Biopsia liquida

Presente e futuro della medicina di laboratorio

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Background

Malignancies shedding cells in circulating blood

- Malignancies, such as leukaemia, lymphoma, myeloma, many epithelial malignancies, and some sarcomas, shed tumour cells into the circulating blood.
- Some tumours shed tumour cells before a primary cancer has become diagnosable, and many before a metastatic locus or recurrence have become detectable by any other known technique

Malignancies shedding cells in circulating blood

- Mandel e Metais 70 years ago identified nucleic acid in plasma.

Mandel, P, Metais, P. (1948). Les acides nucleiques du plasma sanguin chez l'Homme. CR Acad Sci Paris, 241-243.

- For years no follow up. cf-DNA was re-discovered in the serum of LES patients

Tan, E.M., Schur, P.H., Carr, R.I. & Kunkel, H.G. (1966). Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J Clin Invest, 45, 1732-40.

Malignancies shedding cells in circulating blood

Cell-free DNA (cfDNA) was first identified in human plasma in 1948 and is believed to be released from cells throughout the body into the blood stream

It has been known for quite a long time that, in a rat model, mammary tumours shed $3.2–4.1 \times 10^6$ cells (CTC) per day per gram of tissue, most of which are destroyed within minutes in the circulation by anoikis.

In a mouse model, approximately 2.5% of CTCs forms micrometastases, most of which subsequently disappear over time, and 0.01% of CTCs progress to form macrometastases.

The clinical implications of CTC and ctDNA in human tumours is a recent and rapidly developing research topic

Butler TP and Gullino PM. Cancer Res (1975) 35(3):512–516.
Luzzi KJ, et al; Am J Pathol (1998) 153(3):865–873
Sun YF et al. J Cancer Res Clin Oncol (2011) 137(8):1151–73

Circulating tumour DNA as biomarker

- Cell-free DNA circulating in serum is a **candidate molecular biomarker** for malignant tumours.
- DNA released from dead cancer cells **varies in size**.
- Uniformly truncated DNA is released from apoptotic/anoikic cells.
- Serum DNA integrity. The **ratio of longer fragments to total DNA**, may be clinically useful for detecting cancer progression.

Circulating tumour DNA as biomarker

- CfDNA/ctDNA expression in blood samples seem to be dependent upon tumour type.
- CtDNA was detected in over 75% of patients with advanced pancreatic, ovarian, colorectal, bladder, gastroesophageal, breast, melanoma, hepatocellular, and head and neck cancers.
- However, in primary brain tumours, kidney, prostate, and thyroid cancers, levels below 50% were found.

Improve Cancer Patient Outcomes Using Liquid biopsies

Blood is a readily obtained, repeatable clinical sample.
Whereas serial tumour biopsy is often challenging, more expensive and not without risk.

Prediction (within 2019*)

- Blood tests will **stratify most cancer** patients for sequential personalised therapies
- Blood tests will **detect relapse early** and identify **drug resistance** mechanisms.

Blood tests for early detection of cancer…. **It may take some longer time**

Circulating tumour DNA
Circulating tumour cells - more technically challenging (but more information)
Circulating miRNA – up and coming

* Carter L Rothwell D Mesquita B Smowton C Leong H et. Al.; Nature medicine, 2017 vol: 23 (1) pp: 114-119

Liquid Biopsy

ctDNA: circulating tumour DNA shed by tumour cells into peripheral blood

CTCs: circulating tumour cells

Liquid Biopsy: terminology

cf (ccf) DNA: cell free (circulating cell free) DNA, Circulating DNA not bound to cell structures

