Liquid Biopsies:
Circulating tumour cells and circulating DNA

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Overview

• CTCs
  – CellSearch & other techniques
  – Data in breast, prostate and colorectal cancer
  – Molecular characterization

• cfDNA
  – How it is measured
  – Early studies & comparison to CTCs
  – Data in colorectal, lung, breast cancers
Why liquid biopsies?

• Tissue is the issue
• Difficulties in obtaining tissue
  – Meric-Bernstam JCO 2015 at MDACC found 23% of patients referred for studies were ineligible due to tissue inadequacy for genomic testing
• Biopsy related complications
• Cost of biopsies vs CTCs/ctDNA
• Liquid biopsies -> the stethoscope for the next 200 years  Eric Topol, Wall St Journal, 2015
Targeting oncogenic drivers

- Kris et al JAMA 2014
- 1009 pts for 1-10 genes in NSCLC (KRAS, EGFR, ALK, ERBB2, BRAF, PIK3CA, MET, NRAS, MEK, AKT)
- Actionable drivers detected in 64%
- Survival improved if targeted therapy used for oncogenic driver (3.5yrs vs 2.4yrs)
Circulating tumour cells

- Cancer cells that circulate in the peripheral blood
- First documented in 1869 – “cells identical with those of the cancer itself being seen in the blood may...throw some light upon the mode of origin of multiple tumours existing in the same person”
CellSearch

- First FDA approved and validated detection method
- 7.5ml blood draw
- Nanoparticles with magnetic core and surface EpCAM antibodies incubated with the specimen
- EpCAM: epithelial cell adhesion molecule
  - Overexpressed in primary and metastatic adenocarcinomas
  - Expression can decrease during development of metastases and epithelial to mesenchymal transition (EMT)
CTC identification:
• CK +
• EpCAM +
• DAPI +
• CD45 -
Screenshot from the CellTracks Analyzer
CTCs and staining in the 4\textsuperscript{th} channel

<table>
<thead>
<tr>
<th>DAPI/CK-PE</th>
<th>CK-PE</th>
<th>DAPI</th>
<th>CD45-APC</th>
<th>Stathmin</th>
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<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<td><img src="image10.png" alt="Image" /></td>
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Other EpCAM-based techniques

• Gilupi nanodetector; Saucedo-Zeni 2012
  – Seldinger guidewire coated with anti-EpCAM in the cubital vein of patients for 30 minutes

• Microchip; Nagrath Nature 2007
  – Mediated by interaction of target CTCs with antibody-coated microposts under controlled flow Conditions
  - Positive in high & low EPCAM expressing cell lines
**Electric charge – DepARRAY**

- dielectrophoretic field flow fractionation (depFFF)
- Differences in cell phenotype and membrane capacitance
- Capture of cells by ‘electric cages’ on a microelectronic chip
- Allows molecular analysis
CTCs in Cancer vs Healthy subjects

• EpCAM based: Allard et al CCR 2004

• Healthy donors (145), pts with non-malignant disease (199), metastatic carcinomas (964)

• Rare in healthy subjects and non-malignant disease
  – 8 (5.5%) healthy had 1 CTC, 0 had ≥2 CTCs
  – 14 (7.5%) disease pts had 1 CTC, 1 had ≥2 CTCs

• 36% had ≥2 CTCs per 7.5ml blood – 57% prostate, 37% breast, 37% ovarian, 30% colorectal, 20% lung, 26% other
Cristofanilli et al. NEJM 2004

- 177 pts with mBC pre-new treatment and at 1st f/u
- 145 normal and 200 benign breast/other conditions (2 pts had CTC 2)
- CTC ≥2 detected in 61% with mBC
- Training set (102 pts) – used to define CTC ≥5/<5 threshold
- Validation set (75pts)
- Can give a estimate of disease progression prior to imaging (3-4 wks vs 8-12wks)
- Strongest predictor of PFS and OS on multivariate analysis
  -> FDA approval
Probabilities of PFS (A,B,C) and OS (D,E,F) based on CTC ≥5 vs CTC<5 per 7.5ml blood before initiation of a new line of therapy.
CTCs vs imaging in mBC

