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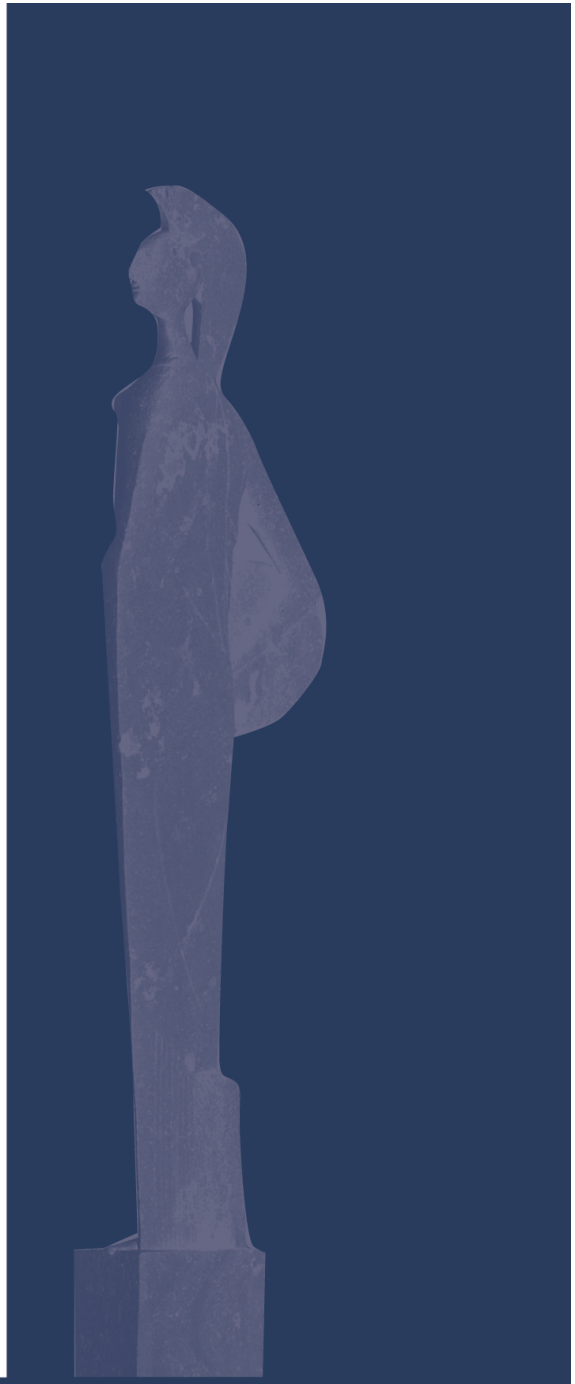

cfDNA Analysis

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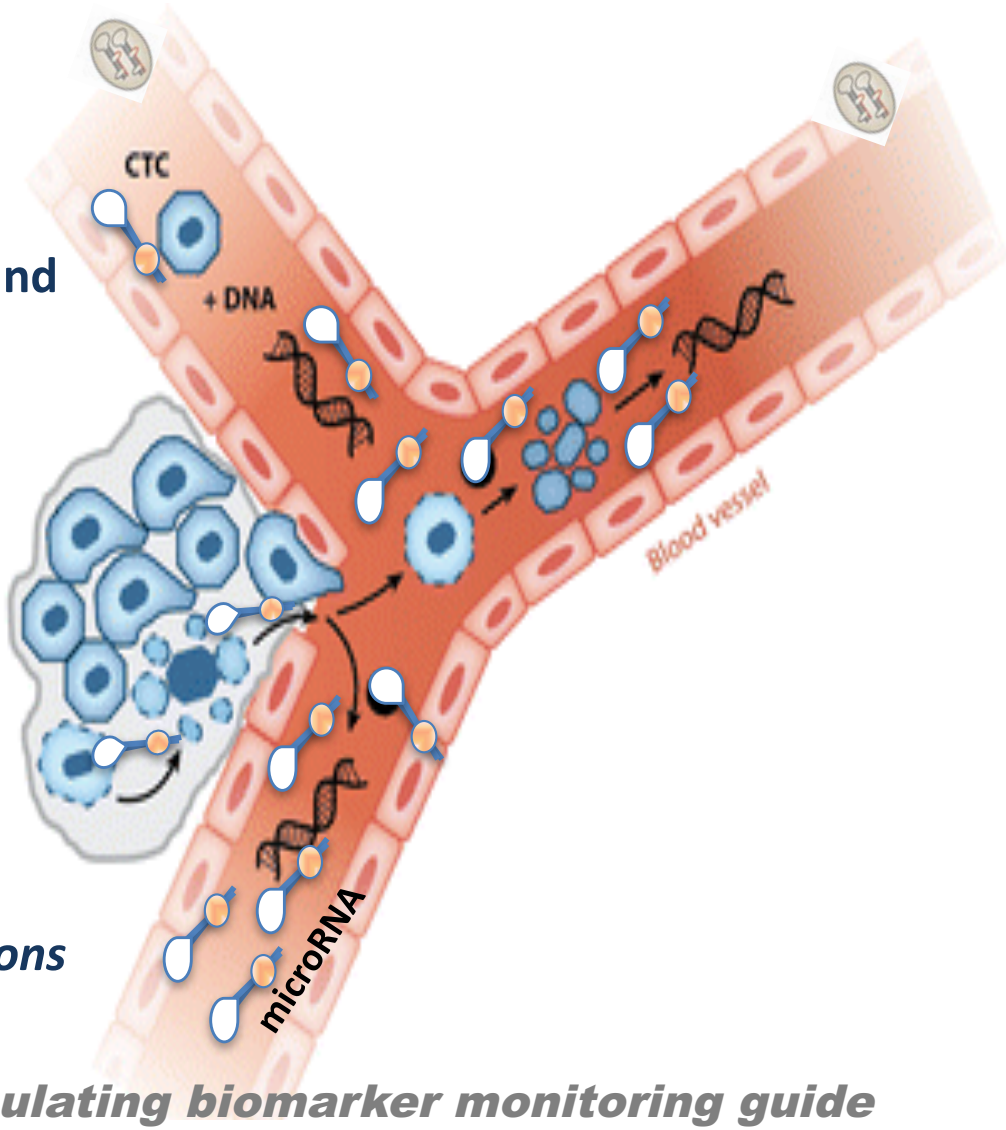
Liquid Biopsies