ct DNA: circulating tumour DNA

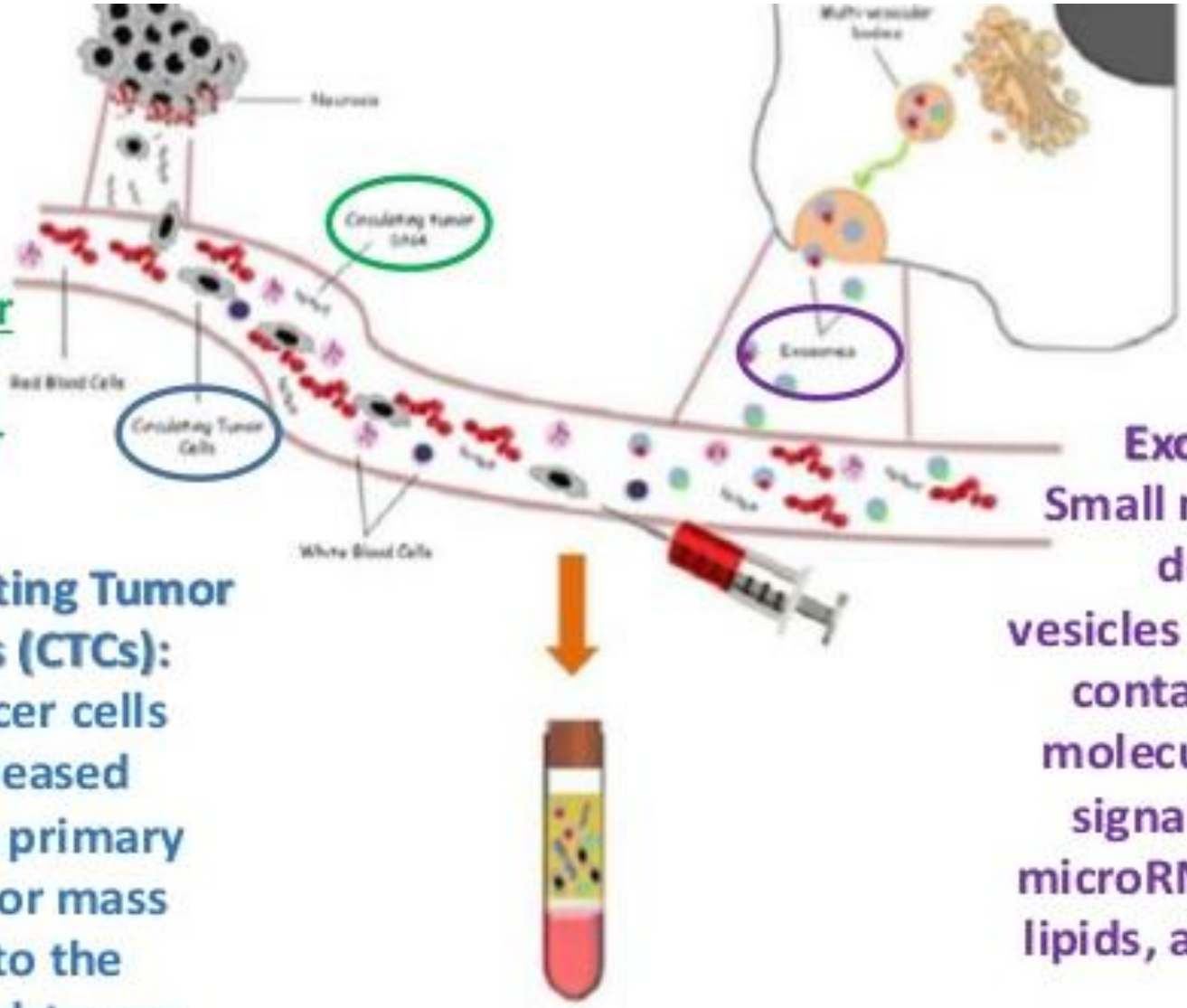
CTC : circulating tumoral cells

Ccff DNA: circulating cell free foetal DNA

scfDNA: seminal cell free DNA

Liquid biopsy

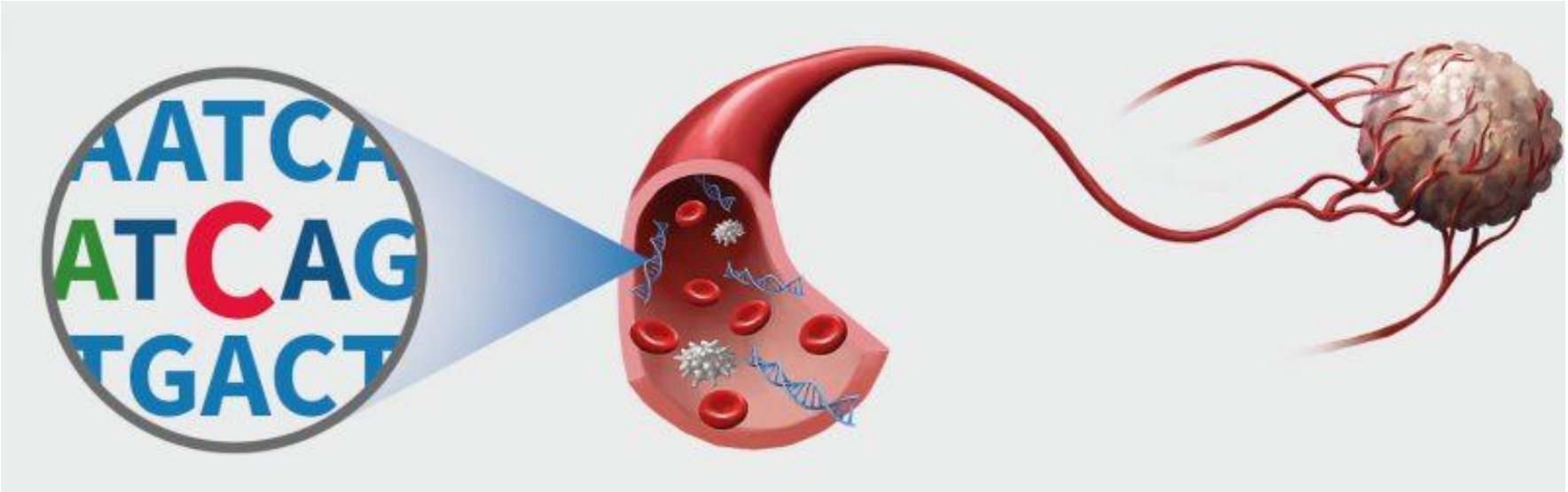
Circulating Nucleic Acids: Mainly ctDNA (circulating tumor DNA), miRNAs, mRNA, long non-coding RNA



Circulating Tumor Cells (CTCs): Cancer cells released from primary tumor mass into the bloodstream

Exosomes: Small membrane-derived vesicles (40–100 nm) contain various molecules such as signal proteins, microRNAs, mRNAs, lipids, and exoDNA.

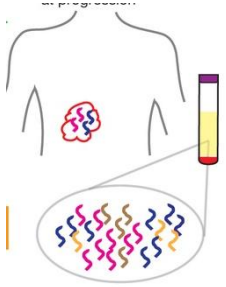
Liquid biopsy



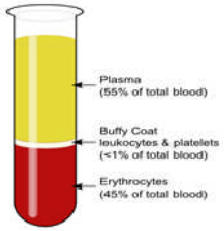
cf/ctDNA

- Origins: necrotic, apoptotic and anoikic tumour cells. Fragmented genome released from dying tumour cells of the primary tumour and/or metastases and/or CTC
- Analytes: DNA
- Technologies: DNA quantification (UV spectrophotometry (NanoDrop), fluorochrome reactions with the single and/or double DNA helix (Qubit) PCR; Next Generation Sequencing)

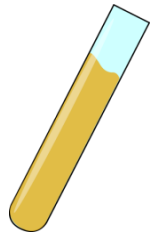
ctDNA Workflow



Blood sample taken in preservative tubes



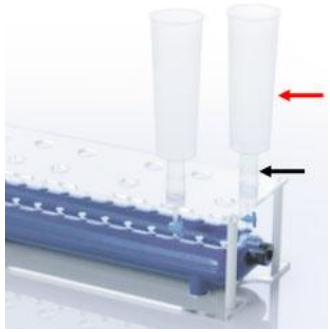
Sample arrives in lab and spun to isolate the plasma



Plasma is stored at -80ºc

Sample is extracted on the same day as the downstream process set up due to ctDNA instability

ctDNA is extracted from the plasma



Set up:
Quantitative PCR
NGS

Circulating Tumour Cells

- Relevant questions:
  - Are CTCs associated with prognosis?
  - Can CTCs be characterised for predictive biomarkers ?
  - Can serial CTCs analysis act as a surrogate of tumour response ?
  - Can CTCs inform
    - on mechanisms of dissemination
    - on tumour heterogeneity and evolution
  - Can CTCs lead to discovery of drug sensitivity/resistance?
  - Can CTCs based mouse models further our understanding of the disease?

