Erasmus MC
Universitair Medisch Centrum Rotterdam

Josephine Nefkens Institute and
Cancer Genomics Center

mRNA and microRNA profiling of CTCs from metastatic breast and colorectal cancer patients

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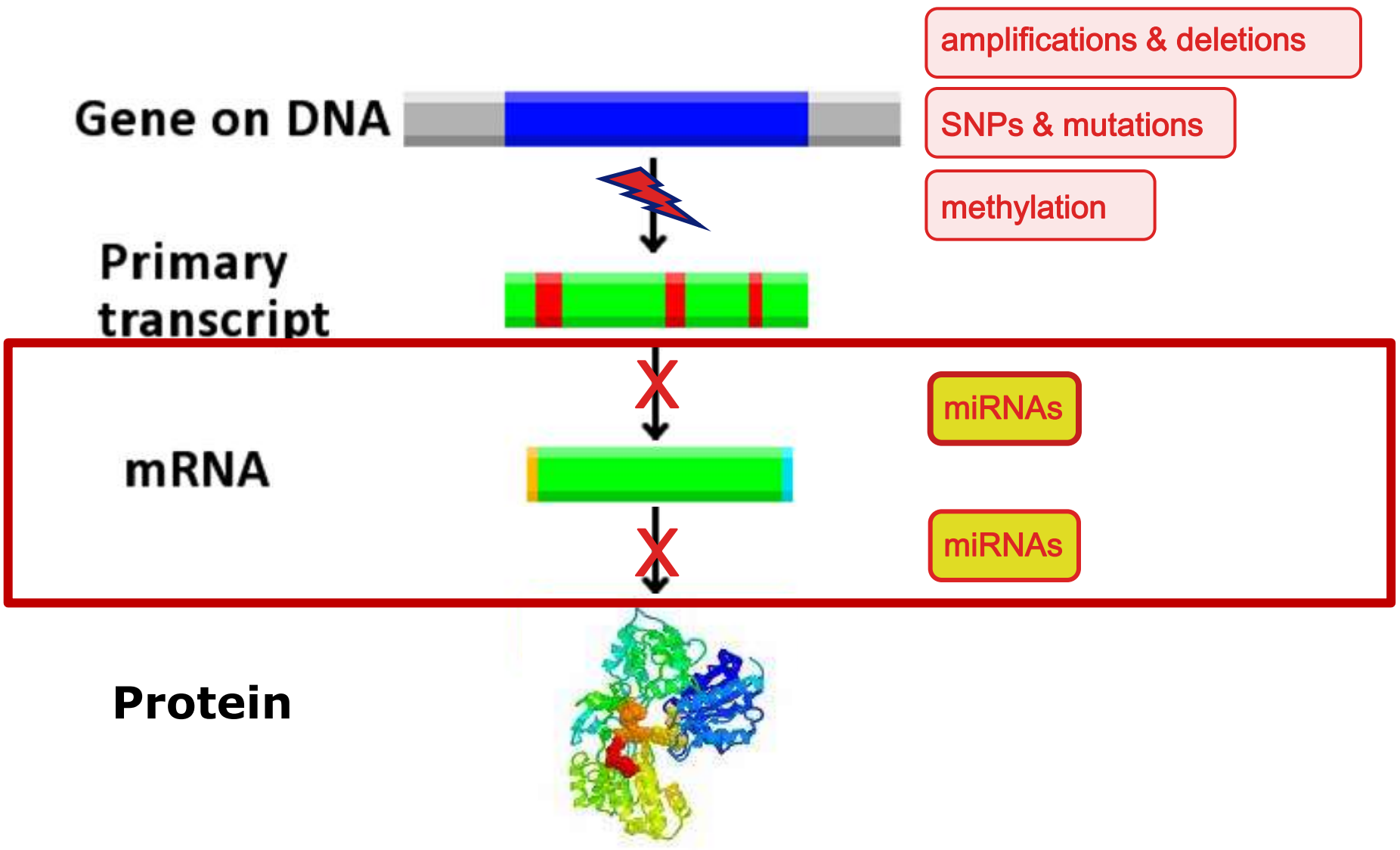
9th ISMRC September 25-27, 2013

Measuring RNA's in the circulation

- Various methods and techniques for isolating and measuring RNAs in the circulation
- Potential advantages of measuring RNA expression in the circulation over assessment in primary tumor tissues
- Future applications of RNAs measured in the circulation [CTCs/plasma/serum]

Gene expression control levels

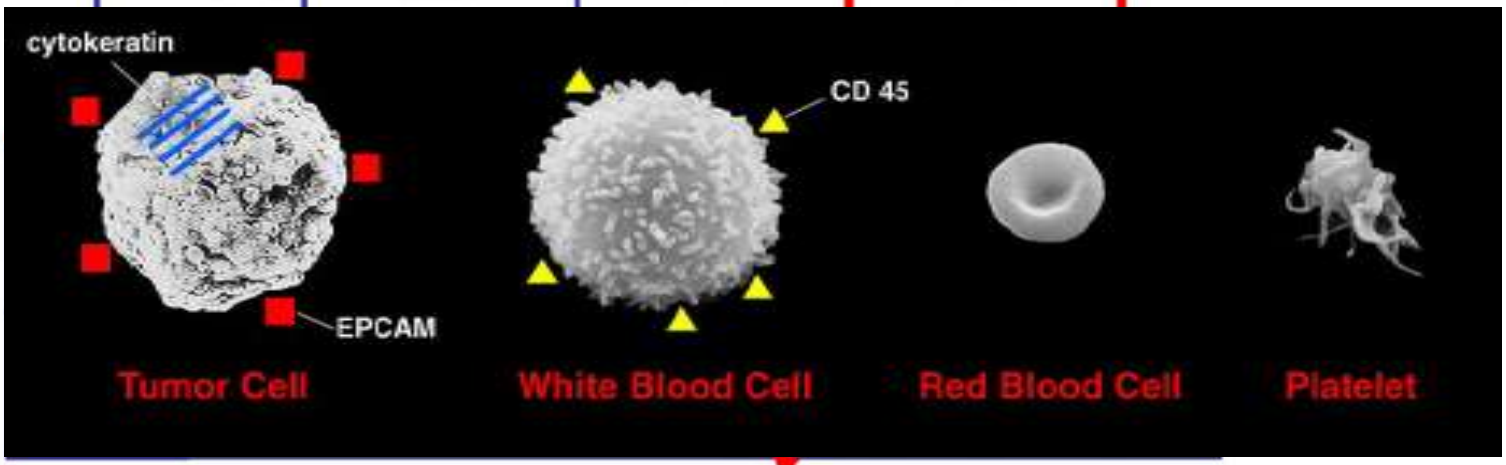


RNA isolation and detection in the circulation

1. Origin of the isolated RNA's

10-100 cells per mL

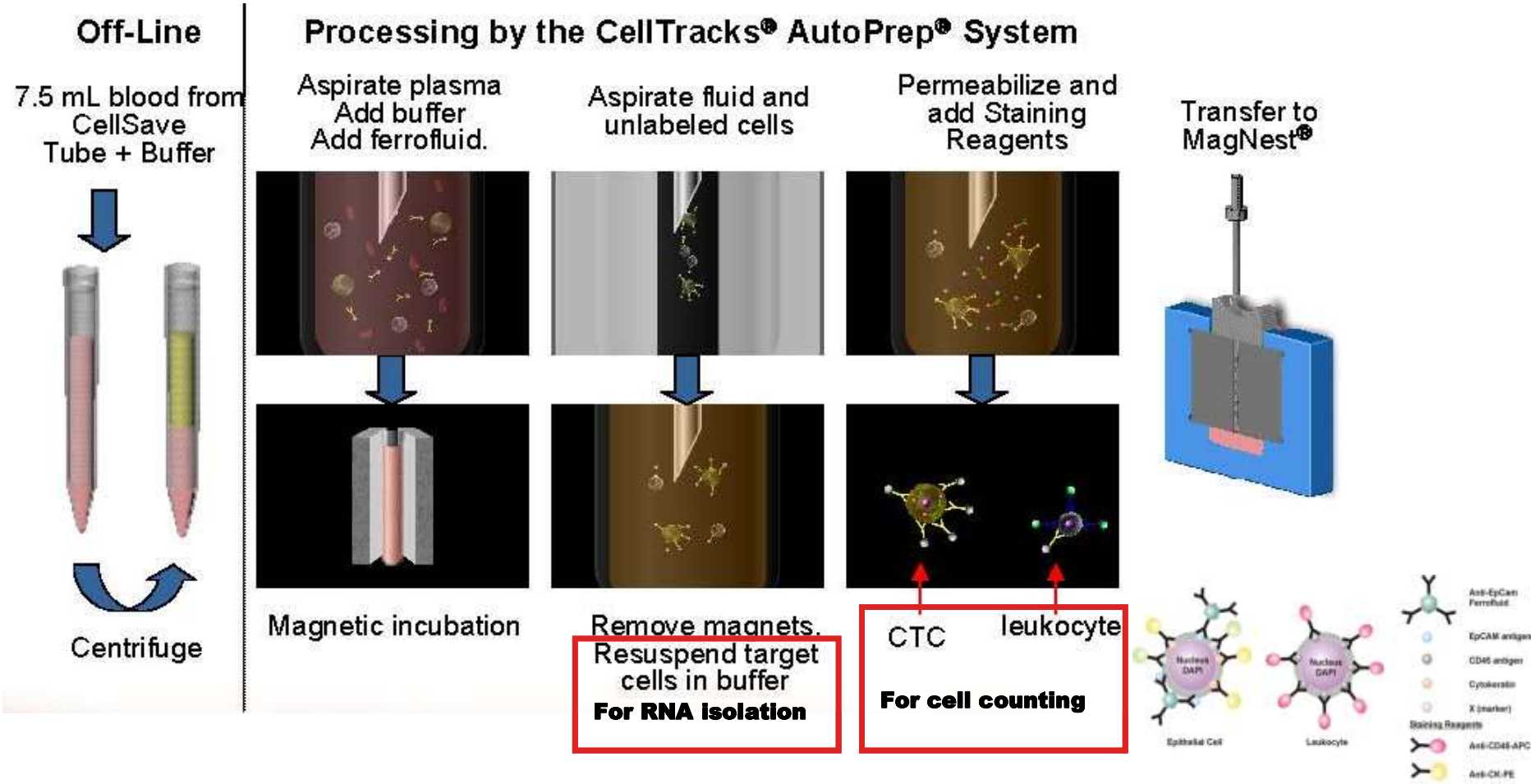
Cell Type	CTC	Erythrocyte	Leukocyte
Size (µm)	12-25	5-7	7-15