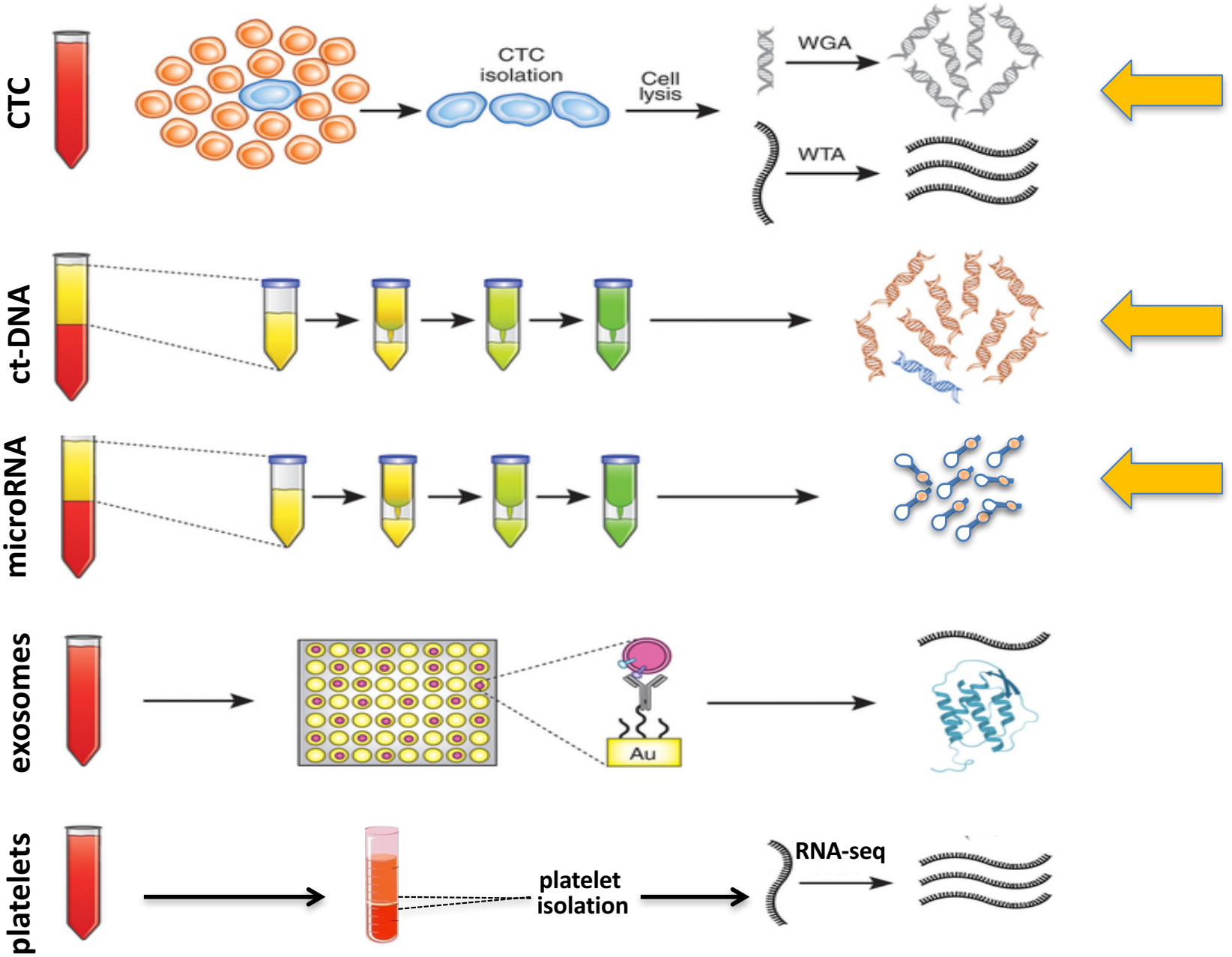
The liquid biopsy concept

- Peripheral blood contains CTCs, nucleic acids, proteins, microvescicle and exosomes, etc., derived from primary and metastatic lesions
- Easy accessibility, with minimally invasive approaches
- Dynamic and real-time monitoring feasible and reliable for:
  - ✓ *early detection of disease/disease progression*
  - ✓ *monitoring of minimal residual disease*
  - ✓ *monitoring of tumor dynamics*
  - ✓ *assessment of the overall molecular heterogeneity*
  - ✓ *evaluation of early treatment response*
  - ✓ *rapid assessment of drug-resistance conferring mutations*
  - ✓ *identification of new actionable mutations*

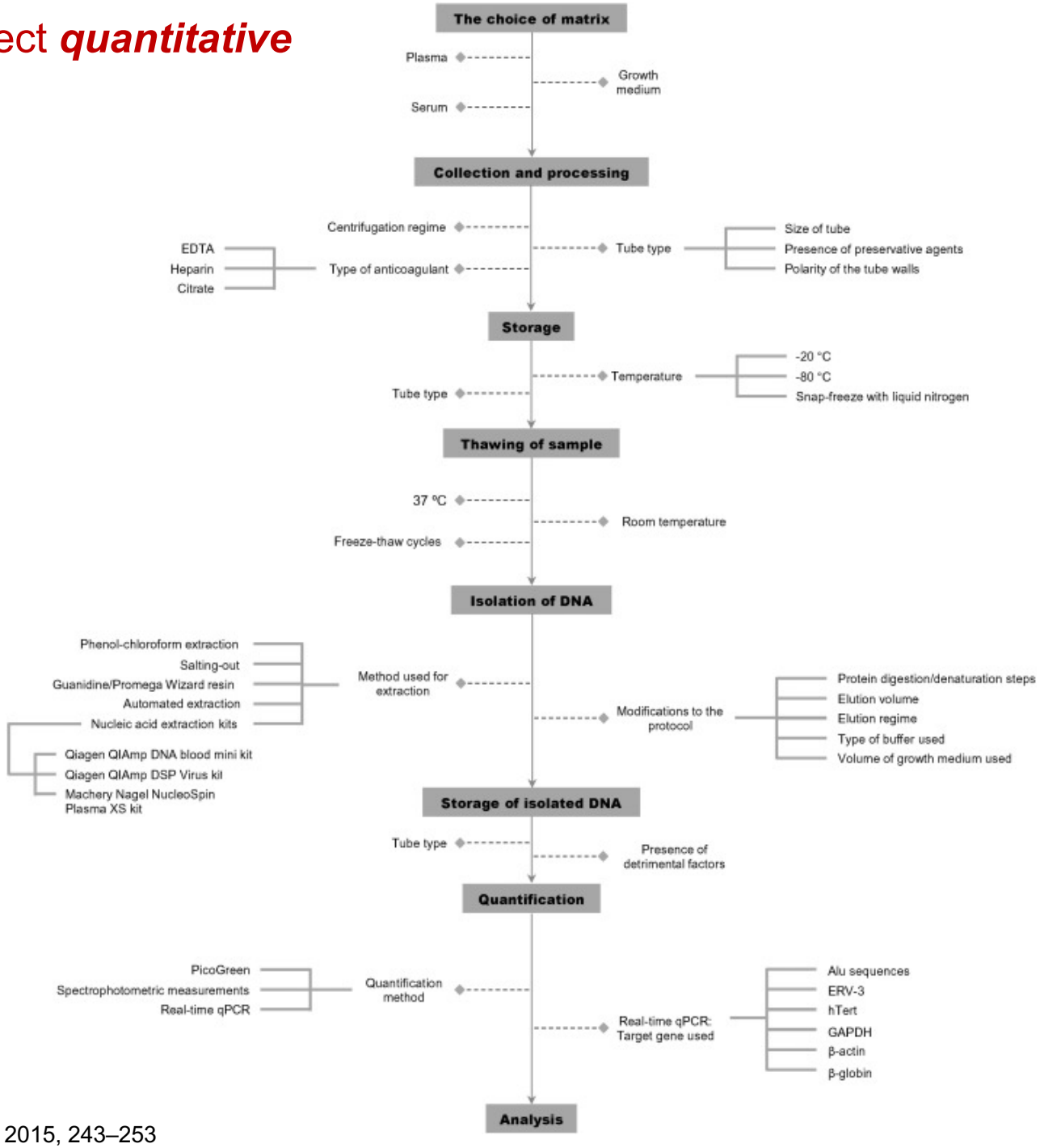


Solidifying liquid biopsies: can circulating biomarker monitoring guide treatment selection in solid tumors?

