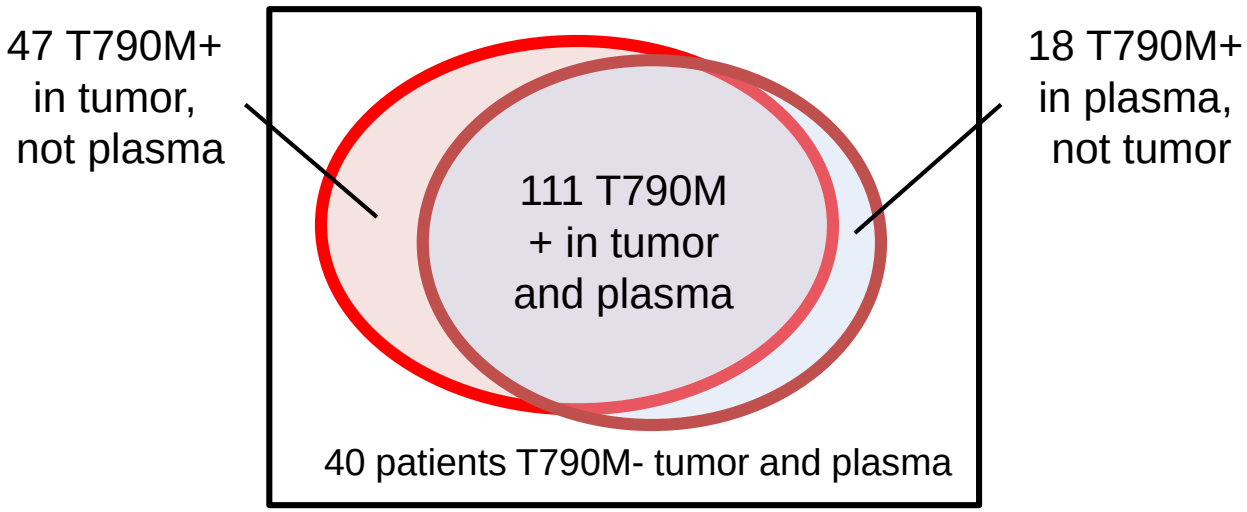




Oxnard et al, JCO, 2016

Plasma *EGFR* T790M

- Plasma from AURA trial sent for BEAMing
  - Paired tumor and plasma available for 216 patients



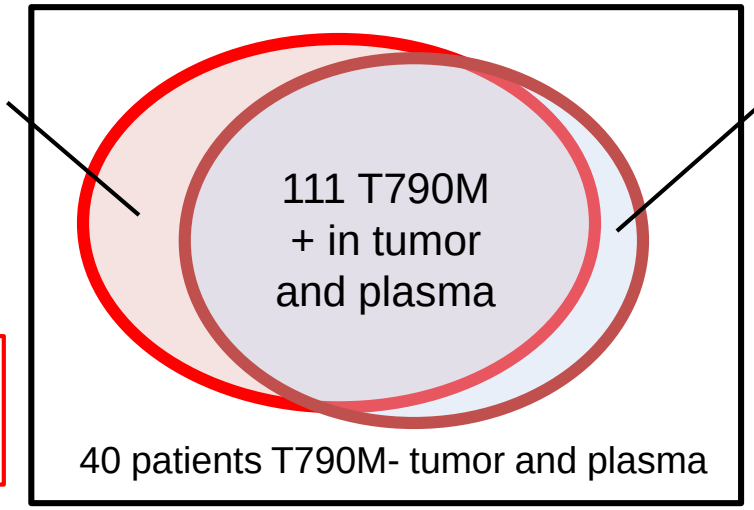
Oxnard et al, JCO, 2016

Plasma *EGFR* T790M

- Plasma from AURA trial sent for BEAMing
  - Paired tumor and plasma available for 216 patients