Cell-free circulating tumour DNA: problems

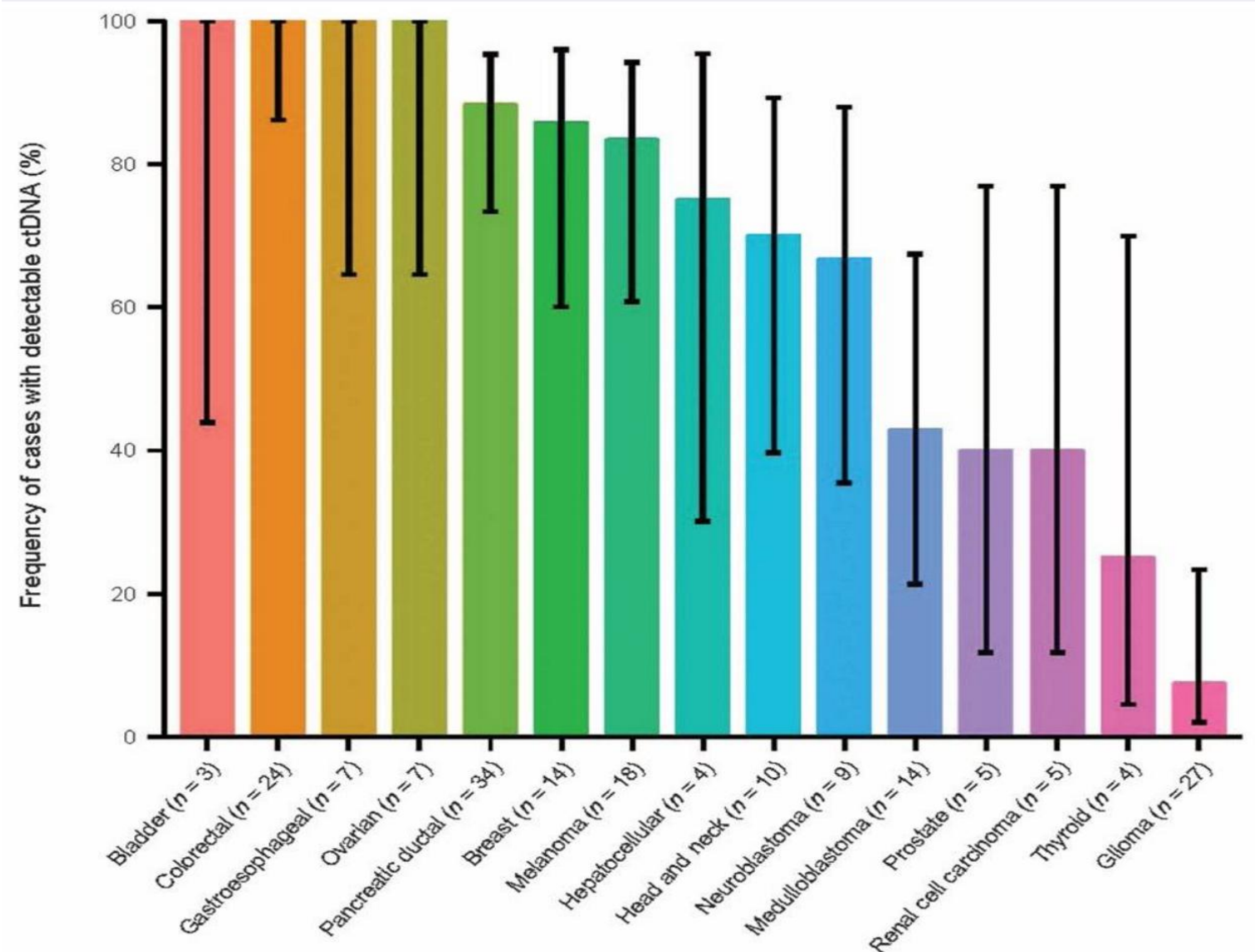
- Masking of ctDNA by variable amounts of normal cfDNA released by dying normal cells (e.g., during chemotherapy, surgery, infections) might lead to false-negative results.
- Many individuals have benign tumours (e.g., skin tumours) that carry cancer-associated mutations and that may cause false-positive findings for cancer screening.
- The use of cfDNA analysis for primary detection of cancer requires very large cohorts of patients with cancer, and matched control individuals need to be analysed prospectively over extended periods of time (>10 years).
- A possible way to circumvent this problem could be the focus on patients at high risk to develop cancer.

Problems with ctDNA

- Due to the unstable nature of ctDNA, the sample has to be collected and processed correctly.
- Only 30ng of cfDNA per 5ml plasma extraction.
- The amount of ctDNA is related to the tumour burden and varies between patients.
- It is difficult to discriminate ctDNA from normal cfDNA.
- The technique used must be sensitive enough to pick up the low level variants.

