**Liquid biopsy to test new treatment strategies in breast cancer: are we there yet?**

Breast cancer is the most common cancer in women in Europe [1]. Recommendations for systemic adjuvant therapy are currently based on the analysis of primary tumor characteristics [2]. For example, the administration of trastuzumab in women with breast cancer relies on the analysis of HER2 protein overexpression or HER2 gene amplification in the primary tumor [3]. However, the target of adjuvant systemic therapy is thought to be the minimal residual disease (MRD), which is present after primary surgery but undetectable by currently used conventional imaging approaches [4]. Recent technological advances have enabled the detection and characterization of bone marrow disseminated tumor cells (DTCs) and peripheral blood circulating tumor cells (CTCs), which are thought to be surrogate markers of MRD.

Clinical trials studying the effect of different treatments on DTCs or CTCs have been recently reported. In two studies, the effect of a bisphosphonate, zoledronic acid, on bone marrow DTCs, was examined [5, 6]. The investigators used novel end points such as the difference in the detection rate of DTCs after a predefined treatment period between a control arm of (neo) adjuvant chemotherapy and an experimental arm of the same chemotherapy plus zoledronic acid. Interestingly, both studies showed that the detection rate of DTCs was lower after treatment with zoledronic acid, supporting the hypothesis that zoledronic acid antitumor efficacy might partly depend on DTC elimination. Both clinicians and patients more easily accept a blood draw than a bone marrow aspiration. Therefore, CTCs could potentially be used as a liquid biopsy to assess new treatment strategies.

This innovative approach was used in the study by Georgoulias et al. [7], in which the decision about secondary adjuvant treatment was based on the results of a liquid biopsy rather than primary tumor analysis. The authors should receive credit for initiating such a study in 2003. In the trial, they used a RT-PCR to detect cytokeratin (CK) 19 messenger RNA (mRNA) in blood samples from patients with early breast cancer who were receiving adjuvant chemotherapy [7]. They screened 378 patients to identify 100 (26%) with detectable CK19 mRNA both before and after they received chemotherapy. Only patients whose blood was CK19 mRNA positive at both timepoints were randomized between six cycles of trastuzumab versus observation.

The authors reported the analysis for patients with HER2-negative primary tumors (36 randomized to trastuzumab and 39 to observation). Of the women who received trastuzumab, 27 (75%) tested CK19 mRNA negative, compared with only 7 (18%) in the observation arm. At a median follow-up of 67.2 months, patients on the observation arm had an almost four times higher risk of relapse than the patients who had received treatment [7]. The authors also carried out double immunofluorescence staining for CK and HER2 in peripheral blood mononuclear cells (PBMCs) cytopsin from a subset of the patients showing CK19 mRNA positivity. They found that 51 (89%) of those 57 patients had CK-positive/HER2-positive cells, suggesting that the trastuzumab benefit was associated with their presence.

Although the results of this trial are intriguing, it should be noted that only 75 patients were randomized, and independent validation is needed. Moreover, there are concerns about the methodology used for CTC detection. No independent laboratories have validated the sensitivity and specificity of the method for CTC detection in early breast cancer using the same procedure to identify CK19 mRNA in peripheral blood. Another group of investigators have shown that CK19 mRNA can be expressed in normal PBMCs, particularly in the lymphocyte population. By using immunomagnetic enrichment for epithelial cells, these investigators were able to reduce the level of background signals to <5% in the PBMCs of healthy donors [8]. However, in the study by Georgoulias et al., no such immunomagnetic enrichment for epithelial cells was carried out. Instead, CK19 mRNA was detected by RT-PCR in RNA extracted from PBMCs, hence raising concerns about the specificity of this method for CTC detection.