16.5m PFS

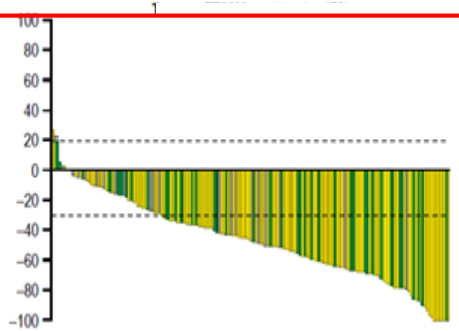
47 T790M+ in tumor, not plasma



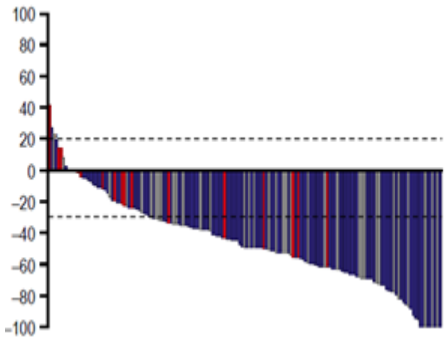
18 T790M+ in plasma, not tumor

4.3m PFS

T790M+ in tumor: 62% RR, 10m PFS



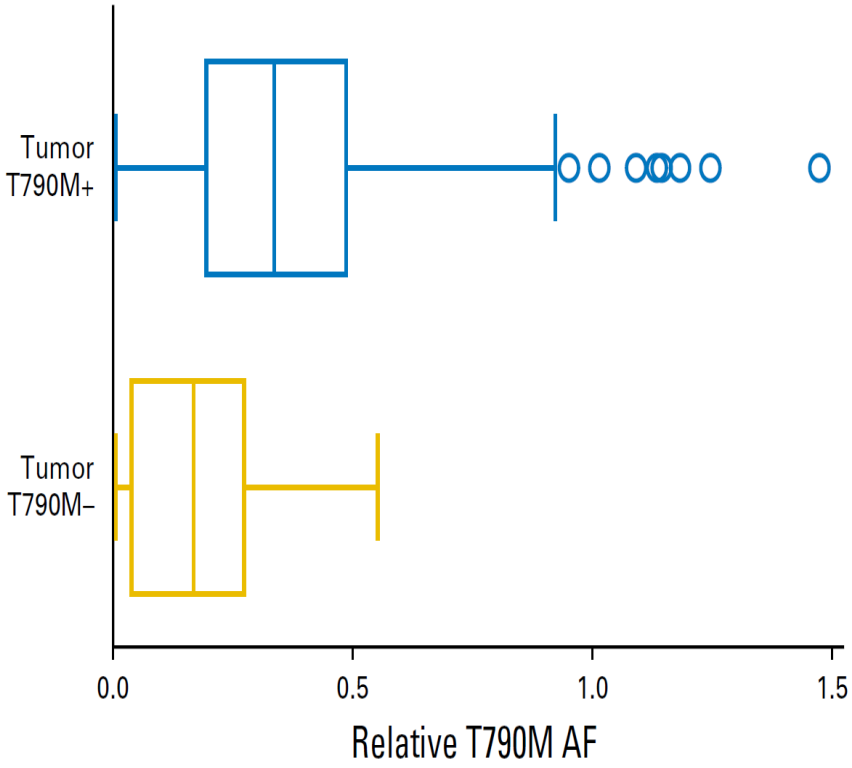
T790M+ in plasma: 63% RR, 10m PFS



Oxnard et al, JCO, 2016

Plasma *EGFR* T790M

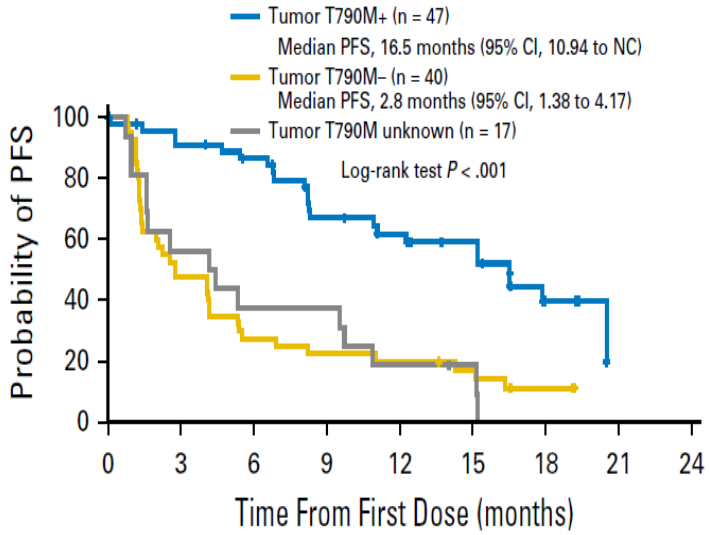
- Plasma from AURA trial sent for BEAMing
  - Paired tumor and plasma available for 216 patients
- Tumor T790M-/plasma T790M+ has lower relative levels of T790M



Oxnard et al, JCO, 2016

Plasma *EGFR* T790M

- Plasma T790M- is a mix of true negative and false negative



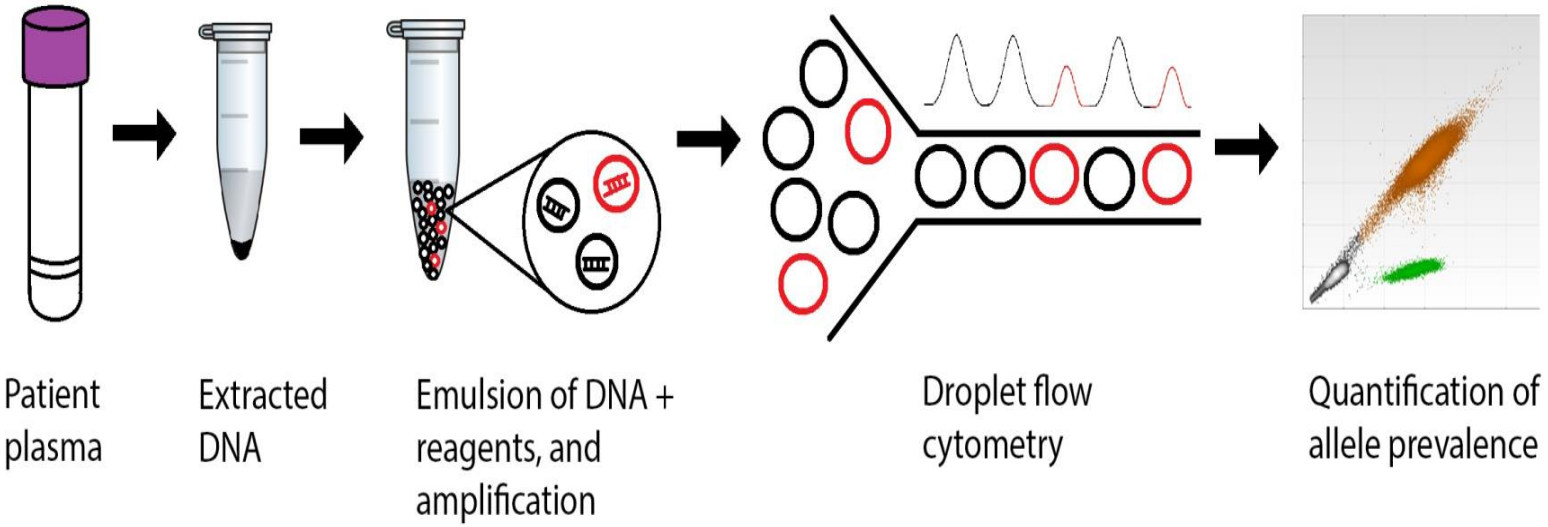
- Proposed algorithm

  where plasma genotyping is a screening test prior to biopsy:
  - If you have tumor, test the tumor
  - If you don't have tumor, plasma is a convenient first pass test
  - If plasma doesn't reveal an actionable finding, tumor genotyping is needed
  - Tumor result trumps plasma result

Oxnard et al, JCO, 2016

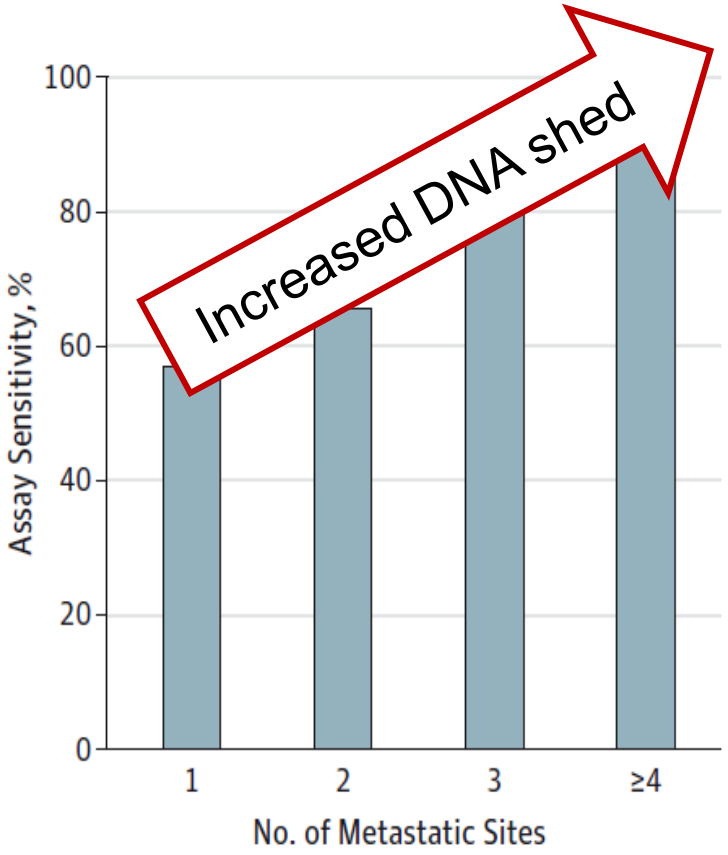
Plasma ddPCR

- Droplet digital PCR is a highly sensitive, quantitative assay for detection of hotspot mutations (e.g. EGFR, KRAS, BRAF, etc)



Plasma ddPCR

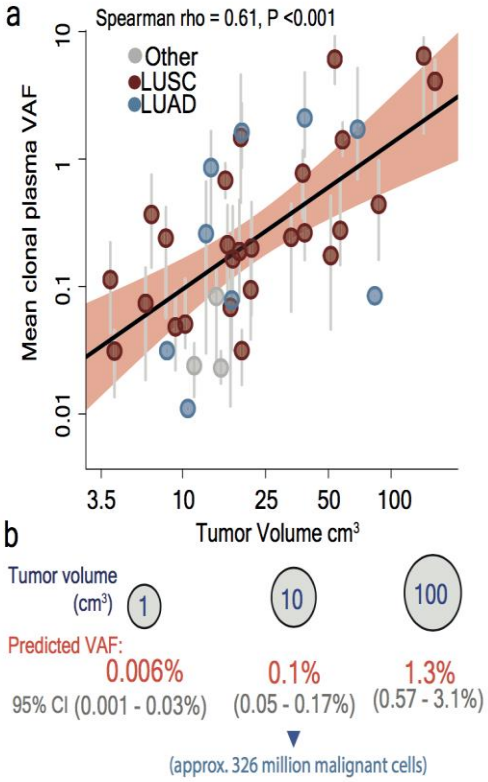
- Completed a prospective validation across 180 patients with NSCLC and known tumor genotype
  - Tested for key mutations in EGFR and KRAS
  - Median TAT ~3 days
  - Sensitivity of 64-82% for detection of known tumor genotype
  - Rate of detection increases with increased tumor burden (increased DNA *shed*)



