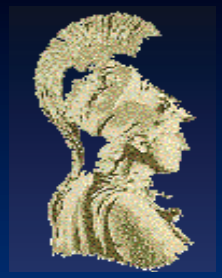


"Challenges in CTC Molecular characterization"

9th ISMRC meeting

Paris, 24 – 27 September 2013

Evi S. Lianidou, Ph.D.

University of Athens

Greece

Outline

- Molecular characterization of CTC :
- Gene expression
- DNA methylation
- DNA mutations
- Immunofluorescence - Single cell level
- Quality Control issues - Comparison between CTC assays
- Clinical implications of the molecular characterization of CTC
- Conclusions - Future perspectives

Molecular characterization of CTC

CTC Enumeration vs Molecular characterization

- Despite the development of numerous CTC platforms using various combinations of enrichment and detection steps, the CellSearch system, developed in the early 2000s, is still the only FDA cleared system for use in the clinical setting

- Using the CellSearch it has been clearly demonstrated that CTCs are correlated with patient outcome and that the change in CTC number during treatment is predictive of therapy response, often sooner than currently utilized techniques such as imaging

- However, simple enumeration of CTCs is not enough to get their full potential as biomarkers of metastatic disease.

Clinical Chemistry 58:5
936–940 (2012)

Brief Communication

Circulating Epithelial Cells in Patients with Benign Colon Diseases

Klaus Pantel,[1] Eric Denève,[2] David Nocca,[2] Amandine Coffy,[3] Jean-Pierre Vendrell,[4] Thierry Maudelonde,[5] Sabine Riethdorf,[1] and Catherine Alix-Panabières[3,4,5*]

[1] Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [2] Department of Digestive Surgery, University Medical Centre, Saint-Eloi Hospital, Montpellier, France; [3] University Institute of Clinical Research

patients with diverticulosis and Crohn disease. All positive events lacked expression of CD45, a common leukocyte antigen.

CONCLUSIONS: These results indicate that patients with benign inflammatory colon diseases in particular can harbor viable circulating epithelial cells that are detectable with current CTC assays. This finding points to the need for further molecular characterization of circulating epithelial cells and has important implications for the use of CTC testing.

In conclusion, the findings of this study have important implications for the use of CTC testing in the clinical laboratory and point to the need for molecular characterization of these cells. Molecular characterization of CTCs is absolutely necessary; simple enumeration will not suffice. The future use of CTCs lies in the molecular characterization of these cells. CTC technologies that are complementary, such as imaging and molecular-characterization methods, should be used in combination to provide a complete view of the malignant nature of these cells. Moreover, standardization of protocols for isolating and detecting CTCs, cross-validation of findings between laboratories, and universal internal and external QC systems for CTC detection and enumeration are necessary and needed.

Clinical Chemistry 58:5
805–807 (2012)

Editorials

Circulating Tumor Cells—New Challenges Ahead

Evi S. Lianidou[1*]

Table 2. Clinical relevance of CTC in metastatic BC

CTC detection	Number of patients	Clinical relevance – conclusions	References
CellSearch assay	177 MBC patients	CTC represent an independent prognostic factor for PFS and OS in patients with metastatic BC. The number (⩾5) of CTC at baseline and at the first follow-up visit were the most significant predictors of PFS and OS.	Cristofanilli *et al*, 2004
AdnaTest BreastCancer	39 MBC patients (226 blood samples)	A major proportion of CTC of metastatic BC patients shows EMT and tumour stem cell characteristics.	Aktas *et al*, 2009
CellSearch assay *vs* AdnaTest BreastCancer	254 MBC patients	The rate of BC patients with HER-2-negative primary tumours but HER-2-positive CTC was 32% using the CellSearch and 49% using the AdnaTest BreastCancer.	Fehm *et al*, 2010
CellSearch assay	235 MBC patients median follow-up: 18 months	The prognostic information provided by CTC count may be useful in patient stratification and therapy selection, particularly in the CTC-positive group, in which various therapeutic choices may procure differential palliative benefit.	Giuliano *et al*, 2011
CellSearch assay	517 MBC patients	Risk of death increased linearly with increasing CTC count in all molecular tumour subtypes, but was higher in ER+ and triple-negative MBC than in HER2+. CTC' prognostic effect was less evident in HER2+ MBC patients treated with targeted therapy.	Giordano *et al*, 2012
Double immunofluorescence (Ariol system)	25 MBC and 25 early BC patients	CTC expressing Twist and vimentin, suggestive of EMT, were identified. EMT is involved in the metastatic potential of CTC.	Kallergi *et al*, 2011
Various systems	Meta-analysis (1990–2012), $n=3069$	Detection of CTC is a reliable prognosticator in patients with metastatic BC: PFS: HR 1.78, 95% CI 1.52–2.09; OS: HR 2.33, 95% CI 2.09–2.60.	Zhang *et al*, 2012
CellSearch and AdnaTest Breast Cancer	254 MBC patients	The prognostic relevance of CTC detection in metastatic BC patients depends on the test method.	Muller *et al*, 2012
CellSearch assay	267 MBC patients	Elevated CTC before the second cycle was an early predictive marker of poor PFS and OS, and could be used to monitor treatment benefit CTC decrease under treatment was stronger with targeted therapy.	Pierga *et al*, 2012

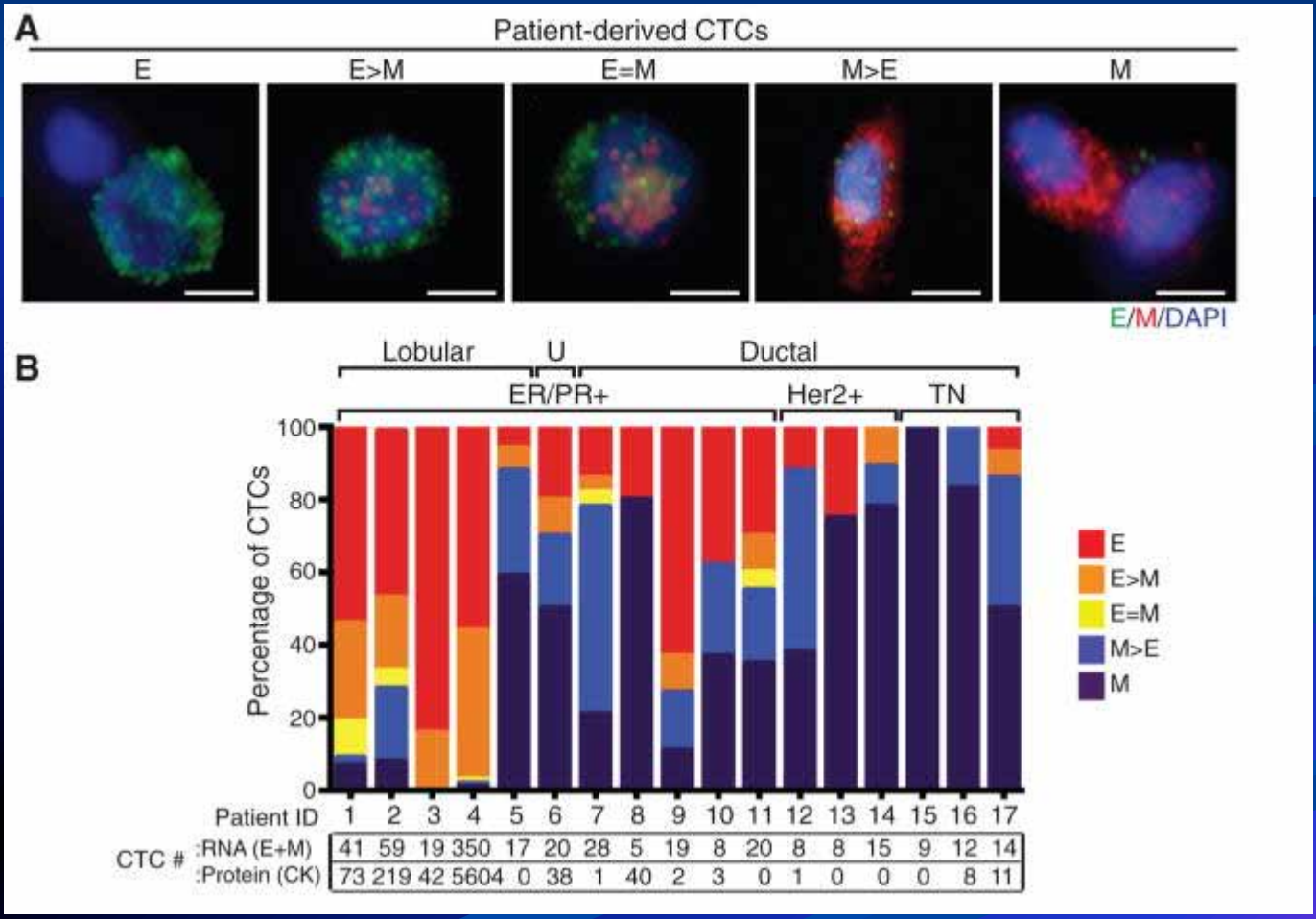
Lianidou et al., (minireview) Br J Cancer, 2013

Science. 2013 Feb 1;339(6119):580-4. doi: 10.1126/science.1228522.

Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition.

Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, Isakoff SJ, Ciciliano JC, Wells MN, Shah AM, Concannon KF, Donaldson MC, Sequist LV, Brachtel E, Sgroi D, Baselga J, Ramaswamy S, Toner M, Haber DA, Maheswaran S.

Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA 02129, USA.



CANCER

Circulating Tumor Cells

Vicki Plaks, Charlotte D. Koopman, Zena Werb

Much remains to be learned about CTCs and their clinical potential as biomarkers and therapeutic targets.

Science, Perspectives, 13 September, 2013

The potential clinical value of CTCs is clear: Early detection and treatment of metastatic spread are key for disease outcome, and CTCs offer the ability to target metastasis in real time.

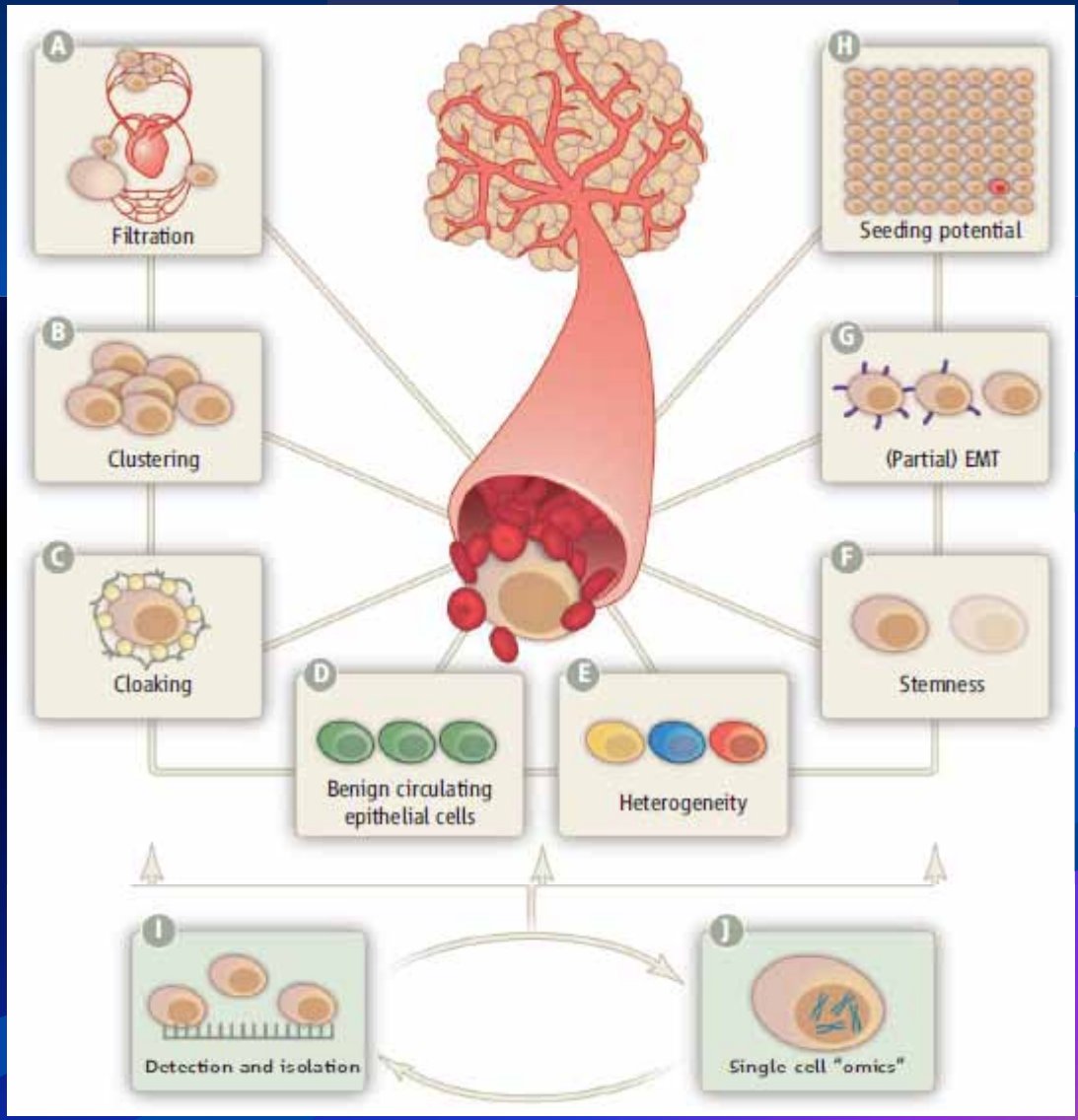
Although CTCs are not yet proven to be the metastatic cells, there is no evidence that they are incapable of being so.

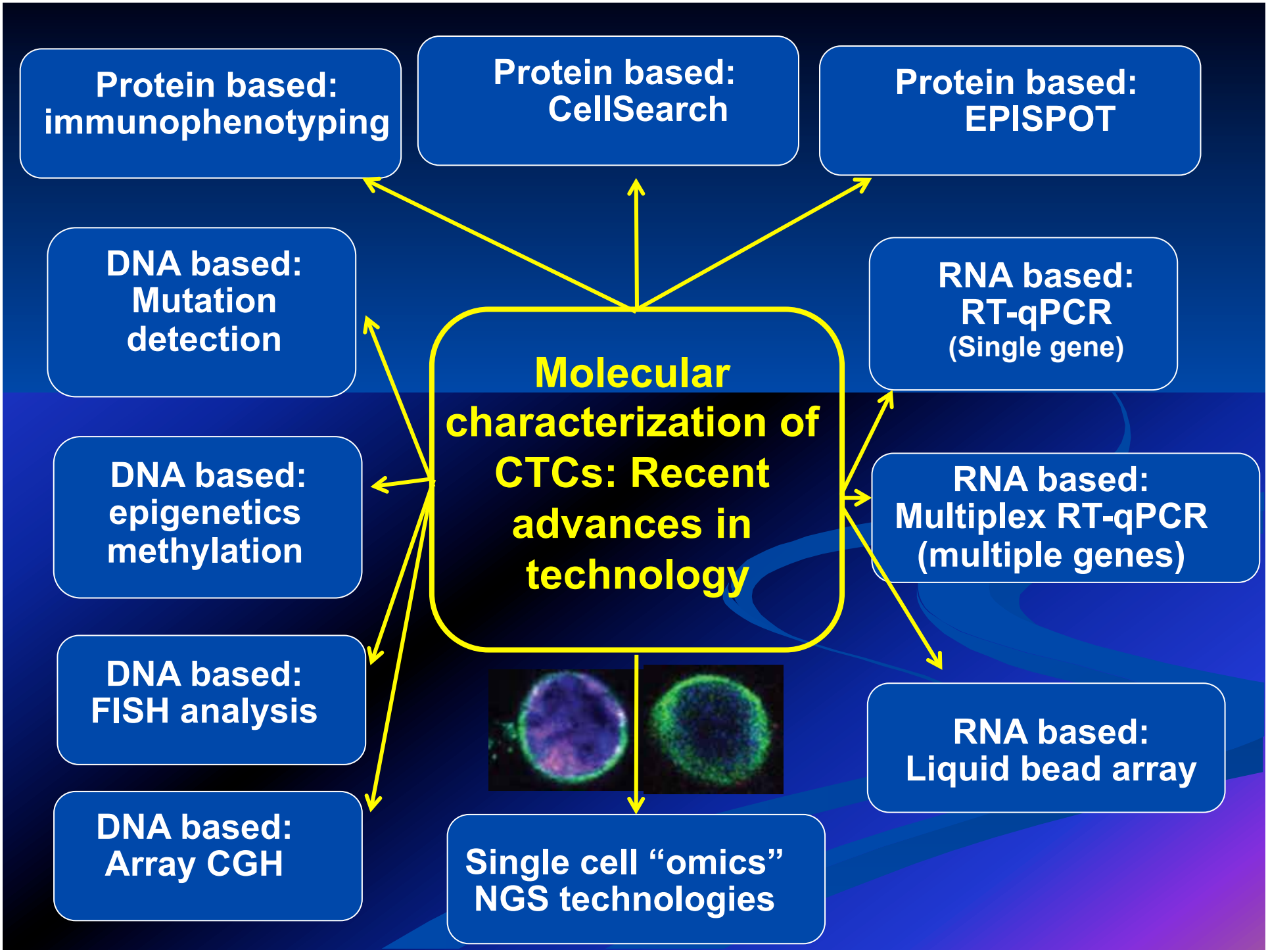
However, a simple enumeration of CTCs without molecular characterization may lead to wrongful clinical assumptions and consequences.

Elucidating CTC biology will also help standardize detection and isolation of the potentially metastatic subpopulation of CTCs.

The next frontier in the CTC field is their characterization using the constantly improving single cell "omics" techniques

This will ultimately determine the clinical value of CTCs as biomarkers and therapeutic targets.