Budd et al.  CCR 2006

- 138 pts
- Imaging at 10 weeks (2 readers) cf CTC at 4 wks (local & central lab)
- Interreader variability for radiology 15.2% vs CTC 0.7%
- Radiologic non-progression:
  - CTC ≥5 (9%) vs CTC<5 (60%), OS 15.3m vs 26.9m
- Radiologic progression:
  - CTC ≥5 (16%) vs CTC<5 (14%), OS 6.4 vs 19.9m
- CTCs may act as an earlier, more reproducible indicator of disease status
CTCs in early breast cancer

Rack et al. JNCI 2014

- 2026 pts pre adj chemo and 1492 pts post
- f/u for median 35m (0-54m)
- Pre chemo – CTCs in 21.5%
- Post chemo – CTCs in 22.1%
- Associated with poor DFS, distant DFS, BCSS, OS
- Luminal pts with CTCs had worse DFS and OS
CTCs before chemotherapy and relationship to
A: disease free survival
B: overall survival
Ongoing CTC trials in breast cancer

• A Randomized Phase III Trial to Test the Strategy of Changing Therapy Versus Maintaining Therapy for Metastatic Breast Cancer Patients Who Have Elevated Circulating Tumor Cell Levels at First Follow-Up Assessment

• CirCe01 Study: Evaluation of the Use of Circulating Tumour Cells to Guide Chemotherapy From the 3rd Line of Chemotherapy for Metastatic Breast Cancer
Prostate cancer

Shaffer et al, CCR 2007

• 63 pts with mPC
• 80% were CTC+
• 65% had ≥5 CTCs per 7.5ml blood, median 16 cells
• Cells isolated from cartridge and could perform IHC, ISH -> EGFR expression, AR gene amplification
• Possible reflection of tumour heterogeneity - eg. Different tumour cell populations in CTCs
Fig. 3. Quantitation of EGFR expression in CTCs by the automated immunofluorescent assay. The percentage of CTCs positive for EGFR was analyzed in samples with >5 CTCs (top). In the bottom, image 27 shows an individual CTC that stained positive for EGFR expression. Image 177 shows a CTC from the same patient with no EGFR staining. These images are representative for patient 20, and both cells showed positive cytokeratin staining and no CD45 staining, whereas 4',6-diamidino-2-phenylindole showed intact nuclei.
Metastatic Prostate Cancer

De Bono, CCR 2008

• 231 pts sampled pre-chemo & monthly thereafter
  – Defined favourable and unfavourable groups
  – Effect of conversion b/t favourable and unfavourable
  – Post treatment CTC count is superior to PSA in predicting OS

• Accurate and independent predictor of OS -> FDA approval
OS of CRPC depending on CTC conversion before and after therapy
Scher et al. JCO 2015.

- CTCs and LDH as a surrogate for survival was a secondary objective of COU-AA-301: abiraterone and prednisone for post-chemo mPC
- CTCs measure at baseline, 4,8,12 wks
- 711 pts
- Defined 3 risk groups:
  - CTC <5/any LDH
  - CTC ≥5/LDH ≤250
  - CTC ≥5/LDH >250
- 1 and 2yr survival for CTCs<5 vs CTCs ≥5/LDH>250U/L at 12 wks was 82% and 46% vs 25% and 2%
Colorectal cancer

Cohen et al. JCO 2008

• 430 pts at baseline and after 1\textsuperscript{st}, 2\textsuperscript{nd} or 3\textsuperscript{rd} line therapy

• Favourable CTC<3 and unfavourable CTC ≥3 per 7.5ml blood

• Conversion at 3-5 wks associated with longer PFS and OS than CTC ≥3 at both time points; PFS 6.2v1.6m and OS 11 vs 3.7m, p=0.0002

• For radiological non-progression, PFS 18.8 vs 7.1m for CTC <3 vs CTC ≥3
Progression-free survival (PFS) and overall survival (OS) of metastatic colorectal cancer patients with < three and ≥ three circulating tumor cells (CTCs) in 7.5 mL of blood (A, B) before therapy, (C, D) 1 to 2, 3 to 5, 6 to 12, and 13 to 20 weeks after initiation of therapy, and (E, F) by circulating tumor cell status at baseline and 3 to 5 weeks.