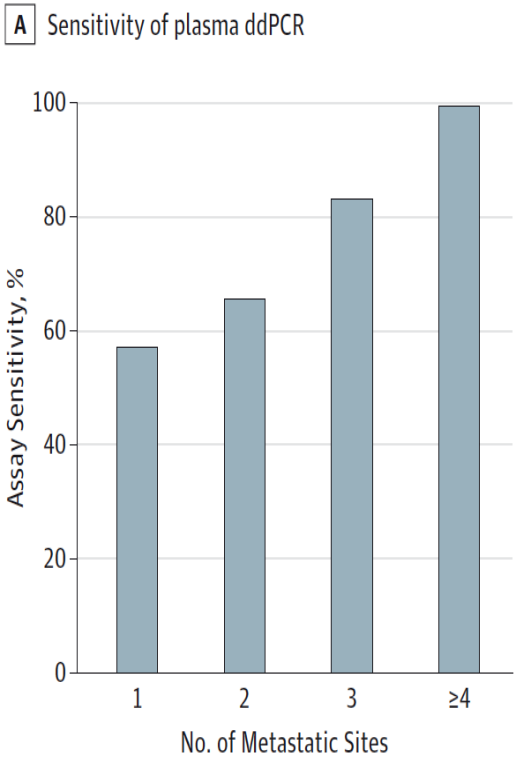
Sacher et al, JAMA Onc, 2016

Are there disease characteristics that may improve performance of plasma-based testing?

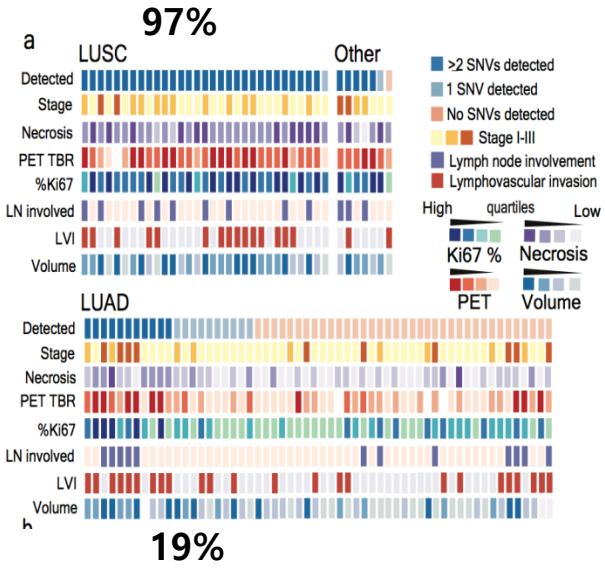
Tumor size



Metastatic burden



Tumor features

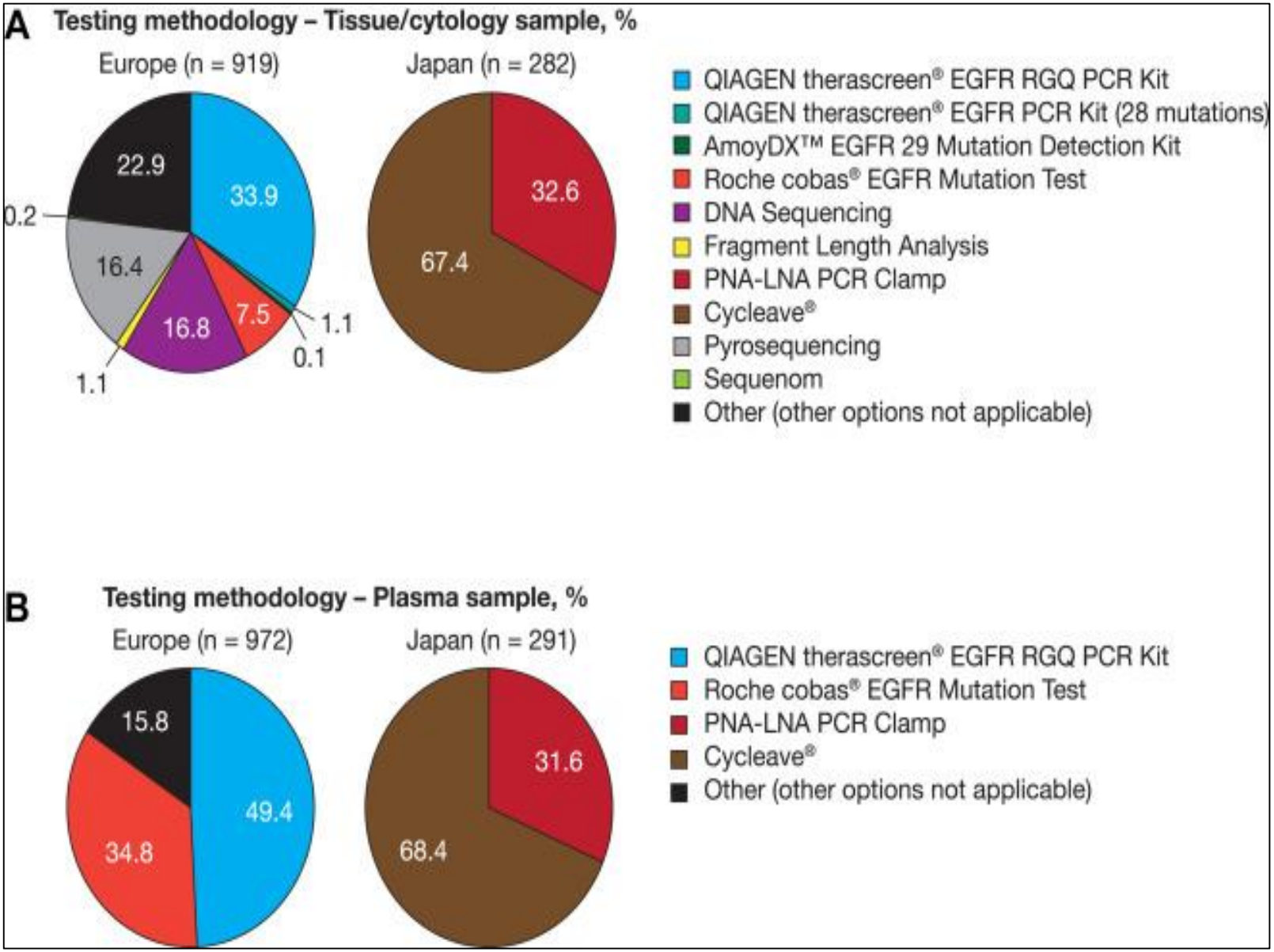


GGO 10x lower DNA concentration

Abbosh et al. Nature 2017; Sacher et al. JAMA Oncology 2016; Chen et al. Scientific Report 2016