Protein based: immunophenotyping
Protein based: CellSearch
Protein based: EPISPOT
DNA based: Mutation detection
RNA based: RT-qPCR (Single gene)
Molecular characterization of CTCs: Recent advances in technology
DNA based: epigenetics methylation
RNA based: Multiplex RT-qPCR (multiple genes)
DNA based: FISH analysis
RNA based: Liquid bead array
DNA based: Array CGH
Single cell "omics" NGS technologies

Molecular CTC assays/ single gene expression

Real-time quantification of CK-19 mRNA positive cells in peripheral blood of breast cancer patients using the LightCycler system

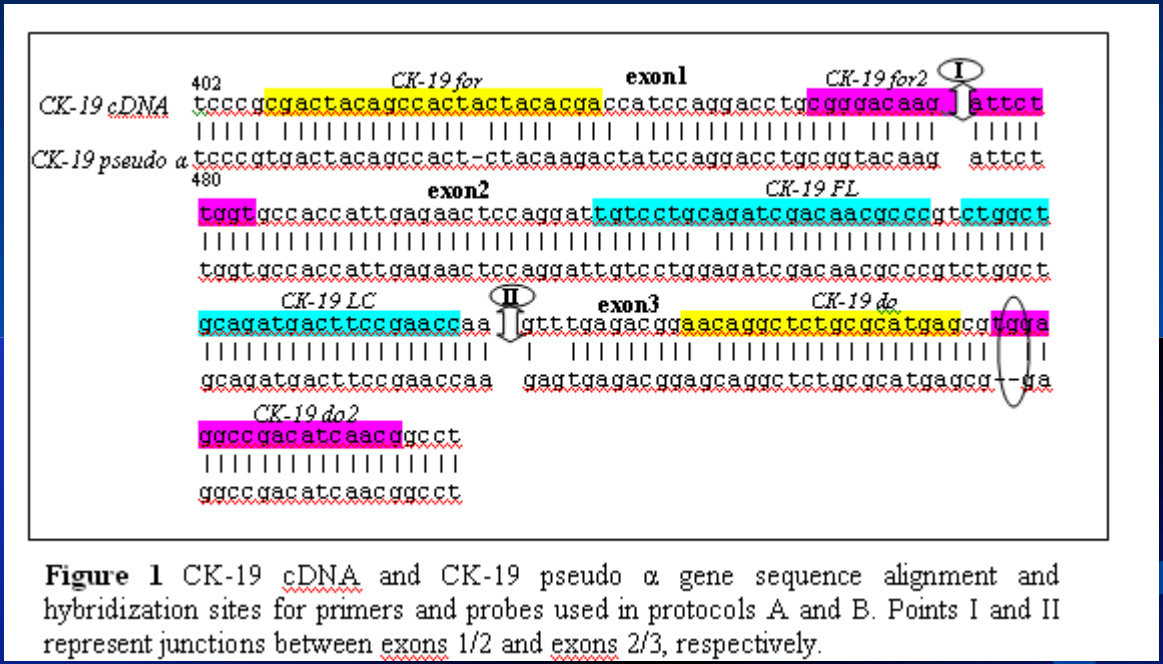
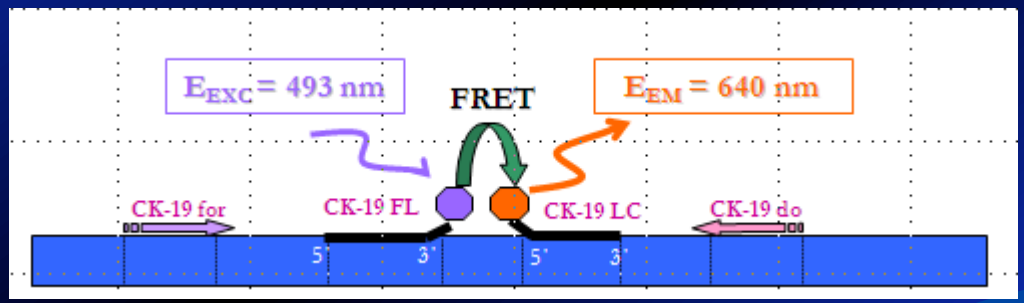


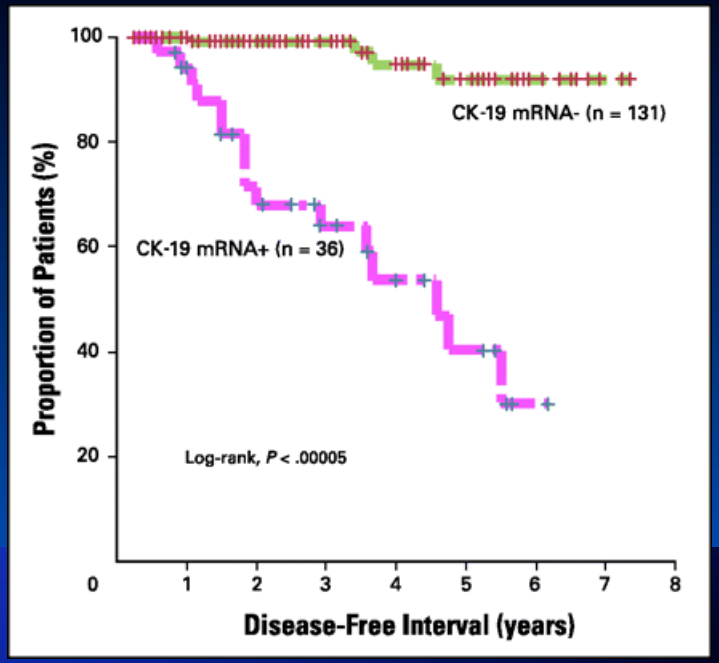
Figure 1 CK-19 cDNA and CK-19 pseudo α gene sequence alignment and hybridization sites for primers and probes used in protocols A and B. Points I and II represent junctions between exons 1/2 and exons 2/3, respectively.

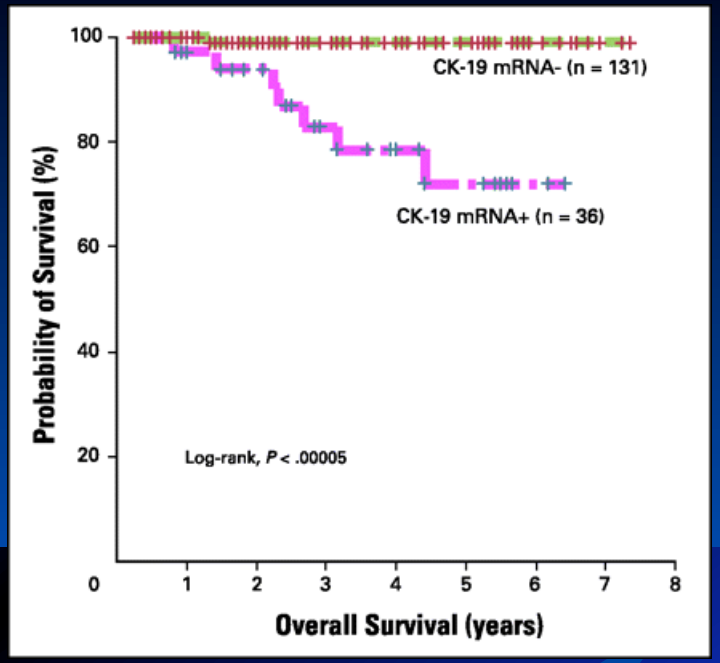


EpCAM independent assay for epithelial CTC detection

Stathopoulou A, et al. Clin. Cancer Res., 2003

Stathopoulou A, et al. Int J Cancer, 2006





Xenidis et al JCO 2006: ***node negative breast cancer before chemotherapy***

Xenidis et al Ann of Oncology 2007: ***during chemotherapy***

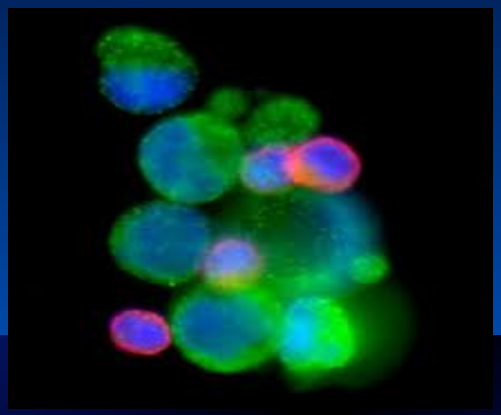
Xenidis et al JCO 2009: ***after chemotherapy***

Molecular CTC assays/

multiplex RT-PCR assays for gene expression in CTCs

Epithelial Markers
Cytokeratins
(CK-8, CK-18, CK-19)
EpCAM, MUC-1

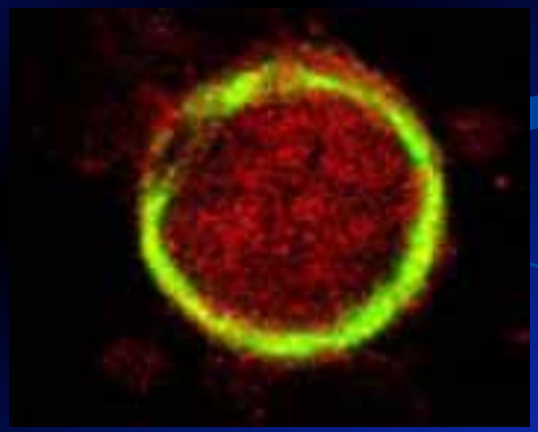
HER-2



ER/PR

DNA methylation
(CST6, BRMS1, SOX17)

Stemness and EMT markers
CD44, CD24, ALDH1, TWIST-1, vimentin, N-cadherin



mammaglobin

angiogenesis
VEGF, VEGF2, HIF1-alpha

PIK3CA pathway
(EGFR, HER2, PIK3K, pFAK)

Lianidou et al, Cancer and Metastasis Reviews, 2012

multiplex PCR assays for the molecular characterization of circulating tumor cells

1: Breast Cancer Res. 2009 Aug 10;11(4):R59. [Epub ahead of print]

Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells.

Fehm T, Hoffmann O, Aktas B, Becker S, Solomayer EF, Wallwiener D, Kimmig R, Kasimir-Bauer S.

1: Breast Cancer Res. 2009 Jul 9;11(4):R46. [Epub ahead of print]

Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients.

Aktas B, Tewes M, Fehm T, Hauch S, Kimmig R, Kasimir-Bauer S.

Clin Cancer Res. 2011 Jun 1;17(11):3600-18. doi: 10.1158/1078-0432.CCR-11-0255. Epub 2011 Apr 19.

mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients.

Sieuwerts AM, Mostert B, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, Dirix LY, van Dam PA, Van Galen A, de Weerd V, Kraan J, van der Spoel P, Ramírez-Moreno R, van Deurzen CH, Smid M, Yu JX, Jiang J, Wang Y, Gratama JW, Sleijfer S, Foekens JA, Martens JW.

Department of Medical Oncology, Josephine Nefkens Institute and Cancer Genomics Centre, Rotterdam, The Netherlands.

Multiplex RT-qPCR for gene expression in CTC

20mL peripheral blood

ficol gradient centrifugation

epithelial enrichment

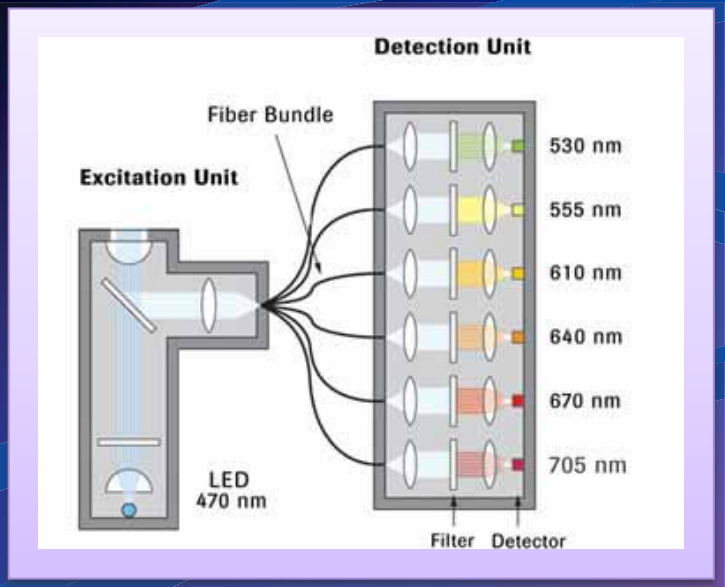
CTC fraction

PBMC fraction

mRNA isolation (oligo dT beads)

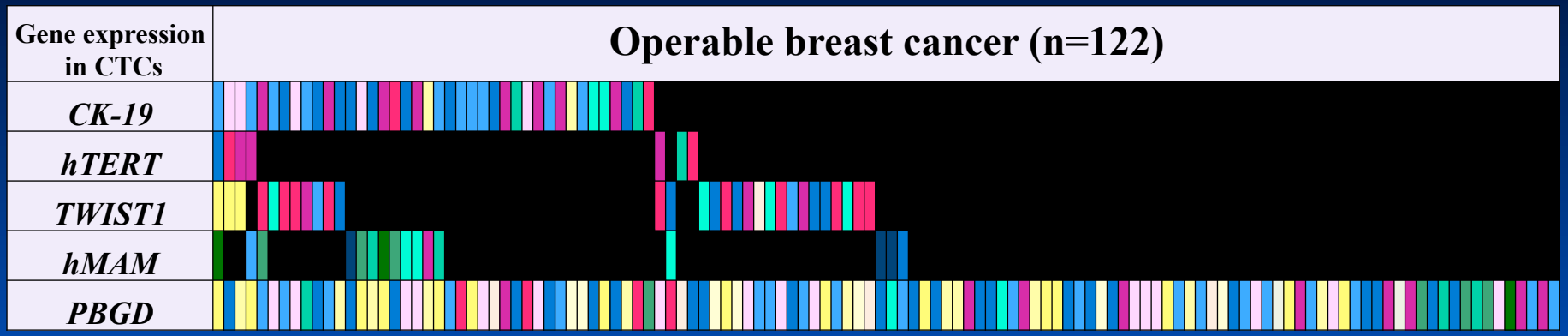
cDNA synthesis

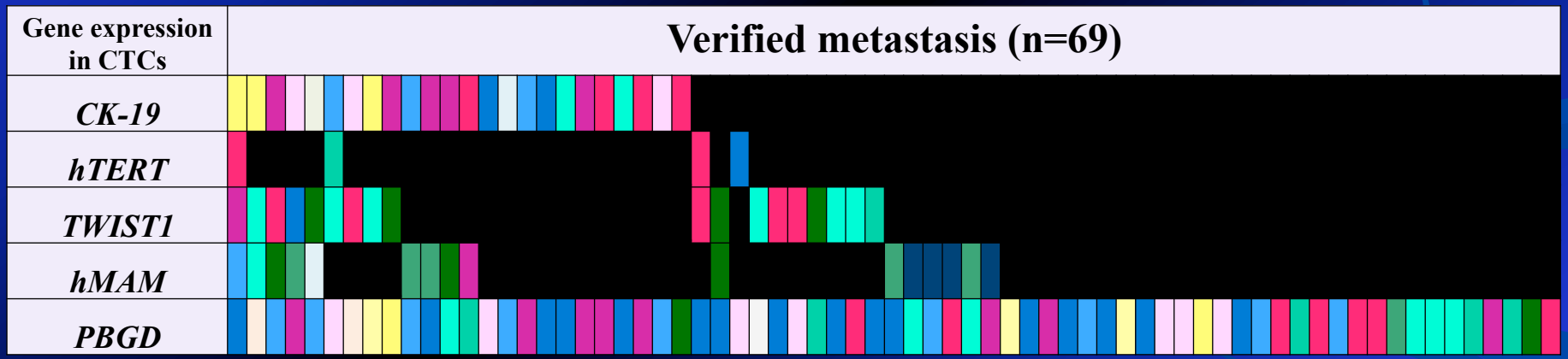
RT-qPCR



A. Strati et al, BMC Cancer, 2011

Same methodology, more samples
A. Strati et al, unpublished results





Cq legend	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41

Molecular CTC assays/

Liquid bead array assays for gene expression in CTCs

Molecular characterization of CTCs in breast cancer by liquid bead array hybridization assay

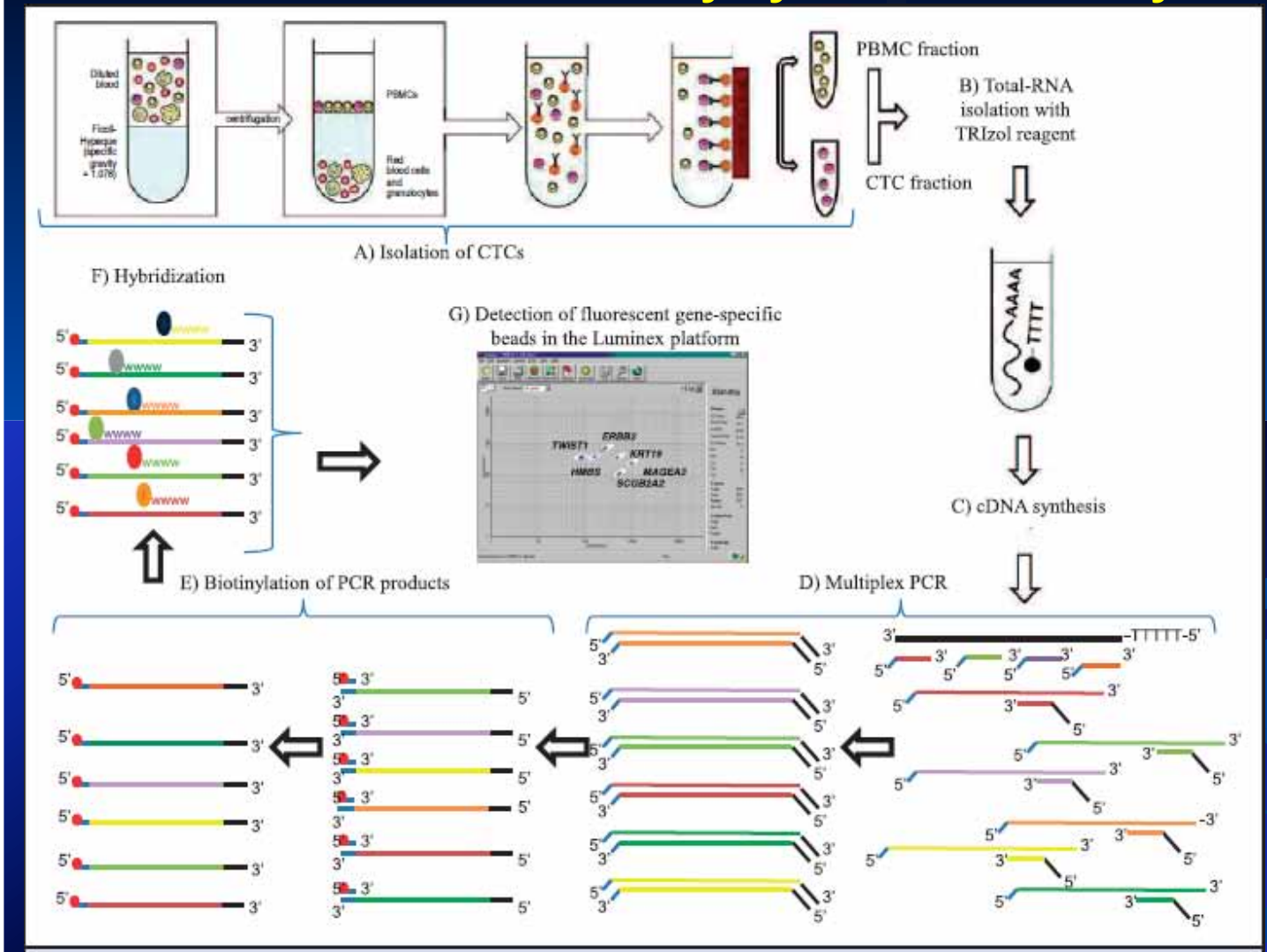
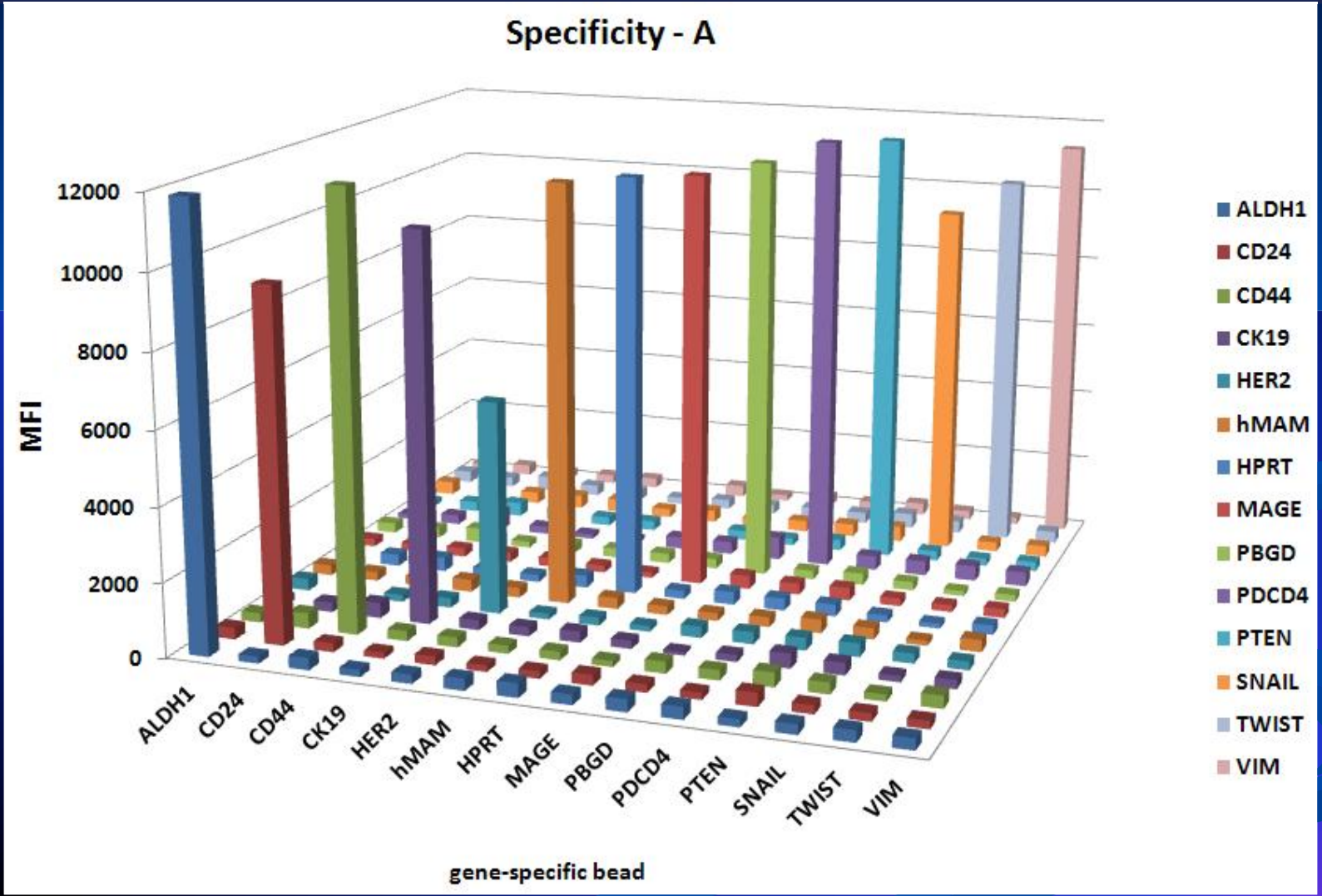


Fig. 1. Outline of the PCR-coupled liquid bead array.