1,000,000 cells per mL

RNA isolation and detection in the circulation

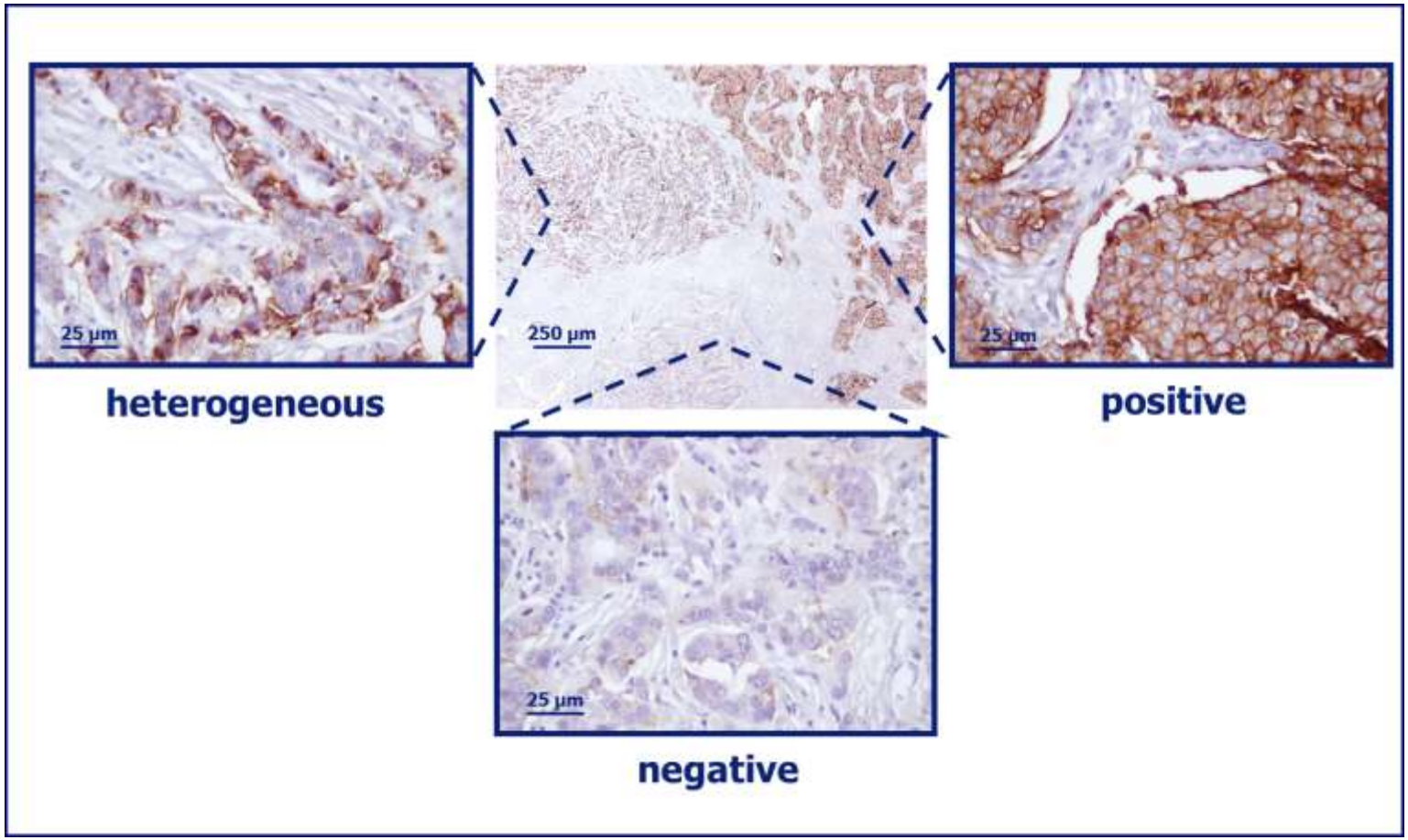
1. Origin of the isolated RNA's



Limitation to marker-specific isolations

1. Origin of the isolated RNA's

Heterogeneous EPCAM staining in breast tumors



Sieuwerts et al. JNCI, 2009

Limitation to marker-specific isolations

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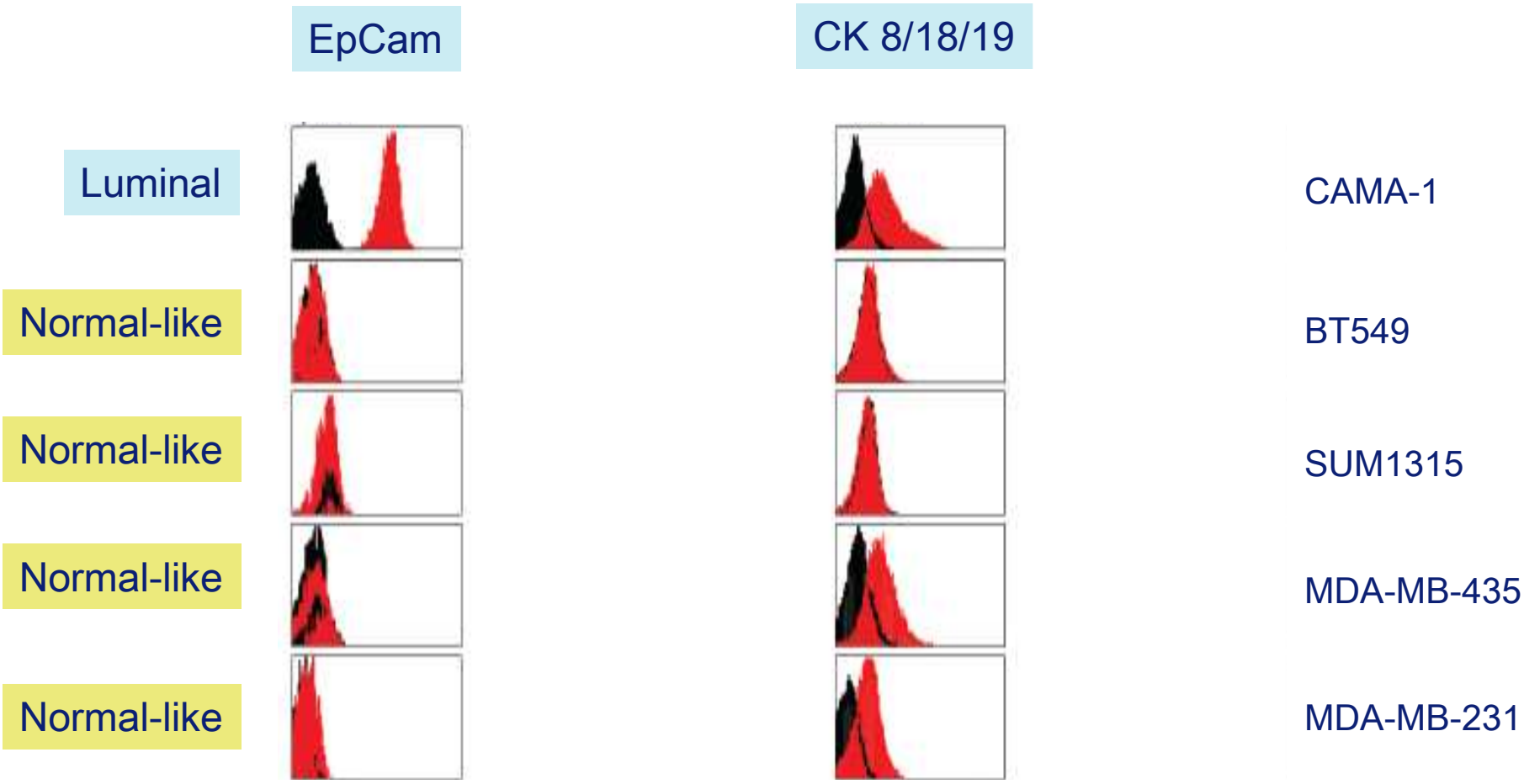
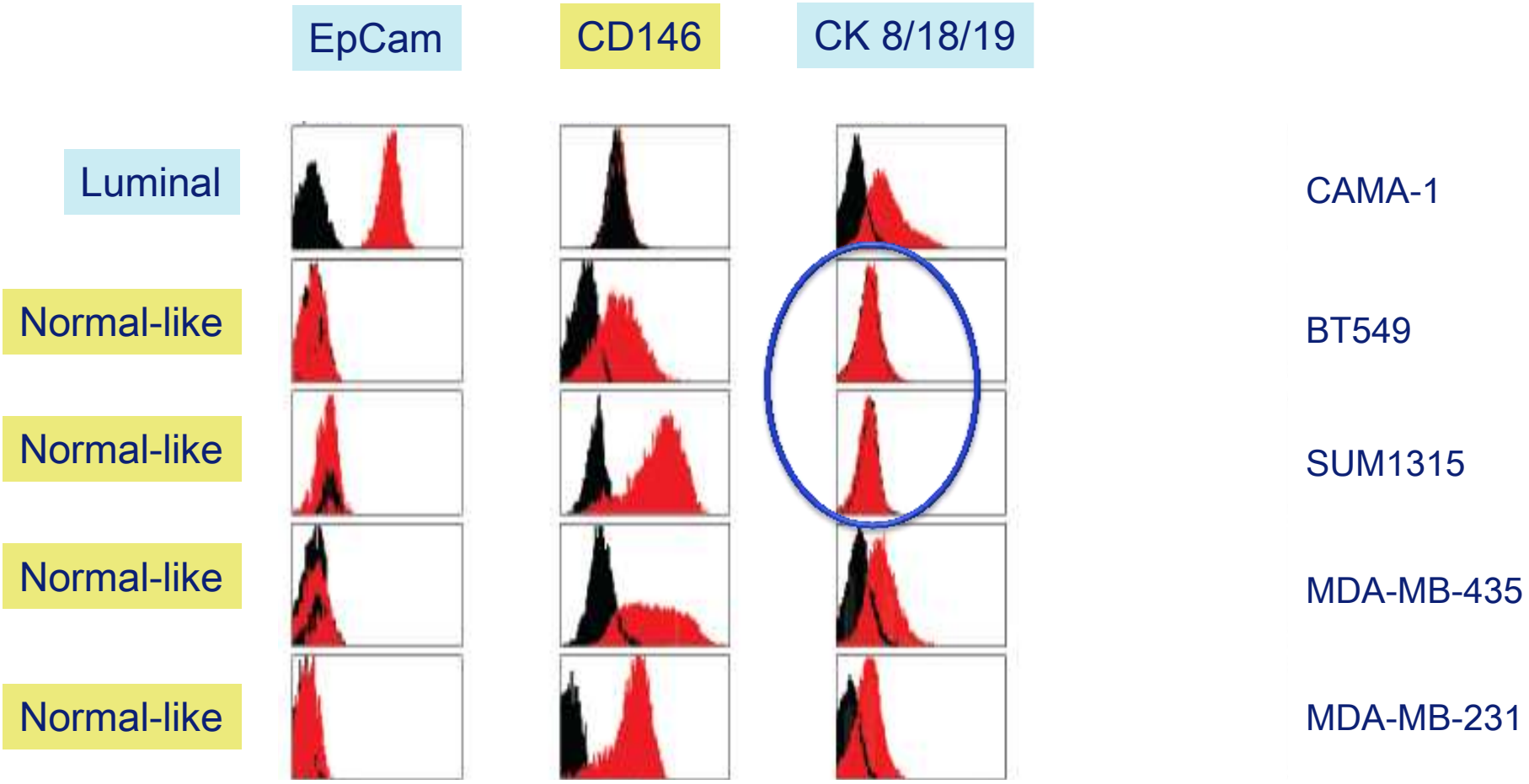