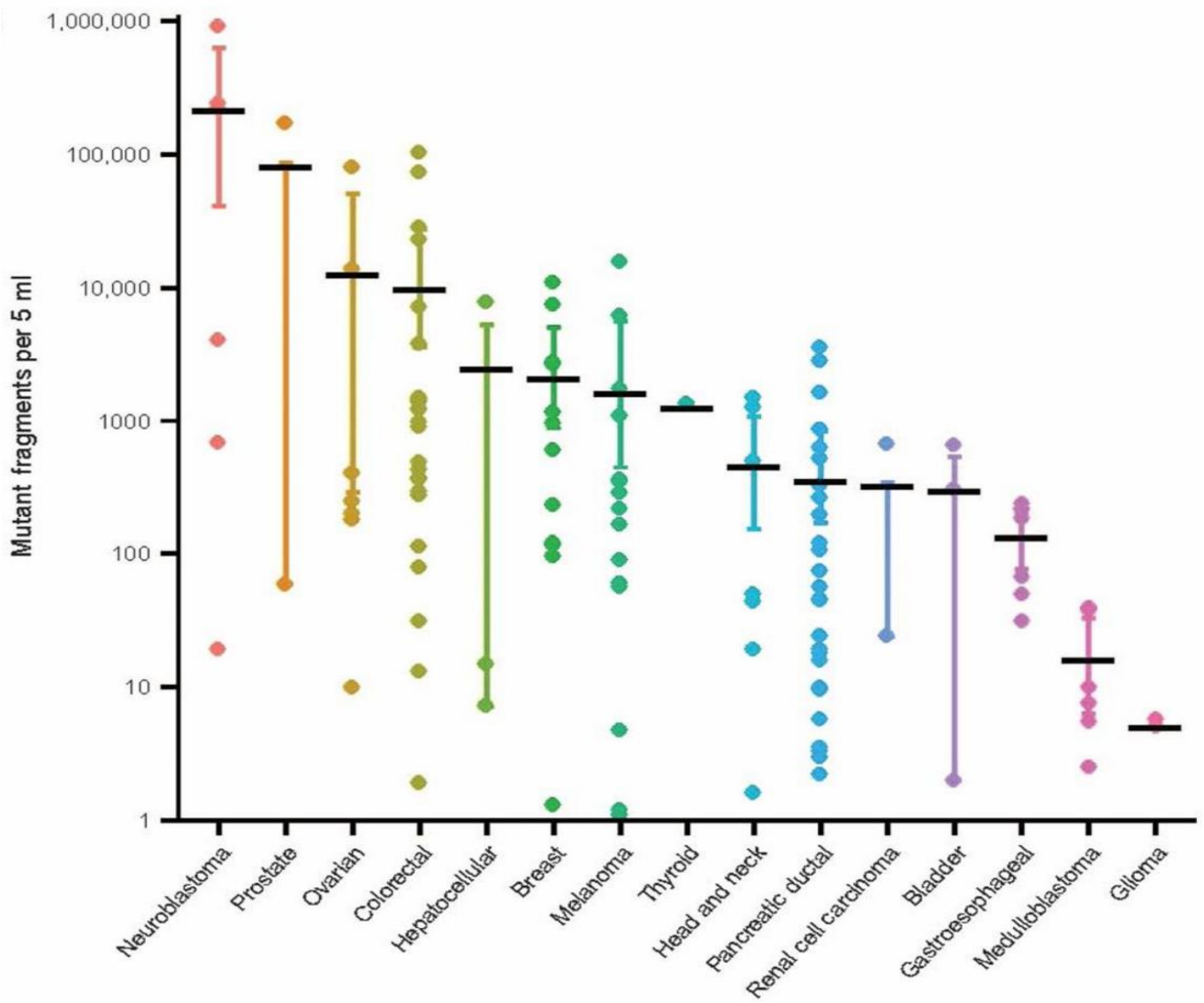
Diaz and Bardelli, 2014 Journal of Clincial Oncology 32, 2014

Detection frequency of ctDNA in advanced malignancies



C. Bettegowda et al. Sci Transl Med. 2014 Feb 19; 6(224): 224ra24.

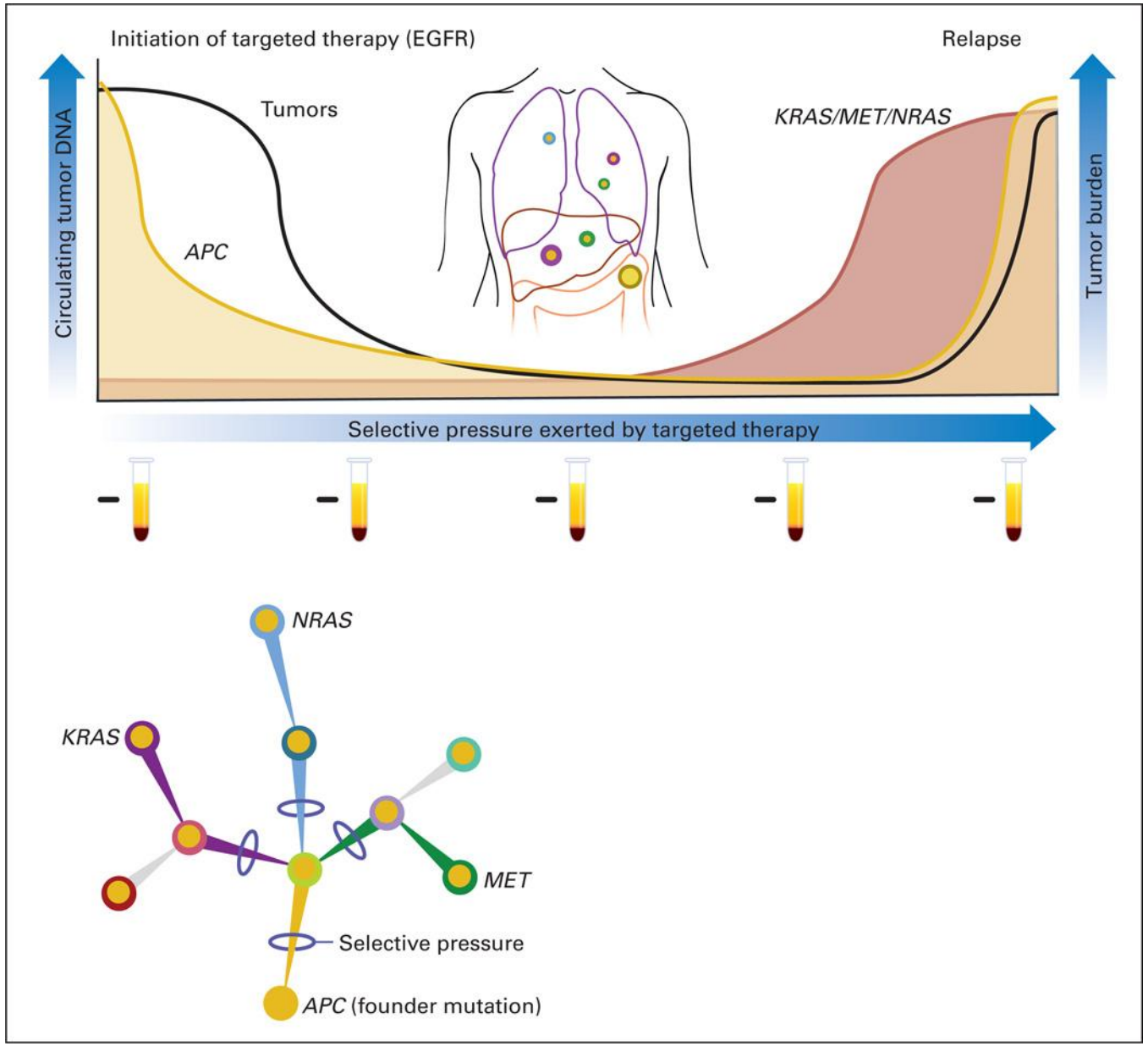
Mutation frequency of ctDNA in advanced malignancies