Isolation of CTCs (A), isolation of total RNA with TRIzol reagent (B), cDNA synthesis (C), multiplex PCR (D), biotinylation of PCR products (1 step for all gene targets) (E), hybridization on gene-specific Luminex beads (F), and detection of fluorescent gene-specific beads on the Luminex platform (G). All forward primers in the multiplex PCR had a common extension sequence (T7), and all reverse primers had a common extension sequence (T3).

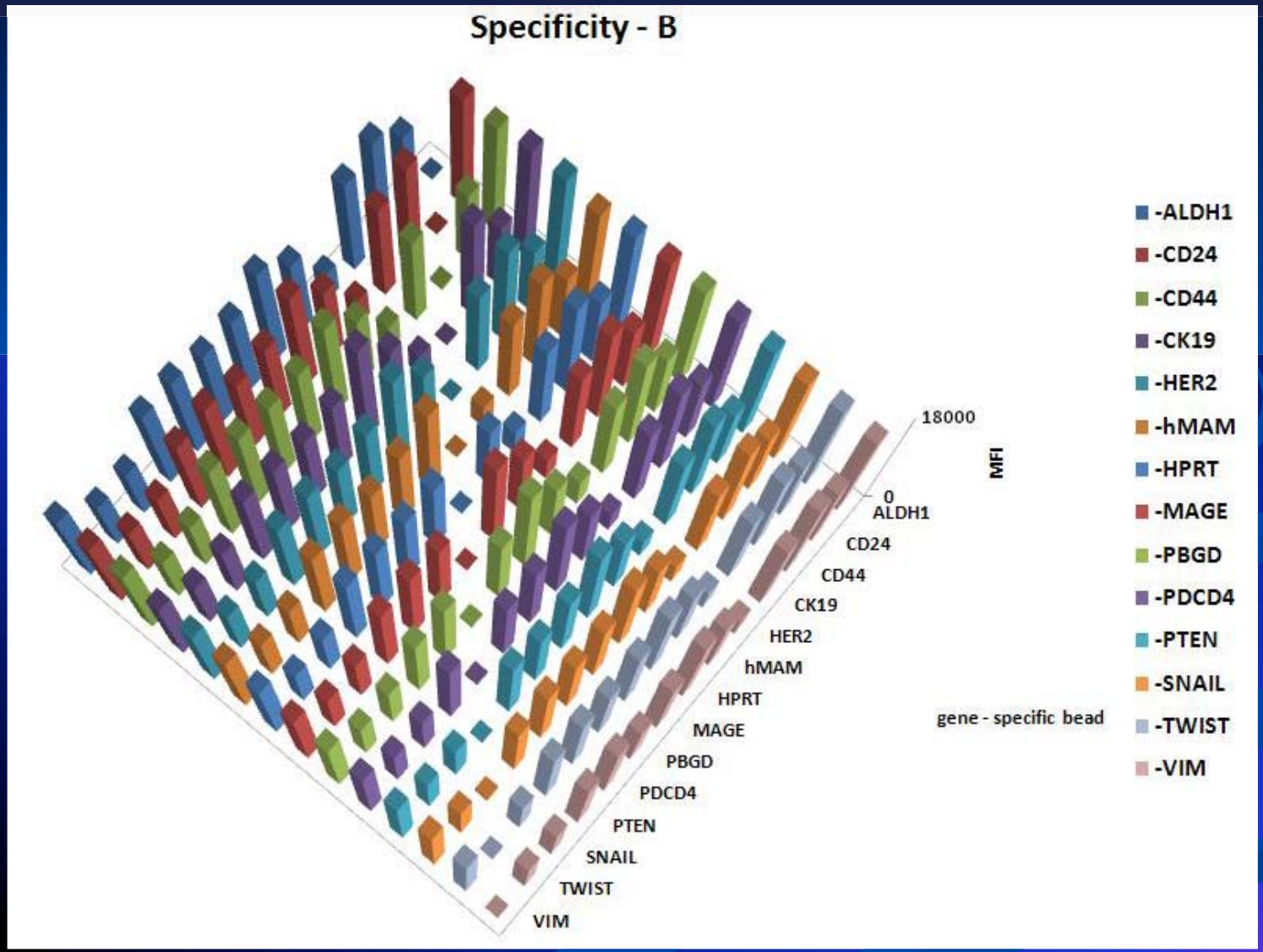
A. Markou et al, Clin Chem,, 2011

Liquid bead array hybridization assay for studying simultaneously the expression of 14 genes



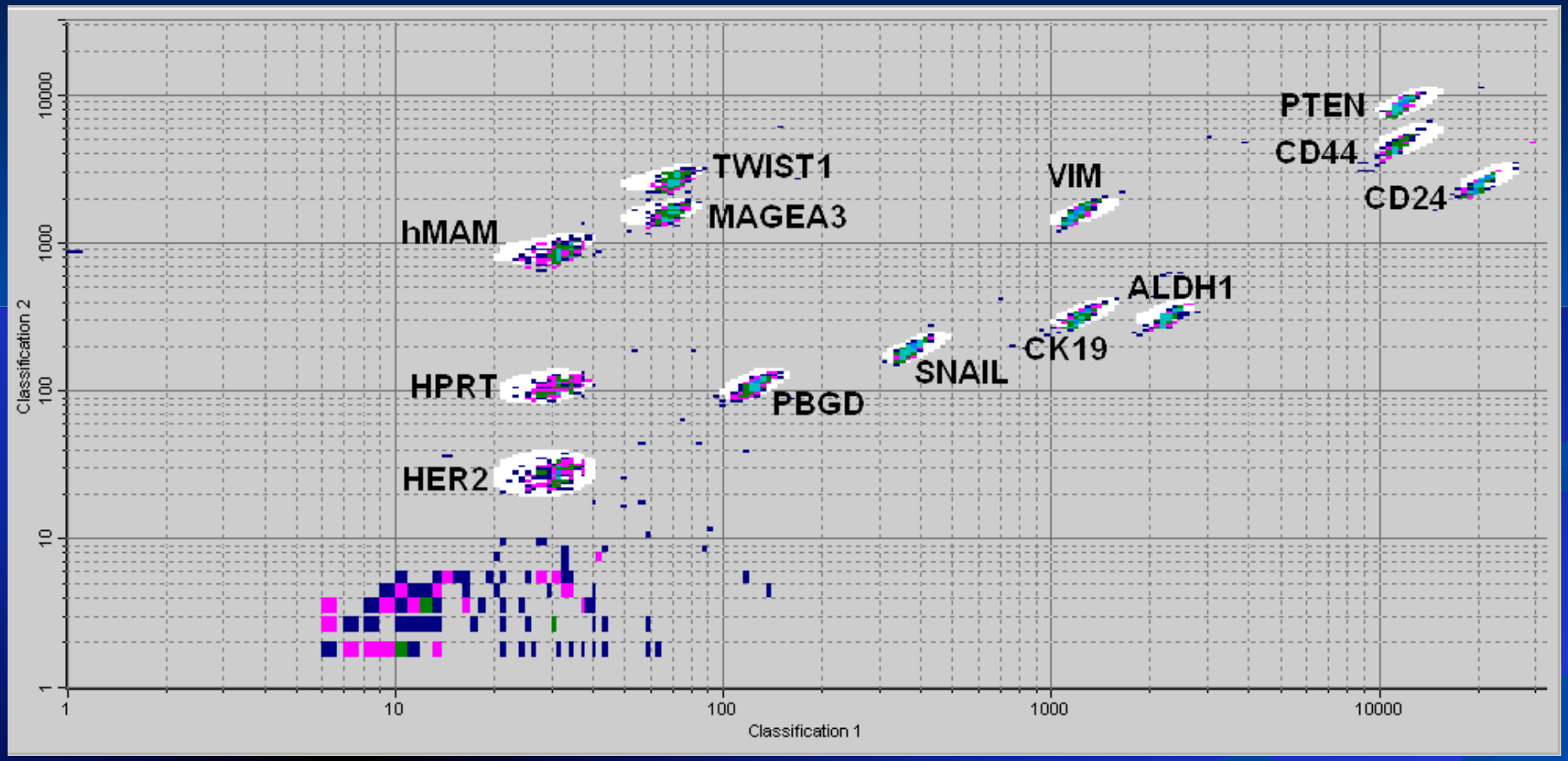
Kleo Parisi et al, manuscript in preparation

Liquid bead array hybridization assay for studying simultaneously the expression of 14 genes



Kleo Parisi et al, manuscript in preparation

Liquid bead array hybridization assay for studying simultaneously the expression of 14 genes



Kleo Parisi et al, manuscript in preparation

CTC are highly heterogeneous even within the same patient

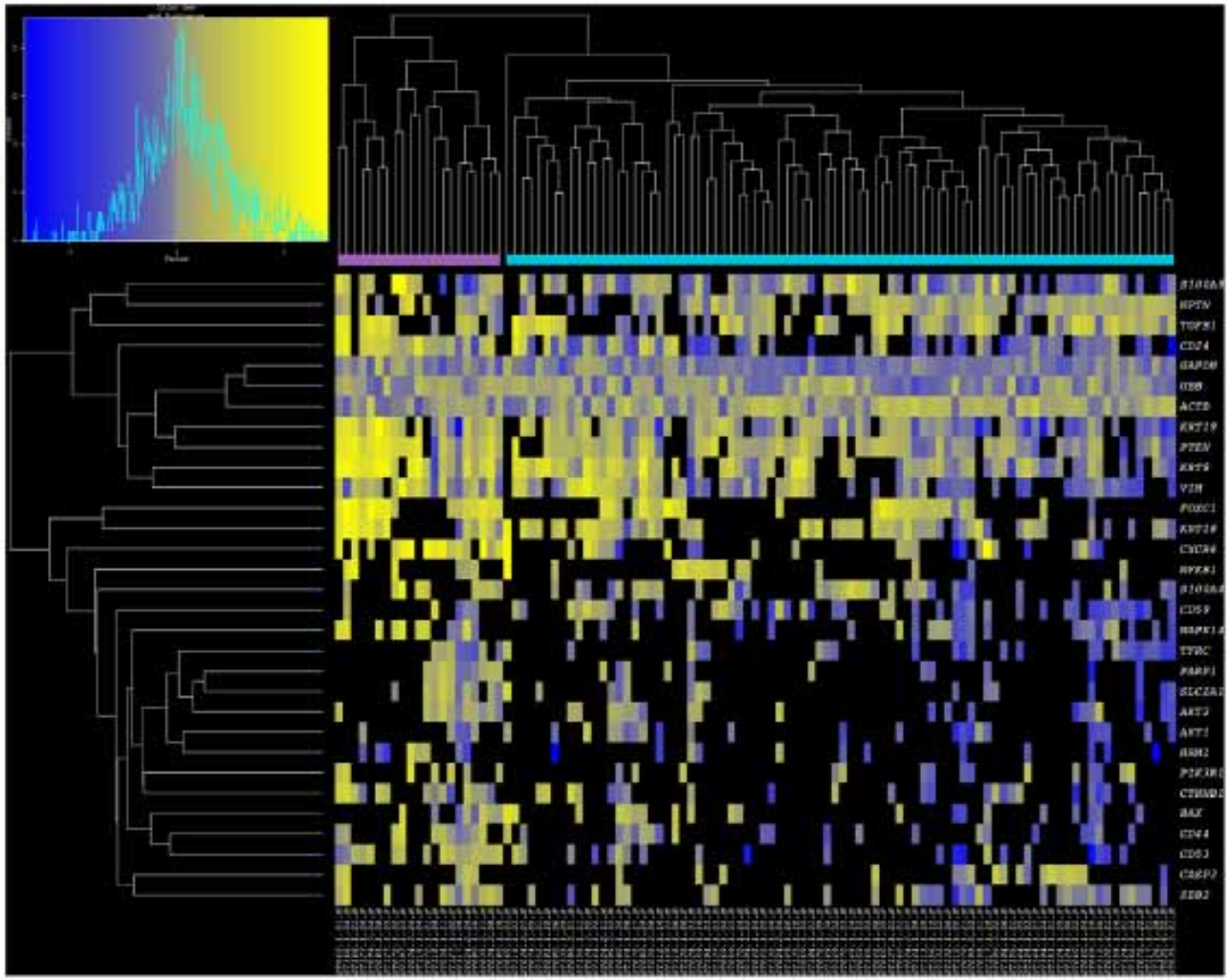


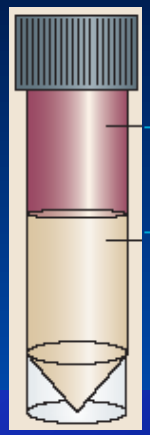
Figure 4. High dimensional single cell analysis and clustering of CTCs isolated from patients with breast cancer. Heatmap of single cell gene expression for 31-gene subset data derived from 105 CTCs isolated from patients with primary and metastatic breast cancer. Yellow indicates high gene expression; gray is median expression; blue indicates low expression; and black represents undetectable expression. The samples reveal two robust clusters for CTCs (lavender: Cluster I; turquoise blue: Cluster II). In addition to epithelial markers, these genes include pathways associated with EMT, metastasis, and AKT/mTOR signaling.
doi:10.1371/journal.pone.0033788.g004

Powell et al, PLOS one, 2012

Molecular characterization of CTC

DNA based: methylation studies

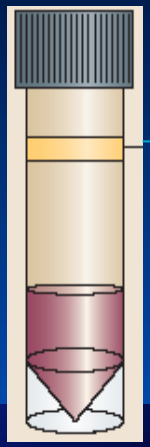
DNA methylation of tumor suppressor and metastasis suppressor genes in circulating tumor cells



20 mL Peripheral blood

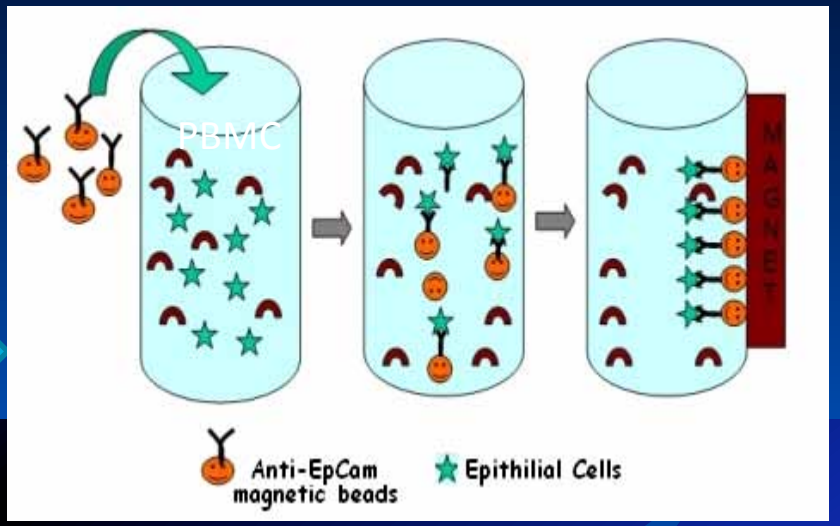
Ficoll

Gradient centrifugation



PBMC fraction

Collection of PBMC



Collection of Epithelial cells

DNA isolation

Genomic DNA extraction

gDNA from CTCs

Bisulfite Treatment

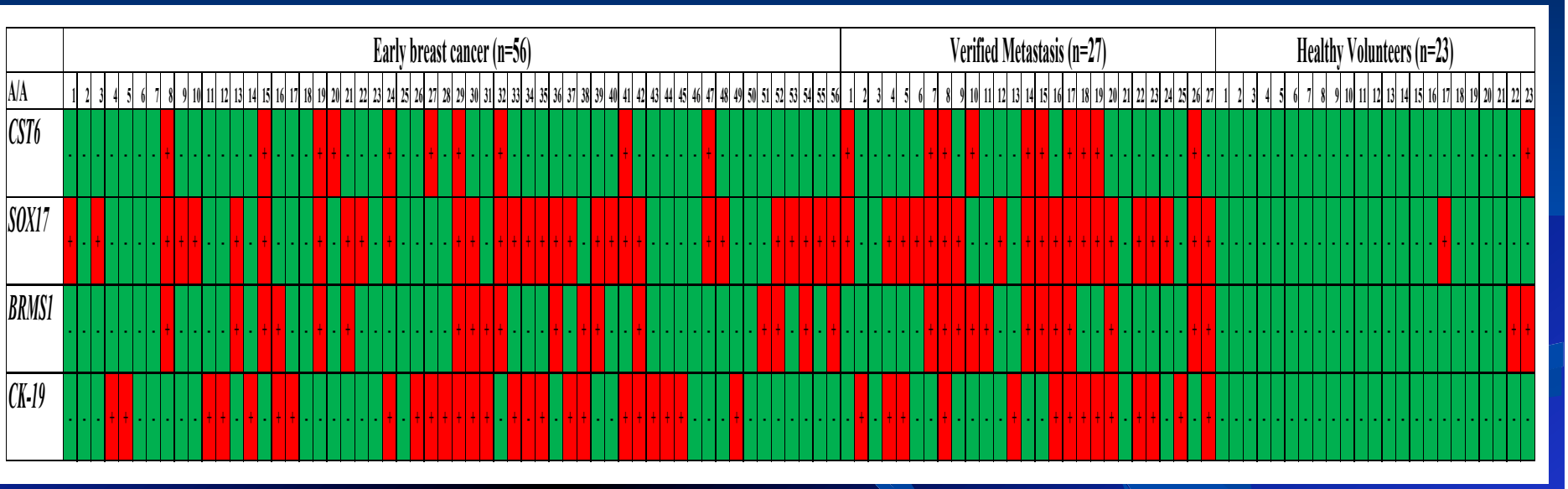
MSP

Group of patients studied

- 56 Early breast cancer
- 27 Verified metastasis
- 23 healthy

Maria Chimonidou et al, Clin. Chem., 2011

Heat map: DNA methylation in CTCs by Methylation Specific PCR (MSP)



Maria Chimonidou et al, Clin. Chem., 2011

Mol Cancer Res. 2013 Jun 6. [Epub ahead of print]

Breast Cancer Metastasis Suppressor-1 promoter methylation in primary breast tumors and corresponding Circulating Tumor Cells.