Sieuwerts et al. JNCI, 2009

Limitation to marker-specific isolations

1. Origin of the isolated RNA's

improve CTC enumeration using CD146 (*MCAM*)?



Mostert et al., BRCT, 2010

Limitation to marker-specific isolations

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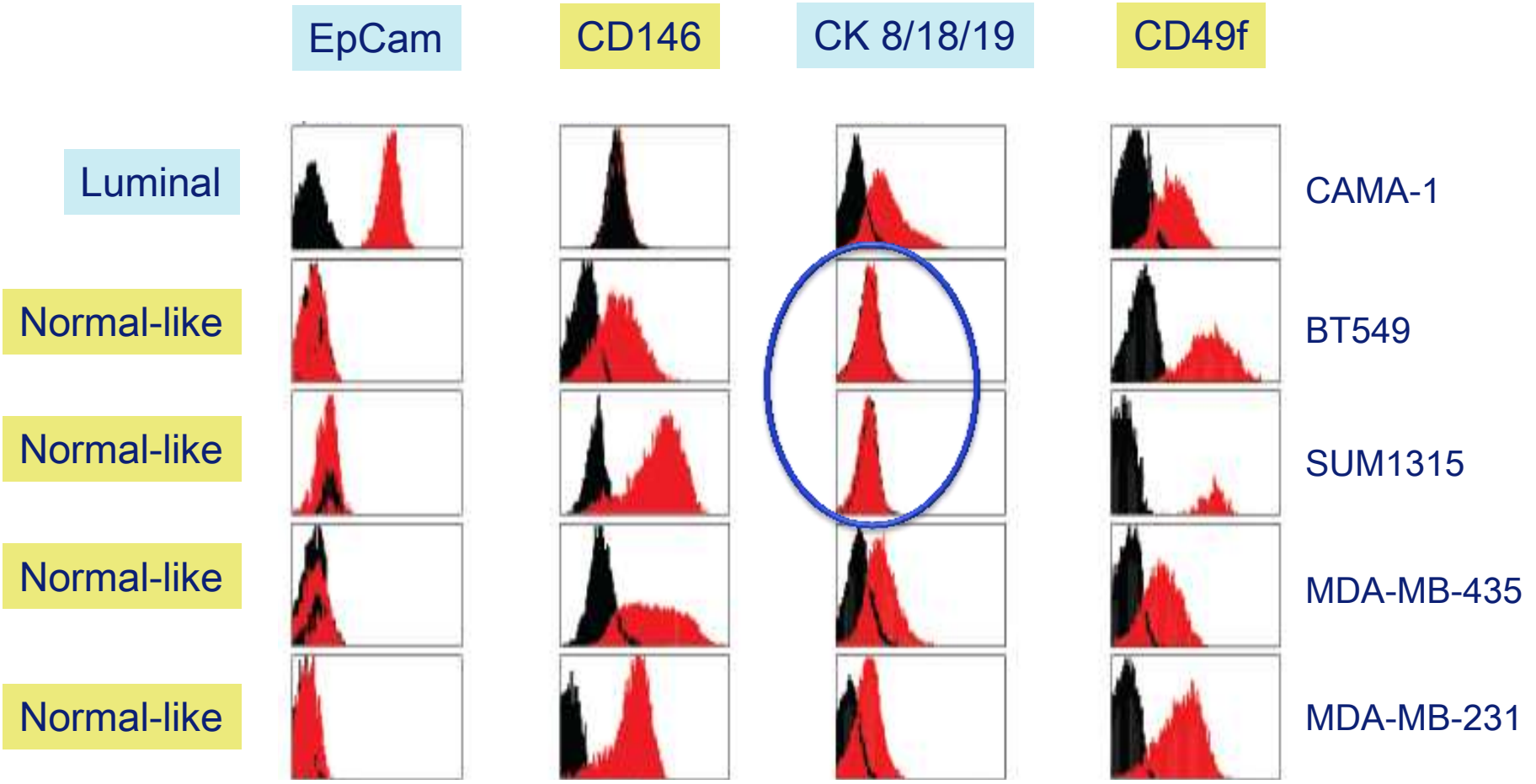
improve CTC enumeration using CD146 (*MCAM*)?



Mostert et al., BRCT, 2010

Limitation to marker-specific isolations

1. Origin of the isolated RNA's

- EpCAM-based enrichment overlooks CTCs with EMT/CSC features (normal-like/caudin-low)

Opportunities: CTC enumeration using CD146 (*MCAM*)+CD49f

- CD146 might be a good marker to capture these EMT/CSC-like CTCs
- Combining CD146 with EpCAM improves recovery but still not all of the breast cancer cells are retrieved…

→ Clinical evidence of improved recovery is being gathered in a BOOG trial (NEOZOTAC)

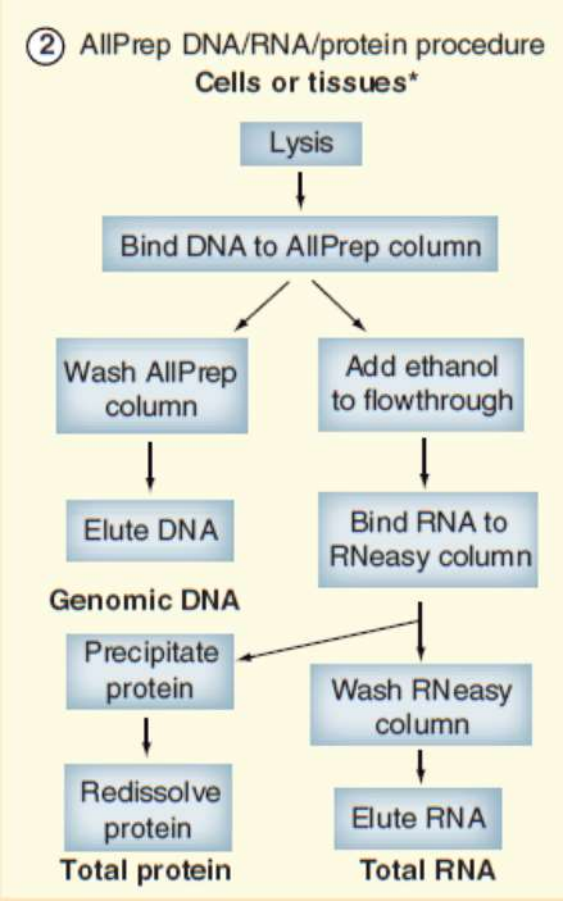
→ Additional markers will be added to recover all breast cancer CTCs (first in cell lines than in clinical material)

RNA isolation and detection in the circulation

2. RNA isolation method

- Total RNA, including microRNA's
  - RNA-B / TriZol
  - Norgen Total RNA
  - NucleoSpin
  - AllPrep/miRNeasy

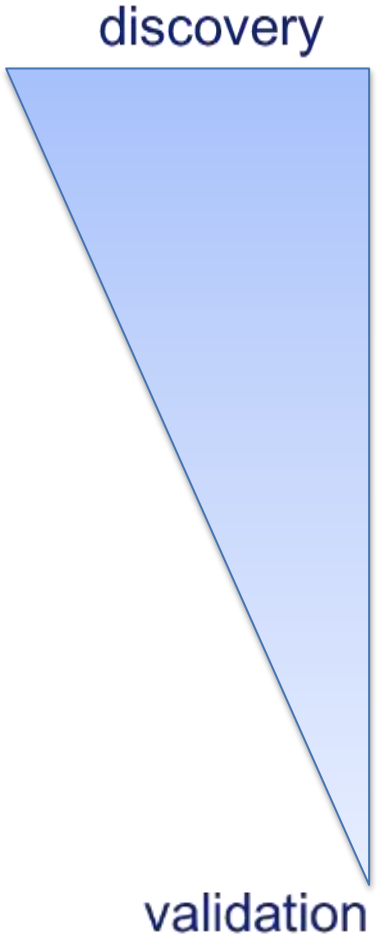
- miRNA/mRNA specific kits
  - PureLink [column-based]
  - mirPremier [column-based]
  - mirVana [organic + column-based]
  - High Pure [organic + column-based]
  - Sequence-specific Magnetic beads