C. Bettegowda et al. Sci Transl Med. 2014 Feb 19; 6(224): 224ra24.

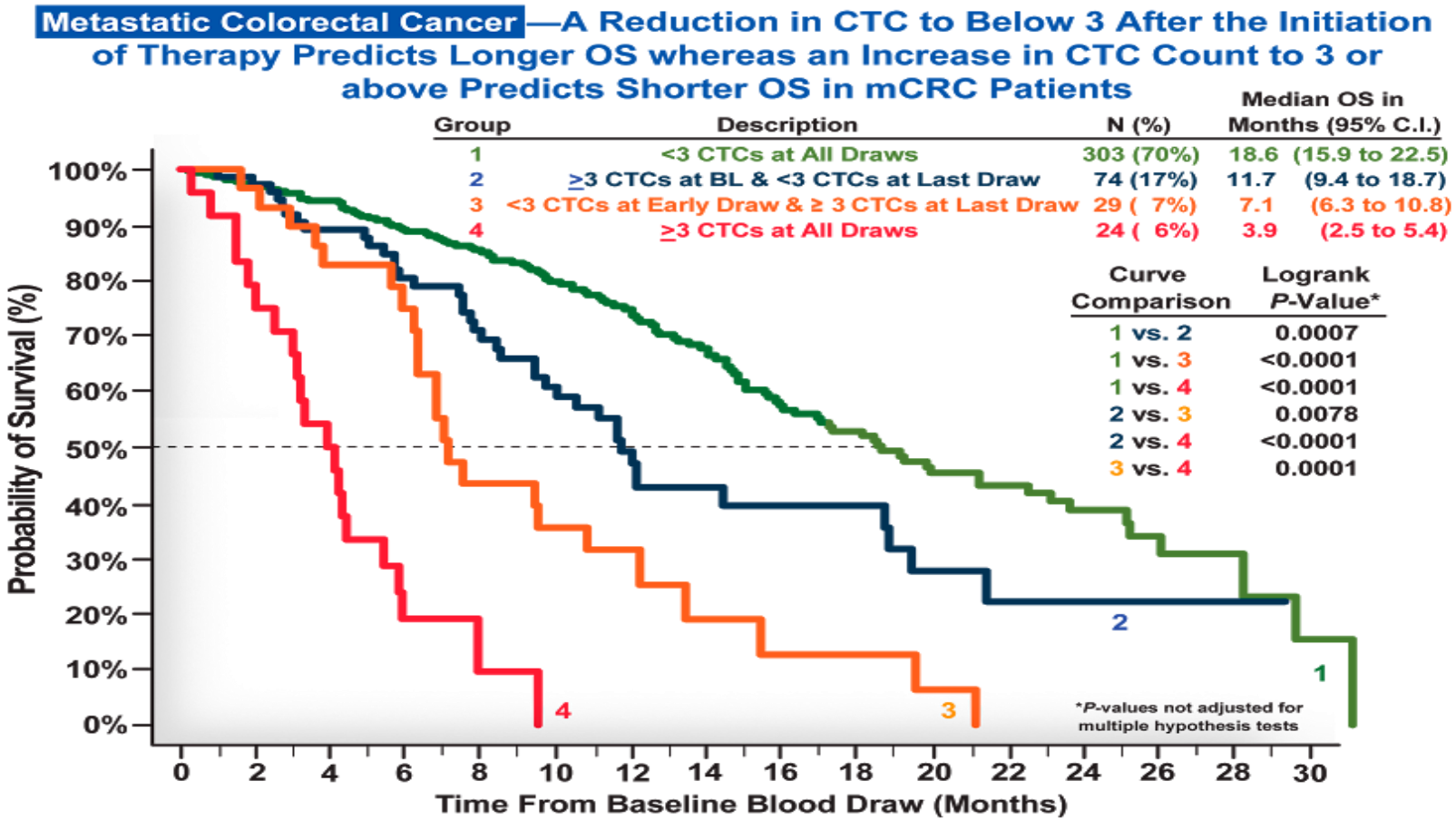
ctDNA Clinical applications in summary:

Cancer screening	**Localised cancer**	**Metastatic cancer**	**Refractory cancer**
Early diagnosis and early intervention	Identifying specific genomic alterations to guide therapeutic selection, monitoring tumour burden and therapeutic responses, detecting minimal residual disease, assessing risks of dissemination and recurrence.	Early identification of relapse and treatment resistance, guidance of treatment selection, monitoring therapeutic responses.	Understanding mechanism of resistance, efficacy of new treatments



L.A.Diaz and A. Bardelli, J Clin Oncol. 2014; 32(6): 579–586

Clinical utility of CTCs



ctDNA analysis

Principle of detection	Method	Type of alteration	Advantage(s)	Limitation(s)
PCR-based	Nested real-time PCR ARMS/Scorpion PCR PCR-SSCP Mutant allele-specific PCR Mass spectrometry Bi-PAP-A amplification	Known point mutations such as *KRAS*, *EGFR*, and *PIK3CA* hotspot alterations	Ease of use, lowest cost	Lower sensitivity, only detect limited genomic loci
Digital PCR	BEAMing Droplet-based digital PCR Microfluidic digital PCR	Known point mutations, genomic rearrangements	High sensitivity	Only detect limited genomic loci
Targeted deep sequencing	SafeSeq TamSeq Ion-AmpliSeq™ CAPP-Seq OnTarget	Selected SNVs, CNVs, and rearrangements across targeted regions	High sensitivity, relatively inexpensive	Less comprehensive than WES methods
Whole-genome sequencing	Digital karyotyping PARE	Genome-wide SNVs, CNVs, and rearrangements	Broad application	Expensive

PCR polymerase chain reaction, ARMS amplified refractory mutation system, SSCP single-strand conformation polymorphism, Bi-PAP-A amplification bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification, BEAMing beads, emulsion, amplification, and magnetics, SafeSeq safe sequencing system, TamSeq tagged amplicon deep sequencing, CAPP-Seq cancer personalized profiling by deep sequencing, PARE personalized analysis of rearranged ends, KRAS Kirsten rat sarcoma viral oncogene homolog, EGFR epidermal growth factor receptor, PIK3CA phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha, SNV single-nucleotide variants, CNVs copy number variations, WES whole-exome sequencing. Z. Qin et al, Chin. J. Cancer 2016,35:36

Before implementation of cfDNA analysis into the clinical decision

several issues need to be addressed:

- Because cfDNA represents mainly the genome of dying tumour cells, the time point of blood sampling during treatmen is important to discover genetic alterations present in the resistant tumour cell clones.
- Genomic screening using NGS technologies has made enormous advances but has also led to the discovery of many genomic aberrations without known clinical relevance.
- Clinical intervention studies with established endpoints (e.g., overall survival) in which decisions are made on the basis of cfDNA analysis are needed to demonstrate that the patient will benefit from cfDNA measurements.

Diagnostic Test Validation

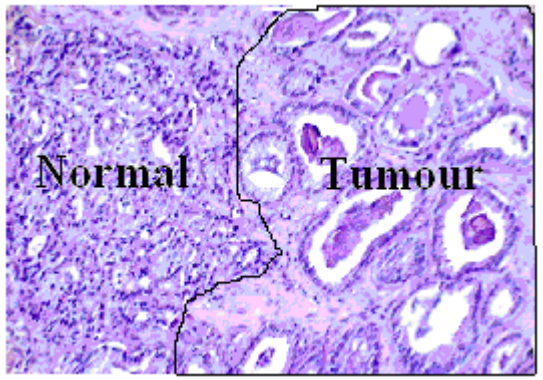
- **Learning set** (identify serum biomarker patterns)
- **Validation set (**screen subsequent patients)

- **Prospective diagnostics trial**
  - Example:
    - Trial of 200 patients proceeding to biopsy
    - Serum from 50 biopsy negatives (normal)
    - Serum from 50 biopsy positives (disease)
    - Apply rules to next 100 patients in the trial

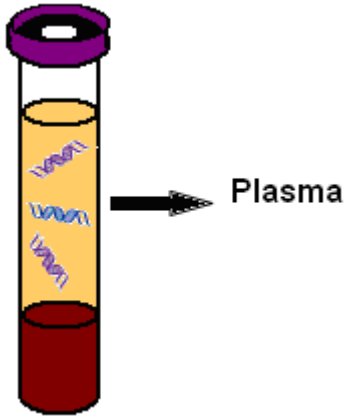
- Validate new serum-based, “home-brew” diagnostic test
- Save lives and reduce unnecessary biopsies

FFPE versus ctDNA

- **FFPE Samples**
- Tumour DNA extracted from fixed biopsy samples or tumour resections
- Problems with quality of DNA due to fixation
- Mixture of normal and tumour DNA
- Long time to process by histopathologists.
- Macrodissected to enrich tumour content
- Some patients have no tumour sample available
- The sample represents the tumour at one fixed



- **ctDNA Samples**
- ctDNA shed directly from tumour
- Extracted from the plasma component of whole blood
- Large fragment sizes possible
- Small quantities extracted ~ 30ng/ 5ml plasma
- Separate out plasma within a few hours of receipt of blood sample.
- Serial samples can be taken at various time points during th t



Kirsty Hambridge

Prostate cancer

Prostate cancer

- Prostate cancer is one of the most common cancers in Europe, accounting for 23% of all new cases of cancer in males. It is also estimated to be the third leading cause of cancer mortality.
- Prostate cancer therefore constitutes a significant public health burden, and like many other cancers, the burden is likely to increase due to the continued growth in the ageing population.
- The principal cause of death from prostate cancer is due to recurrences or metastases, particularly to the skeleton, causing debilitating bone pain and pathological fractures.

Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J. W. W., Comber, H., Bray, F. (2013). Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. *European Journal of Cancer*, *49*(6), 1374–1403.

Prostate cancer

- The use of prostate-specific antigen (PSA) as a serum marker has revolutionised PCa diagnosis.
- However, this antigen is not cancer-specific and may be elevated in association with other non-malignant conditions, such as benign prostatic hyperplasia (BPH) and prostatitis.
- Several new biological markers such as BRCA2, TMPRSS2-Erg fusion and PCA3 and four-kallikrein panel have been shown to add sensitivity and specificity to PSA.
- Current evidence regarding these biomarkers is too limited for the development of recommendations.