Steven J. Cohen et al. JCO 2008;26:3213-3221
©2008 by American Society of Clinical Oncology
(A) Overall survival in metastatic colorectal cancer patients by imaging response, (B) circulating tumor cell (CTC) yield within ± 1 month of imaging, and (C) both imaging response and circulating tumor cell yield within ± 1 month of imaging.
Molecular characterization of CTCs (1)

• Fourth channel
  – EGFR
    • Grisanti 2014: EGFR expression identified in 45% CTC+ HNC pts (not compared to tissue)
  – HER2
    • Fehm 2010: HER2+ CTCs present in HER2- patients
      – 122 from 245 pts had ≥5 CTCs, HER2+ in 50 (41%)
      – 25 from 78 pts with HER2- tumours were CTC HER2+
Molecular characterization of CTCs (2)

- **Prostate cancer**: Attard et al, Cancer Res 2009
  - Isolate CTCs to identify androgen receptor gene amplification, ERG, AR and PTEN gene rearrangement

- **Colorectal cancer**: Gasch et al, Clin Chem 2013
  - EGFR gene amplification, KRAS, BRAF and PIK3CA mutation status

- **Lung**: Maheswaran et al, NEJM 2008.
  - NGS can identify EGFR mutations on CTCs that match the tumour tissue
### CTCs

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
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<tbody>
<tr>
<td>Validated system</td>
<td>Not found in all cancers</td>
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<tr>
<td>Surrogate marker proven</td>
<td>Metastases associated with EMT and lower EpCAM expression</td>
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<tr>
<td>72 day window for processing/shipping samples</td>
<td>Molecular characterization limited to those with CTCs</td>
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<td>Low interreader variability reported</td>
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Circulating free DNA

- DNA fragments from apoptotic or necrotic cells that release DNA into the circulation
- Described by Mandel and Metais in 1948
- ctDNA relies on detection of somatic mutations not present in normal DNA
- Improvements in technology now allow better detection and amplification of ctDNA
- Better detected from plasma than serum

Diaz LA & Bartelli A. Liquid biopsies: genotyping circulating tumour DNA. JCO 2014; 32: 579-586
How is it measured?

• Quantitative PCR amplification methods
  – Requires primers specific for the detection of certain mutations
  – Lowest cost and ease of use
  – Limited sensitivity
• Digital PCR
  – Absolute quantification of allele of interest
  – Highest accuracy and sensitivity
  – Limited genomic loci
• Targeted deep sequencing and NGS
  – High-sensitivity
  – Broad range of genomic coverage
ctDNA Platforms

Commercial entities – over 60 in USA!
Comparison of ctDNA detection methods

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Commercial test</th>
<th>Analytical sensitivity</th>
<th>Analytical specificity</th>
<th>Cost ($USD)</th>
<th>TOT (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Next-generation sequencing</td>
<td>Guardant 360</td>
<td>&gt;85%</td>
<td>99.99%</td>
<td>$5600</td>
<td>14</td>
</tr>
<tr>
<td>Foundation ACT</td>
<td></td>
<td>&gt;95%</td>
<td>99%</td>
<td>$5800</td>
<td>14</td>
</tr>
<tr>
<td>Digital PCR</td>
<td>Biodesix</td>
<td>&gt;85%</td>
<td>100%</td>
<td>$1800</td>
<td>3</td>
</tr>
<tr>
<td>Quantitative PCR &amp; sanger</td>
<td>Biocept</td>
<td>97%</td>
<td>99.5%</td>
<td>$1900</td>
<td>7</td>
</tr>
<tr>
<td>Quantitative PCR &amp; NGS</td>
<td>Trovagene</td>
<td>93%</td>
<td>99%</td>
<td>$1500</td>
<td>14</td>
</tr>
</tbody>
</table>