RNA isolation and detection in the circulation

3. RNA detection method

- Hybridization-based methods
  - Deep sequencing or NGS
  - Microarrays
  - In situ hybridization with LNA probes
- Amplification-based methods
  - RT-PCR
  - Enzymatic luminescence

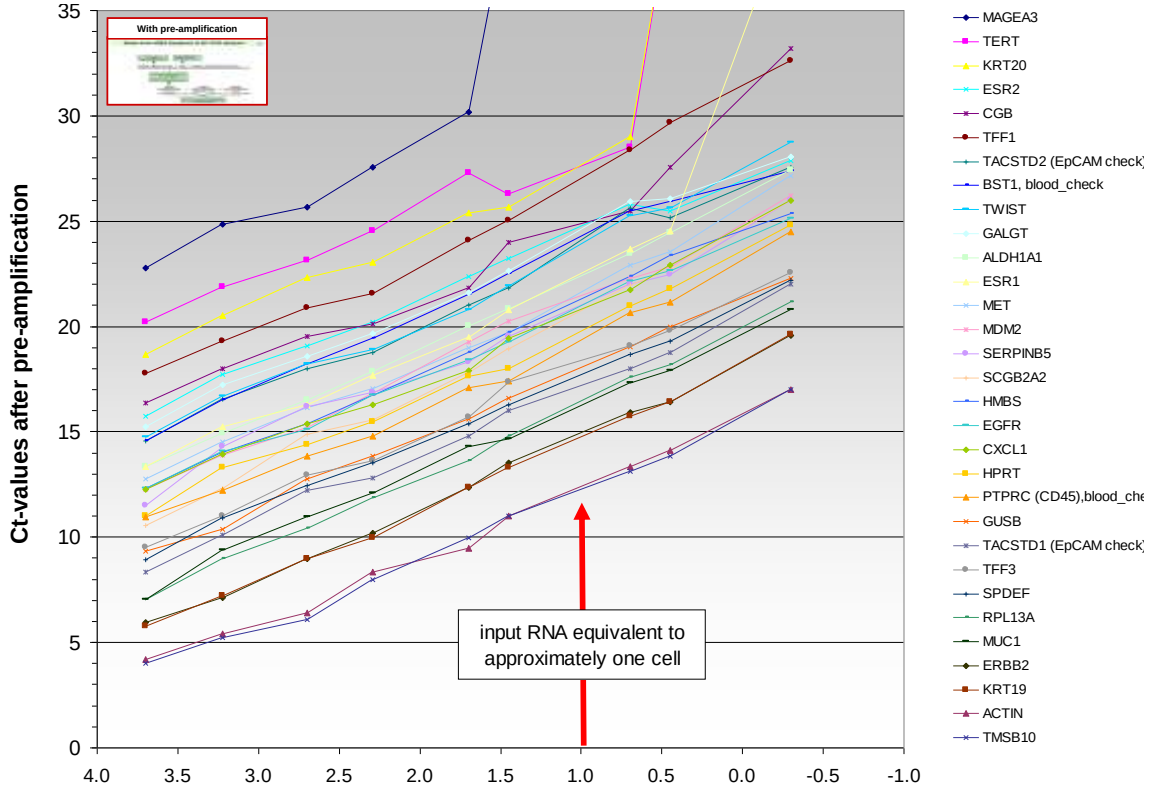


Planell-Saguar et al., Clinical Biochemistry, 2013

RNA isolation and detection in the circulation

4. Analyze multiple transcripts in limited material

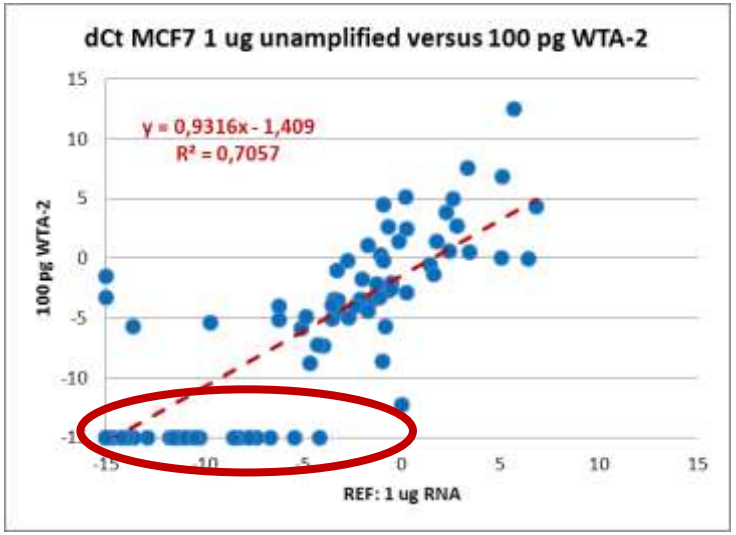
One cell contains ~10-30 pg total RNA of which ~1 pg is mRNA. Linear pre-amplification required.
Gene-specific amplification of up to 96 gene transcripts from as little as one cell has been successful in our hands.

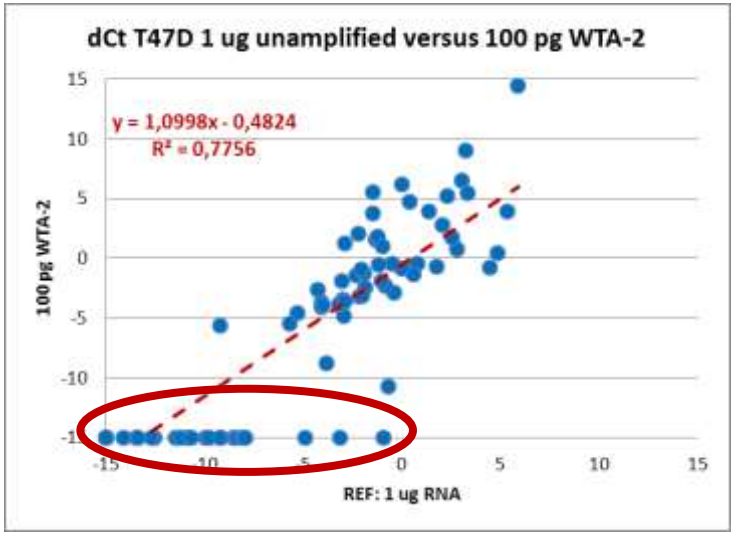


Sieuwerts et al., BCRT, 2009, CCR, 2011

RNA isolation and detection in the circulation

4. But how far are we with Whole Transcriptome Amplification [WTA] methods, required e.g. for NGS?





Sample	SKBR3						
RNA amplification and cDNA synthesis method	None Fermentas	WTA-2			SMARTer		
RNA input	2 ug	1000 pg	100 pg	10 pg	1000 pg	300 pg	30 pg
ACTB	9.33	10.92	6.93	7.80	4.59	7.08	9.49
ERBB2	9.74	16.68	19.79	23.54	14.64	15.50	27.57
KRT7	9.93	13.92	14.76	16.16	10.42	13.58	13.80
CTSD	10.72	17.57	21.35	25.44	11.34	14.98	26.04
CITED2	13.48	19.78	23.21	29.19	19.93	24.44	No Ct
MYC	14.02	20.06	23.40	26.37	13.72	24.05	28.30
COMT	14.74	19.56	18.39	26.67	13.03	15.22	16.49
CD24	15.47	21.84	26.01	25.49	16.74	19.61	34.74
TACSTD1	15.54	19.44	15.37	27.30	18.30	23.93	30.14
E2F1	15.62	22.50	26.09	No Ct	23.19	25.56	30.11
MUC1	15.75	19.36	17.76	27.21	15.56	23.77	30.13
CKAP4	15.88	22.39	26.06	28.18	23.11	28.02	No Ct
HRAS	16.05	19.73	23.14	19.41	16.86	24.20	38.19
HMBS	16.15	25.58	28.08	30.36	13.79	15.48	32.72
HPRT1	16.43	22.11	26.50	27.74	13.31	16.28	28.89
ERBB3	16.58	23.87	27.58	28.73	14.89	24.00	36.52
HSD17B2	16.93	19.18	25.54	No Ct	13.23	27.77	No Ct
CRYZ	18.03	24.40	28.26	No Ct	14.98	18.02	No Ct
NCOR2	18.33	23.87	26.79	28.28	25.20	28.41	No Ct
FN1	18.55	27.12	29.67	No Ct	17.97	30.85	No Ct
ORC6L	18.59	24.98	27.38	No Ct	16.80	28.01	37.15
PLAUR	19.20	25.21	30.28	No Ct	15.51	30.71	34.93
TIMP1	19.60	20.86	10.43	No Ct	16.68	24.68	37.69
SULT2B1	19.82	26.08	No Ct	No Ct	19.08	27.87	36.69
HSD17B1	20.26	28.91	No Ct	No Ct	27.11	No Ct	No Ct
CDH5	20.28	27.96	27.83	No Ct	28.66	No Ct	No Ct
IGF1R	20.47	25.99	No Ct	No Ct	27.61	No Ct	No Ct
B4GALNT1	21.48	28.61	27.45	No Ct	27.68	No Ct	No Ct
FOXC1	21.58	27.86	No Ct	No Ct	26.73	25.70	No Ct
CEACAM1	23.60	No Ct	No Ct	No Ct	No Ct	31.20	No Ct
PLAU	24.96	No Ct	29.01	No Ct	No Ct	No Ct	No Ct
FAS	26.11	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
GSTA1	26.19	No Ct	No Ct	No Ct	No Ct	30.27	No Ct
KRT5	26.46	30.14	No Ct	No Ct	No Ct	No Ct	No Ct
BST1	26.66	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
VWF	26.90	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
CSPG2	27.32	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
PGR	30.25	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
SULT1E1	30.62	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
SOX4	33.20	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
CDH2	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
HOXB13	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
IGF1	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
CDH1	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
COL1A1	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
LEP	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
TWIST1	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct

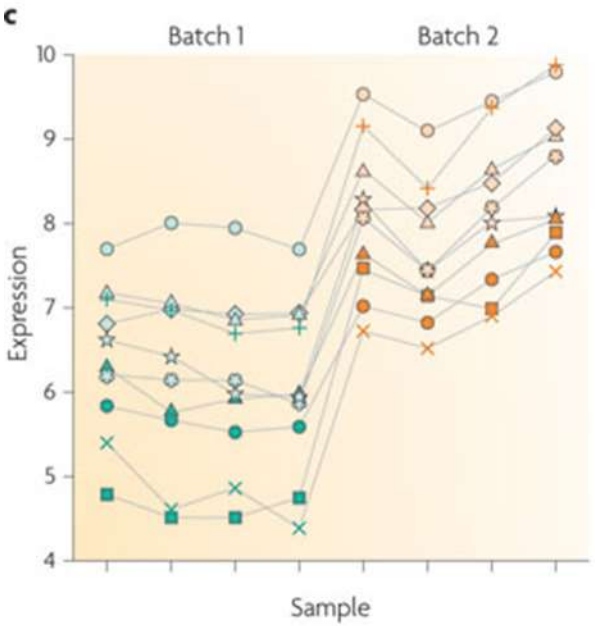
RNA isolation and detection in the circulation

5. Normalization procedures

OPINION

Tackling the widespread and critical impact of batch effects in high-throughput data

Jeffrey T. Leek, Robert B. Scharpf, Héctor Corrada Bravo, David Simcha, Benjamin Langmead, W. Evan Johnson, Donald Geman, Keith Baggerly and Rafael A. Irizarry



Exploratory analyses

Hierarchically cluster the samples and label them with biological variables and batch surrogates (such as laboratory and processing time)

↓

Plot individual features versus biological variables and batch surrogates

↓

Calculate principal components of the high-throughput data and identify components that correlate with batch surrogates

Downstream analyses

Do you believe that measured batch surrogates (processing time, laboratory, etc.) represent the only potential artefacts in the data?

Yes ↓ Use measured technical variables as surrogates for batch and other technical artefacts

No ↓ Estimate artefacts from the high-throughput data directly using surrogate variable analysis (SVA)

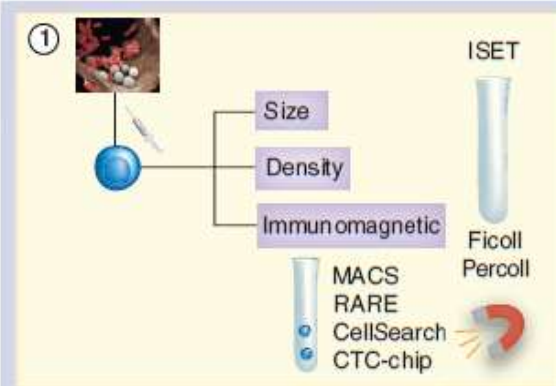
↓

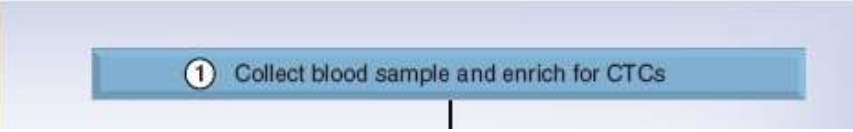
Perform downstream analyses, such as regressions, t-tests or clustering, and adjust for surrogate or estimated batch effects. The estimated/surrogate variables should be treated as standard covariates, such as sex or age, in subsequent analyses or adjusted for use with tools such as ComBat

Diagnostic analyses

Use of SVA and ComBat does not guarantee that batch effects have been addressed. After fitting models, including processing time and date or surrogate variables estimated with SVA, re-cluster the data to ensure that the clusters are not still driven by batch effects

RNA isolation and detection in the circulation





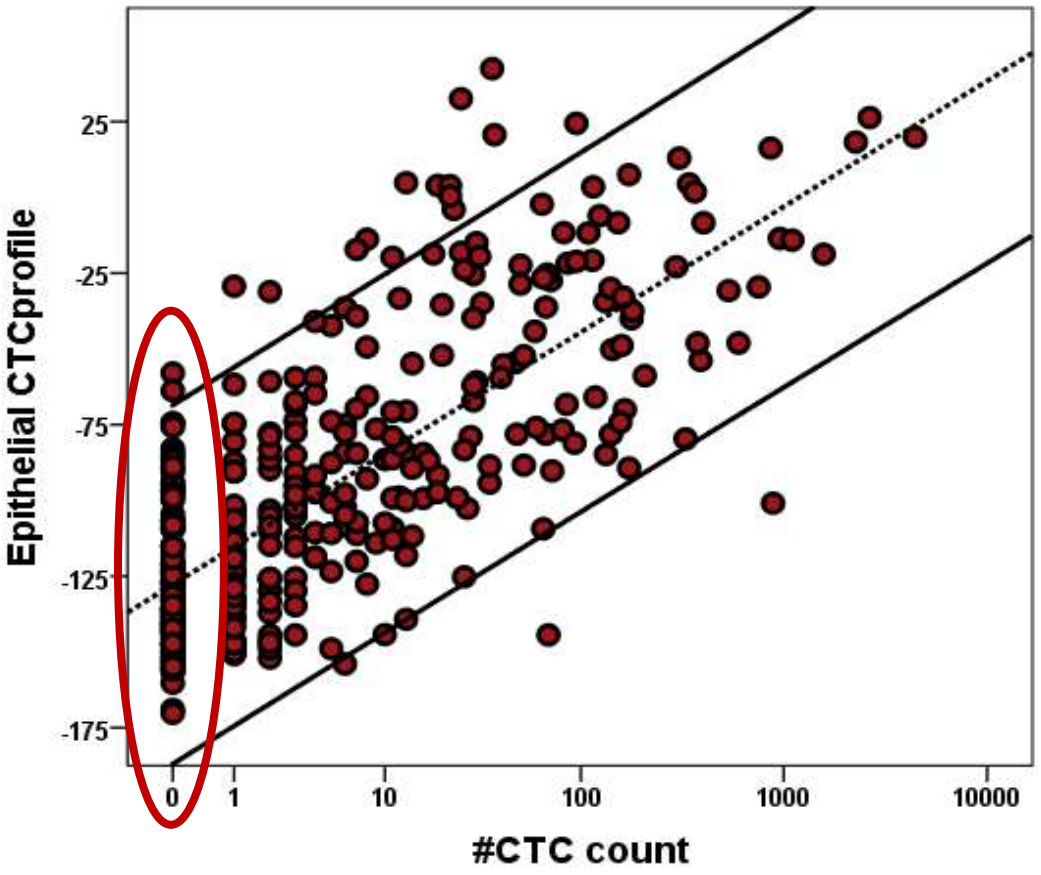
Mostert et al., Expert Rev Mol Diagn, 2011

Applications measuring RNAs in the circulation

1. Useful markers to associate levels measured in CTCs with CTC counts?

12 Epithelial-specific mRNA levels measured in EpCAM-enriched CTC fractions of n=397 MBC patients.
Mean ± 95% CI levels - after correction for the leukocyte contribution - with CellSearch derived CTC count.

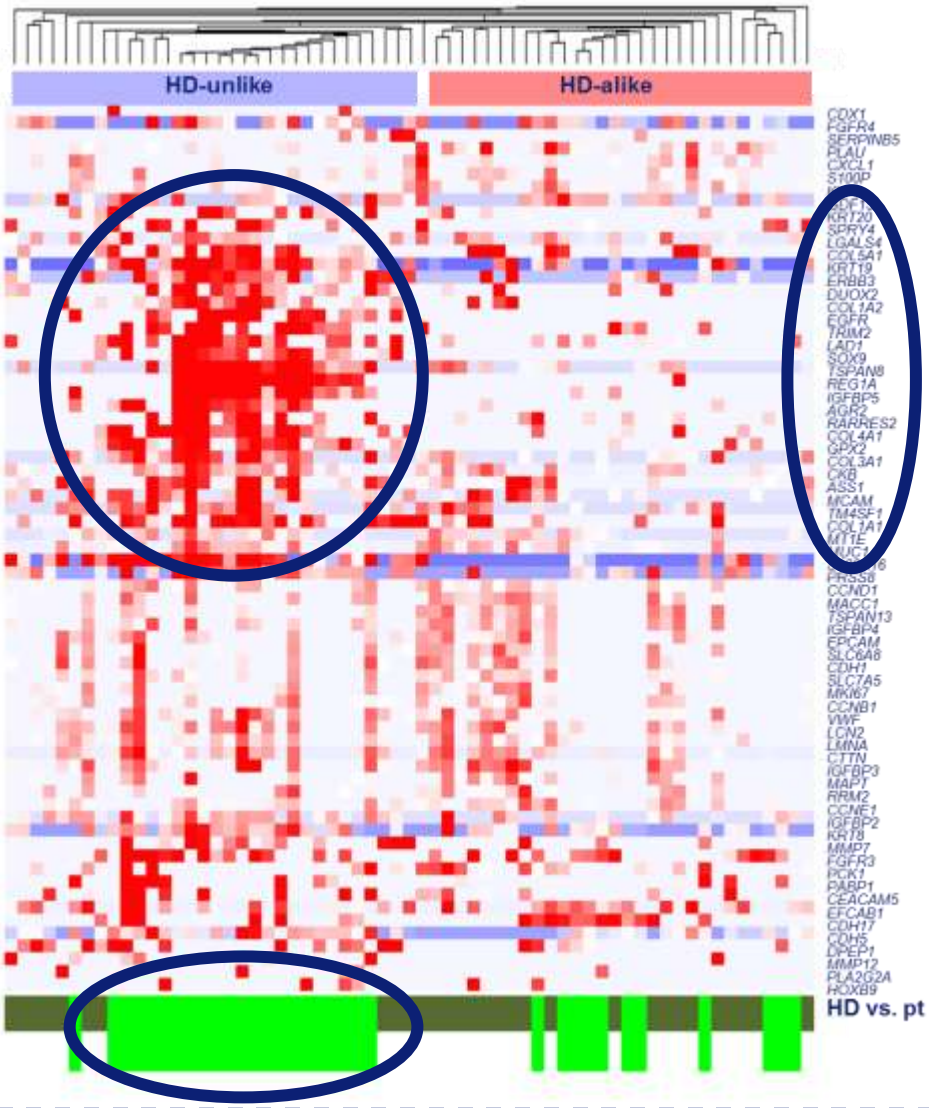
Epithelial mRNA levels measured in CTCs vs CTC count



Spearman rs = 0.74
P < 0.0001
n=397

Applications measuring RNAs in the circulation

2. Useful markers to identify colon cancer patients with an HD-unlike profile?



1. 30 HDs

2. 33 patients with metastatic colon cancer, without CellSearch detectable CTCs.