Minimally invasive approaches to monitor tumor genome evolution
(modified from Speicher & Pantel, Nature Biotechnol 2014)



Preanalytical factors that affect ***quantitative*** measurements of cfDNA



Bronkhost et al Clinica Chimica Acta, Volume 450, 2015, 243–253

Background

- *cfDNA is usually at very low concentration*

- *cfDNA in the plasma of cancer patients ranges between just a few ng/mL to several thousand ng/mL, which overlaps with the concentration range of healthy humans*

- *cfDNA levels are not constant, but fluctuate within healthy and diseased individuals. This could be attributed to a web of cellular responses to various environmental cues and stressors (such as the circadian rhythm, heavy smoking, non-malignant diseases, exercise, heart dysfunction and medicinal status, for instance).*

cfDNA profile can be influenced by:

1. ***inadequate blood collection procedures***
2. ***inappropriate storage/transport conditions,***
3. ***plasma separation***
4. ***cfDNA extraction procedures.***

The release of gDNA after collection from WBC can change the cfDNA profile impacting the validity of the analytical test results.

*Plasma samples show **less variability** and are less likely to suffer from cellular contamination,*

Plasma is the source of tfDNA for liquid biopsy analyses, because the lower levels of background wild-type DNA would facilitate the detection of mutated DNA.

Furthermore, vesicles with which cfDNA is associated (virtosomes, HDL, argonaute 2, exosomes) can bind to fibrin and fibrinogen. Removal of this fraction from blood to obtain serum could thus result in the loss of crucially informative tfDNA. Platelets have been shown to be the main producers of microparticles that play a key role in thrombin generation. Defibrination of plasma would thus result in the loss of platelet-derived microparticles, and consequently the loss of more cfDNA

The prerequisite for analyses of cfDNA ***is uncontaminated starting material. = material free from cells that may release cellular DNA at any pre-analytical step.***

European Committee for Standardization
Comité Européen de Normalisation
Europäisches Komitee für Normung

- *Technical Specification has been published for processing of venous whole blood specimens intended for circulating cell free DNA (ccfDNA) analysis during the preanalytical phase, before a molecular assay is performed.*

Molecular in-vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood — Isolated circulating cell free DNA

SELECTION OF THE VENOUS WHOLE BLOOD COLLECTION TUBE

Without stabilizers

With stabilizers

Tubes without stabilizers

EDTA blood collection tubes should be used

@EDTA prevents clotting

@EDTA allows extensive storage

@ The date and the time of blood collection should be included in the documentation on primary blood sample

@The primary blood samples should be transferred immediately to 2 °C to 8 °C to minimize the release of gDNA from WBC into the blood.

@Storage duration needs validation. Some reports indicate storage at 2-8°C up to 6h without remarkable effects.

Tubes without stabilizers

EDTA blood collection tubes

@Samples should not be frozen

@Samples should not be shaken vigorously

@ Storage in the blood collection facility contributes to the total duration of storage.

@Blood transport should be documented (duration and temperature)

Tubes with stabilizers

@Their use is suggested to prevent the lysis of WBC and epithelial cells and to preserve plasma DNA integrity for several days at room temperature or at 2-8°C.

@Follow the instruction of the providers.

@ Different products in commerce

@Their use is optimized with specific isolation kits for extracting cfDNA

@Maximum storage duration and temperature is provided by the manufacturer.

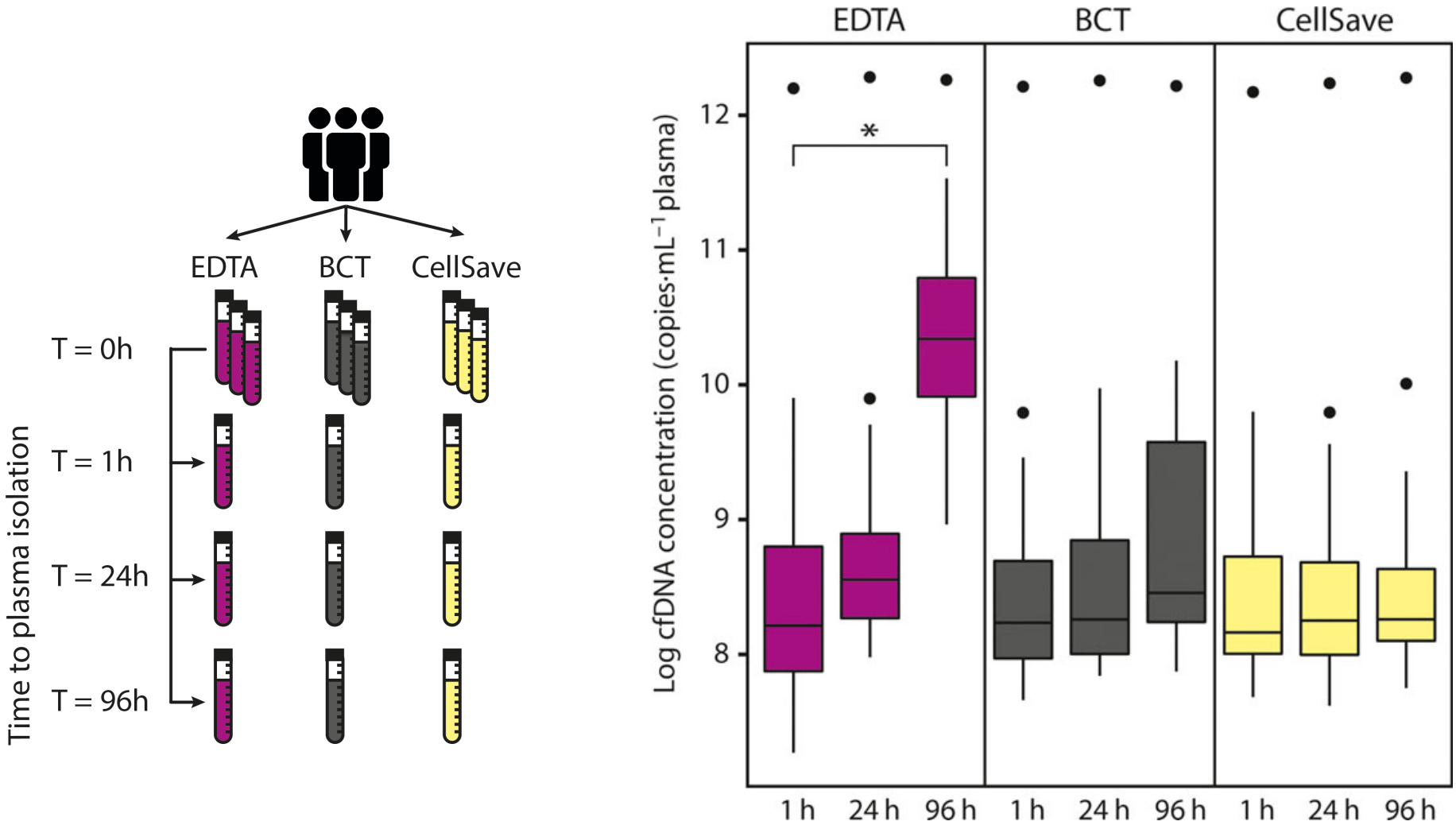
Tubes with stabilizers

@Tube storage temperature varies with providers, ranging from room temperature to 2-8°C.