From Johnson ML, ASCO 2016 education session: Biomarkers, Blood-based testing and the heterogenous tumour.
Tumour burden and prognosis

41 pts. Tumour DNA pre and post mastectomy
PCR based assessment of 6 microsatellite markers and TP53 mutations
Plasma DNA associated with vascular invasion, , >3LN+, high grade and disease-free survival

44% pre-op  --> 19.5% detected post-op

Pre-op  Post-op  Follow-up

70 pts, cfDNA, Invitrogen DNA DipStick Kit
100% pts had cfDNA 20x healthy donors  -->  decreased post-op  -->  stable if DF
  -->  increase with recurrence
### TABLE 1. Cell-free circulating DNA quantification by the Dipstick method in 20 healthy donors and 70 colorectal cancer patients

<table>
<thead>
<tr>
<th></th>
<th>Number of subjects</th>
<th>$t_0$: day at surgery</th>
<th>$t_1$: 4 months follow-up</th>
<th>$t_2$: 10 months follow-up disease-free patients</th>
<th>$t_2$: 10 months follow-up with recurrence patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy donors</td>
<td>20</td>
<td>10.3 ± 10.5 median: 5 range: 5–50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer patients</td>
<td>70</td>
<td>495.7 ± 357.6 median: 450 range: 100–1,750</td>
<td>170.6 ± 160.1 median: 110 range: 15–500</td>
<td>240.9 ± 276.8 median: 110 range: 5–1,000</td>
<td>136.2 ± 160.2 median: 62.5 range: 5–500</td>
</tr>
</tbody>
</table>

Results are expressed as ng DNA/mL plasma.
CTC vs cfDNA studies

- EGFR in NSCLC
- Maheswaran NEJM 2008
  - DNA extracted from CTCs (microchip) vs plasma DNA -> PCR for identification of EGFR mtns
  - 31 pts; all had CTCs – EGFR mtn in 92% CTCs and only 30% matched cfDNA
  - CTCs corresponded with tumor response, progression and new EGFR mts in some cases
- Punnoose CCR 2012
  - 41 pts; PCR preamplification and mtn detection by TaqMan genotyping assays for EGFR, KRAS, PIK3CA, BRAF, NRAS
  - CTCs in 78% pts; correlated with RECIST and FDG-PET response
  - Mutation sensitivity was greater in ctDNA (CTC DNA did not reliably detect EGFR mtn)
CRC ctDNA to predict response and resistance

- DNA extraction and BEAMing (bead emulsion, amplification and magnetics)
  - test relevant mutation from tumour in amplified plasma DNA via quantitative rtPCR using a mutation-specific probe
- Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy
Detection of circulating KRAS mutant DNA in a patient with acquired resistance to cetuximab therapy.

**a**, Size of liver metastasis (blue bars) and carcinoembryonic antigen (CEA) levels in blood (blue line) at the indicated time points, showing an initial response to cetuximab followed by progression (patient 8). PR, partial response; PD, progressive disease. **b**, Quantitative analysis of KRAS(Q61H) mutant DNA in plasma, as assessed by BEAMing. **c**, Two-dimensional dot plot showing quantitative analysis of the KRAS(Q61H) mutation in plasma using BEAMing at individual time points. **d**, Mutational analysis of KRAS on tumour samples collected before cetuximab treatment and at the time of disease progression.

NGS of cfDNA in NSCLC

- Oxnard et al. CCR 2014
- Droplet digital PCR; 77 pts
- Assays for EGFR and KRAS mtns in NSCLC using non-overlapping genotypes as positive and negative controls
  - Testing for false positives for EGFR mtn in a KRASmt population
- Identified a reference range for EGFR L858R and exon 19 deletions via testing in KRAS-mutant patients
- Serial plasma genotyping on pts on erlotinib -> pretreatment detection of EGFR mutations, plasma response and emergence of T790M mutations
Plasma genotyping using droplet digital (dd)PCR.

cfDNA is extracted from a plasma specimen and emulsified with oil into thousands of droplets, each containing approximately 0 to 1 molecules of target DNA. PCR is performed to endpoint in each droplet.