Correlation of EGFR mutation status between matched tissue and ctDNA

Study	Method	Matched Samples	Results
Assess Study (Reck et al JTO 2016)	Variable	**1162**	**Concordance = 89%** **Sensitivity = 46%** **Specificity = 97%** **PPV = 78%** **NPV = 90%**
IFUM (Douillard et al JTO 2014)	Therascreen	**652**	**Concordance = 94.3%** **Sensitivity = 65.7%** **Specificity = 99.8%** **PPV = 99%** **NPV = 94%**
FASTACT-2 (Mok et al Clin Cancer Res 2015)	**Cobas**	**238**	**Concordance = 88%** **Sensitivity = 75%** **Specificity = 96%** **PPV = 94%** **NPV = 85%**



Reck et al, JTO 2016

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Tissue and plasma = similar response rates (76.9% and 70%)

Correlation of EGFR mutation status between matched tissue and ctDNA

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Tissue and plasma = similar clinical out comes

Correlation of EGFR mutation status between matched tissue and ctDNA

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Detection of DNA Methylation

Table 4 Methods of detection of DNA methylation in circulating cells

Detection type	Method	Description	Refs.
Site-specific detection	Conventional MSP	Requiring a sample spot (5 ml of peripheral blood); Able to be used in the detection of certain methylated genes in the plasma of serum; using specific PCR primers for methylated sequences	[156–158]
	Fluorescence-based real-time MSP	Facilitating quantitative detection; sensitive; requiring prior knowledge of the methylated sequences	[174]
	QDs-FRET	Able to reduce the background for detecting targets at low concentration; greater sensitivity; limited FRET efficiency; impractical for challenging samples such as serum and plasma	[163]
	MOB	Easy to handle; increased detection throughput; providing efficient, sensitive methylation detection in diagnosis; able to be used in blood samples	[160,164]
	cMethDNA	High sensitivity, specificity, reproducibility, dynamic range, and quantitative advantages; detecting methylated site at low levels in cell-free circulating serum DNA; promising new liquid biopsy tool	[165]
Genome-scale detection	Conventional bisulfite conversion-based methods	Gold standard for the detection of DNA methylation; requiring a relatively large amount of sample; focused on CpG islands or promoter regions	[166–168]
	Conventional enrichment-based methods	No conversion treatment; requiring a high concentration of DNA; likely ignoring other methylated sites when using antibody against 5 mC or 5 mCG	[169–171]
	Short-gun massively parallel bisulfite sequencing	Detecting with high sensitivity and specificity even at a low sequence depth with 10 million sequencing data; requiring 4 ml plasma only	[172]
	MCTA-seq	Working well with ctDNA samples as small as 7.5 pg; able to simultaneously detect thousands of hypermethylated CpG islands in cfDNA	[173]

Note: MSP, methylation-specific PCR; QDs-FRET, quantum dots-fluorescence resonance energy transfer; MOB, methylation on beads; MCTA-seq, methylated CpG tandem amplification and sequencing; ctDNA, circulating tumor DNA; cfDNA, cell-free DNA.

Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer

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ABSTRACT

Aberrant DNA methylation is a common phenomenon in human cancer. The aims of this study were to investigate the methylation profiles of non-small cell lung cancer (NSCLC) in the Chinese population. Twenty tumor suppressor genes (TSGs) were determined of the methylation status using methylation-specific PCR in 78 paired NSCLC specimens and adjacent normal tissues, as well as in 110 Stage I/II NSCLC and 50 cancer-free plasmas. The results showed that, nine genes (APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RARβ and $p16^{INK4A}$) demonstrated a significantly higher frequency of methylation in NSCLC compared with the normal tissues ($P \leqslant 0.001$), while the others (RUNX3, hMLH1, DAPK, BRCA1, $p14^{ARF}$, MGMT, NORE1A, FHIT, CMTM3, LSAMP and OPCML) showed relatively low sensitivity or specificity. Furthermore, methylation of multiple genes was more frequent in cancerous tissue, CpG island methylator phenotype positive (CIMP+) cases were detected in 65.38% of (51/78) NSCLC while only in 1.28% (1/78) of adjacent normal tissues ($P < 0.001$), and CIMP+ was associated with advanced stage ($P = 0.017$), lymphatic metastasis ($P = 0.001$) and adverse 2-year progression-free survival ($P = 0.027$). The nine genes validated in tissues also showed a significantly higher frequency of tumor-specific hypermethylation in NSCLC plasma, as compared with the cancer-free plasmas, and a 5-gene set (APC, RASSF1A, CDH13, KLK10 and DLEC1) achieved a sensitivity of 83.64% and a specificity of 74.0% for cancer diagnosis. Thus, the results indicated that methylated alteration of multiple genes plays an important role in NSCLC pathogenesis and a panel of candidate epigenetic biomarkers for NSCLC detection in the Chinese population was identified.

Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer

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[b] *Department of Cardiothoracic Surgery, Jinling Hospital, Nanjing 210002, China*

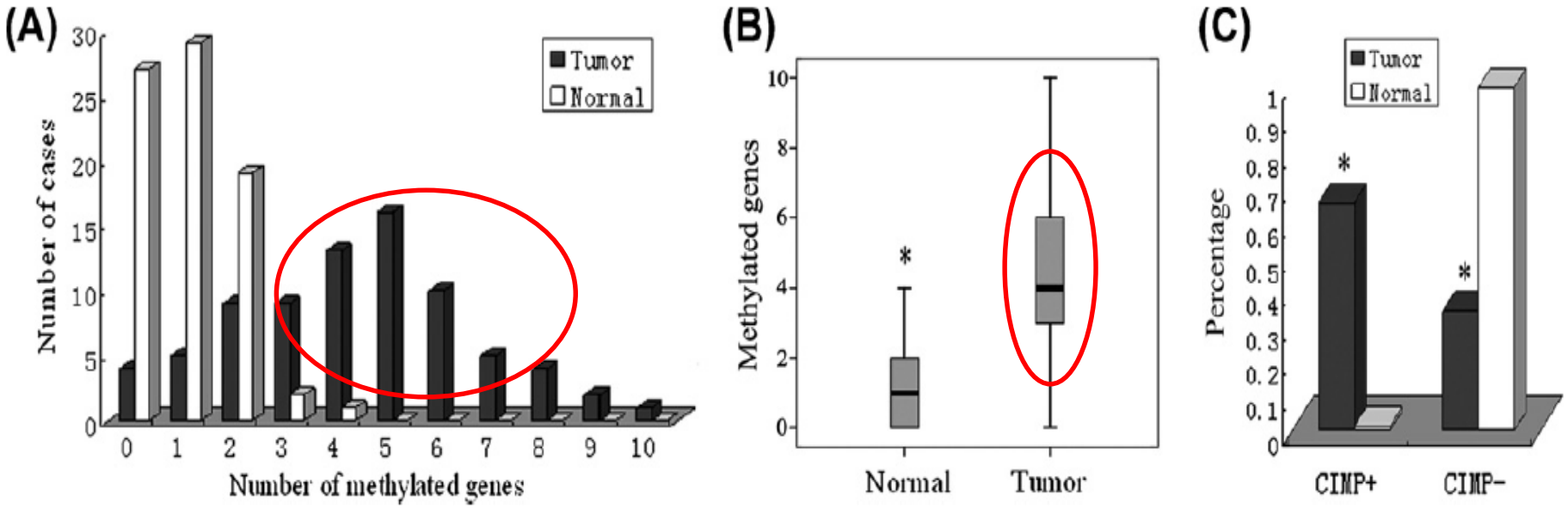

Fig. 2. Histogram of 15 methylated genes distribution. (A) Number of methylated genes per sample in 78 paired NSCLC and normal tissues. (B) Average methylated-gene numbers is significantly different between NSCLC and adjacent normal tissues. $^{*}P < 0.001$. (C) Frequency of the CpG island methylator phenotype (CIMP) in NSCLC and adjacent normal tissues, CIMP+ (more than three methylated genes); CIMP– (3 or 3 less methylated genes). CIMP+ was observed in 65.38% (51/78) of NSCLC, and 1.28% (1/78) of normal lung tissues. CIMP– was observed in 34.62% (27/78) of NSCLC, and 98.72% (77/78) of normal lung tissues. $^{*}P < 0.001$.

Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer

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Methylation profiles in plasma of Stage I/II NSCLC and cancer-free controls.

Genes	Cancerous plasma cases (%), $n = 110$	Non-cancer plasma cases (%), $n = 50$	*P*-value[a]
APC	52 (47.27)	5 (10.0)	<0.001
CDH13	37 (33.64)	2 (4.0)	<0.001
KLK10	32 (29.09)	2 (4.0)	<0.001
DLEC1	28 (25.45)	1 (2.0)	<0.001
RASSF1A	40 (36.36)	4 (8.0)	<0.001
EFEMP1	24 (21.82)	3 (6.0)	0.012
SFRP1	26 (23.64)	2 (4.0)	0.002
RAR β	22 (20.0)	3 (6.0)	0.033
p16INK4A	25 (22.73)	4 (8.0)	0.027

[a] Chi-square test or Fisher's exact test.

Concordance of DNA methylation pattern in plasma and tumor DNA.

Plasma sample		NSCLC tissue		Kappa value
		M	U	
APC	M	24	3	0.758
	U	4	27	
CDH13	M	20	2	0.784
	U	4	32	
KLK10	M	17	1	0.772
	U	5	35	
DLEC1	M	15	0	0.835
	U	4	39	
RASSF1A	M	18	2	0.847
	U	2	36	
EFEMP1	M	15	1	0.834
	U	3	39	
SFRP1	M	13	1	0.820
	U	3	41	
RAR β	M	13	0	0.906
	U	2	43	
$p16^{INK4A}$	M	12	1	0.812
	U	3	42	

M, methylated; U, unmethylated.

Limitation as Screening Test

Logistic regression analysis of risk factors associated with CIMP + NSCLC patients ($n = 78$).

Characteristics	Category	CIMP+	RR (95% CI)	*P*-value
Gender	Male ($n = 58$)	37	1.086 (0.193–6.115)	0.925
	Female ($n = 20$)	14		
Age	<60 ($n = 22$)	12	1.284 (0.296–5.572)	0.739
	⩾60 ($n = 56$)	39		
Histological type	Squamous cancer ($n = 36$)	25	0.449 (0.118–1.715)	0.242
	Adenocarcinoma ($n = 30$)	18	(Squamous *vs.* non-squamous cancer)	
	Others ($n = 12$)	8		
Differentiation	Well ($n = 13$)	6	0.666 (0.185–5.457)	0.702
	Moderate ($n = 46$)	30	(Well/moderate *vs.* poor/undifferentiated)	
	Poor/undifferentiated ($n = 19$)	15		
Tumor size	⩽3 cm ($n = 25$)	14	1.026 (0.255–4.124)	0.971
	>3 cm ($n = 53$)	37		
Stage	I/II ($n = 58$)	33	8.419 (1.472–48.157)	0.017*
	III/IV ($n = 20$)	18		
Lymph metastasis	N_0 ($n = 44$)	24	25.047 (3.966–158.168)	0.001*
	$N_1/N_2/N_3$ ($n = 34$)	27		
Smoking habit	Never ($n = 29$)	17	0.627 (0.170–2.313)	0.483
	Smoker ($n = 49$)	34		

CIMP+: CpG island methylator phenotype positive; RR: Relative risk; 95% CI: 95% confidence interval.

* $P < 0.05$.

Limitation as Screening Test

Table 2 The DNA methylation for cancer detection

Cancer type	Marker	Sensitivity	Specificity	Refs.
Colorectal cancer	*MLH1*	3/18 (17%)	N/A	[128]
	CDKN2A (INK4A)	14/52 (27%)	44/44 (100%)	[129]
	ALX4	21/58 (36%)	N/A	[130]
	CDH4	25/30 (83%)	36/52 (70%)	[131]
	NGFR	32/46 (70%)	17/17 (100%)	[127]
	RUNX3	68/133 (51%)	150/179 (84%)	[132]
	SEPT9	11/17 (65%)	10/10 (100%)	[133,134]
	TMEFF2	87/133 (65%)	123/179 (69%)	[132]
Breast cancer	*CDKN2A (INK4A)*	5/35 (14%)	N/A	[135]
Lung cancer	*CDKN2A (INK4A)*	3/22 (14%)	N/A	[136]
	DAPK1	4/22 (18%)	N/A	
	GSTP1	1/22 (5%)	N/A	

Note: Table was adapted from Jin et al. [126] with permission. MLH1, mutL homolog 1; CDKN2A (INK4A), cyclin dependent kinase inhibitor 2A; ALX4, ALX homeobox 4; CDH4, cadherin 4; NGFR, nerve growth factor receptor; RUNX3, Runt related transcription factor 3; SEPT9, septin 9; TMEFF2, transmembrane protein with EGF-like and two follistatin-like domains 2; DAPK1, death associated protein kinase 1; GSTP1, glutathione *S*-transferase Pi 1.

Limitation as Screening Test

Representative receiver operating characteristics (ROC) of the informative sets for NSCLC detection.

No.	Gene sets	TP/FN	FP/TN	Sensitivity (%), TP/(TP + FN)	Specificity (%), TN/(TN + FP)
1	APC	52/58	5/45	47.27	90.0
2	APC, RASSF1A	68/42	9/41	61.82	82.0
3	APC, RASSF1A, CDH13	79/31	10/40	71.82	80.0
4	APC, RASSF1A, CDH13, KLK10	87/23	12/38	79.09	76.0
5	APC, RASSF1A, CDH13, KLK10, DLEC1	92/18	13/37	83.64	74.0
(5)	APC, RASSF1A, CDH13, KLK10, SFRP1	92/18	14/36	83.64	72.0
(5)	APC, RASSF1A, CDH13, KLK10, $p16^{INK4A}$	91/19	15/35	82.73	70.0
6	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1	95/15	15/35	86.36	70.0
(6)	APC, RASSF1A, CDH13, KLK10, DLEC1, RAR β	93/17	15/35	84.55	70.0
(6)	APC, RASSF1A, CDH13, KLK10, DLEC1, EFEMP1	94/16	15/35	85.45	70.0
(6)	APC, RASSF1A, CDH13, KLK10, DLEC1, $p16^{INK4A}$	95/15	16/34	86.36	68.0
7	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, $p16^{INK4A}$	97/13	17/33	88.18	66.0
(7)	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, EFEMP1	97/13	17/33	88.18	66.0
(7)	APC, RASSF1A, CDH13, KLK10, DLEC1, $p16^{INK4A}$, EFEMP1	97/13	18/32	88.18	64.0
8	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, $p16^{INK4A}$, EFEMP1	99/11	19/31	90.0	62.0
9	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, $p16^{INK4A}$, EFEMP1, RAR β	99/11	21/29	90.0	58.0

TP, true positive; FN, false negative; FP, false positive; TN, true negative.
No. was number of the genes in each set.