@Products: Cell-Free DNA collection Tube (Roche), Cell free DNA BCT (Streck), PAXgene Blood ccfDNA Tube (PreAnalytix), Cell- Save Preservative tubes (Janssen Diagnostics)……*

@ After blood withdrawal mix by inversion 10x (all tubes)

COMPARISON



Molecular Oncology 11 (2017) 295–304

Overall

@The formation of small DNA fragments (180– 200 bp lengths) is related to apoptosis,

@During cell lysis or necrosis intact gDNA and thus much larger DNA fragments (50– 300 kbp) can be detected

@Time dependent increment of longer fragment are indicative of WBC lysis

PLASMA ISOLATION

@When using blood collection tubes with stabilizers, the manufacturers' instructions must be followed to obtain plasma.

@EDTA blood samples should be centrifuged at 1600 g to 2500 g at 2 °C -8 °C for 10 min. Plasma must be transferred into a new tube without disturbing the plasma- cellular interface layer to ***avoid contamination*** *from gDNA and cellular RNA from leucocytes.*
A second centrifugation *should be performed at 14000 g -16000 g at 2 °C t- 8 °C for 10 min. After that step, the supernatant must be transferred into a new tube without disturbing the pellet.*

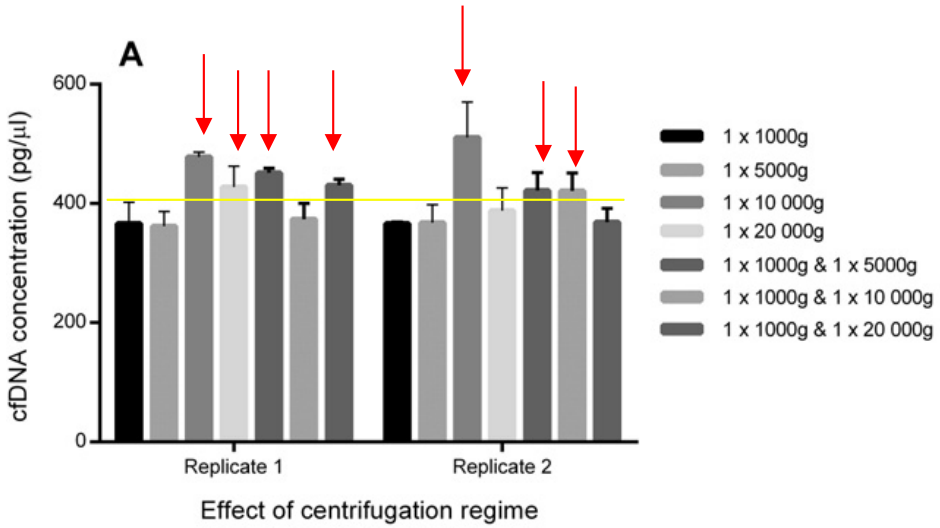
PLASMA ISOLATION

@If high g-force centrifugation is not possible, the analytical test must be validated carrying out the second centrifugation at a lower g-force, 3000 g -5000 g for 20 min at 2 °C - 8 °C.

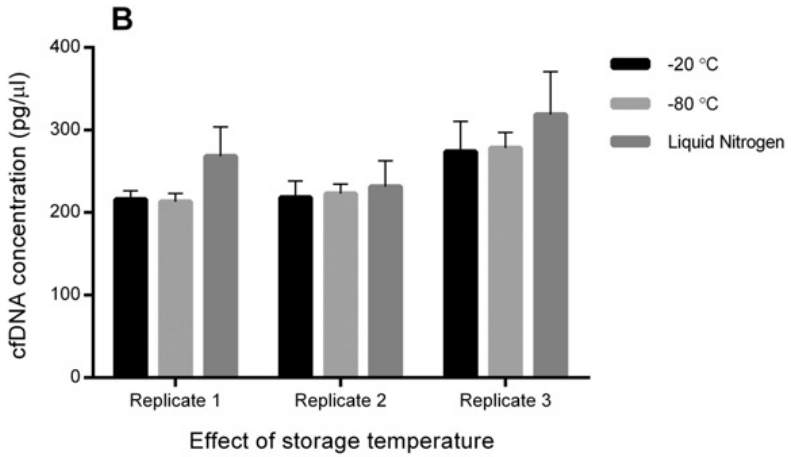
@ For short term storage, plasma may be stored at 2 °C - 8 °C for a maximum of 24 h. ***For long-term storage plasma should be stored frozen at -20 °C or below.***

@ Aliquoting plasma samples is compulsory. Thawing shall not be made more than once.