Chimonidou M, Kallergi G, Georgoulias V, Welch DR, Lianidou ES.

We evaluated *BRMS1* promoter methylation as prognostic biomarker in primary breast tumors and studied *BRMS1* promoter methylation in a subset of corresponding Circulating Tumor Cells (CTC) for the first time.

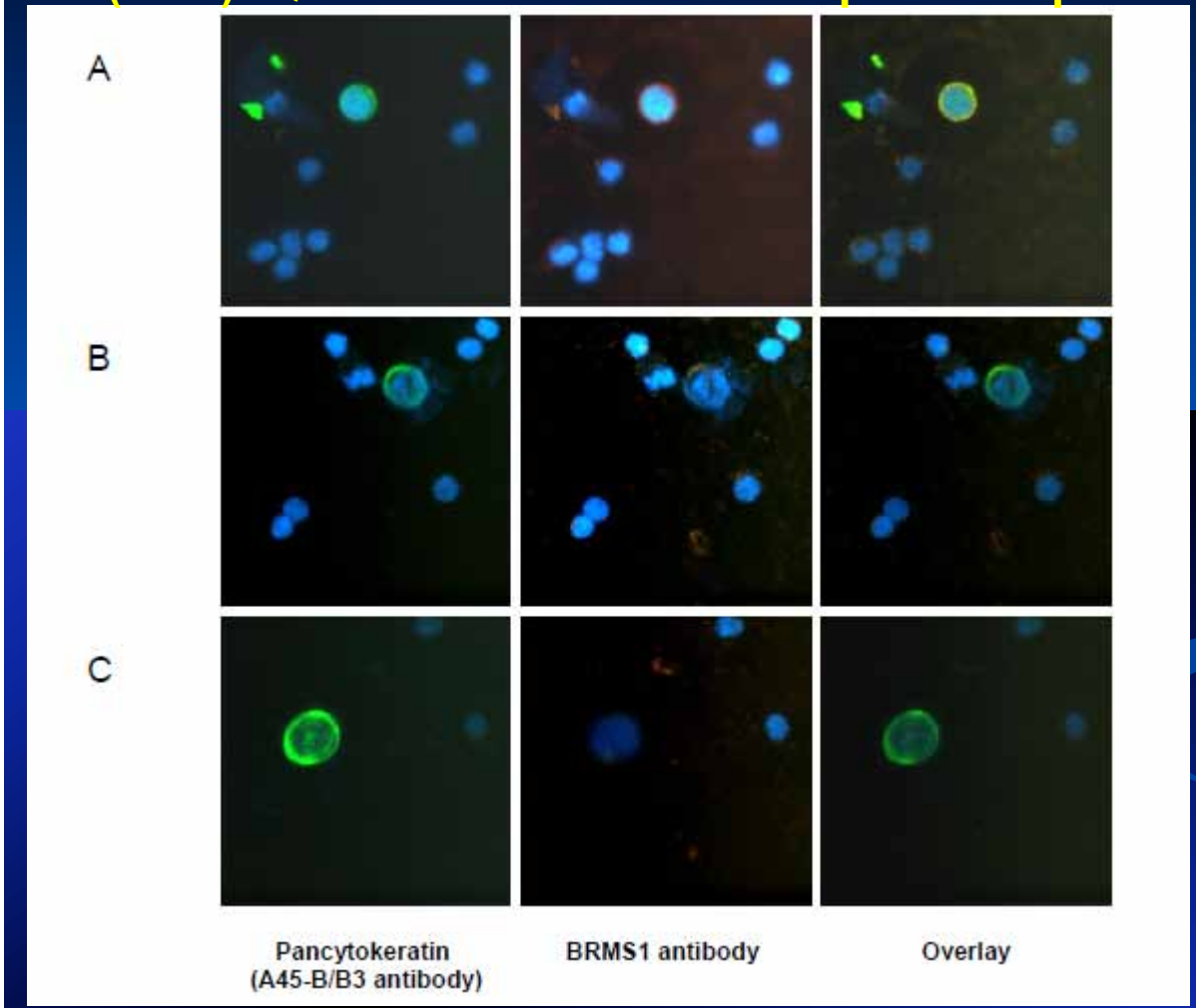
BRMS1 promoter methylation in primary breast tumors provides prognostic information for DFS.

Most CTC (80.5%) were negative for BRMS1 or maintained low expression, implying that BRMS1 is down regulated in these cells.

BRMS1 promoter methylation was observed in 5/39 (12.8%) CTC samples.

BRMS1 expression in CTC was highly heterogeneous, between patients and even in the same patient.

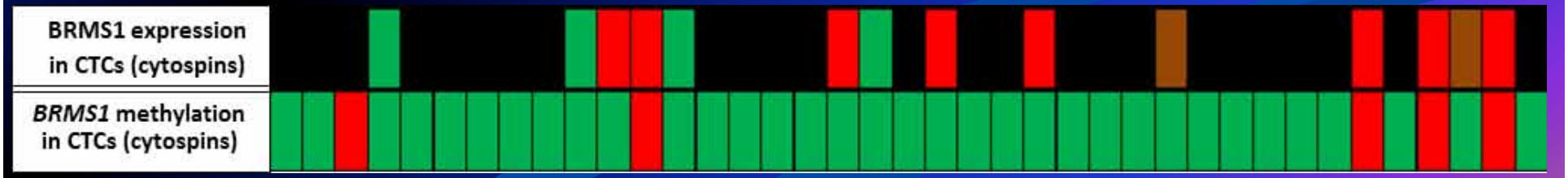
BRMS1 expression in cytospin stained CTC of early breast cancer patients. Cells were stained with A45-B/B3 antibody (green), BRMS1 antibody (red) and Dapi (blue). Quantification of the samples was performed with the ARIOL system



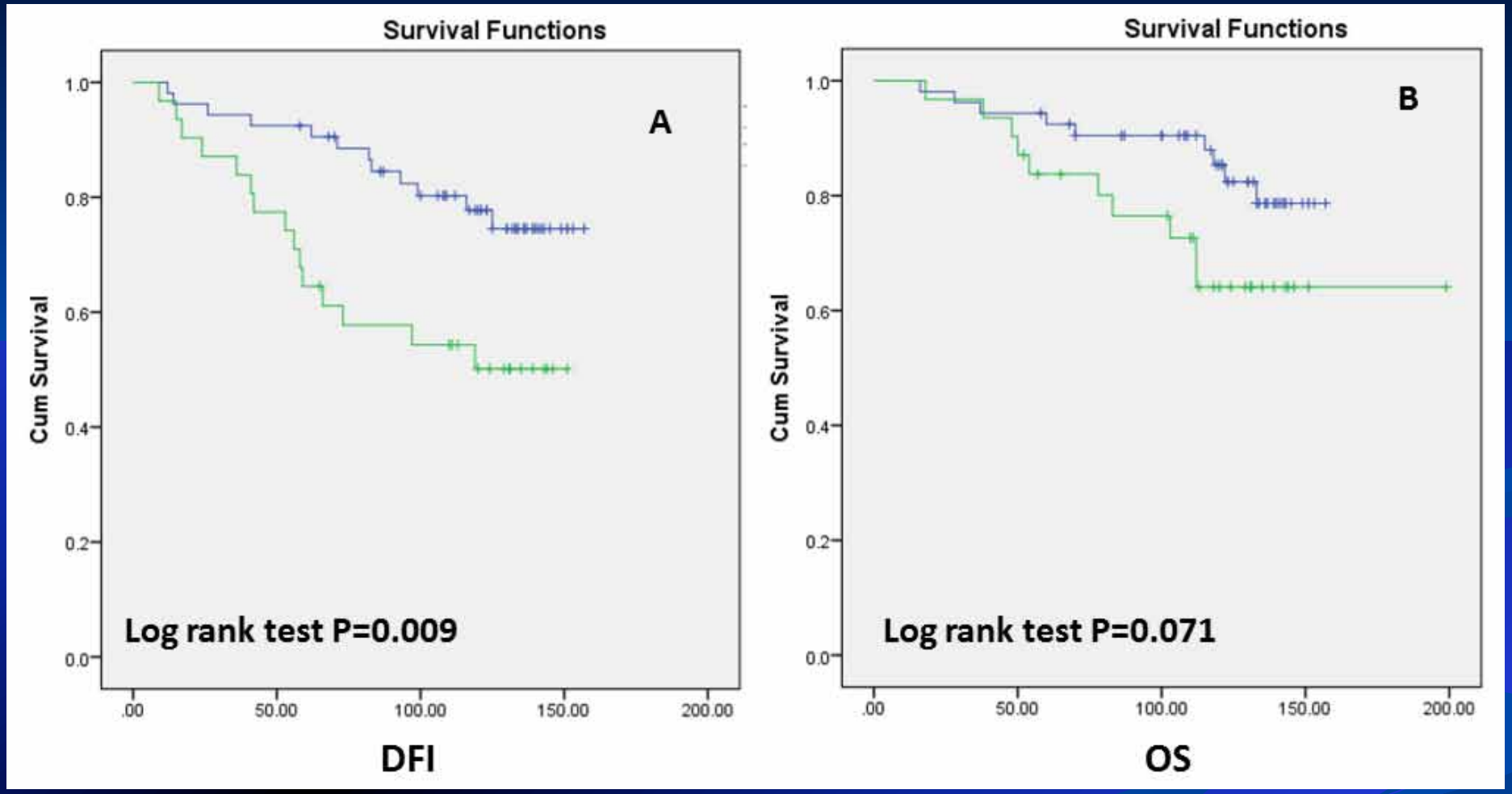
A):BRMS1 high expression,

(B): BRMS1 low expression,

C) negative for BRMS1



Kaplan-Meier estimates of DFI and OS for early breast cancer patients with (green) or without (blue) BRMS1 promoter methylation



Is there any correlation between CTC and cell free DNA???

Question:

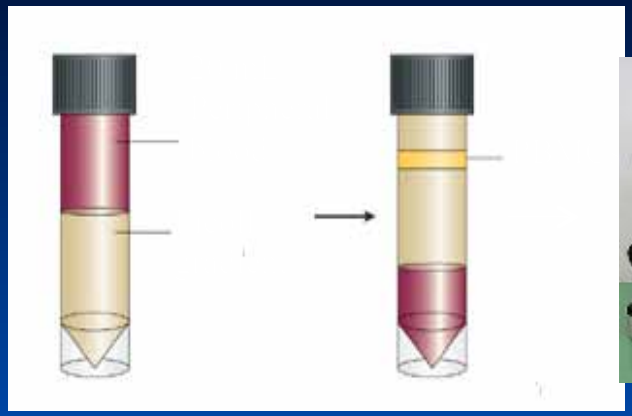
Is there a direct connection between the presence of CTCs and cell free DNA in patients with operable breast cancer where the primary tumor has already been resected?

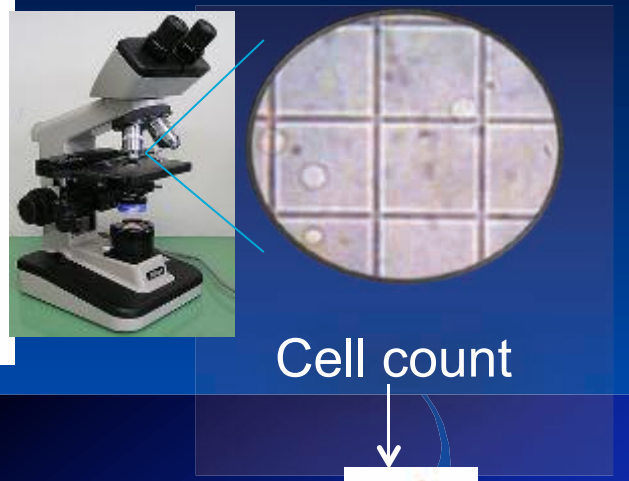
To address this question, we have chosen to use the same marker and the same methodology in matched clinical samples.

We evaluated whether *SOX17* promoter methylation in CTCs was associated with the methylation pattern of this gene in matched cfDNA isolated from plasma of patients with breast cancer.

Maria Chimonidou et al, Clin. Chem., 2013

Methodology

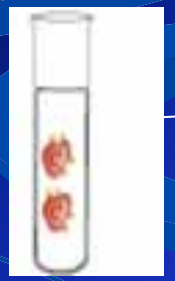




Cell count

Apply magnet

DNA extraction From CTCs



Positive selection (EpCAM)

CTCs isolation

CellFreeDNA isolation

Plasma

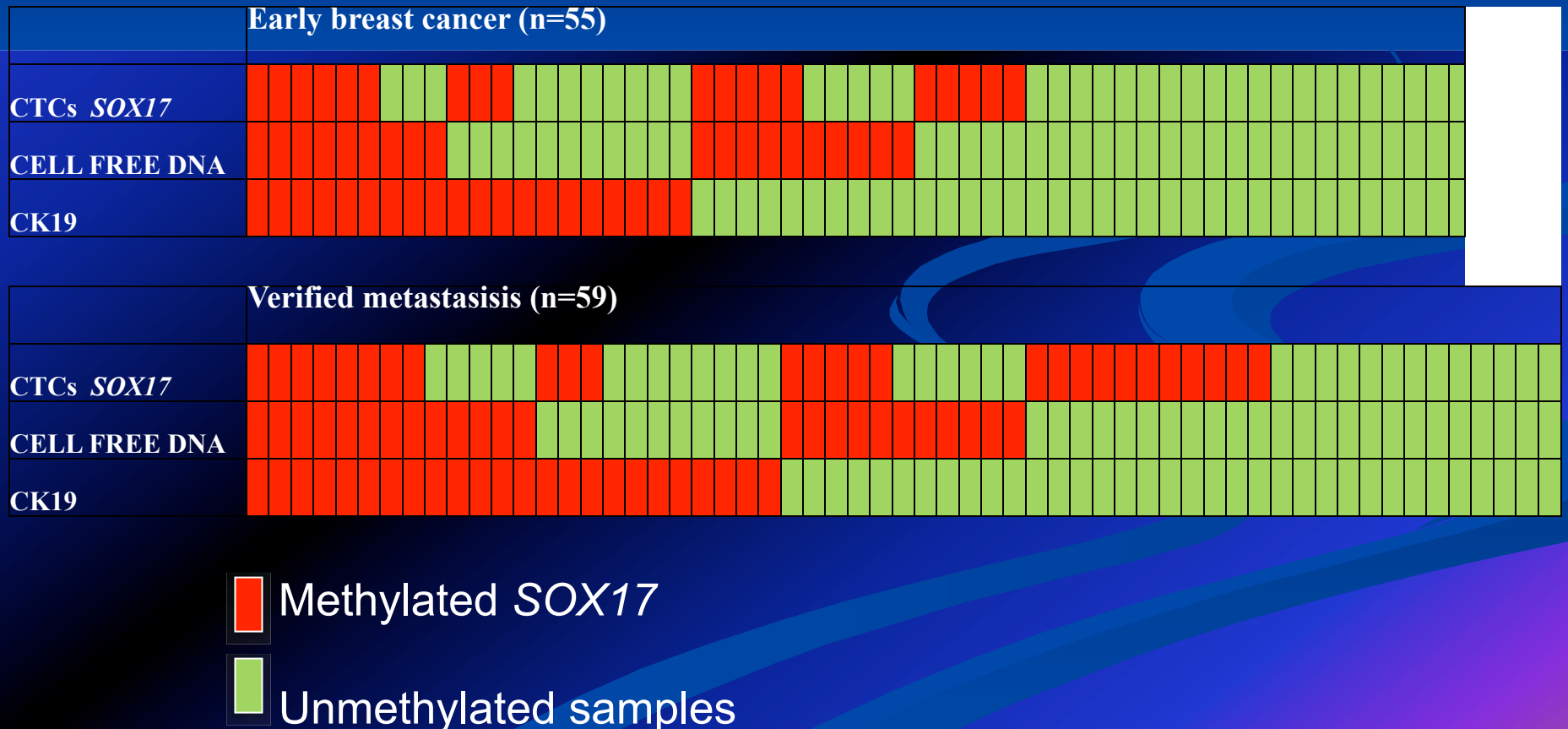
Outline of the extraction of cell free DNA and CTCs.

Maria Chimonidou et al, Clin. Chem., 2013

Maria Chimonidou et al, Clin. Chem., 2013

RESULTS

Heat map of *SOX17* promoter methylation in matched samples:
CTCs Fraction and cfDNA of patients with: (a) operable breast cancer (n=55), (b) verified metastasis (n=59)
In parallel, the expression of the epithelial marker *CK-19* is shown in CTCs fraction



ISMRC Paris 2013

Maria Chimonidou et al : DNA Methylation of Tumor Suppressor and Metastasis Suppressor Genes in Circulating Tumor Cells and corresponding circulating tumor DNA

Oral Presentation, Friday, session 7, 13:30

Is there a direct connection between the presence of CTCs and cell free DNA in patients with breast cancer (early or metastatic) when the primary tumor has already been resected??

To address this question, we have chosen to use the same markers and the same methodology in matched clinical samples

We evaluated whether *SOX17, CST6, and BRMS1* promoter methylation in CTCs was associated with the methylation pattern of the same genes in matched ctDNA isolated from plasma of patients with breast cancer.

Molecular characterization of CTC

DNA based: mutation analysis

ARTICLE *…..M Stratton, Nature, 22 Aug 2013*

doi:10.1038/nature12477

Signatures of mutational processes in human cancer

…..Here we analysed 4,938,362 mutations from 7,042 cancers and extracted more than 20 distinct mutational signatures. …….

In addition to these genome-wide mutational signatures, hypermutation localized to small genomic regions, 'kataegis', is found in many cancer types. ……..

The results reveal the diversity of mutational processes underlying the development of cancer, with potential implications for understanding of cancer aetiology, prevention and therapy.

DNA mutations in CTC

Cancer Cell. 2005 Sep;8(3):227-39.