Figure 5. Plasma levels of mutant *EGFR* in 9 patients receiving first-line erlotinib until objective disease progression (PD) by RECIST. In all patients (A–I), plasma levels of the *EGFR* sensitizing mutation (solid line) drop in response to treatment, with 8 patients (B–I) having a complete plasma response. In 6 patients, plasma genotype levels reemerge up to 4 months before disease progression, and a lower concentration of T790M (dashed line) is also detected. In 3 patients (G–I), plasma genotype was not detected at the time of disease progression; all 3 had indolent progression in the chest only.
Prospective validation of EGFR mt testing

- Sacher and Oxnard JAMA Oncol 2016
- 180 pts either newly diagnosed or planned for rebiopsy at resistance to EGFRi
- ctDNA tested via ddPCR for EGFR exon 19 del, L858R, T790M and KRAS mutations
- Turnaround times for plasma ctDNA vs tissue is 3 (1-7) vs 12/27 (1-146) days
- High specificity – 100% for all mtns except T790M 79%
- Sensitivity 72-84% for EGFR mts and 64% for KRAS mtns

![Graphs and diagrams showing sensitivity and dynamic range of plasma genotyping](chart.png)
FDA approval of EGFR mtn test

- Cobas EGFR mutation test v2 for exon 19 deletion or exon 21 (L858R) substitution mutations
- Tumor biopsy recommended if not detected in the blood
Tracking mutations in breast, ovarian and lung cancers


- ctDNA used to track markers of resistance in breast, ovarian and lung cancers
- 6 pts followed over 1-2 yrs & sampled at intervals >3wks.
- 2pts had synchronous biopsies to confirm plasma DNA findings
- Exome sequencing on ctDNA performed when allele fraction of tumour mutations in plasma was high to improve sensitivity
- Mutant alleles detected in therapy resistance
  - PIK3CA mtn on paclitaxel
  - T790M mtn on gefitinib
  - RB1 mtn on cisplatin
  - MED1mtn on tamoxifen and trastuzumab
Breast cancer: ctDNA vs CTCs

- Dawson et al. NEJM 2013
- Compared ctDNA, CA15-3, CTCs and imaging in 30 women (from 52 recruited)
- Performed targeted or whole genome sequencing and designed personalized assays to quantify ctDNA
- ctDNA detected in 97%, Ca15-3 in 78% and CTCs in 87%
- ctDNA showed greater correlation with tumour burden and earliest measure of treatment response in 53%
- ctDNA provided the earliest measure of treatment response in 10 from 19 women
- Specific and sensitive marker of mBC
ctDNA levels, CTC numbers, CA 15.3 levels and radiological response for 4 patients.
Orange dashed line: 5 CTCs per 7.5ml blood
Green dashed line: CA 15.3 threshold of 32.4U/ml
Figure 2. Monitoring Multiple Point Mutations and Structural Variants in Circulating DNA.
Panels A, B, and C show plasma levels of circulating tumor DNA (ctDNA) for three patients (one per panel), quantified in parallel by means of a digital polymerase-chain-reaction (PCR) assay across multiple time points. In Panels B, C, and D, the use of endocrine or cytotoxic therapy is indicated by colored shading, and disease status at various times (as ascertained on computed tomography) is shown. Panel A shows three structural variants (deletions) and a point mutation in PIK3CA. The three deletions occurred in the setting of a complex rearrangement associated with amplification. Panel B shows six point mutations, all of which showed similar dynamic patterns. Panel C shows point mutations in PIK3CA and TP53; the TP53 mutation was dominant in the circulation as compared with the PIK3CA mutation. Panel D shows plasma levels of ctDNA for a fourth patient, with point mutations in PIK3CA and TP53 quantified by means of tagged-amplicon deep sequencing. The TP53 mutation was identified in plasma only, and levels remained elevated after paclitaxel chemotherapy despite a fall in the PIK3CA mutation level in the presence of stable disease. ND denotes not detected.
# CtDNA Pros and Cons