Circulating tumor RNA (miRNA)

- In blood, miRNAs are highly stable, because most of them are included in apoptotic bodies, microvesicles, or exosomes and can withstand known mRNA degradation factors
- miRNAs are ~21 nt, have potential to serve as diagnostic markers
- miRNA expression is frequently deregulated in cancer
- Long non-coding RNAs (lncRNAs) play regulatory roles in cancer progression and metastasis
- The long non-coding PCA3 RNA-based urine test is the first FDA-approved test for the diagnosis of prostate cancer patients

Nat. Rev. Cancer 11 (2013) 426
Biomed. Res. Int. 2014;591703:17

miRNAs in Lung Cancer

microRNAs	Gene Targets	Biological Processes
Tumor suppressor microRNAs with down-regulation in lung cancer		
let-7 family	*RAS, HMGA2, CDK6, MYC, DICER1*	(i) Cell proliferation (*RAS, MYC, HMGA2*) (ii) Cell cycle regulation (*CDK6*) (iii) microRNA maturation (*DICER1*)
miR-34 family	*MET, BCL2, PDGFRA, PDGFRB*	TRAIL-induced cell death and cell proliferation
miR-200 family	*ZEB1, ZEB2, E-cadherin (CDH1), vimentin (VIM)*	Promotion of EMT and metastasis
Oncogenic microRNAs with up-regulation in lung cancer		
miR-21	*PTEN, PDCD4, TPM1*	Apoptosis, cell proliferation, and migration
miR-17-92 cluster	*E2F1, PTEN, HIF1A*	Cell proliferation and carcinogenesis
miR-221/222	*PTEN, TIMP3*	Apoptosis and cell migration

TRAIL: TNF-related apoptosis-inducing ligand; EMT: epithelial-mesenchymal transition.

Inamura, J Clin Med 2016

Comparison of Different Cancer Detection Methods

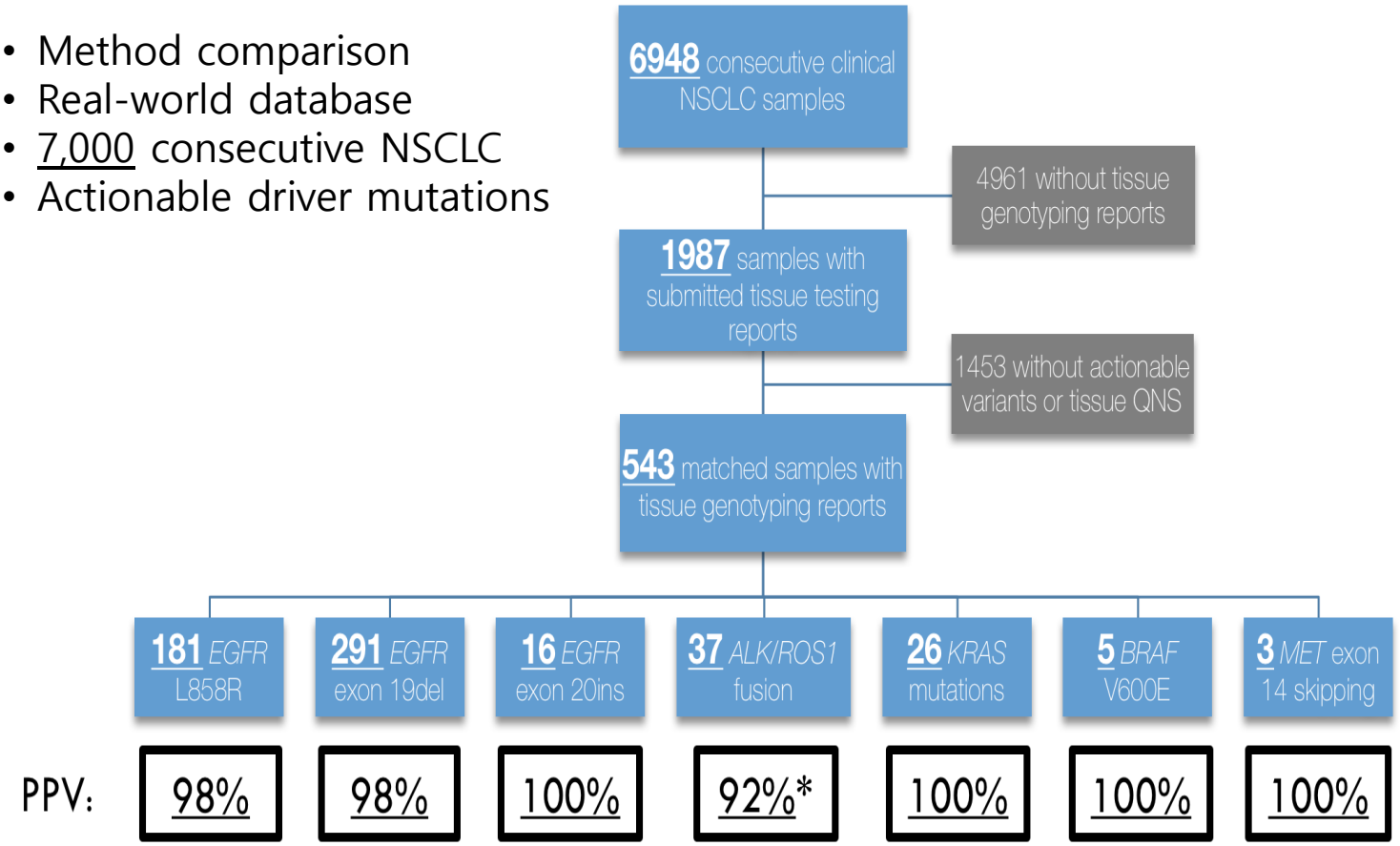
Table 1 Comparison of different cancer detection methods for their clinical utilities

Detection method		Strengths	Limitations	Refs.
Imaging-based methods (CT, MRI, PET, *etc.*)		Rapid; easy to use; displaying solid tumor visually	Unable to detect minimal residual disease; exposing patients to additional ionizing radiation	[19–21]
Solid biopsy		Reflecting certain histological issues; short operating time	Unable to represent the entre tumor due to the intra- and inter-tumor heterogeneity; serial biopsy often impractical; discomfort suffered by the patient; not accessible for some tumors	[22–25]
Liquid biopsy	Protein (CA-125, CEA, PSA, *etc.*)	Non-invasive; easy to obtain	Low specificity; Unable to be detected in vast majority of patients with advanced cancers	[26–28]
	CTCs	Non-invasive; high specificity; demonstrating colocalization of signals; evaluating protein expression; potentially addressing tumor heterogeneity	Low signal-to-noise; affected by heterogeneity on selection methods	[7,11,29]
	ctDNA	Non-invasive; high specificity and sensitivity; providing personalized snapshot of disease; fully representing tumors	Low signal-to-noise; lack of colocalization, protein expression, and functional studies	[10,30,31]
	Circulating cfRNA	Non-invasive; stable; demonstrating distinct gene expression patterns from particular tumor	Lack of large-scale studies; lack of correlations between tumor behavior and findings	[32–34]
	Exosomes	Non-invasive; stable within exosomes; easy to isolate or enrich	Lack of large-scale studies; hard to define	[35–37]

Note: CT, computed tomography; MRI, magnetic resonance imaging; PET, positron emission tomography; CA-125, carcinoma antigen-125; CEA, carcinoembryonic antigen; PSA, prostate-specific antigen; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; cfRNA, cell-free RNA.