Genomic analysis of single cytokeratin-positive cells from bone marrow reveals early mutational events in breast cancer.

Schardt JA, Meyer M, Hartmann CH, Schubert F, Schmidt-Kittler O, Fuhrmann C, Polzer B, Petronio M, Eils R, Klein CA.

Institut für Immunologie, Ludwig-Maximilians Universität München, Germany.

N Engl J Med. 2008 Jul 24;359(4):366-77. doi: 10.1056/NEJMoa0800668. Epub 2008 Jul 2.

Detection of mutations in EGFR in circulating lung-cancer cells.

Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, Inserra E, Diederichs S, Iafrate AJ, Bell DW, Digumarthy S, Muzikansky A, Irimia D, Settleman J, Tompkins RG, Lynch TJ, Toner M, Haber DA.

Massachusetts General Hospital Cancer Center, Boston 02129, USA.

Int J Cancer. 2012 Dec 12. doi: 10.1002/ijc.27987. [Epub ahead of print]

KRAS and BRAF mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue.

Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-de Vries J, Biermann K, Kraan J, Lalmahomed Z, van Galen A, de Weerd V, van der Spoel P, Ramírez-Moreno R, Verhoef C, Ijzermans JN, Wang Y, Gratama JW, Foekens JA, Sleijfer S, Martens JW.

Department of Medical Oncology, Erasmus University Medical Center, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands.

Cancer Res. 2013 Mar 7. [Epub ahead of print]

Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing.

Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, Lax S, Waldispuehl-Geigl J, Mauermann O, Lackner C, Höfler G, Eisner F, Sill H, Samonigg H, Pantel K, Riethdorf S, Bauernhofer T, Geigl JB, Speicher MR.

Institute of Human Genetics, Medical University of Graz.

PIK3CA mutations in CTC

Clin Chem. 2013 Jan;59(1):252-60. doi: 10.1373/clinchem.2012.188557. Epub 2012 Nov 7.

Heterogeneity of epidermal growth factor receptor status and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer.

Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeh M, Seifert AM, Mauermann O, Izbicki JR, Pantel K, Riethdorf S.

Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

……PIK3CA mutations were detected in 14 of 36 CTCs from 4 patients.....

Mol Oncol. 2013 Jul 13. pii: S1574-7891(13)00092-6. doi: 10.1016/j.molonc.2013.07.007. [Epub ahead of print]

Analysing the mutational status of PIK3CA in circulating tumor cells from metastatic breast cancer patients.

Schneck H, Blassl C, Meier-Stiegen F, Neves RP, Janni W, Fehm T, Neubauer H.

Department of Obstetrics and Gynecology, Eberhard Karls University of Tuebingen, Calwerstr. 7, 72076 Tuebingen, Germany. Electronic address: Helschn84@gmail.com.

44 Metastasis verified patients: 7/44 (15.9%)

Development and validation of a novel and highly sensitive method for the detection of *PIK3CA* hotspot mutations in CTC

ISMRC Paris 2013
Metastasis biology
Poster # 22
Athina Markou et al

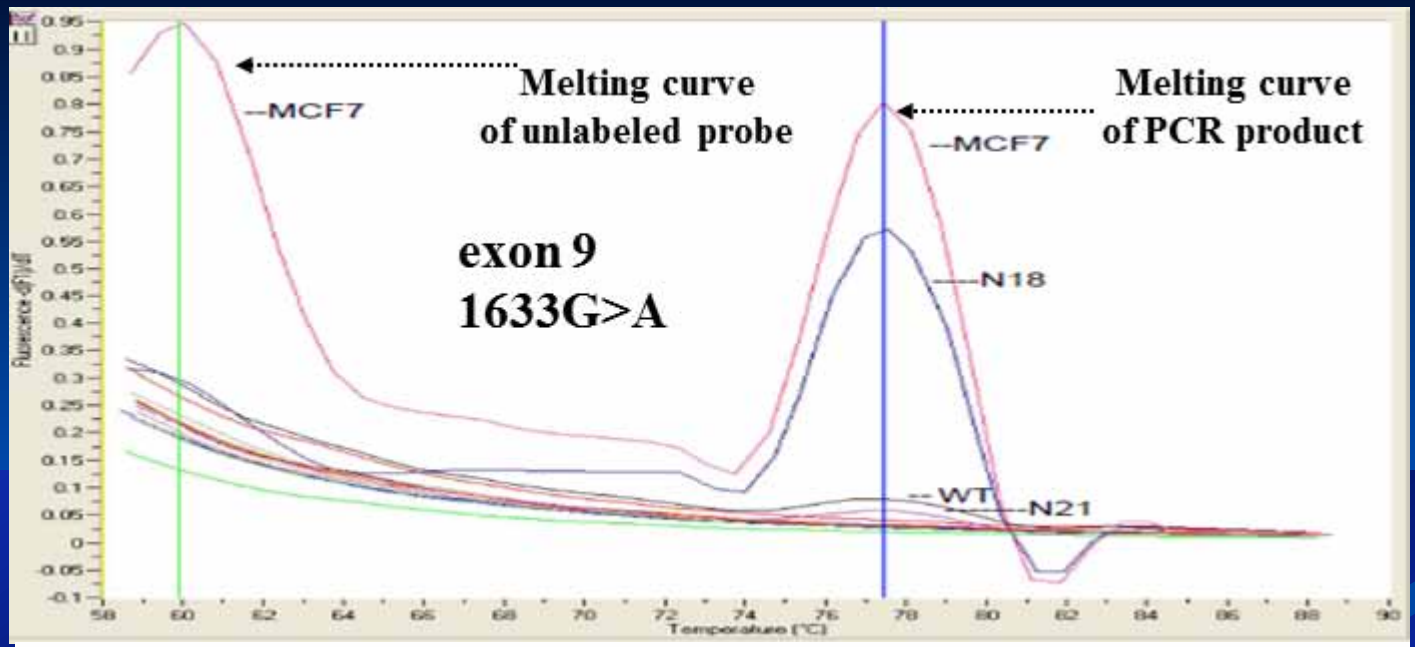
- To detect *PIK3CA* mutations (exons 9 and 20) in CTC we developed and validated an ultra-sensitive methodology, based on a combination of allele-specific, asymmetric rapid PCR and melting analysis

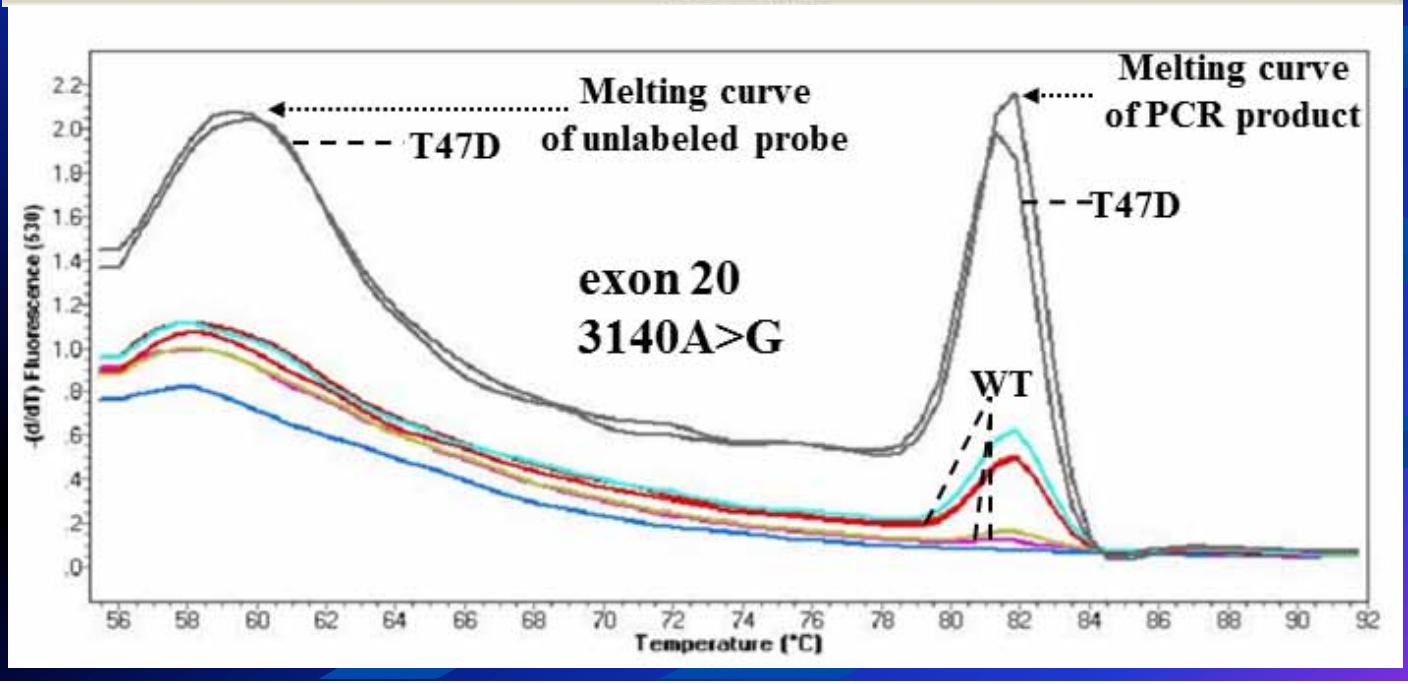
- We performed the developed method for the detection of *PIK3CA* hotspot mutations in DNA isolated from the EpCAM positive CTC fraction of breast cancer patients

- We compared the *PIK3CA* mutational status in CTC and corresponding primary tumours

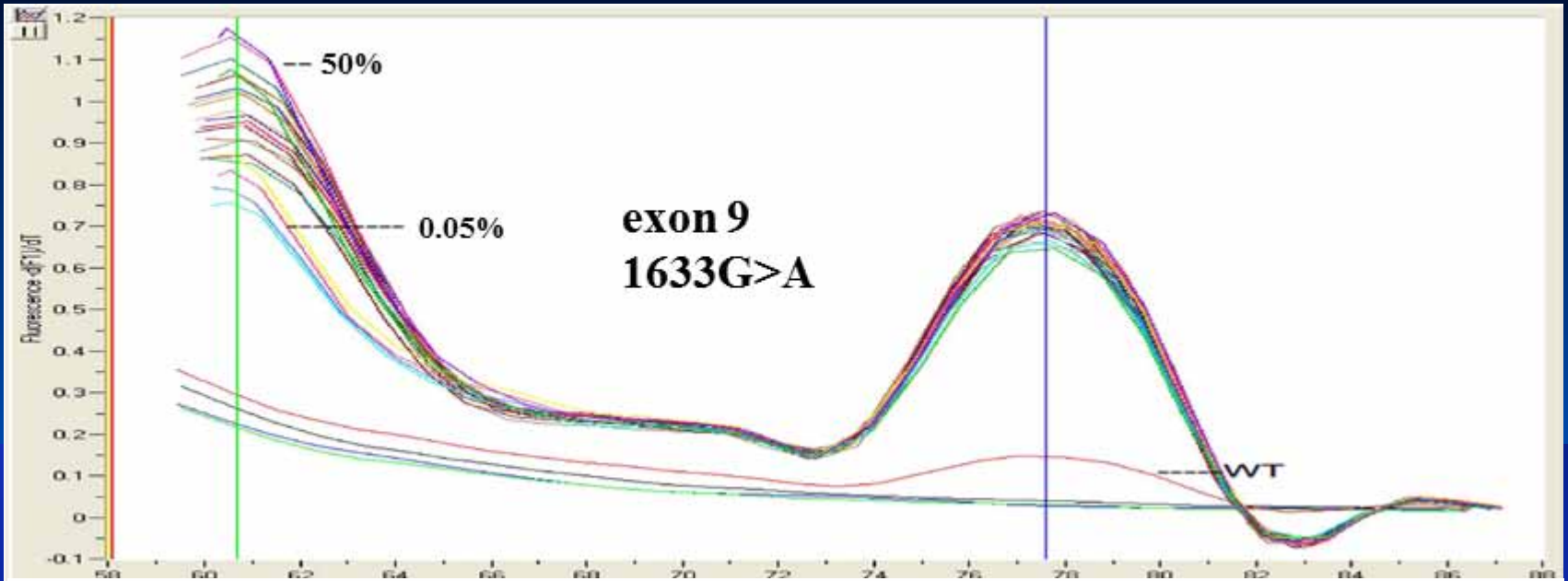
Markou et al, manuscript submitted

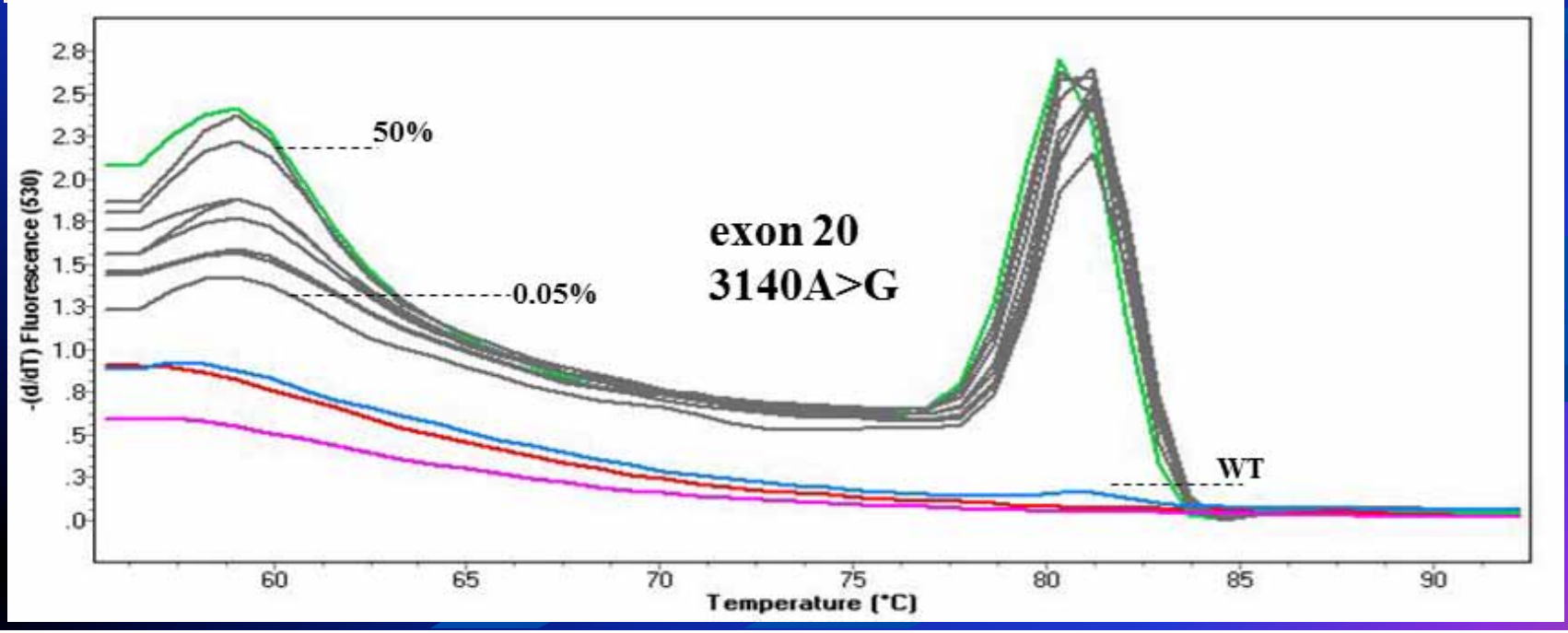
PIK3CA mutation assay – Analytical Specificity - (0/26 healthy controls)



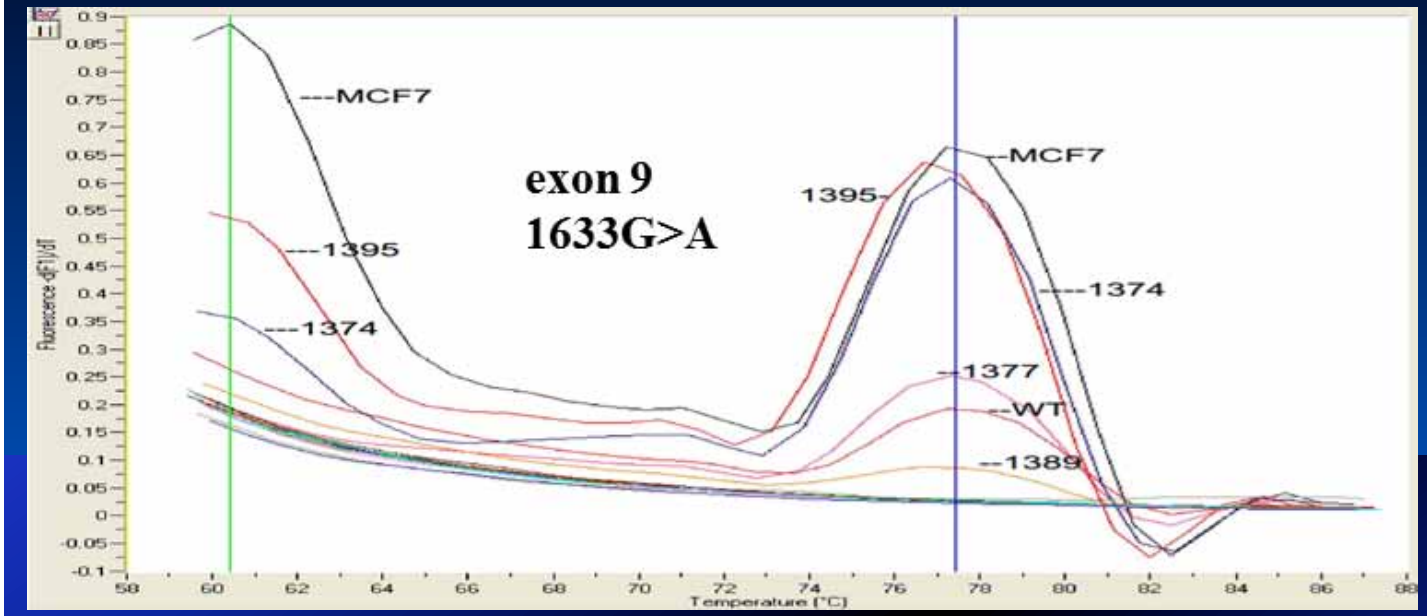


PIK3CA mutation assay – Analytical Sensitivity - (MCF-7 and T47D dilutions)