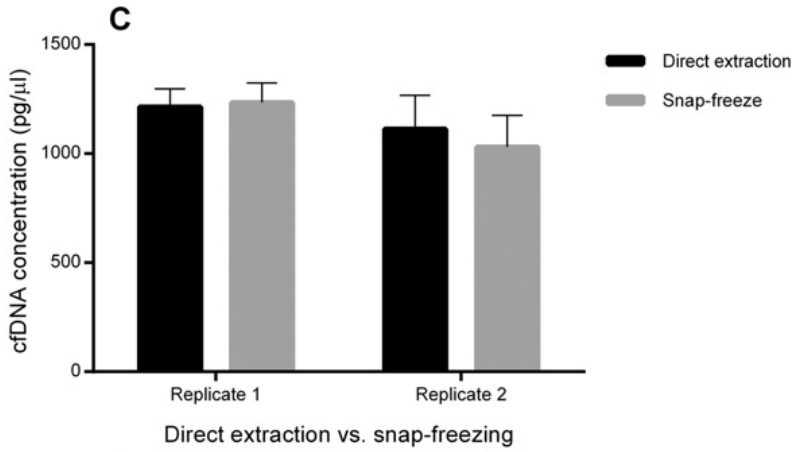
Effect of Centrifugation



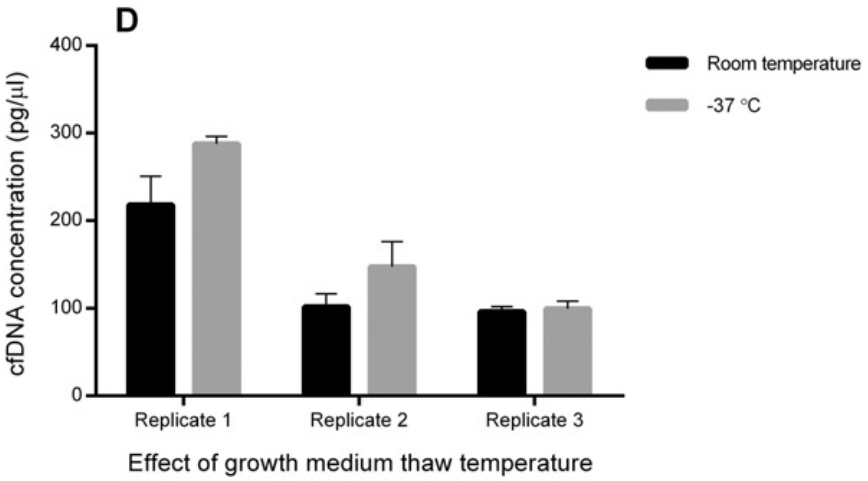
Storage Temperature



Effect of Storage



Effect of thawing temperature



Bronkhorst et al Clinica Chimica Acta 450 (2015) 243–253

OPEN QUESTIONS

- *Blood collection- fasting? Avoid blood withdrawal close to major meals with lipid content?*
- *Plasma processed within 2h- is it reliable in most Hospitals? Is the use of tube with stabilizer compulsory for cfDNA processing?*
- *When ultracentrifugation is not possible? What is the performance of 22µm filtration?*
- *Plasma samples storage at 2 ° C to 8 ° C for a maximum of 24 h is suggested. For long-term storage should plasma be stored frozen at -20 ° C or below?*

Outcome of studies on cell-free DNA in breast cancer

Quantitative alterations:

❖ It has been reported that the concentration of DNA in the bloodstream of patients with breast cancer varied from 153 to 549 ng/ml in serum and from 122 to 462 ng/ml in plasma, whereas that of healthy controls ranged between 63–318 and 3–63 ng/ml, respectively (Fleischhacker, 2007).

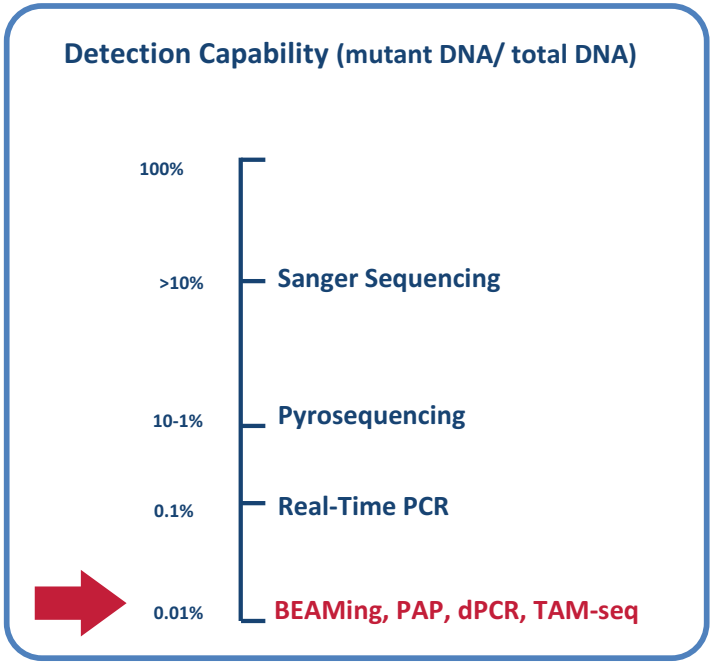
❖ Kohler et al. (2008) showed **significantly higher levels of cell-free DNA in patients with breast cancer** in comparison to the patients with benign breast tumors and healthy individuals (81% sensitivity and 69% specificity).

Qualitative alterations:

Cf-DNA alterations	Molecular alterations
	Breast
Mutation	TP53 (28–31,37) PIK3CA (32–34,37)
DNA integrity	Serum DNA integrity (40,41)
Microsatellite alterations	LOH and MSI (9,11,42,43)
Methylation	RASSF1A, APC, DAPK, ESR1, BRCA1, MGMT, GSTP1, Stratifin, MDR1, HSD17B4, HIC1, NEUROD1 (48–53)

LOH, loss of heterozygosity; MSI, microsatellite instability.

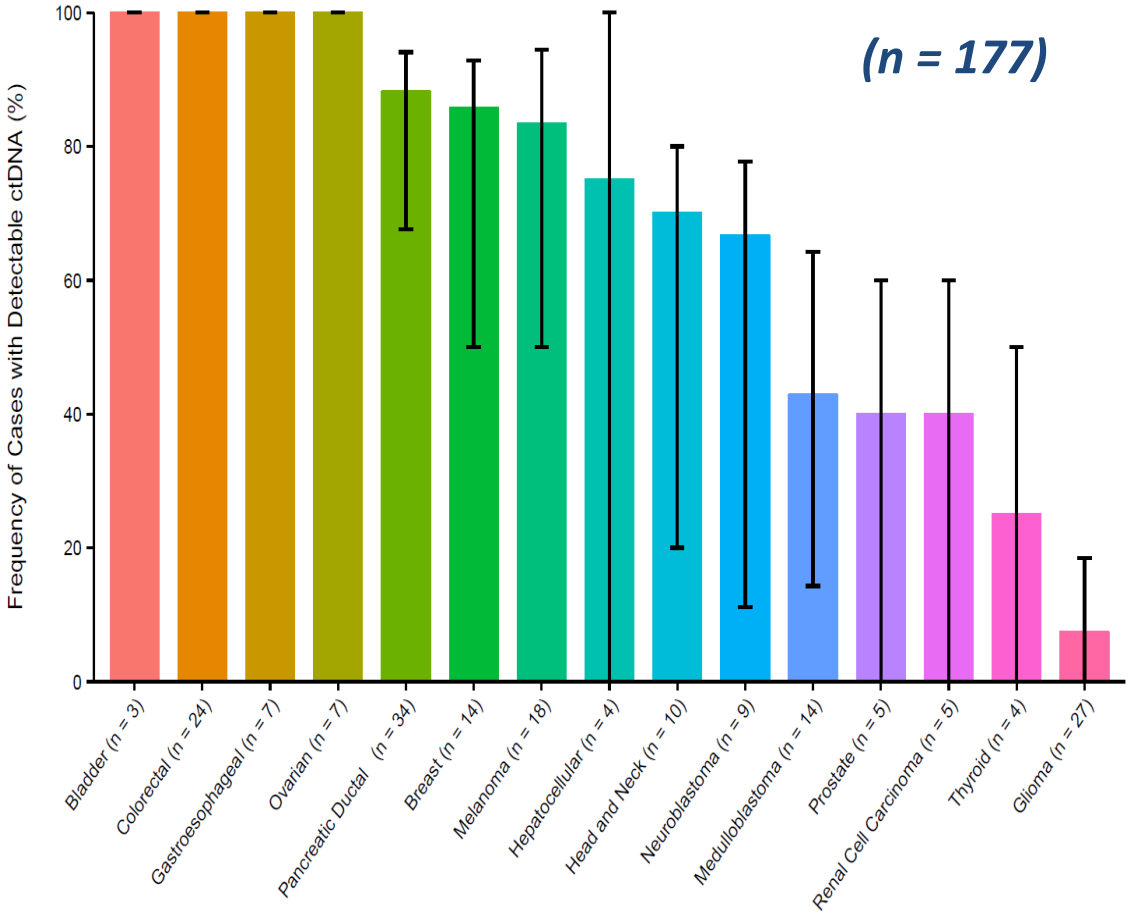
A. Esposito et al./Cancer Treatment Reviews xxx (2013) xxx–xxx