The only technology that has received Food and Drug Administration approval for CTC detection as an aid in monitoring women with metastatic breast cancer is CellSearch® (Veridex, Warren, NJ) [9]. This is a semi-automated system that first uses immunomagnetic separation to isolate epithelial cell adhesion molecule (EpCAM)-positive cells from whole blood followed by an immunofluorescent staining of captured cells with antibodies specific for CKs 8, 18, 19 (pan-CK) and CD45 (specific for leucocytes). The same cells are also stained with 4′,6-diamidino-2-phenylindole-2 (DAPI; to confirm the presence of a cell nucleus). A CTC is defined as an EpCAM-positive cell staining for pan-CK and DAPI but not for CD45. Two independent studies have validated the analytical performance of CellSearch® for clinical use in metastatic breast cancer [10, 11]. Data on the role of CTC detection and characterization using CellSearch® in nonmetastatic cancer are only recently emerging [12–15]. An international collaborative study involving 30 readers from 15 academic and 2 industry labs is currently being carried out to harmonize CTC...
enumeration and HER2 characterization on CTCs using CellSearch® technology in nonmetastatic breast cancer [16]. This crucial step must be taken before using the technology to test new treatment strategies in the nonmetastatic setting.

Despite the concerns about the method for CTC detection used, the intriguing results of the Georgoulias et al. trial [7] concur with those from subset analyses of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 and North Central Cancer Treatment Group (NCCTG) N9831 trials, suggesting that benefit from adjuvant trastuzumab may not be confined to patients with tumors identified as immunohistochemistry (IHC) 3-positive or FISH-positive [17, 18]. Several hypotheses may explain these results. Recent data have reinforced the idea that the therapeutic effect of anti-HER2 antibody therapy depends on immune-related mechanisms [19]. Therefore, one could assume that trastuzumab might also work through the eradication of MRD using immune-related mechanisms. The detection of CTCs is a surrogate for MRD, so their presence could be associated with benefit from trastuzumab and thus explain the results of this study [7]. Another hypothesis concerns the heterogeneity of tumor cell populations with respect to HER2 amplification. Specifically, one cannot exclude the possibility that rare undetectable subpopulations of HER2-amplified tumor cells in the primary tumor drive the benefit from trastuzumab. Finally, it may also be hypothesized that lower levels of HER2 expression are sufficient for benefit from trastuzumab.

Currently, the NSABP B47 (NCT01275677) phase III trial aims to randomize 3260 women with HER2 non-amplified breast cancer (HER2 1+ and HER2 2+ by IHC and FISH/CISH-negative) to standard chemotherapy or chemotherapy plus trastuzumab, the primary end point being invasive disease-free survival. A CTC detection substudy will be carried out within the trial.

Similarly, the European Organization for Research and Treatment of Cancer is planning to launch the 'Treat CTC' trial. In this study, women with HER2 non-amplified primary breast cancer (HER2 0, 1+ and HER2 2+ by IHC and FISH/CISH-negative) who, after completing (neo)adjuvant chemotherapy and surgery, have detectable CTCs in peripheral blood using CellSearch® will be randomized in a 1:1 ratio to either six cycles of trastuzumab or to observation. The primary end point will compare the two arms for CTC detection, whereas the secondary end point will analyze the recurrence-free interval. Different laboratories across Europe that use CellSearch® will be responsible for screening women for CTCs. Prior to randomization, a central review will be carried out on both the HER2 status of the primary tumor and the Cellsearch® CTC images. Whereas the NSABP B47 trial will investigate whether there is any role for trastuzumab in HER2 non-amplified breast cancer, the phase II 'Treat CTC' trial is a proof of concept study to investigate whether currently available CTC detection technology can be used for patient selection to address the same question.

As technologies become more rapidly available for the detection and molecular characterization of CTCs—even at the single-cell level [4]—it is critical that they undergo appropriate analytical validation before moving into clinical testing and thereafter continuous quality control. In the future, more studies are expected to test the clinical utility of using CTCs as a liquid biopsy to monitor tumor genotypes and to contribute to personalized treatment strategies in breast cancer.