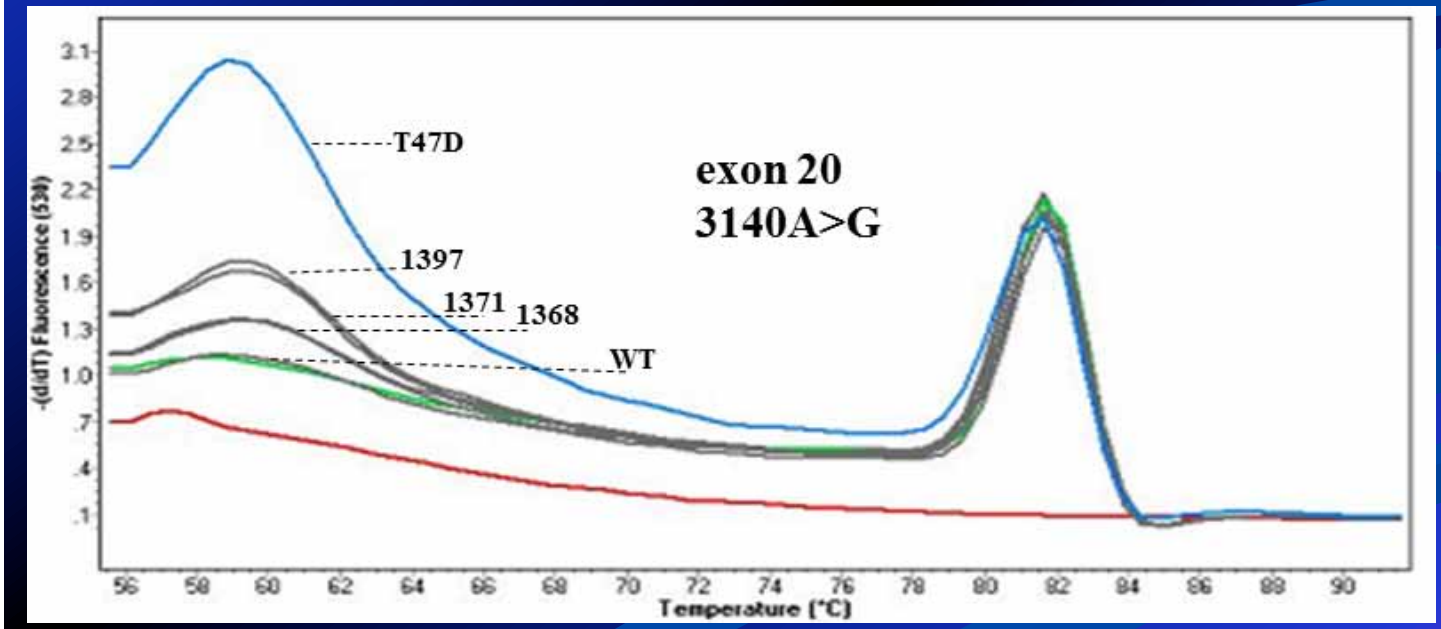


PIK3CA mutations in CTC: early breast cancer



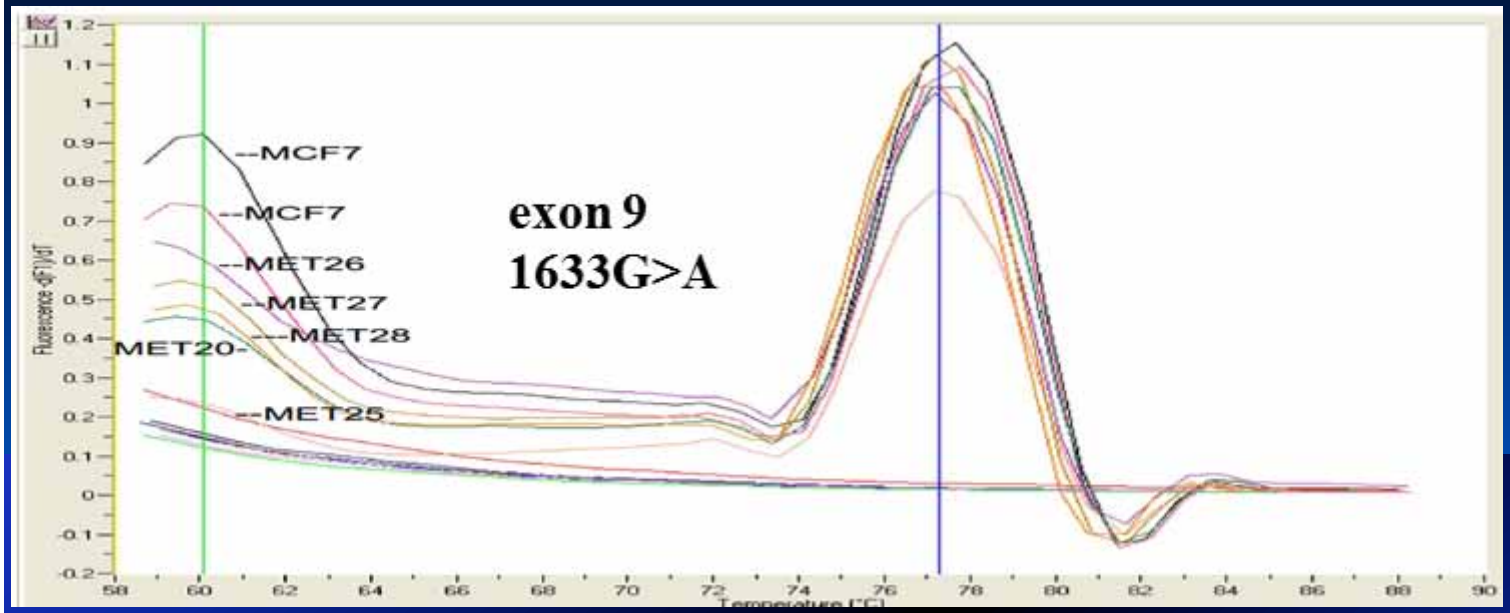
In total:
24/118 (20.3%)

Exon 9
3/118 (2.5%)



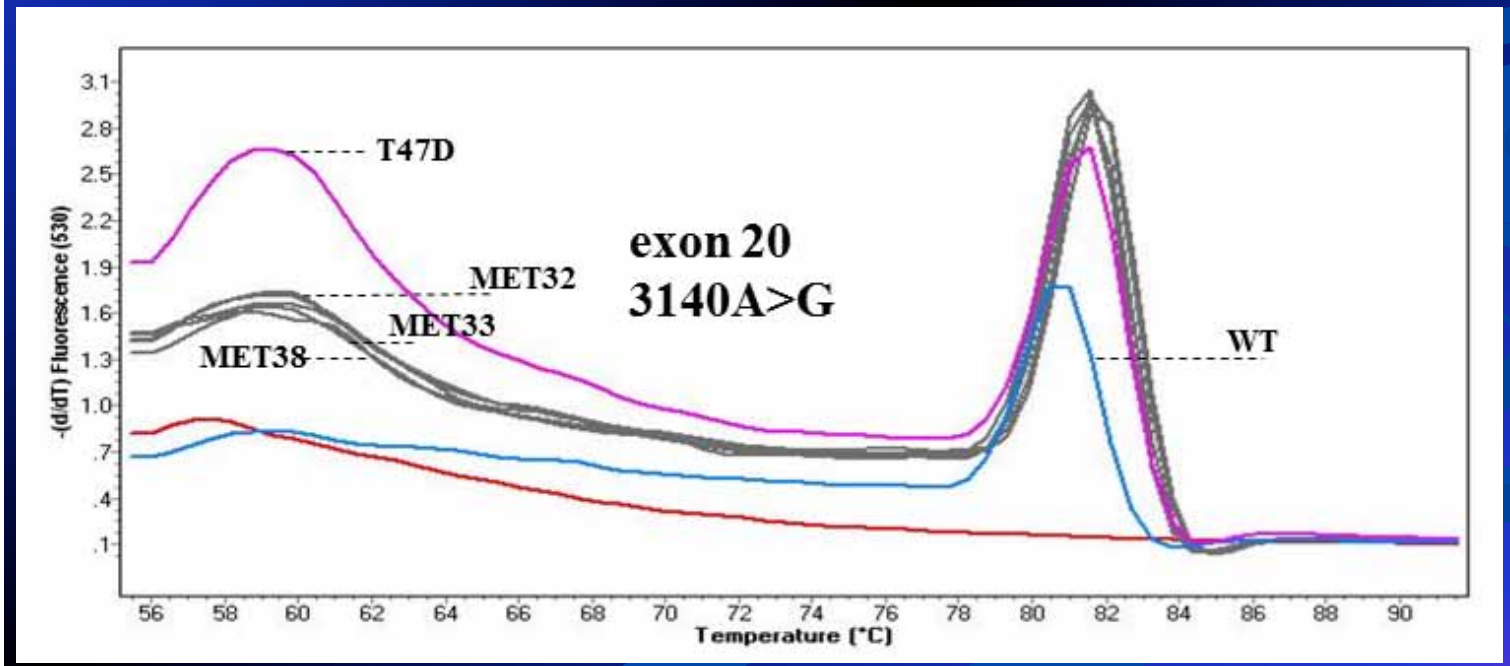
Exon 20
21/118 (17.5%)

PIK3CA mutations in CTC: metastatic breast cancer



In total:
20/57
(35.1%)

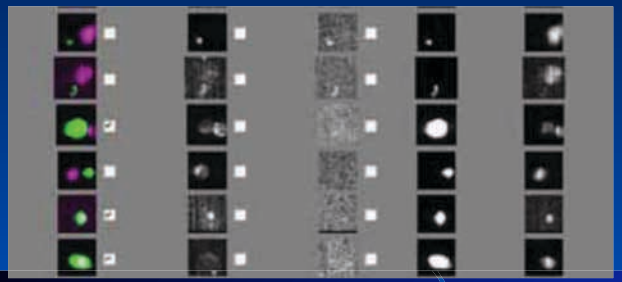
Exon 9
8/57 (14.0%)



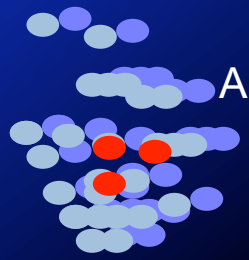
Exon 20
12/57 (21.1%)

KRAS Mutation Status Analysis in Primary Tumor and CTCs in Patients with mCRC

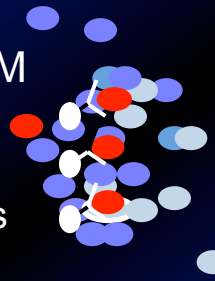
(Al. Voutsina et al, Univ of Crete, manuscript submitted)

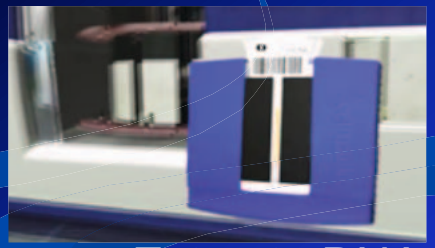


7.5ml

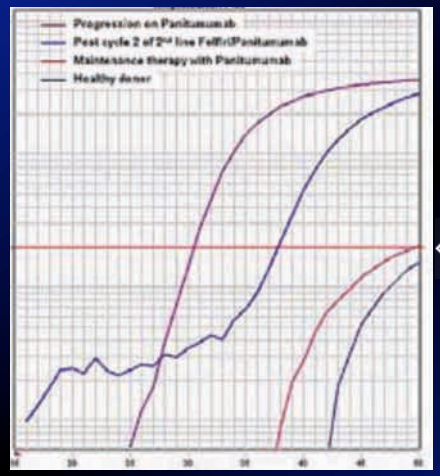


Anti-EpCAM coated ferrofluids

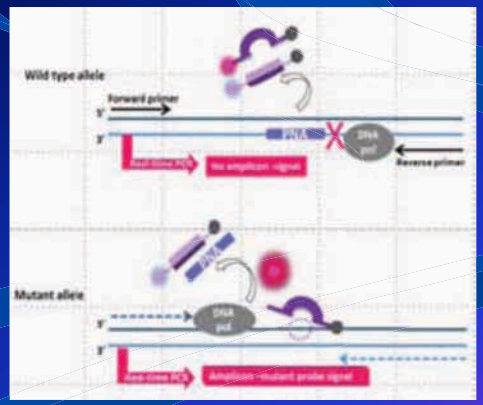




Extract gDNA



Allele-specific TaqMan PCR



Mutation assay

KRAS mutation status of CTCs may substantially differ from that of the corresponding primary tumor

(Al. Voutsina et al, Univ of Crete, manuscript submitted)

Table 3 - *KRAS* mutation status in primary tumor and corresponding CTCs-enriched samples in *KRAS* wild type mCRC patients.

	Primary tumor	CTC-enriched fraction		
Patient ID	*KRAS* status	CTC count [a]	*KRAS* status	Time point
15	wt	3	NVD	Maintenance therapy with Panitumumab
		42	c.35G>A; p.G12D	Progression on Panitumumab
		10	c.35G>A; p.G12D	Post cycle 2 of 2nd line Folfiri/Panitumumab
16	wt	7	c.34G>T; p.G12C	Post cycle 1 of 1st line Folfiri/Bevacizumab
18	wt	10	NVD	Without treatment
19	wt	660	NVD	Prior 1st line Folfox/Bevacizumab
20	wt	3	NVD	Prior 1st line Folfox/Bevacizumab
25	wt	173	NVD	Post cycle 2 of 1st line Folfiri/Bevacizumab
29	wt	4	NVD	Progression on 1st line Folfox/Panitumumab
30	wt	3	NVD	Progression on 1st line Folfiri/Panitumumab

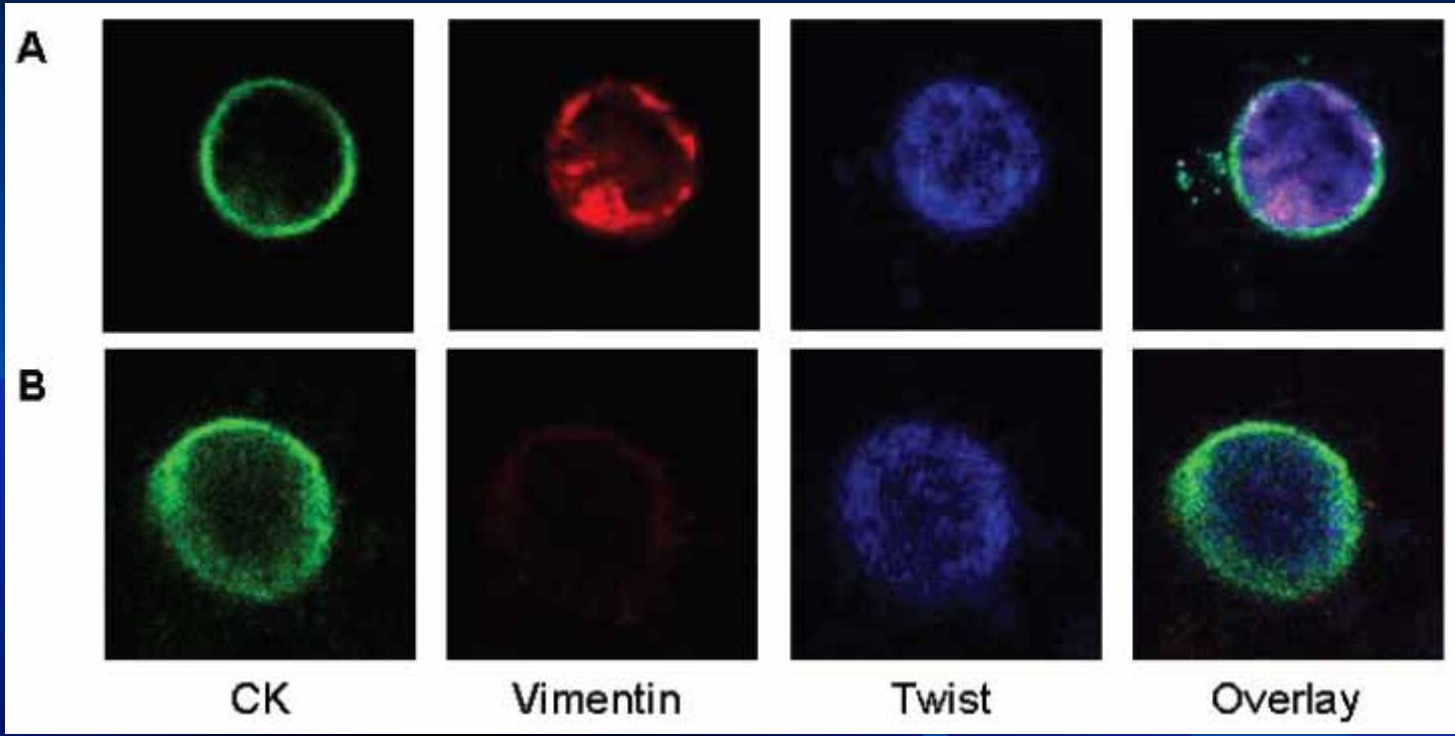
a CTC count using CellSearch system depicted as number of cells per 7.5 mL whole blood; NVD, non variant detected; c, coding DNA reference sequence; p, protein reference sequence.

a CTC count using CellSearch system depicted as number of cells per 7.5 mL whole blood; NVD, non variant detected; c, coding DNA reference sequence; p, protein reference sequence.

Molecular characterization of CTC at the protein level

Immunofluorescence
Single cell analysis

EMT in CTC
Coexpression of CK, Twist and vimentin in the same cell



Representative confocal laser-scanning photomicrographs of CTC cytospin after negative immunomagnetic separation in a patient with metastatic breast cancer.

Cells were triple-stained with:

- **Original magnification, x600 pan-CK A45-B/B3 antibody/Zenon Alexa Fluor 488 (green)**
- **Twist anti-mouse/Alexa Fluor 633 anti-mouse antibody**
- **vimentin anti-rabbit/Alexa Fluor 555 anti-rabbit antibody (orange)**

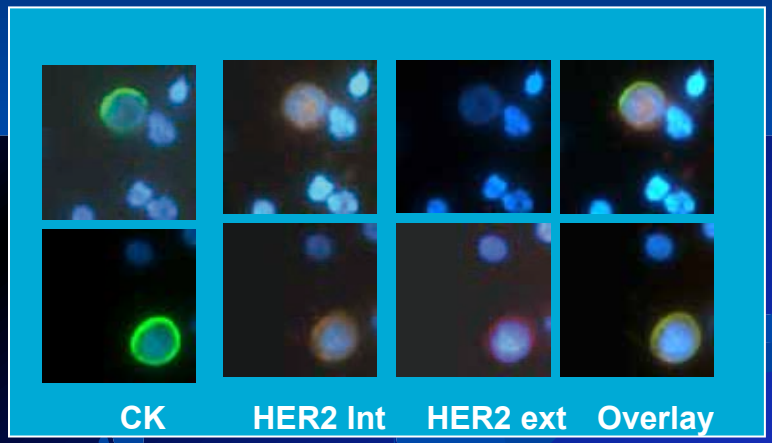
Kallergi et al Breast Cancer Res 2011: 13: R59

Identification of truncated HER2 receptor on Circulating Tumor Cells (CTCs) before and after Trastuzumab treatment in metastatic breast cancer patients.

Galatea Kallergi, Maria Papadaki, Dimitris Mavroudis, Vassilis Georgoulias, Sofia Agelaki

ISMRC Paris 2013
Mol Char. Poster #5
Galatea Kallergi et al

Aim: To identify the expression of p95HER2 on CTCs before and after trastuzumab-based first-line treatment in order to investigate the possibility of its involvement in the development of resistance.