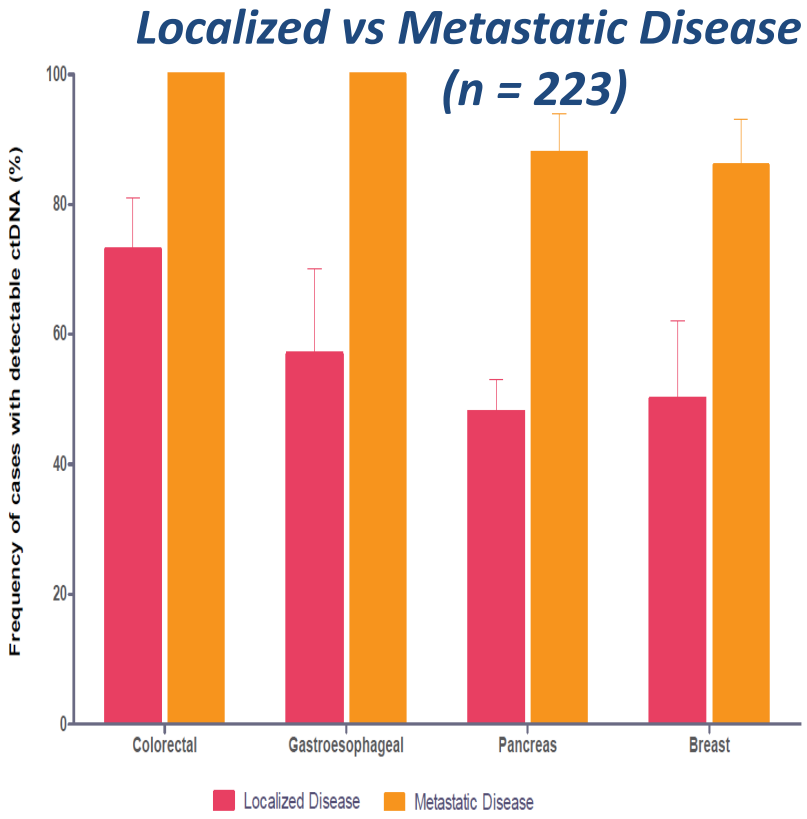


Diehl F, et. al. Gastroenterology. 2008 Aug;135(2):489-98.

Outcome of studies on ctDNA in solid tumors

CtDNA is MORE detectable in late stage cancers



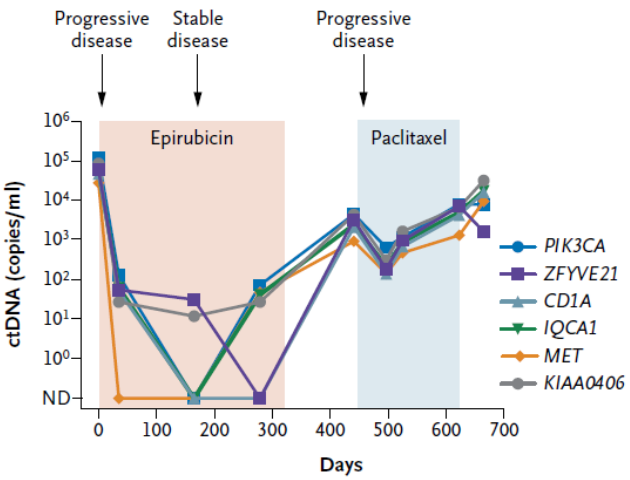


Bettegowda et al, Sci Tran Med Feb 2014

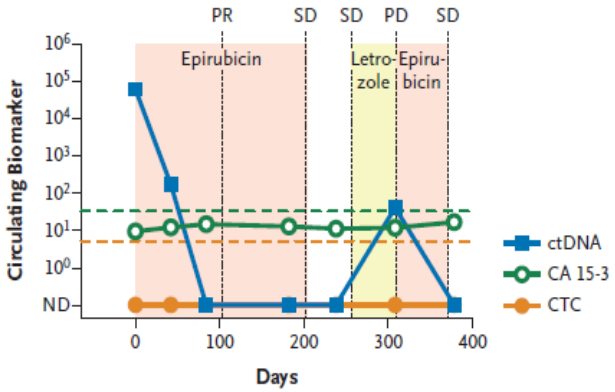
Tumor type	Genomic alteration	Potential indications
Breast	*ESR1* mutation	Tracking emerging resistance mechanism
	PIK3CA mutation	Prediction of response to agents targeting the PI3K/AKT pathway, Prognostic evaluation
Colorectal	*KRAS* mutation	Prediction of response to anti-EGFR treatment, Tracking emerging resistance mechanism
	NRAS mutation	Prediction of response to anti-EGFR treatment, Tracking emerging resistance mechanism
	BRAF	Prediction of response to anti-EGFR treatment, Tracking emerging resistance mechanism
Esophagogastric	*APC* methylation	Prognostic evaluation
	RASSF1A methylation	Association with tumor burden
	HER2 amplification	Prediction of response to anti-HER2 treatment
Pancreas	*KRAS* mutation	Prognostic evaluation
Lung	*EGFR* mutation	Prediction of response to anti-EGFR treatment, Tracking emerging resistance mechanism
Prostate	*GSTP1* methylation	Prognostic evaluation
Ovarian	*ERCC1* mutation	Prediction of response to platinum-based chemotherapy, Prognostic evaluation
Melanoma	*BRAF* mutation	Prediction of response to anti-BRAF treatment
Glioma	*MGMT* methylation	Prognostic evaluation
Gastro-Intestinal Stromal Tumor	*KIT* mutation	Prediction of response to treatment with tyrosine kinases inhibitors
	PDGFR mutation	Prediction of response to treatment with tyrosine kinases inhibitors
Neuroblastoma	*ALK* mutation	Prediction of response to treatment with ALK inhibitors