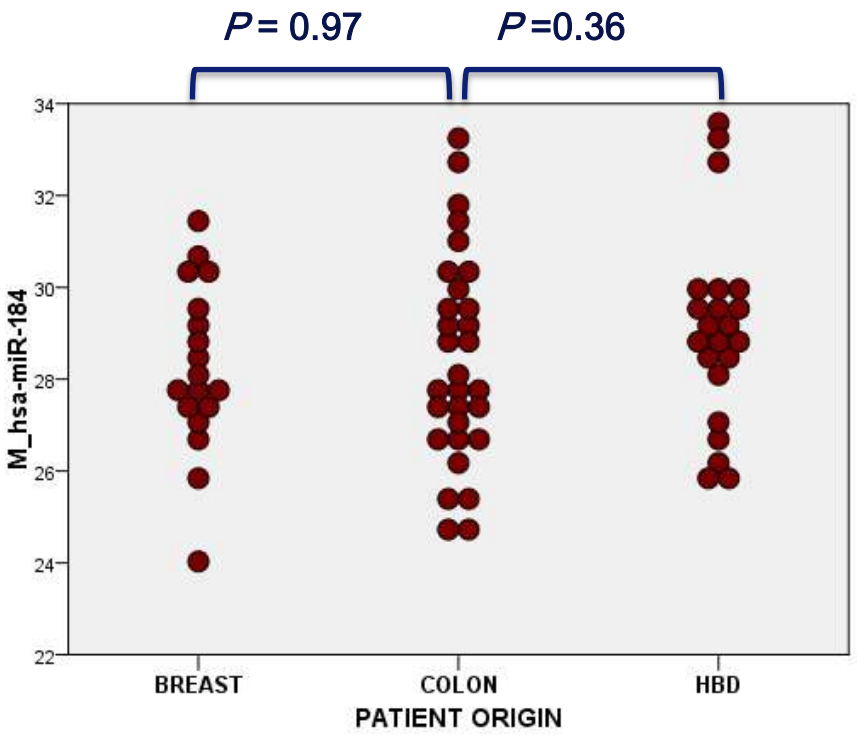
Unsupervised hierarchical cluster analysis comparing mRNA gene expression profiles in CTC-enriched fractions

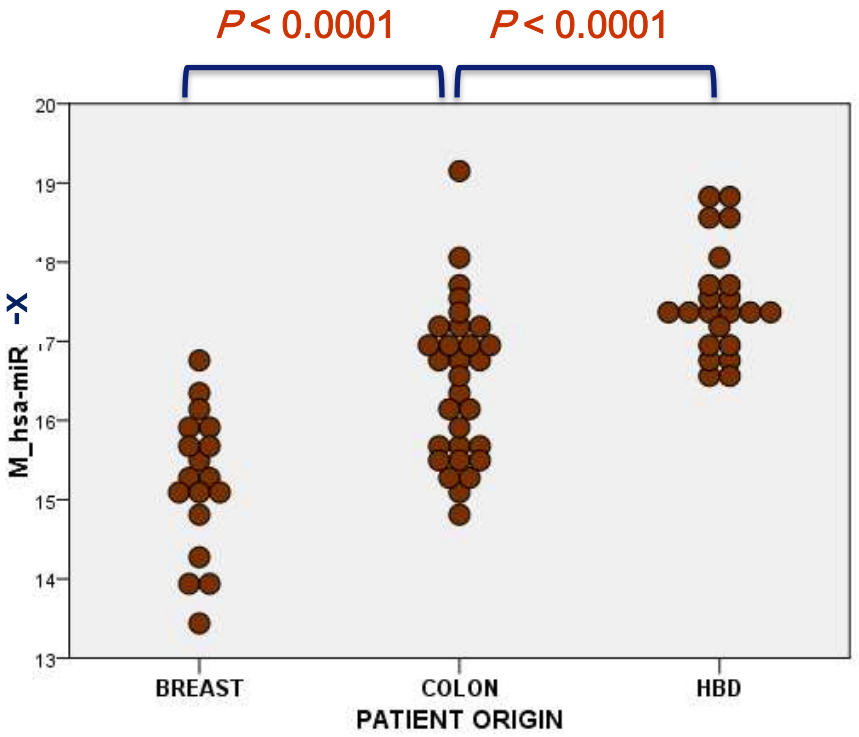
Mostert et al., AACR 2012

Applications measuring RNAs in the circulation

3. Useful markers to identify the type of primary tumor based on characteristics at metastatic disease?

miRNAs isolated from plasma of n=18 metastatic breast cancer patients, n=30 metastatic colon cancer patients and n=22 healthy blood donors.





Applications measuring RNAs in the circulation

4. Useful markers to understand the biology behind the disease?

mRNA levels of 96 genes measured in EpCAM-enriched CTC fractions of n=50 MBC patients.
Unsupervised hierarchical clustering after correction for leukocyte contribution.

Metastatic disease

CellSearch enriched CTCs

Start 1st line treatment

Epithelial CTC-specific gene expression patterns in CTC-enriched samples from blood of 50 metastatic BC patients

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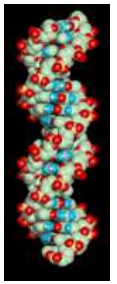
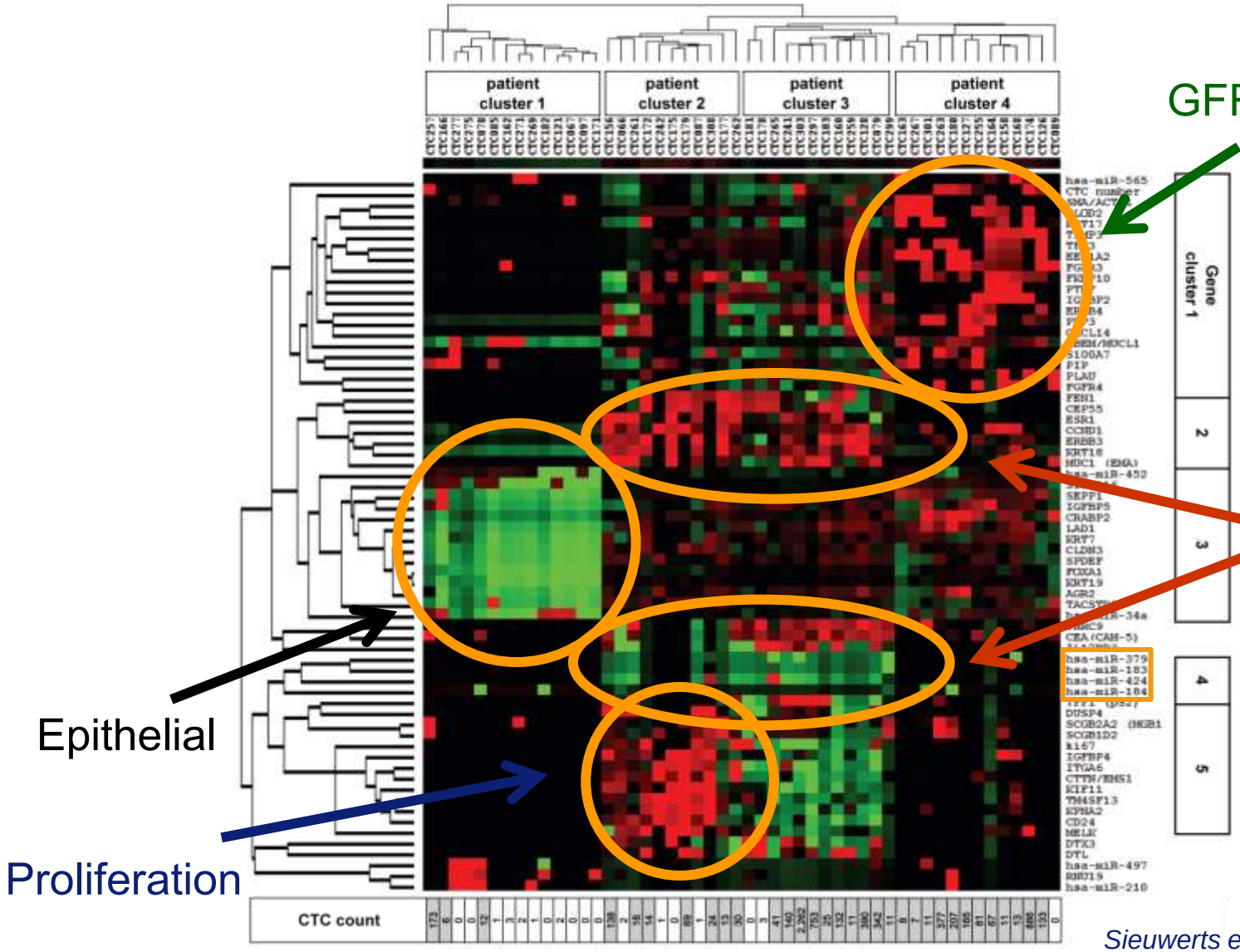
Unsupervised hierarchical cluster analysis



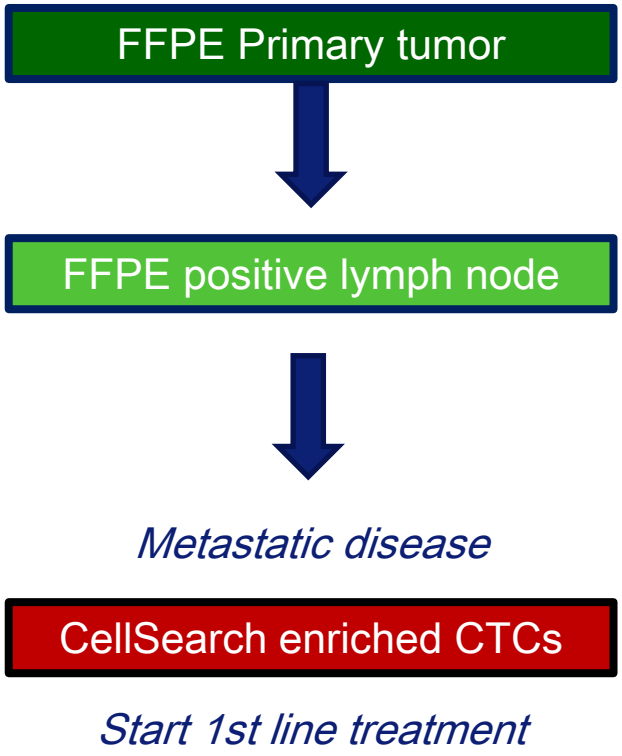


Sieuwerts et al., Clin Cancer Res, 2011

Applications measuring RNAs in the circulation

4. Useful markers to understand the biology behind the disease?

mRNA levels of 96 genes measured in EpCAM-enriched CTC fractions and matched primary FFPE's of n=72 MBC patients. Unsupervised hierarchical clustering after correction for leukocyte contribution and ComBat normalization to correct for CTC/FFPE batch effects.