High PPV for NGS panels

- Method comparison
- Real-world database
- 7,000 consecutive NSCLC
- Actionable driver mutations



Zill et al. ASCO 2016

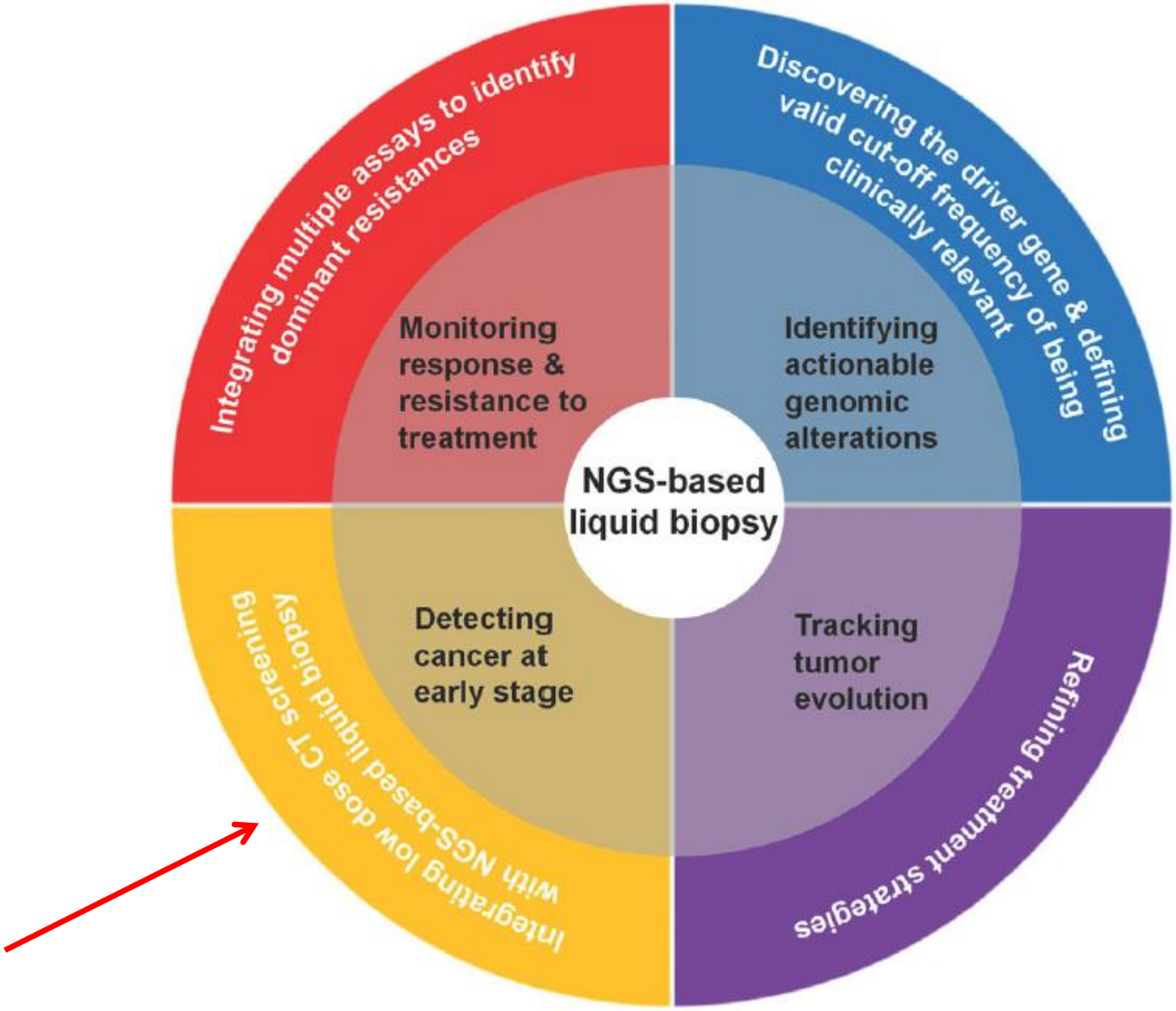
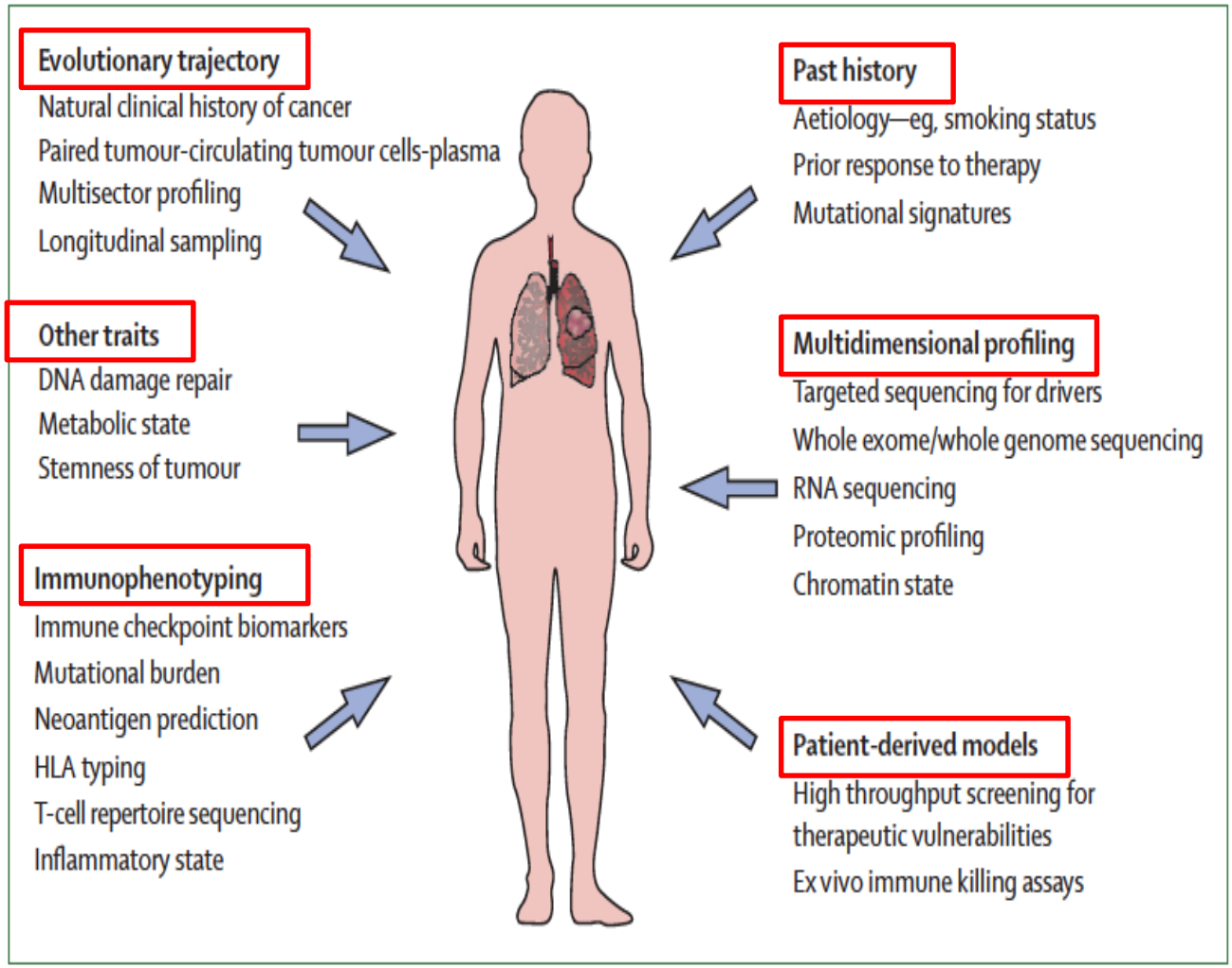