Monitoring metastatic breast cancer by ctDNA

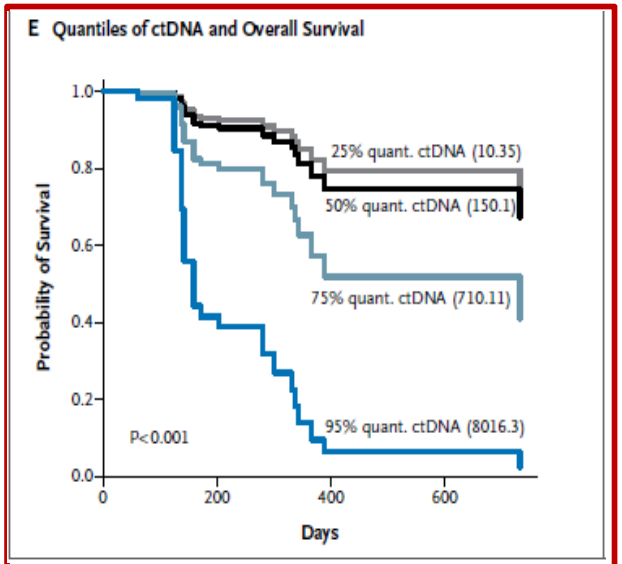
Identification of tumor somatic alterations by NGS (targeted or whole-genome) in tissues and quantification of ctDNA by digital PCR (dPCR) in serially collected plasma specimens



Mutations identified in tumor-tissue samples generally showed similar dynamic patterns in plasma and their variations correlated with responses to treatment



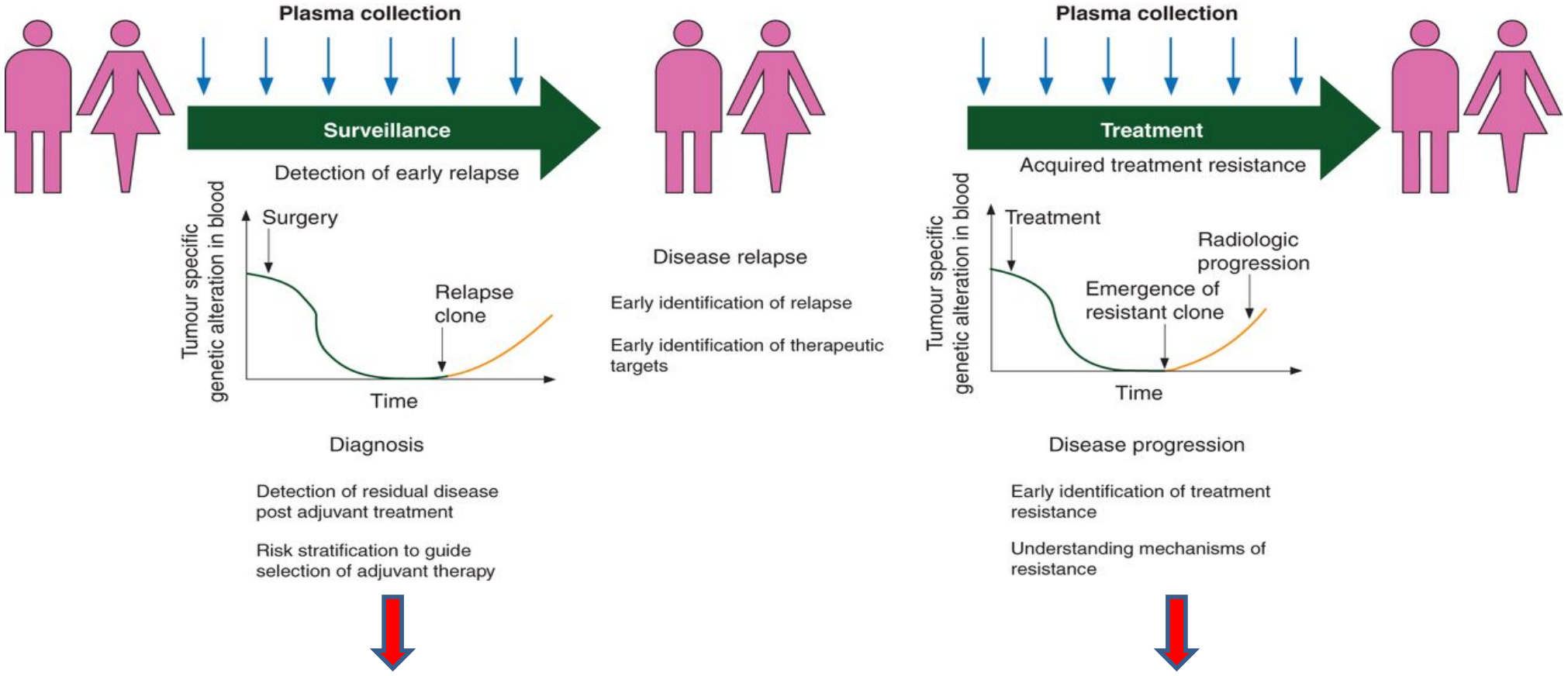
ctDNA showed superior sensitivity than CA15-3 and CTC and has a greater dynamic range that correlates with changes in tumor burden



Among the measures tested, circulating tumor DNA provided the earliest measure of treatment response in 10 of 19 women (53%).

Dawson et al., NEJM 2013, 368:1199-1209

Potential clinical applications of ctDNA analysis



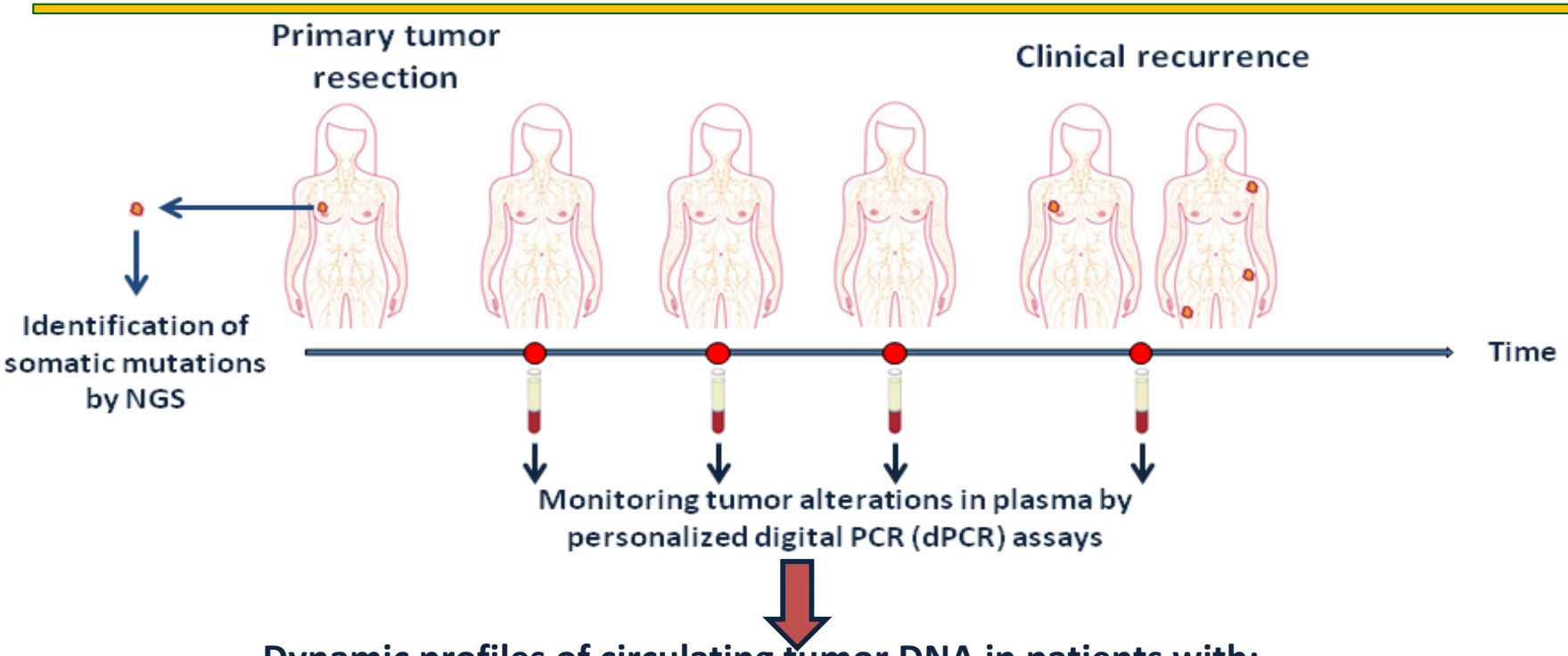
Point mutations, identified on the basis of a tumor-resection sample subjected to next-generation sequencing (NGS), could be used to monitor tumor burden or recurrence after surgery or chemotherapy