Gene expression patterns in CTC-enriched samples from blood of 72 metastatic BC patients and their corresponding primary tissues

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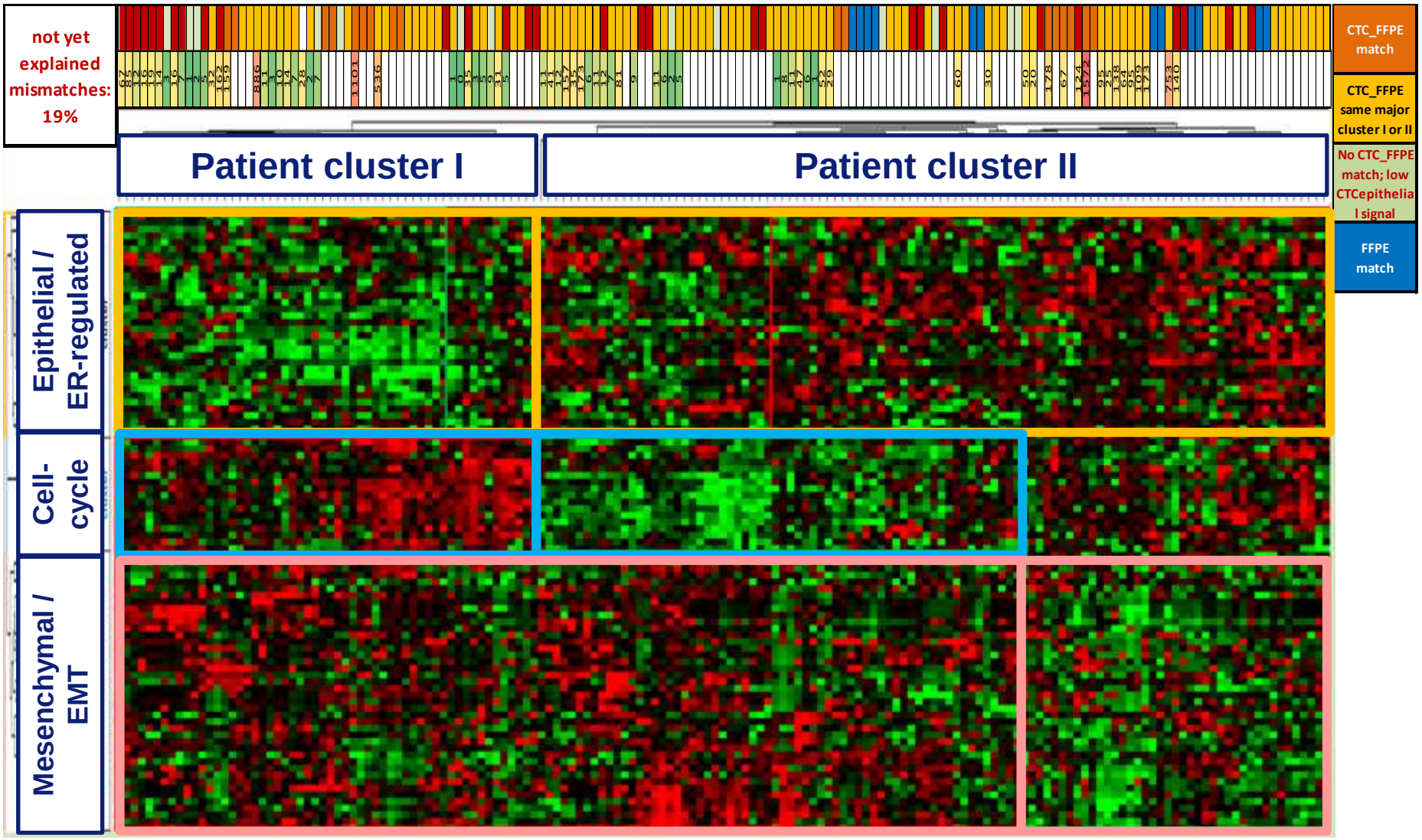


Limitations: primary and metastatic tissue

- Patients die from metastatic disease but are treated based on their primary tumor characteristics

- Primary and metastatic lesions are not identical

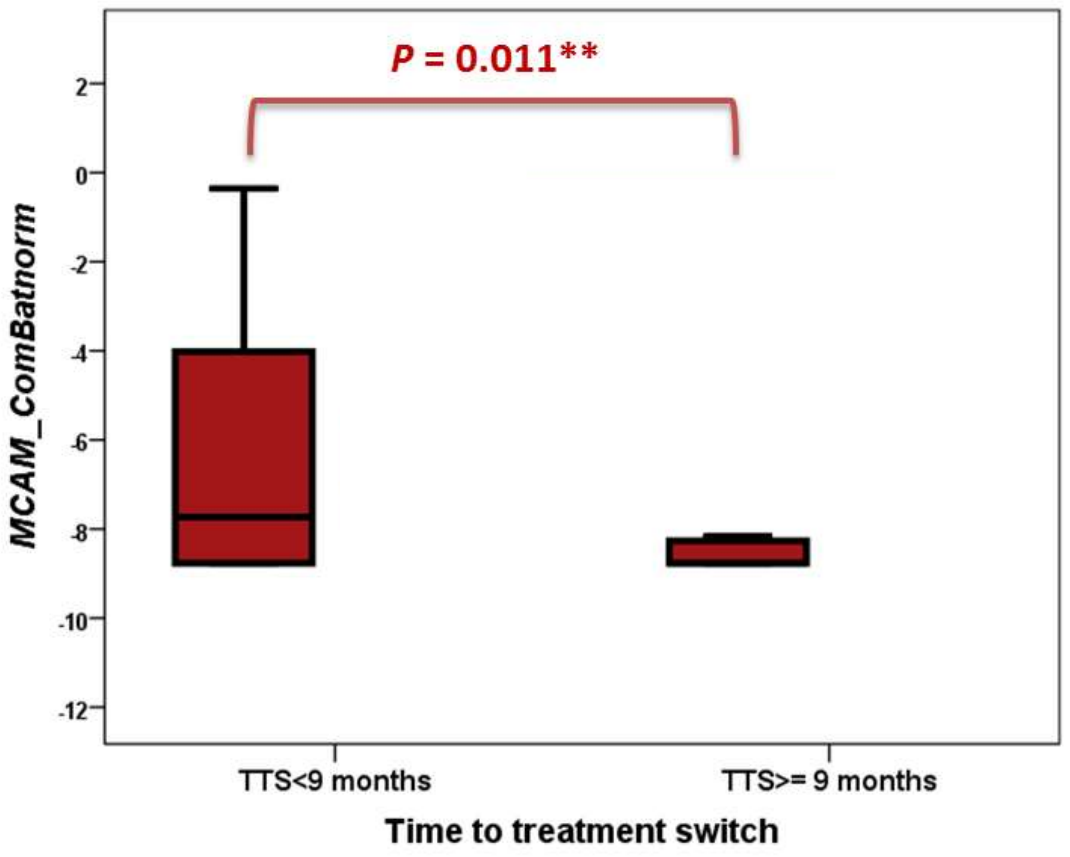
Q: Is this why treatment based on primary tumor characteristics shows poor response rates?

Q: Should we start treating based on characteristics of metastatic tissue, and can the information stored in CTCs be of help in this respect?

Applications measuring RNAs in the circulation

4. Useful markers to associate levels measured in CTCs with response to therapy?

CD146/MCAM mRNA levels measured in EpCAM-enriched CTC fractions and matched primary FFPE's of n=72 MBC patients. Box Whisker plot after correction for leukocyte contribution and ComBat normalization to correct for CTC/FFPE batch effects.