Prostate cancer early diagnosis

- The advent of liquid-biopsy, such as blood circulating free DNA (cfDNA), may have important applications in PCa diagnosis and management.

- Seminal plasma cfDNA (scfDNA) assessment may be able to assist in risk stratification, diagnosis and therapeutic monitoring of PCa patients.

Circulating DNA in prostate cancer: results of our study

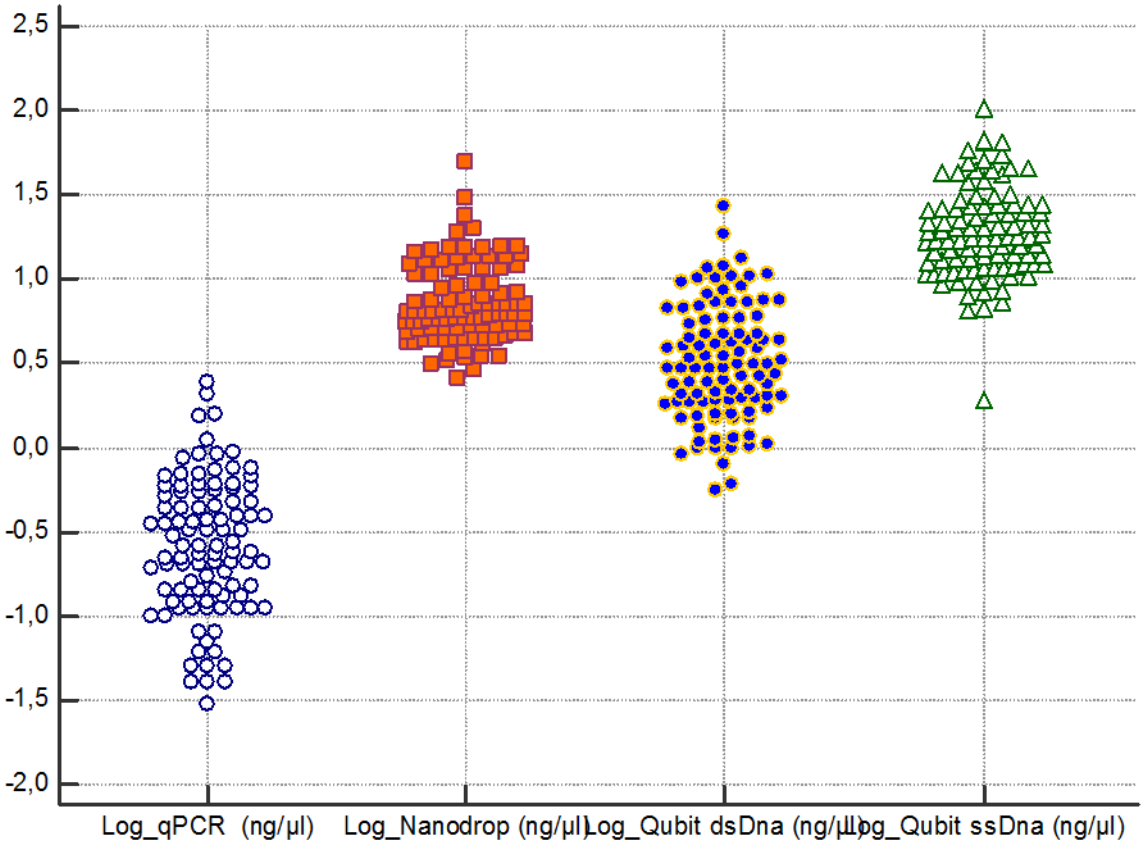
- Identify tumour-specific ctDNA biomarkers in the plasma of 90 patients with histologically confirmed prostate cancer and 15 healthy donors, and to investigate their clinical relevance as a diagnostic and prognostic tool.
- Multicentre cohort study (University of Modena and Akdeniz University - Antalya) prospectively for the evaluation and characterization of the role of tumour cfDNA assets in populations of patients suffering from prostate cancer.

		PCa (n = 96)
Median age (years)		**67**
PSA (range ng/mL)		**0.20 – 25.00**
Gleason Score		
	G < 7	**44**
	G ≥ 7	**52**
Tumour clinical stage		
	T1 **T2** **T3**	**26** **58** **12**

STUDY DESIGN

- cfDNA was extracted from plasma through Qiagen kit and Promega automatic extractor.
- Qubit 2.0, Nanodrop techniques were applied for measurements of total amount cfDNA
- The cfDNA amount was confirmed by the qPCR, the gold standard for quantitation of cfDNA.

Plasma study



Boxplots showing the data (Log of the measurements) for the 4 techniquest that were applied for the quantification of cfDNA.

Qubit 2.0 and Nanodrop have the tendency to overestimate cfDNA compared to qPCR which amplified only a short fragment of amyloid protein adopted in this cfDNA assessment.

Non-blood DNA

Biological sources of cfDNA


Saliva


CSF


Blood


Seminal


Pleural


Urinary


Ascites


Stool

Liquid biopsy non-blood DNA sources

- Seminal plasma cfDNA, which can be analysed with cost-effective procedures, may provide powerful information capable to revolutionize prostate cancer (PCa) patient diagnosis and management.
- In the near future, cfDNA analysis from non-blood biological liquids will become routine clinical practice for cancer patient diagnosis and management.