Fig. 2 Current applications and future development of NGS-based liquid biopsy in lung cancer

Ideal profiling for n=1



Tan WL et al. TLO 2016

Liquid Biopsy Test to Potential Cancer Screening Test

Table 1. Comparison of ctDNA Liquid Biopsy Test to Potential Cancer Screening Test

Indication	Tumor Liquid Biopsy (Genotyping, Monitoring)	Early Cancer Detection
Target population	Patients with known diagnosis of cancer	Asymptomatic individuals
Tissue reference	Can be informed by tissue analyses	No prior knowledge of tissue
Key performance characteristics	Sensitivity and specificity for specific actionable genotypes	• Sensitivity and specificity for clinically detectable cancer • Premium on specificity in individuals without detectable cancer • Tissue of origin needed to guide workup
Clinical Endpoint for Utility	Therapeutic benefit with specific therapies	Net outcome improvement with early detection and local treatment of cancer
Genes Covered	10-50	100-1000s
ctDNA Limit of Detection	0.1%	<0.01%
Importance of Novel Variant Detection	Low	High
Amount of Sequencing	1x	100X
Study Size for Clinical Validity and Utility	100's	10,000 - 100,000 s

Ongoing Studies of Circulating Biomarkers

Table 2 Examples of Ongoing Studies of Circulating Biomarkers in NSCLC

Biomarker	Type of Study	Purpose/Clinical Setting	Clinicaltrials.gov Identifier
Circulating Tumor Cells	Observational	Postoperative detection of CTCs at baseline and up to 36 months after surgery	NCT02647164
	Observational	Detection of CTCs in patients undergoing definitive radiotherapy or chemoradiotherapy	NCT02135679
	Observational	Enumeration, phenotypical, and molecular characterization of CTCs in advanced disease	NCT02407327
	Observational	Correlation of EGFR mutation status between cfDNA and CTCs	NCT02422628
	Nonrandomized interventional	Diagnostic accuracy of FISH for ALK rearrangement detection in CTCs captured using ISET	NCT02372448
Circulating DNA	Observational	Mutational profiling of advanced NSCLC	NCT02169349
	Observational	Postoperative detection of ctDNA at baseline and up to 36 months after surgery	NCT02696525
	Observational	Identification and monitoring of ALK rearrangements in ctDNA	NCT02718651
	Observational	Quantitative and qualitative analysis of ctDNA based on known molecular alterations	NCT01930474
	Observational	Prognostic value of ctDNA in advanced disease	NCT02245100
	Phase 2	Evaluation of resistance to osimertinib in ctDNA and CTCs	NCT02771314
	Phase 2	Osimertinib efficacy in patients tested for positive for EGFR mutations and/or T790M mutation in ctDNA	NCT02769286
Micro RNA	Observational	Detection of microRNA signature pattern in NSCLC patients	NCT02445924
	Phase 3	Micro RNA profiling in healthy smokers as first line screening	NCT02247453

Abbreviations: ALK = anaplastic lymphoma kinase; cfDNA = cell-free DNA; CTC = circulating tumor cell; ctDNA = circulating tumor DNA; EGFR = epidermal growth factor receptor; FISH = fluorescence in situ hybridization; ISET = isolated according to size of epithelial tumor cells (RareCell Diagnostics, Paris, France); NSCLC = non–small-cell lung cancer.

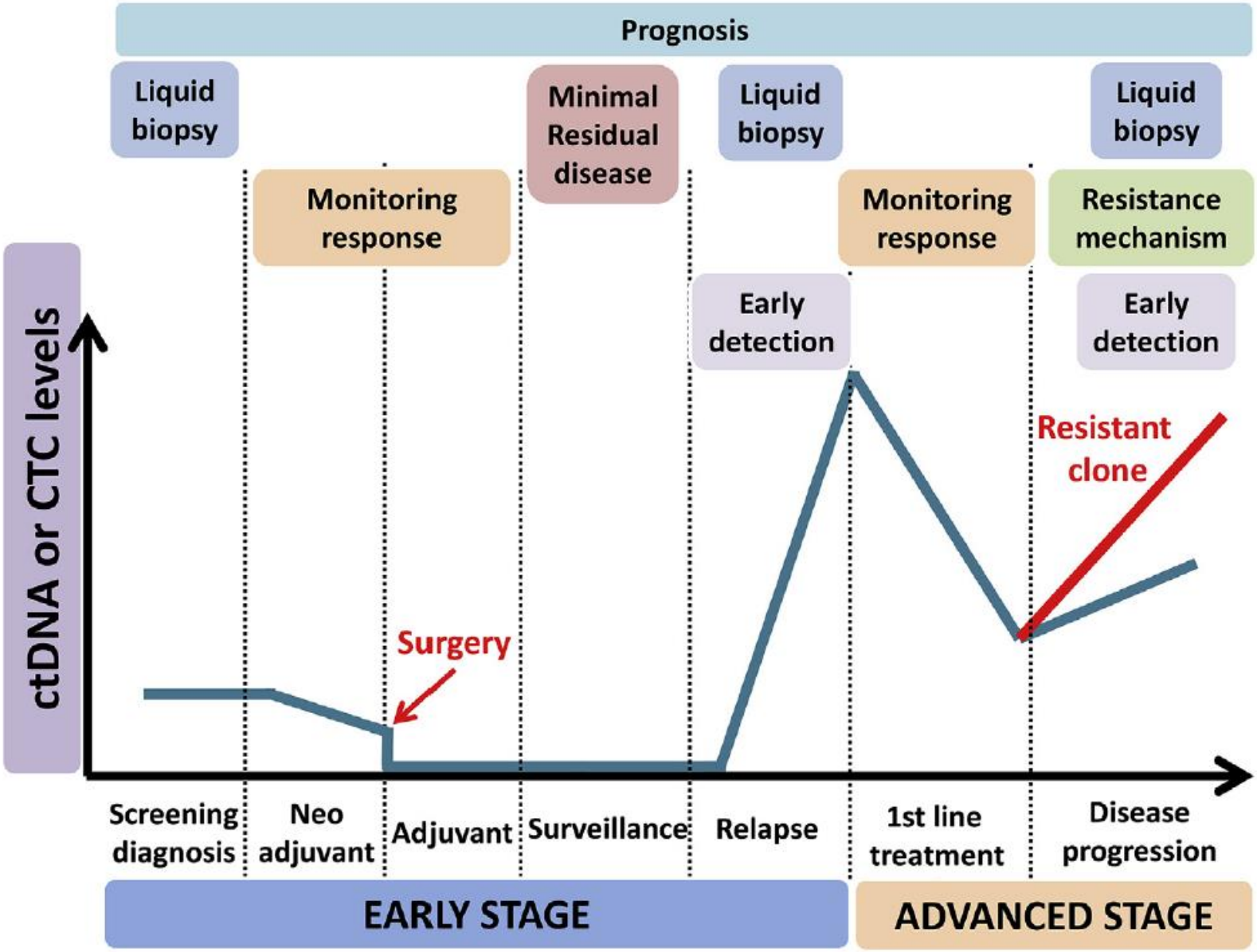


Figure 2. Overview of the potential clinical applications of circulating tumor DNA (ctDNA) and circulating tumor cell (CTC) depending on the clinical setting.

Conclusion

- None of the previously described Circulating Biomarkers to detect lung cancer at an early stage is being used routinely now.

- Before developing such a test for early detection of lung cancer, it is essential to perform multicenter studies on a large cohort of patients and on a control population(high risk pt) in order to validate the test.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