FFPE Primary tumor

Metastatic disease

CellSearch enriched CTCs

Start 1st line treatment

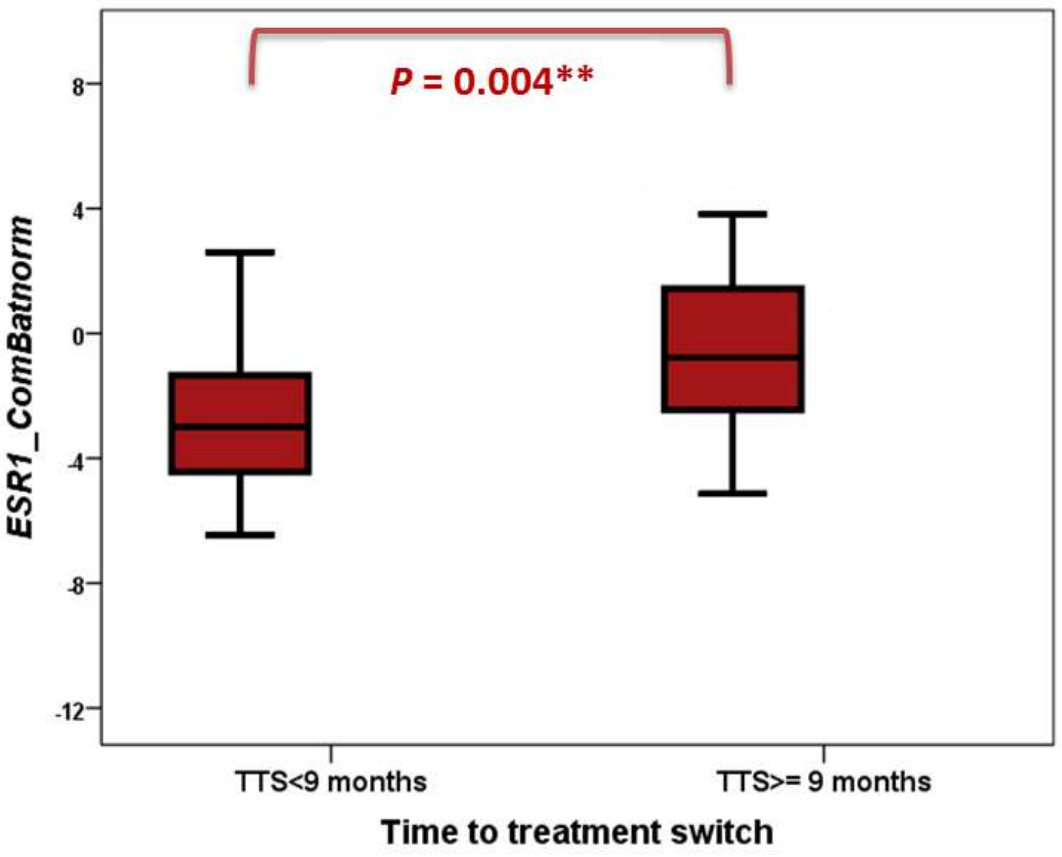
Response failure [TTP]

Start 2nd line treatment [TTS]

Applications measuring RNAs in the circulation

4. Useful markers to associate levels measured in CTCs with response to therapy?

ESR1 mRNA levels measured in EpCAM-enriched CTC fractions and matched primary FFPE's of n=70 MBC patients.
Box Whisker plot after correction for leukocyte contribution and ComBat normalization to correct for CTC/FFPE batch effects.



FFPE Primary tumor

Metastatic disease

CellSearch enriched CTCs

Start 1st line treatment

Response failure [TTP]

Start 2nd line treatment [TTS]

Applications measuring RNAs in the circulation

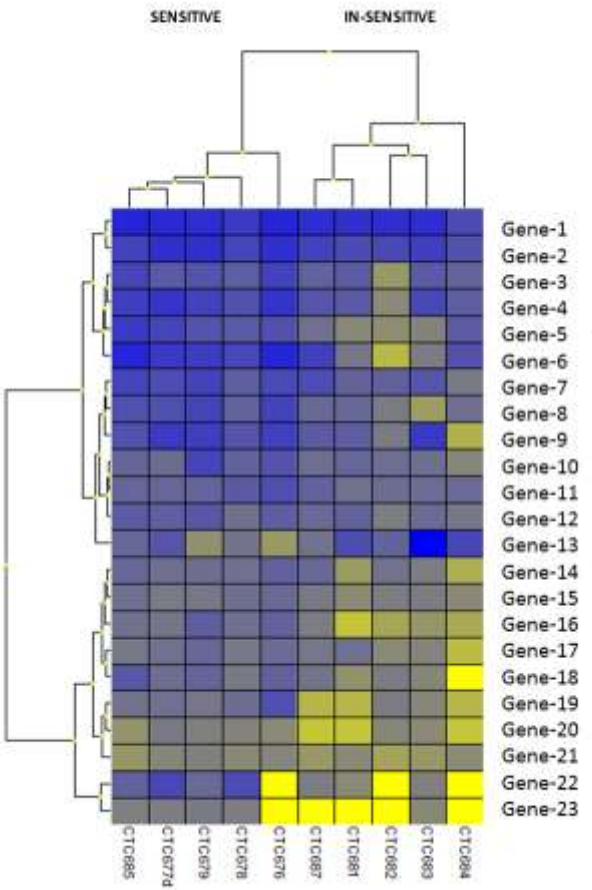
4. Useful markers to associate levels measured in CTCs with response to a new type of therapy?

mRNA levels measured in EpCAM-enriched fractions of cisplatin [cDDP]-sensitive and insensitive cell lines spiked in blood.
Training: 23 differentially expressed genes identified and used to build a cDDP-sensitivity profile. Validation: in MBC patients.

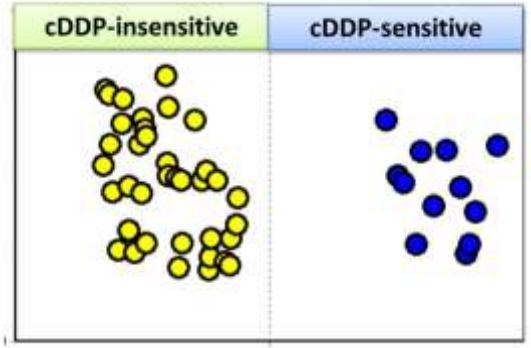
discovery

Table 1: cell lines tested for generatio

	Cell lines
cDDP-sensitive	SUM149PT
	UACC893
	MDA-MB-46
	MDA-MB-17
	SUM185PE
	SUM52PE
cDDP-insensitive	SKBR3
	MPE600
	MCF7
	ZR75-1
	SUM44PE
	BT20



validation



Metastatic disease

CellSearch enriched CTCs

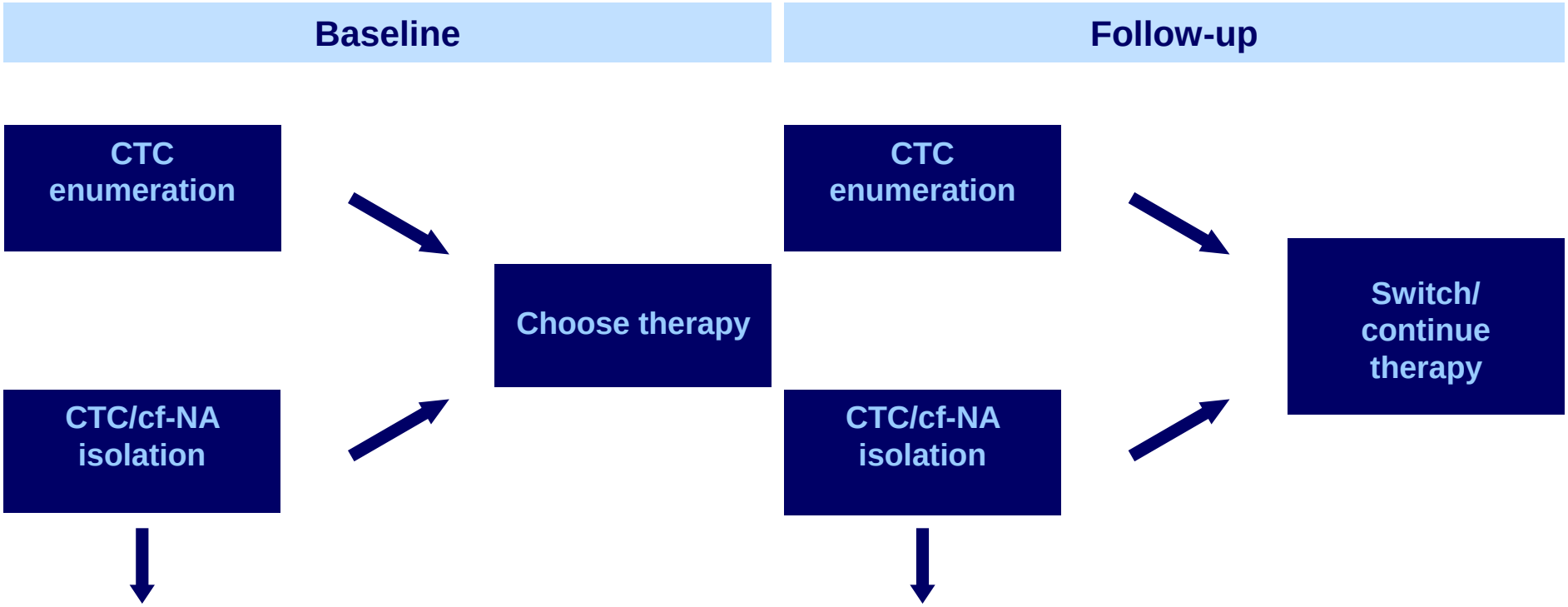
Patients with 5 or more CTCs and favorable cDDP-sensitivity profile, start cDDP treatment

Response rate [RR]

Sister's Hope project, 2013

Applications measuring RNAs in the circulation

one-stop shop



Mostert et al., Expert Rev. Mol. Diagn. 2011

Applications measuring RNAs in the circulation

summary

- There is an urgent need for additional diagnostic, prognostic and predictive markers in oncology
- These markers should preferably be measurable at any time during the course of the disease
- CTCs and cf-NAs provide an unique opportunity to diagnose the origin and type of primary tumor, and to assess prognosis, response to therapy and drug targets non-invasively and repeatedly
- Among these markers, miRNAs are especially promising because of their stability and key regulatory role in carcinogenesis
- The technical challenge is to isolate ALL CTCs and to discriminate between tumor/disease-specific RNAs and RNAs from background leukocytes.

Ongoing CTC and cf-NA profiling projects

- Advanced breast cancer before and after therapy *[06-248]*
- Breast cancer treated with hormonal agents *[09-405]*
- Colon cancer with pre-liver metastatic resection, matched with primary and metastatic tissue; *gene expression and mutation profiles*
- Ovarian cancer (ascites and matched primary tumors); *SNP arrays*
- Breast cancer: cell-line based cisplatin-sensitivity profile
- Prostate cancer: development of a prostate-specific profile
  [in collaboration with Antwerp: genes/re-arrangements and mutations]
- Breast and colon cancer: development of plasma miRNA profiles
  [in collaboration with Toray]

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