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references
X-ACT: An important step on an unfinished journey

The adjuvant chemotherapy treatment of stage III colorectal cancer has saved thousands of lives over the last 20 years since the first evidence of the benefit of 5FU and levamisole was reported [1]. The benefit from 5FU based regimens is risk related. Collated data, analysed by the ACCENT investigators from eight years of follow up for 20,898 patients in 18 randomised trials of 5FU based chemotherapy versus control, showed that the absolute improvement in overall survival was 10% for patients with stage III colorectal cancer [2]. Since those initial studies and the refinement of them to the standard of care in the mid to late 1990s of 6 months therapy with bolus 5FU and leucovorin (5FU/LV) regimens, there have been only two practice changing sets of evidence in this field of practice. The first was the introduction of an oral alternative to the bolus 5FU/LV regimens, using either capecitabine or Uftoral [3,4] and the second the introduction of oxaliplatin [5-7].

In a recent edition of Annals the long term outcomes of the X-ACT trial were reported, which confirm the non-inferiority of the oral 5FU prodrug, capecitabine, in comparison with one of the previous international standards, the Mayo clinic bolus 5FU/LV regimen in the adjuvant treatment of stage III colorectal cancer [8]. The X-ACT trial recruited about 2000 patients from many countries internationally, between 1998 and 2001 and has now been extensively reported. The characteristics of the patients in the study were well balanced, with if anything slightly worse prognostic factors in the capecitabine group, though with no statistically significant differences. Elevated CEA was present in 8.6% of the capecitabine group compared to 7% of control; N2 disease (four or more involved nodes) was present in 30.8% versus 29.4% respectively.

The primary endpoint of the X-ACT trial was 3 year disease free survival (DFS) and the statistical plan required 632 events to exclude a non-inferiority margin of 1.25 with a type 1 error of 2.5%. At the time of original publication, 728 DFS events had occurred and 427 patients had died and the data confirmed the primary hypothesis of non-inferiority of capecitabine compared to 5FU/LV [3]. The selection of such a wide non-inferiority margin is worth consideration. If the upper limit of the 95% confidence interval of the hazard ratio had been 1.24, the primary endpoint of the trial would have been met. However there would have been a chance that the outcome with capecitabine was inferior to 5FU/LV by 24%, sacrificing nearly a quarter of the benefit of adjuvant therapy. Had the results turned out that way there is a question as to whether clinical practice would have changed so significantly on the basis of this trial. In fact, the upper limit of the confidence interval of the hazard ratio was 1.00 (HR 0.87 (0.75–1.00) p < 0.001) and the question is rather whether capecitabine may be superior to 5FU/LV rather than simply non-inferior.

In the long term follow up reported now, the database was closed in June 2007 with 6.9 years median follow up at which time 670 patients had died, 319 patients (32%) in the capecitabine arm and 351 patients (36%) in the 5-FU/FA arm. It is remarkable that it has taken nearly 5 years from database closure to see the results published. For this overall survival analysis, a non-inferiority margin of 1.14 was predefined. The results confirm the findings of the 3 year DFS analysis, in that the hazard ratio for overall survival for capecitabine versus 5-FU/LV was 0.86 (95% CI, 0.74–1.01); the upper limit of the 95% CI was significantly less than the predefined non-inferiority margin of 1.14 (p < 0.001). This trial therefore adds to the growing confidence in 3 year disease free survival as a good surrogate for 5 year overall survival for adjuvant chemotherapy trials in colorectal cancer [9].

So is capecitabine superior to 5FU/LV? From a pure statistical perspective, the answer has to be No on the basis of this analysis. The test for superiority showed only a trend (p = 0.06). However the point estimate of the hazard ratio was 0.86, indicative of a useful effect of a 14% reduction in risk of dying with capecitabine rather than 5FU/LV. The validity of switching the objective of a comparison from a non-inferiority trial to a superiority trial has been discussed by the Committee for Proprietary Medical Products of the European Agency for the Evaluation of Medicinal products (EMEA) [10]. They conclude it is feasible provided the trial has been properly designed and carried out in accordance with the strict requirements of a non-inferiority trial. Further actual p-values...