

ORIGINAL ARTICLE

Blood tumor load: combining biomarkers to increase the proportion of informative patients

E. Dathathri¹, A. Nanou¹, F.-C. Bidard^{2,3,4}, S. Renault^{2,3,4}, J.-Y. Pierga^{2,3,5}, J. de Bono⁶, L. Terstappen^{1,7,8} & F. A. W. Coumans^{8*}

¹Department of Medical Cell Biophysics, TechMed Center, Faculty of Science and Technology, University of Twente, Enschede, The Netherlands; ²Department of Medical Oncology, Institut Curie, PSL Research University, Paris; ³Circulating Tumor Biomarkers Laboratory, Siric, Institut Curie, PSL Research University, Paris; ⁴UVSQ, Paris-Saclay University, Paris; ⁵Université Paris Descartes, Paris, France; ⁶The Institute of Cancer Research and Royal Marsden Hospital, London, UK; ⁷Department of General, Visceral and Pediatric Surgery, Heinrich-Heine University, University Hospital Düsseldorf, Düsseldorf, Germany; ⁸Decisive Science, Amsterdam, The Netherlands



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Background: The load of circulating tumor cells (CTC) and tumor-derived extracellular vesicles (tdEV) strongly correlates with poor clinical outcomes and can be used to evaluate treatment response and the presence of treatment targets. However, the frequency of CTC is low, making an accurate assessment impossible in most patients. Here, we introduce blood tumor load (BTL), in which CTC and tdEV are combined into one value ranging from 0 (low) to 1 (high) to simplify result interpretation and increase the percentage of patients from which a reliable assessment can be made.

Patients and methods: The CTC and tdEV counts were obtained from the ACCEPT analysis of the CellSearch image datasets of 98 metastatic breast cancer patients (mBC) and 157 castration-resistant prostate cancer patients (CRPC). The BTL generated using these counts was used in human epidermal growth factor receptor 2 (HER2) expression assessment in mBC patients. The BTL scores of CRPC patients at baseline and first follow-up time points were evaluated, and a change in BTL, indicating response to therapy, was measured in the patients.

Results: Using 10 CTCs as a threshold, the HER2 positivity could be determined in 34/98 (35%) breast cancer patients, whereas with BTL, the positivity increased to 76/98 (78%). The BTL showed an improved Cox hazard ratio for overall survival in 157 CRPC patients before and at first follow-up points compared with CTC and tdEV alone. A decrease in BTL indicating response to therapy was seen in 45% of CRPC patients, and an increase in BTL was seen in 9%, indicating progression on treatment. The remaining 46% of patients showed no change.

Conclusions: In this study, we demonstrated the applications of BTL in improving the reliability of measuring response to therapy and increasing the proportion of patients from which the presence of a treatment target can be assessed.

Key words: circulating tumor cells, tumor-derived extracellular vesicles, human epidermal growth factor receptor 2 (HER2), image analysis, ACCEPT, blood tumor load (BTL)

INTRODUCTION

The number of biomarkers aiding in the diagnosis, monitoring of treatment efficacy, and choice of treatment in cancer patients is rapidly increasing. Biomarkers present in blood and frequently referred to as ‘liquid biopsies’ are composed of tumor cells traveling through the blood [circulating tumor cells (CTC)], tumor-derived extracellular vesicles (tdEV), circulating tumor DNA, circulating tumor-related micro RNA, and proteins associated with the

presence of cancer.¹⁻³ These biomarkers can improve the ability of the treating physician to make treatment choices. However, the selection of the most optimal predictive and prognostic biomarker is challenging due to the plethora of available biomarkers, each with its advantages and disadvantages. There is an urgent need to tailor therapies to improve patient survival during the course of the disease.^{4,5}

In this study, we propose a method to combine multiple biomarkers into a single biomarker such that the strength of the combined biomarker is amplified over the single biomarkers. We evaluated these combined biomarkers into blood tumor load (BTL) and first explored its feasibility by combining CTC and tdEV for the assessment of the presence of treatment target HER2 in metastatic breast cancer (mBC) and the assessment of prognosis in patients treated for castration-resistant prostate cancer (CRPC).