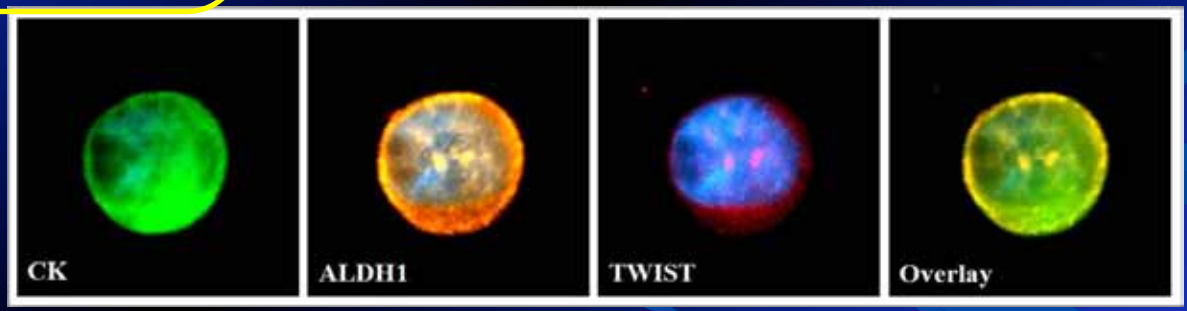
- Loss of the extracellular domain of HER2 receptor was evident in CTCs before and after trastuzumab administration in HER2-positive metastatic breast cancer patients.
- The truncated form of HER2 (p95HER2) was more frequently observed after treatment.
- Exclusively p95HER2 positive CTCs were observed in 10% of metastatic patients before vs 37.5% after treatment.
- These results may provide an explanation for the increased efficacy of the trastuzumab plus lapatinib combination in HER2 positive breast cancer.

Phenotypic characterization of single circulating tumor cells (CTCs) in patients with early and metastatic breast cancer (BC) using stemness and epithelial-to-mesenchymal transition (EMT) markers

Maria A Papadaki, Galatea Kallergi, Zafeiris Zafeiriou, Vassilis Georgoulias, Panayiotis A Theodoropoulos, Sofia Agelaki, Dimitris Mavroudis

ISMRC Paris 2013
Mol Char. Poster #7
Maria Papadaki et al

Aim: Investigation of the co-expression of ALDH1 and TWIST on CTCs of patients with early and metastatic BC, at a single-cell level



- A new assay using ARIOL system is provided for the evaluation of ALDH1 and TWIST co-expression at a single-CTC level.
- The two markers were strongly correlated and frequently co-expressed in the same CTC
- Differential ALDH1 expression levels and TWIST subcellular localization was confirmed on CTCs between early and metastatic breast cancer patients
- CTCs bearing the ALDH1high/TWIST nuclear phenotype were more commonly detected in metastatic compared to early breast cancer, suggesting that this phenotype prevails during disease progression

Molecular characterization of CTC at the single cell level

Br J Cancer. 2013 Apr 2;108(6):1358-67. doi: 10.1038/bjc.2013.92. Epub 2013 Mar 7.

Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting.

Peeters DJ, De Laere B, Van den Eynden GG, Van Laere SJ, Rothé F, Ignatiadis M, Sieuwerts AM, Lambrechts D, Rutten A, van Dam PA, Pauwels P, Peeters M, Vermeulen PB, Dirix LY.

Translational Cancer Research Unit, Oncology Center GZA Hospitals Sint-Augustinus, Oosterveldlaan 24, Antwerp B-2610, Belgium.

Cancer Lett. 2013 Jul 10;335(1):225-31. doi: 10.1016/j.canlet.2013.02.015. Epub 2013 Feb 16.

Detection and recovery of circulating colon cancer cells using a dielectrophoresis-based device: KRAS mutation status in pure CTCs.

Fabbri F, Carloni S, Zoli W, Ulivi P, Gallerani G, Fici P, Chiadini E, Passardi A, Frassineti GL, Ragazzini A, Amadori D.

Biosciences Laboratory, IRCCS Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Meldola, Italy. francesco.fabbri@irst.emr.it

PLoS One. 2013 Sep 18;8(9):e75038. doi: 10.1371/journal.pone.0075038.

Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients.

Babayan A, Hannemann J, Spötter J, Müller V, Pantel K, Joosse SA.

Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

Cancer Discov. 2012 Nov;2(11):995-1003. doi: 10.1158/2159-8290.CD-12-0222. Epub 2012 Oct 23.

Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer.

Miyamoto DT, Lee RJ, Stott SL, Ting DT, Wittner BS, Ulman M, Smas ME, Lord JB, Brannigan BW, Trautwein J, Bander NH, Wu CL, Sequist LV, Smith MR, Ramaswamy S, Toner M, Maheswaran S, Haber DA.

Massachusetts General Hospital Cancer Center, Charlestown, MA 02129, USA.

Molecular characterization of CTC at the single cell level

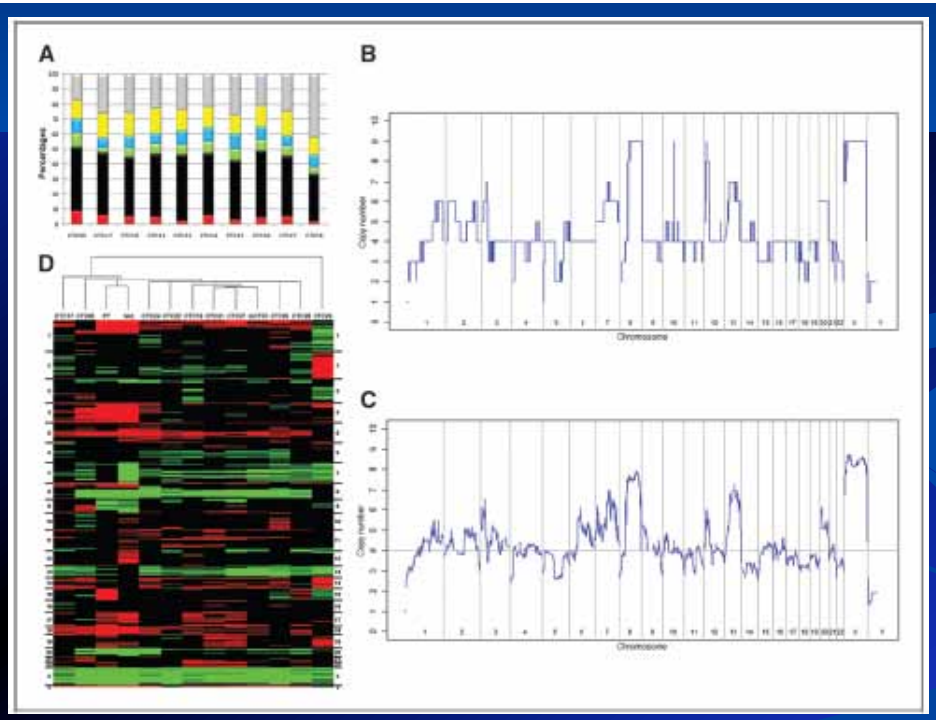
Next generation sequencing

Integrated Systems and Technologies

Cancer Research

Complex Tumor Genomes Inferred from Single Circulating Tumor Cells by Array-CGH and Next-Generation Sequencing

Ellen Heitzer[1], Martina Auer[1], Christin Gasch[6], Martin Pichler[3], Peter Ulz[1], Eva Maria Hoffmann[1], Sigurd Lax[5], Julie Waldispuehl-Geigl[1], Oliver Mauermann[6], Carolin Lackner[2], Gerald Höfler[2], Florian Eisner[3], Heinz Sill[4], Hellmut Samonigg[3], Klaus Pantel[6], Sabine Riethdorf[6], Thomas Bauernhofer[3], Jochen B. Geigl[1], and Michael R. Speicher[1]



metastases, and the corresponding CTCs from two of these patients. Mutations in known driver genes [e.g., adenomatous polyposis coli (APC), KRAS, or PIK3CA] found in the primary tumor and metastasis were also detected in corresponding CTCs. However, we also observed mutations exclusively in CTCs. To address whether these mutations were derived from a small subclone in the primary tumor or represented new variants of metastatic cells, we conducted additional deep sequencing of the primary tumor and metastasis and applied a customized statistical algorithm for analysis. We found that most mutations initially found only in CTCs were also present at subclonal level in the primary tumors and metastases from the same patient. This study paves the way to use CTCs as a liquid biopsy in patients with cancer, providing more effective options to monitor tumor genomes that are prone to change during progression, treatment, and relapse.

Quality control issues:

Comparison studies between different CTC assays

Quality Control issues

- An important question raised in the CTC analysis area is the lack of concordance between these assays.
- This can partially be explained since most of these assays are based on different capture and detection technologies.
- Despite the fact that most CTC isolation and detection methods are highly specific and sensitive, there are not so far extensive studies especially designed to compare their efficacy when using the same clinical samples.
- However, agreement on the standardized isolation and detection of CTC is absolutely necessary. Cross validation of findings between labs and a universal internal and external quality control system for CTC detection, enumeration and molecular characterization is urgently needed before any routine clinical application.

Clinical Chemistry 57:9
1242–1255 (2011)

Circulating Tumor Cells in Breast Cancer: Detection Systems, Molecular Characterization, and Future Challenges

Evi S. Lianidou[1*] and Athina Markou[1]

QC in CTC-Detection Systems: Comparison of Different Methodologies

Clinical results of CTC analysis largely depend on the detection technology used. Despite the fact that most of these methods are analytically sensitive and specific, extensive studies have not been performed that were specifically designed to compare the efficacy of different detection methods when used to analyze the same clinical samples. This is an important issue for their clinical use because, particularly in early disease, differences in analytical sensitivity between these methods play a very critical role. Thus, standardization of micrometastatic cell detection and characterization is important for the incorporation of CTCs into prospective clinical trials to test their clinical utility. Results of numerous single-institution studies suggest that CTCs play an important role for risk stratification and monitoring of therapeutic efficacy. These findings must be evaluated in trials to verify the principle of this concept in the clinical setting.

There is a clear need for an external quality control system for CTC assays and validation of findings

Table 2. Comparison studies between different laboratories and different methodologies for the same clinical samples.

Compared methods	Patients, n, type of breast cancer	Agreement	Reference
CellSearch vs Adnatest	245, Metastatic	50%	Fehm et al. *(48)*
CellSearch vs AdnaTest vs RT-PCR	79, Metastatic	CellSearch vs AdnaTest: 81%	Van der Auwera et al. *(53)*
		CellSearch vs RT-PCR: 57%	
		RT-PCR vs AdnaTest: 50%	
Oncoquick vs CellSearch	61, Metastatic	33/61 (54.1%)	Balic et al. *(50)*
RT-PCR vs ICC (DTCs compared)	385	280/385 (73%)	Becker et al. *(52)*
CellSearch	6 (14 independent laboratories)	Between-laboratory CVs 45%–65%	Kraan et al. *(49)*
		Between-instrument CV <20%	
		Between-assay CV <12%	
CellSearch	92 (3 independent laboratories)		Riethdorf et al. *(32)*

E. Lianidou and A. Markou, (Review) : Clin Chem, 57:9, 1242-1255, 2011

CTC analysis in the clinical lab

We must have in mind the classic steps in biomarker development:

pre-analytical issues: sample volume, collection tubes, stability, storage, shipping……..

analytical validation:

- analytical sensitivity and specificity
- Robustness
- quality control
- repeatability within the same lab (within run, between run)
- comparison between different laboratories, using the same samples and the same methodologies
- comparison between different methodologies, using the same samples

clinical validation: diagnostic sensitivity and diagnostic specificity

clinical qualification for specific use

Parkinson et al. Journal of Translational Medicine 2012, 10:138
http://www.translational-medicine.com/content/10/1/138

JOURNAL OF TRANSLATIONAL MEDICINE

REVIEW Open Access

Considerations in the development of circulating tumor cell technology for clinical use

David R Parkinson[1*], Nicholas Dracopoli[2], Brenda Gumbs Petty[3], Carolyn Compton[4], Massimo Cristofanilli[5], Albert Deisseroth[6], Daniel F Hayes[7], Gordon Kapke[8], Prasanna Kumar[9], Jerry SH Lee[10], Minetta C Liu[11], Robert McCormack[12], Stanislaw Mikulski[13], Larry Nagahara[10], Klaus Pantel[14], Sonia Pearson-White[15], Elizabeth A Punnoose[16], Lori T Roadcap[17], Andrew E Schade[18], Howard I Scher[19], Caroline C Sigman[3] and Gary J Kelloff[10]

Precollection Variables

- Reagent storage temperature
- Reagent storage time
- Patient preparation—for example, fasting status

Sample Collection Variables

- Timing of sample collection in study protocol
- Sample collection method
- Number of specimen collected, including duplicates
- Subject positioning during sample collection
- Specimen transport time
- Specimen storage temperature
- Specimen storage time
- Assay-specific SOPs
- Lot-specific control testing
- Levels of quality control testing (daily, monthly)
- Lab certification
- Assay platform training

Table 3 CTC Assay Clinical Readiness Evaluation

	Assay Validation
Pre-Analytic	How is specimen collected (venous route, body position, draw order, tourniquet time, needle bore, tube type)?
	When is specimen collected (time of day, relative to treatment, relative to infusates)?
	How is specimen stored (time and temperature)?
	How is specimen handled (shipping, transfers)?
Analytic	Sensitivity (lower limit of quantitation)?
	Reportable range?
	Specificity?
	Reproducibility?
	Robustness?
Post-Analytic	How is data reported?
	How is data analyzed?
	What are the reference intervals?
Clinical Feasibility	
• Are there analytically valid results when tested in appropriate preclinical models?	
o with use of clinically relevant/feasible specimen acquisition?	
o with use of clinically relevant specimen handling procedures (both at the point of acquisition and in the receiving laboratory)? These processes should be tracked and recorded.	
o with use of clinically relevant collection scheduling?	
Therapeutic Relevance	
• For predictive biomarkers, is there a relationship between dose/exposure, quantifiable target modulation, and disease outcome?	
• For prognostic biomarkers, is there a relationship between baseline levels and survival?	

Int J Cancer. 2012 Apr 1;130(7):1590-7. doi: 10.1002/ijc.26111. Epub 2011 Nov 30.

Comparison of assay methods for detection of circulating tumor cells in metastatic breast cancer: AdnaGen AdnaTest BreastCancer Select/Detect™ versus Veridex CellSearch™ system.

Andreopoulou E, Yang LY, Rangel KM, Reuben JM, Hsu L, Krishnamurthy S, Valero V, Fritsche HA, Cristofanilli M.

Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. eandreop@mdanderson.org

Table 3. Distribution of AdnaTest Results by CellSearch CTCs (<2 *vs.* ≥2)

		Cell search		*p* value
		<2	≥2	
GA73.3-2	Negative	28	17	<0.01
	Positive	1	9	
Tests concordance rate			67%	
MUC-1	Negative	23	8	0
	Positive	6	18	
Tests concordance rate			75%	
HER2	Negative	23	13	0.02
	Positive	6	13	
Tests concordance rate			65%	
Adna overall result	Negative	20	6	<0.01
	Positive	9	20	
Tests concordance rate			73%	

Table 4. Distribution of AdnaTest Results by CellSearch CTCs (<5 *vs.* ≥5)

		Cell search		*p* value
		<5	≥5	
GA733-2	Negative	33	12	<0.01
	Positive	2	8	
Tests Concordance rate			75%	
MUC-1	Negative	25	6	<0.01
	Positive	10	14	
Tests Concordance rate			71%	
HER2	Negative	26	10	0.07
	Positive	9	10	
Tests concordance rate			65%	
AdnaTest overall result	Negative	22	4	<0.01
	Positive	13	16	
Tests concordance rate			69%	

" The AdnaTest has equivalent sensitivity to that of the CellSearch system in detecting 2 or more CTCs

The immunomagnetic cell capture technology, combined with multiplex RT-PCR in AdnaTest may potentially allow the detection of a broad range of molecular abnormalities in CTCs that can more accurately characterize the biological status of metastatic disease and support design of future studies testing CTCs directed therapies.

AdnaTest complements the Cell-Search system for CTC detection."

Müller et al. Breast Cancer Research 2012, 14:R118
http://breast-cancer-research.com/content/14/4/R118

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

Prognostic impact of circulating tumor cells assessed with the CellSearch System™ and AdnaTest Breast™ in metastatic breast cancer patients: the DETECT study

Volkmar Müller[1†], Sabine Riethdorf[2†], Brigitte Rack[3], Wolfgang Janni[4], Peter A Fasching[5], Erich Solomayer[6], Bahriye Aktas[7], Sabine Kasimir-Bauer[7], Klaus Pantel[2†] and Tanja Fehm[8*†] and on behalf of the DETECT study group

Prognostic impact of the AdnaTest BreastCancer™

When the AdnaTest Breast was performed, 88 out of 221 (40%) patients were CTC-positive. Concordance between the two assays used in our study was 64% ($P < 0.01$, $\kappa = 0.28$) as described earlier [14]. No correlation could be observed between CTC positivity and any of the analyzed clinicopathological factors, except for HER2 status. CTC positivity assessed by the AdnaTest Breast™ had no association with clinical outcome parameters such as PFS or OS. Median PFS values were 8.8 and 10.7 months ($P = 0.230$), and OS values were 19.3 and 23.8 ($P = 0.278$). This was also the case when

Strati *et al. Breast Cancer Research* 2013, **15**:R20
http://breast-cancer-research.com/content/15/2/R20

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

Comparison of three molecular assays for the detection and molecular characterization of circulating tumor cells in breast cancer

Areti Strati[1], Sabine Kasimir-Bauer[2], Athina Markou[1], Cleo Parisi[1] and Evi S Lianidou[1*]