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td><strong>Ease</strong></td>
<td>Assay comparisons limited and problematic</td>
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<tr>
<td><strong>Less invasive than tumour biopsy</strong></td>
<td>Requires standardization and validation of laboratory and preanalytic conditions</td>
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<tr>
<td><strong>Does not rely on detection of intact CTCs</strong></td>
<td>Normal DNA from dying cells after blood collection may contaminate the specimen</td>
</tr>
<tr>
<td><strong>Capture tumour heterogeneity over time</strong></td>
<td>Does DNA from dying cells give the best information on viable therapy-resistant cancer cells</td>
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<tr>
<td><strong>Track selective pressures that lead to resistance</strong></td>
<td>Does ctDNA give make-up of multiple metastatic lesions</td>
</tr>
<tr>
<td><strong>New technologies have increased sensitivity for early stage disease and monitoring minimal residual disease</strong></td>
<td>Detection relies on DNA polymerase – sensitivity of 0.01% -&gt; impact on therapeutic decisions for occult/minimal residual disease</td>
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<tr>
<td><strong>Early detection</strong></td>
<td>Significance of mutations</td>
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**Cons**: Assay comparisons limited and problematic. Requires standardization and validation of laboratory and preanalytic conditions. Normal DNA from dying cells after blood collection may contaminate the specimen. Does DNA from dying cells give the best information on viable therapy-resistant cancer cells? Does ctDNA give make-up of multiple metastatic lesions? Detection relies on DNA polymerase – sensitivity of 0.01% -> impact on therapeutic decisions for occult/minimal residual disease. Significance of mutations.
<table>
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<th>Study</th>
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| Efficacy of palbociclib plus fulvestrant (P+F) in patients (pts) with metastatic breast cancer (MBC) and ESR1 mutations (mus) in circulating tumor DNA (ctDNA).  
Nicholas C. Turner, Royal Marsden Hospital and Institute of Cancer Research |
| Phase (Ph) 1 study of oral seviteronel (VT-464), a dual CYP17-Lyase (L) inhibitor and androgen receptor (AR) antagonist, in patients (pts) with advanced AR+ triple negative (TNBC) or estrogen receptor (ER)+ breast cancer (BC).  
Aditya Bardia, Massachusetts General Hospital Cancer Center, Boston, MA |
| Circulating tumor DNA mutational profiling by targeted next generation sequencing guides personalized treatments in multiple cancer types.  
Yang Shao, University of Toronto, Toronto, ON, Canada |
| Genotype concordance between archival tumor DNA (atDNA) and circulating tumor DNA (ctDNA) in advanced solid malignancies: The Oncopanel Pilot study.  
Hagen F. Kennecke, BC Cancer Agency, Vancouver, BC, Canada |
| Characterization of cell-free circulating tumor DNA in patients with brain metastases.  
Nadia Faiq, UC San Diego Moores Cancer Center, San Diego, CA |
| And many others |
References

- CTCs
  - Allard et al CCR 2004
  - Breast Cancer CTCs
    • Cristofanilli et al. NEJM 2004
    • Budd et al. CCR 2006
    • Rack et al. JNCI 2014
  - Prostate cancer CTCs
    • Shaffer et al, CCR 2007
    • De Bono, CCR 2008
    • Scher et al. JCO 2015.
  - Colorectal Cancer CTCs
    • Cohen et al. JCO 2008

- cfDNA/ctDNA
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    • Silva et al. Ann Surg Oncol 2002
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    • Gevensleben et al CCR 2013.
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  - NSCLC
    • Maheswaran NEJM 2008
    • Punnoose CCR 2012
    • Oxnard et al. CCR 2014