*Correspondence to: Dr Frank A. W. Coumans, Decisive Science, Ertskade 10, 1019BB Amsterdam, The Netherlands. Tel: +31-(6)-44411518
E-mail: frank@decisivescience.com (F. A. W. Coumans).

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Equations 3 and 4 with I_C of 20, 200, and 500 for HER2+, ++, and +++, respectively.

$$\text{Fraction HER2+}_{CTC} = \frac{\sum_{i=1}^N \mathbf{1}(CTC_{i,HER2} \text{ Intensity} > I_C)}{N_{CTC}} \quad (3)$$

$$\begin{aligned} \text{Fraction HER2+} \\ &= \frac{\text{Fraction HER2+}_{CTC} + \beta \text{ Fraction HER2+}_{tdEV}}{1 + \beta}, \text{ with} \\ \beta &= \frac{25}{(CTC + 1)^2} \end{aligned} \quad (4)$$

Factor β expresses the assumption that the HER2 expression on CTC is more reliable than tdEV when the CTC count is high and less reliable when the CTC count is low. For 1 CTC, the HER2 expression is composed of 14% CTC and 86% tdEV. For 4 CTC, the contribution of CTC and tdEV are balanced. For 9 CTC, the HER2 expression is 75% CTC and 25% tdEV.

Statistical analysis

Statistical analysis was carried out in Python 3.9 with the lifelines 0.27.4 package¹⁴ and SPSS (v24.0, SPSS Inc., Chicago, IL). Graphs were generated in OriginPro (v2021b, OriginLab Corporation, Northampton, MA). For correlation and regression, the CTC and tdEV counts were log-transformed with base 10 to mitigate considerable right skew in the distribution. Additionally, 1 was added to all CTC and tdEV counts because of samples with 0 CTC and or tdEV, and the log of 0 is undefined. We computed Pearson's correlation and reported the R^2 value. Cox proportional hazard regression was applied to obtain univariate hazard ratios (HRs) for overall survival, including the 95%

confidence intervals (CIs) with the $\log_{10}(CTC + 1)$, $\log_{10}(tdEV + 1)$, or BTL (Equation 2) as the covariate. Each covariate was tested at baseline and the first follow-up (2-5 weeks). HRs were scaled to the interquartile range to account for differences in the scale of the covariates. Additionally, we tested the change in each covariate between baseline and follow-up. To test whether a change in BTL was significant, we carried out a one-sided paired *t*-test.

RESULTS

Blood Tumor Load using ACCEPT enumerated CTC and tdEV

mBC samples ($n = 98$) were reanalyzed with the ACCEPT software by the application of linear gates to the detected events for classifying CTC and tdEV. The number of ACCEPT tdEV and CTC in a sample was used to compute the BTL (Equation 2). Figure 1A represents the image dataset for the mBC patients, which are processed with ACCEPT to identify CTC and tdEV from features extracted for each event in the image dataset. The distribution of two of the features is shown in Figure 1B and C.

Assessment of HER2 expression on CTC and tdEV

For the ACCEPT-selected CTC and tdEV, we determined the fraction that was at least weakly positive (+), positive (++), or strongly positive (+++) for HER2-FITC in the mBC patients ($n = 98$). Weakly positive (+) was defined as a CTC with a FITC fluorescence signal greater than the background intensity plus 10. The background intensity was defined as the 90-percentile fluorescence intensity of the leukocytes present in the same CellSearch cartridge. For most cartridges, this is equivalent to the 99-percentile on the leukocytes, but for a few cartridges, false positive classifications of White blood cells led to an abnormally high background level at the 99-percentile. Median positive (++) was set at the fluorescence intensity above which 30% of all CTC detected in the 98-breast cancer patients expressed HER2.