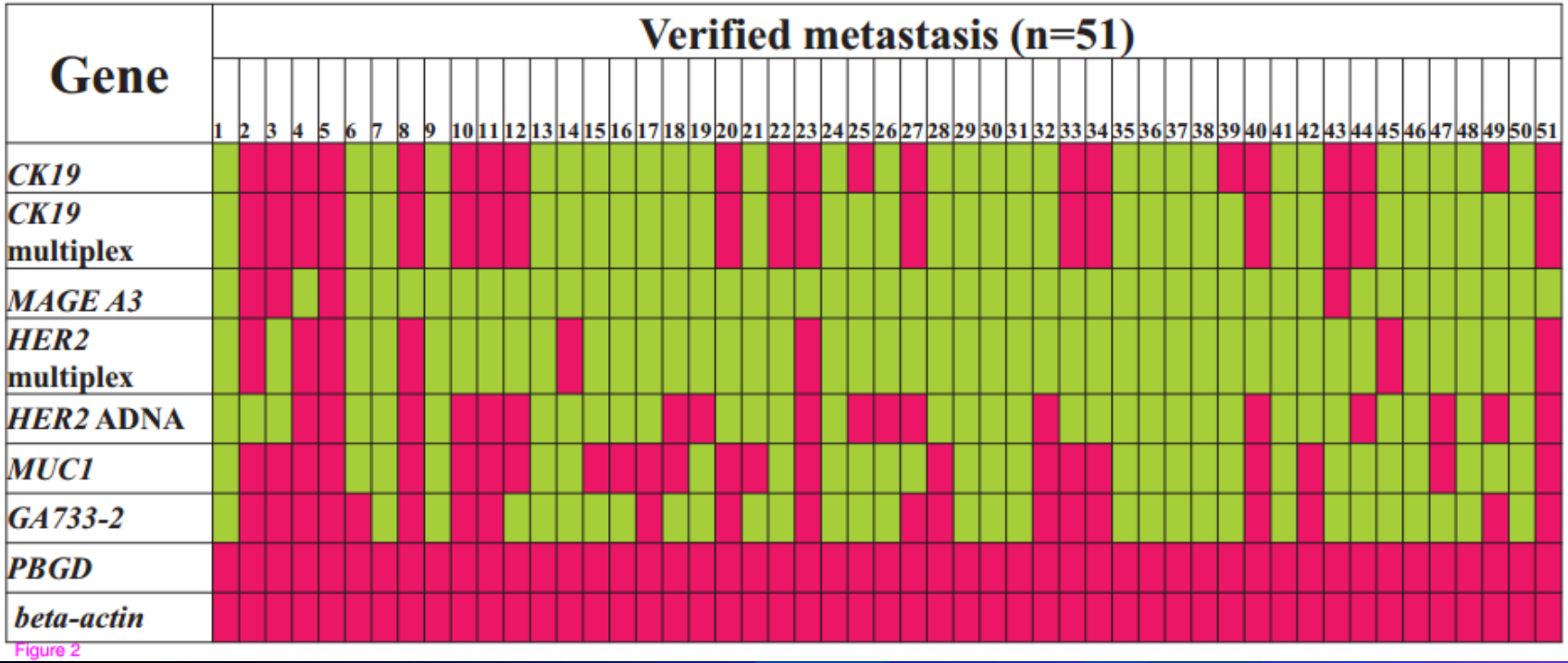


Figure 2

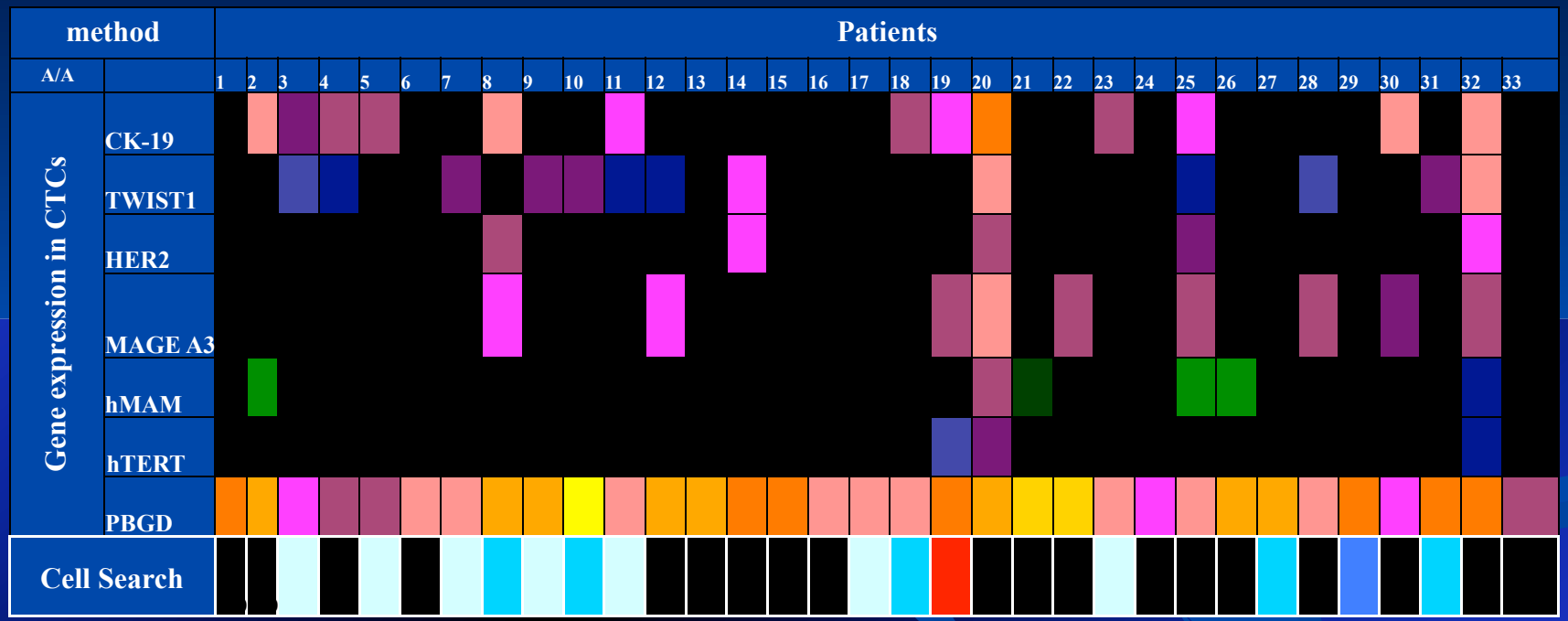
Comparison of three molecular assays for the detection and molecular characterization of CTC in breast cancer

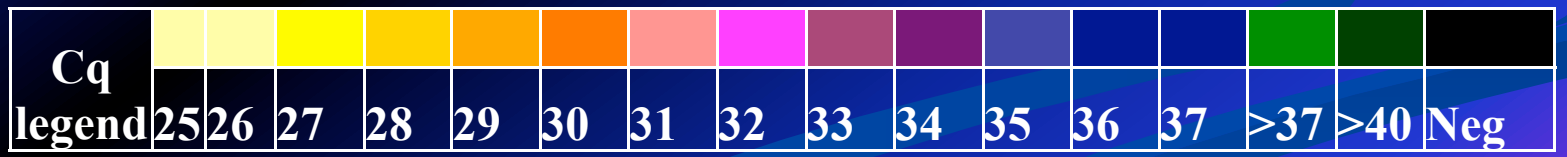
Table 2. Comparison between *CK19* RT-qPCR, multiplex RT-qPCR and *AdnaTest* for the detection of CTC in breast cancer.

Molecular assay	*CK19* RT-qPCR		Multiplex- RT-qPCR		Total
			Early breast cancer, (n=254)		
AdnaTest	**Positive**	**Negative**	**Positive**	**Negative**	**Total (%)**
Positive	4	38	5	37	42 (16.5)
Negative	32	180	53	159	212 (83.5)
Total (%)	36 (14.2)	218 (85.8)	58 (22.8)	196 (77.2)	254
Kappa test	poor agreement: Kappa= -0.059 (P =0.344)		poor agreement: Kappa= -0.114 (P =0.065)		
Concordance (%)	184 (72.4), P^a=0.344		164 (64.6), P^a=0.065		
			Verified metastasis, (n=51)		
AdnaTest	**Positive**	**Negative**	**Positive**	**Negative**	**Total (%)**
Positive	17	11	16	12	28 (54.9)
Negative	4	19	4	19	23 (45.1)
Total (%)	21 (41.2)	30 (58.8)	20 (39.2)	31 (60.8)	51
Kappa test	Moderate agreement: Kappa =0.422 (P =0.002)		Fair agreement: Kappa=0.386 (P =0.004)		
Concordance (%)	36 (70.6), P^a=0.002		35 (68.6), P^a=0.004		

Areti Strati et al., Breast Cancer Research, 2013

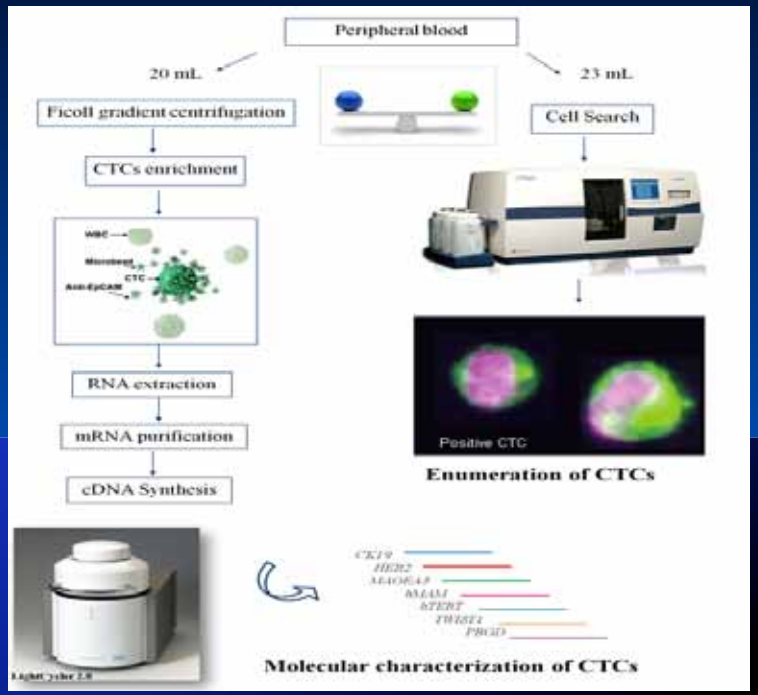
Comparison of multiplex RT-qPCR with CellSearch in early breast cancer (n=33)





(*A. Strati et al, BMC Cancer, 2011*)

"Comparison study between CellSearch and RT-qPCR for the detection of EpCAM-positive CTC in operable breast cancer"



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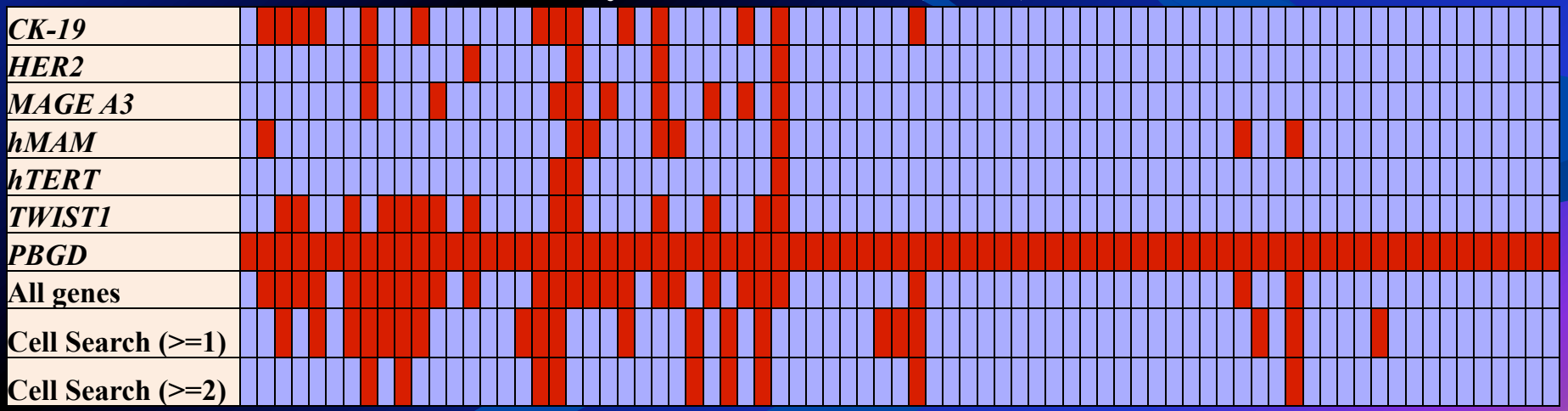
Early breast cancer (n=77)



Clinical significance of CTC molecular characterization

HER2 as a Proof-of-Principle

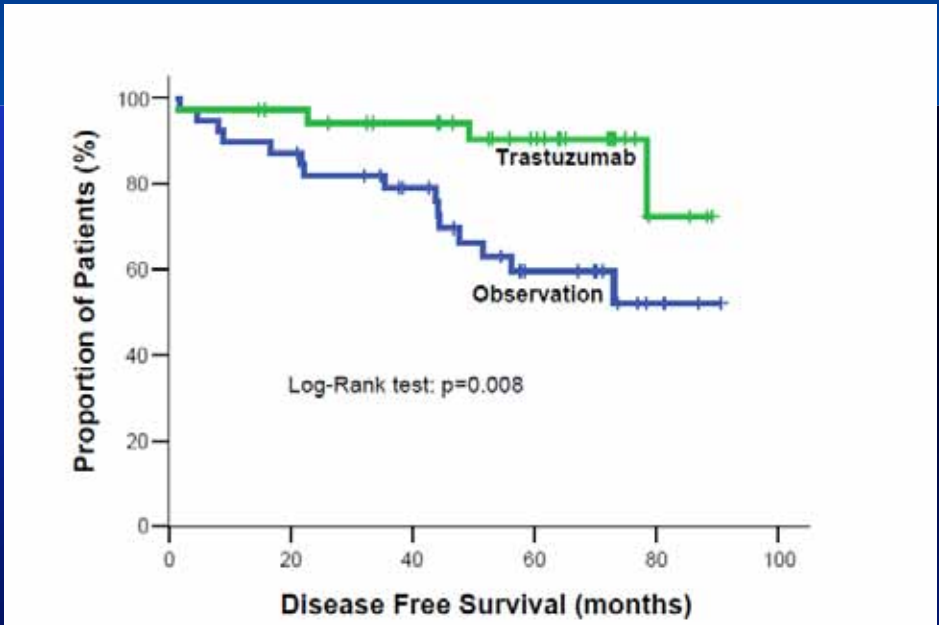
Clinical implications of the molecular characterization of CTC

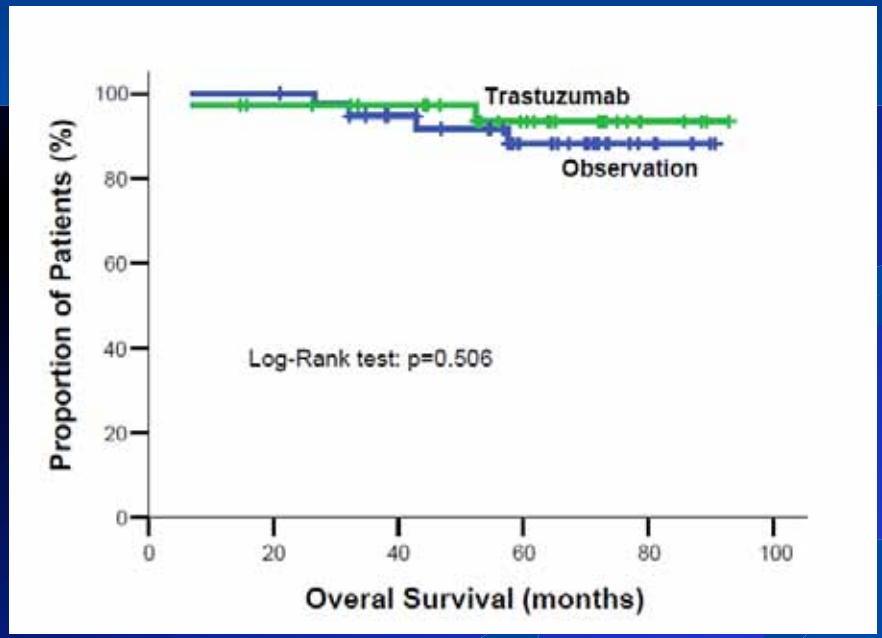
Ann Oncol. 2012 Feb 29. [Epub ahead of print]

Trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting chemotherapy-resistant CK19 mRNA-positive circulating tumor cells: results of a randomized phase II study.

Georgoulias V, Bozionelou V, Agelaki S, Perraki M, Apostolaki S, Kallergi G, Kalbakis K, Xyrafas A, Mavroudis D.

Department of Medical Oncology, University Hospital of Heraklion, Heraklion.





Administration of trastuzumab can eliminate chemotherapy-resistant CK19 mRNA-positive CTCs, reduce the risk of disease recurrence and prolong the DFS.

Georgoulias et al, Annals of Oncology, 2012

Transl Oncol. 2012 Dec;5(6):475-85. Epub 2012 Dec 1.

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Bock C, Rack B, Kuhn C, Hofmann S, Finkenzeller C, Jäger B, Jeschke U, Doisneau-Sixou SF.

Tumorbiologisches Labor der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Campus Innenstadt, Klinikum der Ludwig-Maximilians-Universität, Munich, Germany.

Breast Cancer Res Treat. 2013 Apr;138(2):509-17. doi: 10.1007/s10549-013-2470-9. Epub 2013 Mar 15.

The HER2 status of disseminated tumor cells in the bone marrow of early breast cancer patients is independent from primary tumor and predicts higher risk of relapse.

Hartkopf AD, Banys M, Meier-Stiegen F, Hahn M, Röhm C, Hoffmann J, Helms G, Taran FA, Wallwiener M, Walter C, Neubauer H, Wallwiener D, Fehm T.

Department of Obstetrics and Gynecology, University of Tuebingen, Calwer Strasse 7, 72076, Tuebingen, Germany, andreas.hartkopf@med.uni-tuebingen.de.

Gynecol Oncol. 2013 Jan;128(1):15-21. doi: 10.1016/j.ygyno.2012.09.021. Epub 2012 Sep 24.

Molecular characterization of circulating tumor cells in patients with ovarian cancer improves their prognostic significance -- a study of the OVCAD consortium.

Obermayr E, Castillo-Tong DC, Pils D, Speiser P, Braicu I, Van Gorp T, Mahner S, Sehouli J, Vergote I, Zeillinger R.

Department of Obstetrics and Gynecology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria. eva.obermayr@meduniwien.ac.at

Breast Cancer Res. 2012 Jan 20;14(1):R15.

Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells.

Kasimir-Bauer S, Hoffmann O, Wallwiener D, Kimmig R, Fehm T.

Department of Gynecology and Obstetrics, University Hospital of Essen, University of Duisburg-Essen, D-45122 Essen, Germany. sabine.kasimir-bauer@uk-essen.de

Gynecol Oncol. 2011 Aug;122(2):356-60. doi: 10.1016/j.ygyno.2011.04.039. Epub 2011 May 24.

Comparison of estrogen and progesterone receptor status of circulating tumor cells and the primary tumor in metastatic breast cancer patients.

Aktas B, Müller V, Tewes M, Zeitz J, Kasimir-Bauer S, Loehberg CR, Rack B, Schneeweiss A, Fehm T.

Department of Gynecology and Obstetrics, University of Essen, Hufelandstraße 55, 45122 Essen, Germany. bahriye.aktas@uk-essen.de

Monitoring proliferation and apoptosis markers on circulating tumor cells (CTCs) in serial samples of early breast cancer (BC) patients during clinical dormancy

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Exemestane plus everolimus is effective in decreasing CTC counts in patients with HER2-negative metastatic breast cancer

Ch Nikolaou[1], S. Apostolaki[2], M. Spiliotaki[2], A. Kotsakis[1], D. Mavroudis[1], V. Georgoulias[1], S. Agelaki[1]

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Conclusions - Future perspectives

CTC molecular characterization holds great promise for :

- providing novel biomarkers
- monitoring the course of disease in cancer patients,
- understanding the biology of cancer progression
- individualizing treatment
- monitor response to cancer therapy (companion diagnostics)
- providing novel biomarker assays useful in oncological drug development,

Future Perspectives

The future of CTCs lies in Molecular Characterization

Further research on the molecular characterization of CTCs will contribute to a better understanding of the biology of metastatic development in cancer patients.

However, there is a clear need for an external quality control system for CTC enumeration and molecular characterization and validation of findings for the same samples by different laboratory centers.

Athens Circulating Tumor Cells Group

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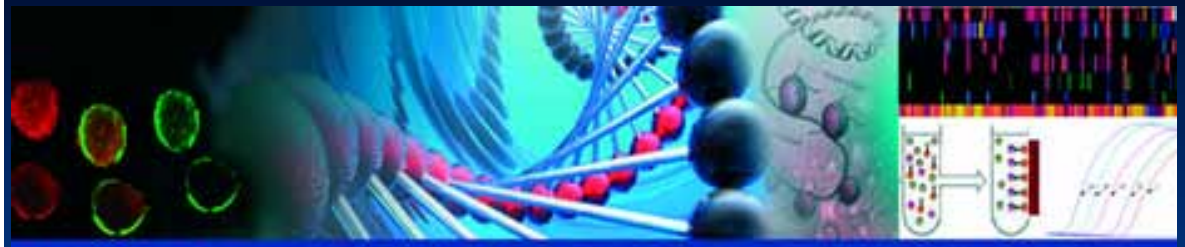

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- *Klaus Pantel, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany*
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Thank you !!!