Direct sequencing of plasma DNA could be used to identify new mutations in ctDNA as a means to the early identification of resistance to targeted therapies and to investigate tumor genetic heterogeneity

M. Ignatiadis, and S.-J. Dawson

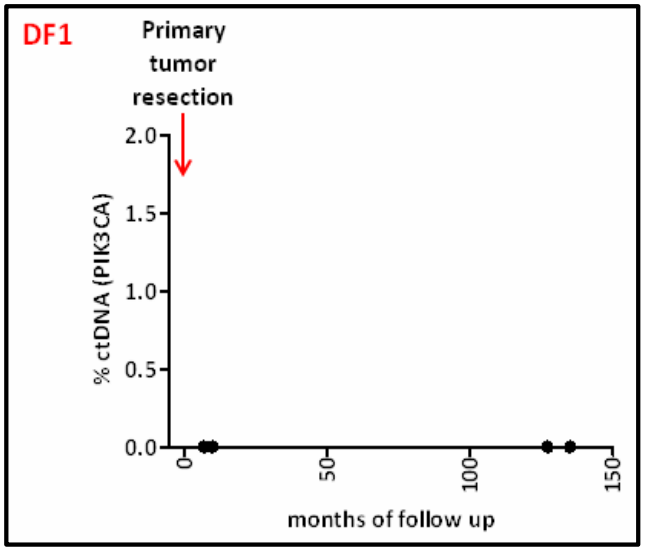
Annals of Oncology

Monitoring early stage breast cancer by ctDNA: an INT pilot study

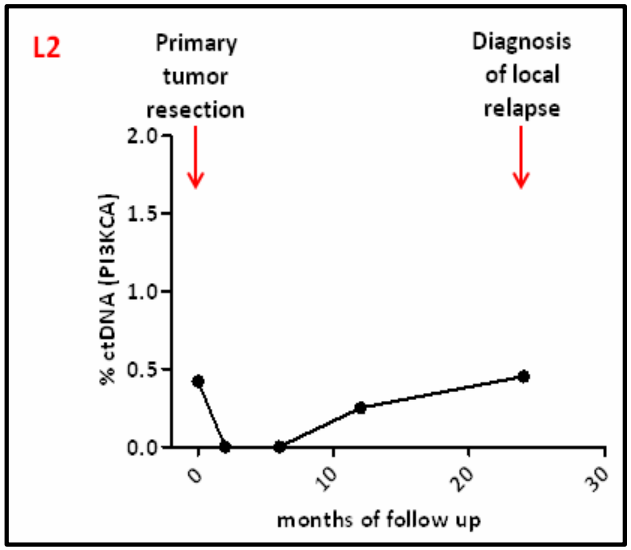


Dynamic profiles of circulating tumor DNA in patients with:

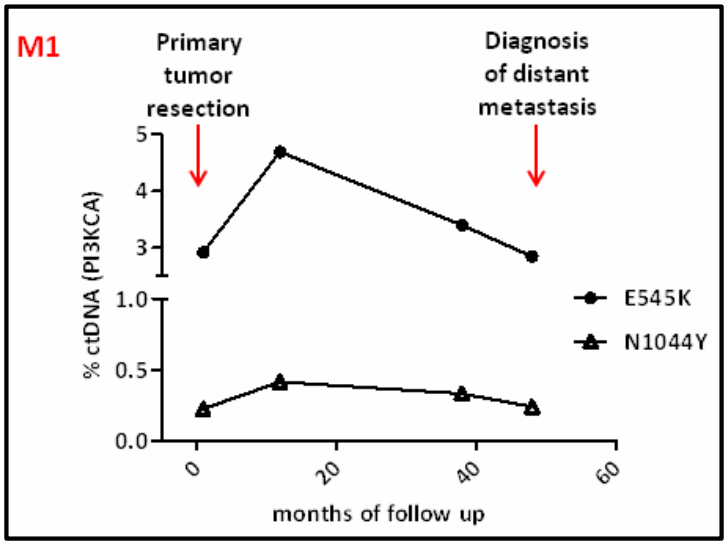
NO EVIDENCE OF DISEASE (4/4)



LOCAL RELAPSE (5/6)



DISTANT METASTASIS (4/4)



Monitoring early stage breast cancer by ctDNA: conclusions from a pilot study

In patients with an early breast cancer (T1-2, N0, M0), ctDNA monitoring during post-operative follow-up can anticipate recurrence events (local relapse and distant metastasis) and may highlight incomplete tumor resection or occult metastasis.

Findings consistent with recent observations:

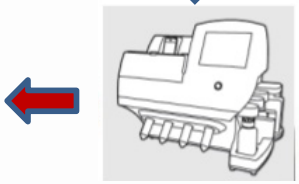
- ctDNA (PIK3CA mutations) can be detected in the blood of early stage breast cancer patients (*Beaver et al., Clin Cancer Res, 2014*)
- ctDNA detection (tumor specific rearrangements) preceded clinical detection of metastasis in non-metastatic (stage I-III) breast cancer patients (*Olsson et al., EMBO Mol Med, 2015*)
- ctDNA tracking predicted metastatic relapse in early breast cancer patients subjected to neoadjuvant therapy (*Garcia-Murilla at al., Sci Transl Med, 2015*)

planning of prospective studies to evaluate the potential of ctDNA in early breast cancer to anticipate disease progression?

Workflow and molecular approaches

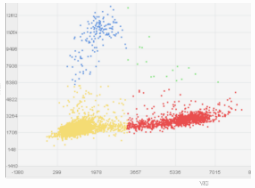
Tumor sample

DNA extraction



Sequencing

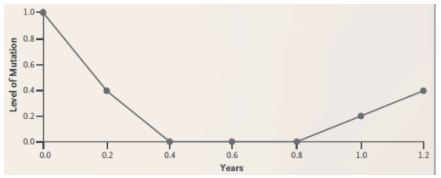
Identification of somatic mutations



Validation by dPCR

Plasma sample

DNA extraction



ctDNA dynamic profile by dPCR