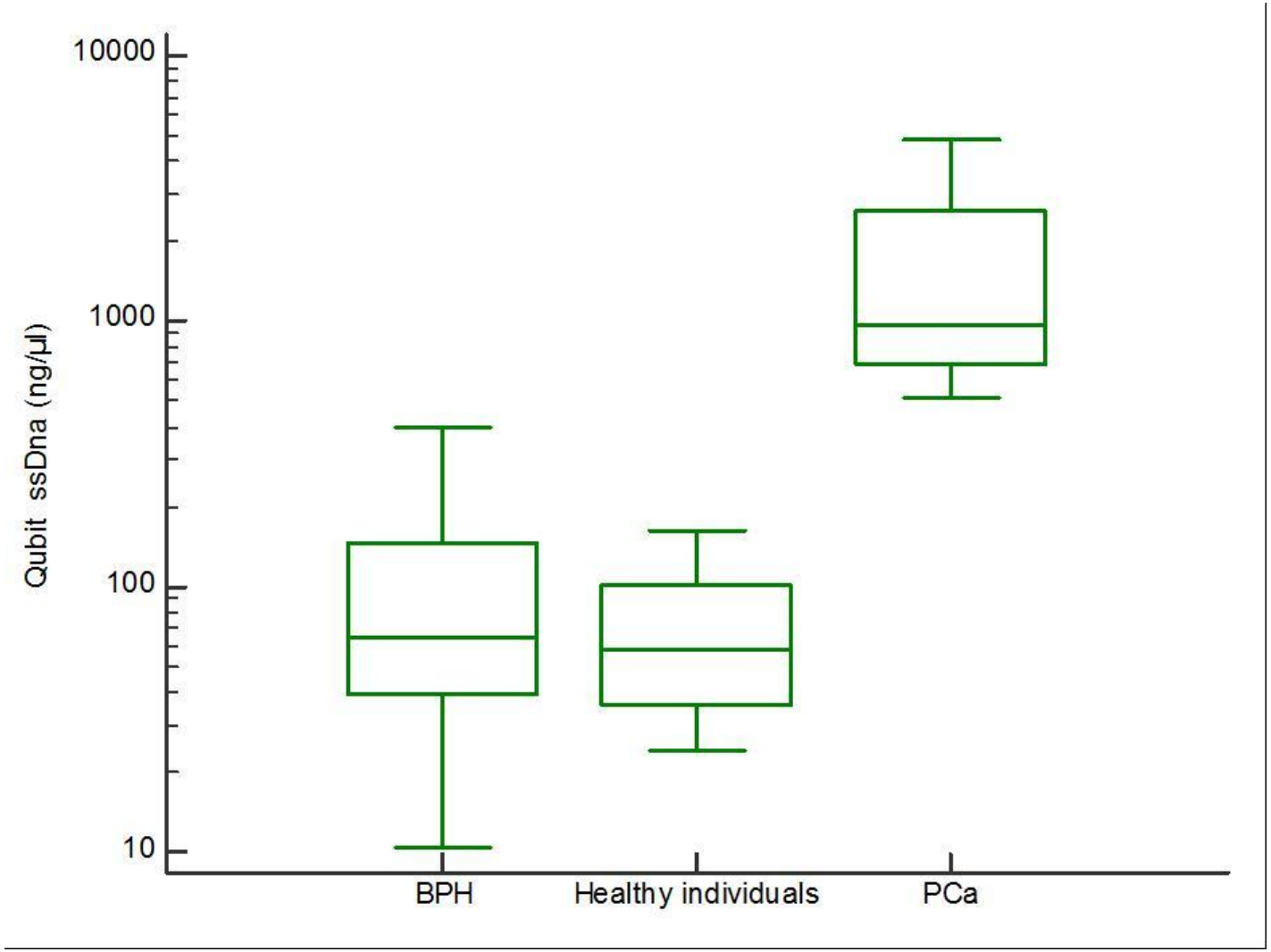
Methods

- A cohort of 31 patients with pathology proven PCa (12 patients) and BPH (19 patients), and 4 healthy age-matched control subjects were enrolled.
- Samples of seminal fluid were processed and analyzed within 2 hours of collection.
- Following cfDNA extraction, Qubit was used for cfDNA quantification.
- Comparative data was assessed with the multivariate ANOVA test and p-value of <0.001 was considered significant.

Results

PC Patients	Age	PSA (ng/ml)	Gleason Score	Qubit ssDna (ng/ml)
Mean	67,18	7,16	Range: 6-8	1721,27
BPH Patients	**Age**	**PSA (ng/ml)**		**Qubit ssDna (ng/ml)**
Mean	60,63	5,64		119,52
Healthy individuals	**Age**	**PSA (ng/ml)**		**Qubit ssDna (ng/ml)**
Mean	52,75	1,4		75,4

Box-Whisker plot of cfDNA concentration levels



Results

- cfDNA in PCa patients derive both from cancer cells and non-cancer cells induced apoptosis by pro-apoptotic cytokines released from prostate cancer cells.

- The measurement of total cfDNA may be useful for cancer detection .

Conclusions

- Several non-blood biologic fluids are potential sources for quantification and characterization of cfDNA in the clinical setting. Among these, seminal fluid is a precious source of nucleic acids, characterized by higher values of cfDNA with respect to blood.

- Seminal fluid, which also contains prostatic secretions, can be adopted as a useful biomarker for differential diagnosis between PCa and BPH patients.

- Seminal fluids of PCa patients were characterized by significantly higher values of cfDNA whilst BPH patients had lower seminal cfDNA concentration, similar to that observed in healthy volunteers.

Conclusions

- ScfDNA analysis can be applied to risk stratification, diagnosis and therapeutic monitoring of Prostate Cancer patients.

- cfDNA concentration is significantly different between PCa patients and BPH patients, being potential biomarkers for PCa diagnosis and screening programs and therapeutic monitoring.

Future Trends

- Clinical applications for the non-invasive diagnosis of several diseases will be forthcoming in the near future .
- It is thus likely that non-invasive genome-wide analysis will play an increasingly important role in the future practice.
- With further advances in technology and reduction in costs, it is possible that methylomic and transcriptomic profiling might eventually become routine clinical tests.

Personalized cancer medicine

- Giving the right drug, to the right patient, at the right dose, from the right moment onwards, till the right moment
- Higher chance to benefit when treated with less toxicity
- Better cost-effectiveness

the project was made possible by:

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