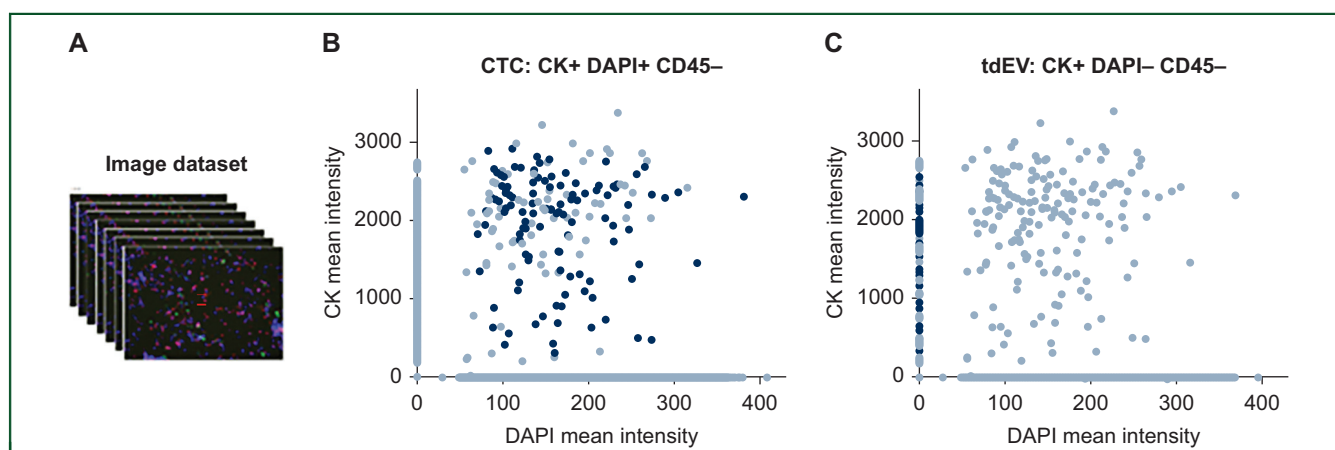


Figure 1. Processing of an image dataset. (A) A stack of multichannel immunofluorescence images representing a single CellSearch cartridge. (B) Scatter plot showing two parameters of the ACCEPT circulating tumor cells (CTC) gate (Table 1) for all events. Blue dots show events that meet all criteria and are counted as CTC. Dots in gray show events that fail at least one of the criteria. For example, an event is not counted as CTC because it expresses CD45. (C) Scatter plot showing two parameters of the ACCEPT tumor-derived extracellular vesicles (tdEV) gate (Table 1). Blue dots show events that meet all criteria and are counted as tdEV. The gray dots fail at least one of the criteria. For example, the area of an event could be too large. CK, cytokeratin; DAPI, 4',6-diamidino-2-phenylindole.

Strongly positive (+++) was set at the fluorescence intensity at which 10% of all CTC expressed HER2.

The percentage of patients who had HER2-CTC was 73.5%, 44.9%, and 9.2% at the +, ++, and +++ expression thresholds, respectively. The percentage of patients who had HER2-tdEV was 73.5%, 42.8%, and 14.3% using the same thresholds. Figure 2A and B illustrates the HER2-FITC expression versus CK-PE expression for CTC and tdEV in a single sample to show the range of expression levels. Figure 2C and D show some examples of HER2 expressing CTC and tdEV.

HER2 BTL assessment

The ACCEPT CTC and tdEV counts have a high positive correlation ($R^2 = 0.77$), see Figure 3A. The fraction of CTC and tdEV expressing HER2 in patients with CTC >10 are plotted in Figure 3B, showing a correlation $R^2 = 0.61$. The ACCEPT and manually identified CTC also correlated well

($R^2 = 0.72$), as shown in Supplementary Figure S1A, available at <https://doi.org/10.1016/j.esmooop.2025.105302>.

The fraction of CTC and tdEV expressing HER2 in patients with at least one CTC correlate poorly ($R^2 = 0.19$). When patients with only high CTC counts (>10 CTC) were considered, the correlation improved ($R^2 = 0.61$). In this study, 11% of patients had zero CTC and no HER2 assessment was possible, whereas 54% had 1-10 CTC and 35% had >10 CTC. To improve the reliability of the HER2 assessment, Equations 3 and 4 were applied to the CTC and tdEV per patient sample.

In Figure 4A, the BTL of the 98 patients ranging from 0 to 1 is indicated, where black dots are samples with CTC + tdEV >10 (78%), blue dots have CTC + tdEV count of 1-10 (21%), and purple diamonds have CTC + tdEV = 0 (1%). The fraction of HER2 and their division into HER2+, HER2++ and HER2+++ sorted according to the HER2 fraction for each of the 98 patients is indicated in the Figure. In Figure 4B, the fraction of HER2+ CTC is shown,

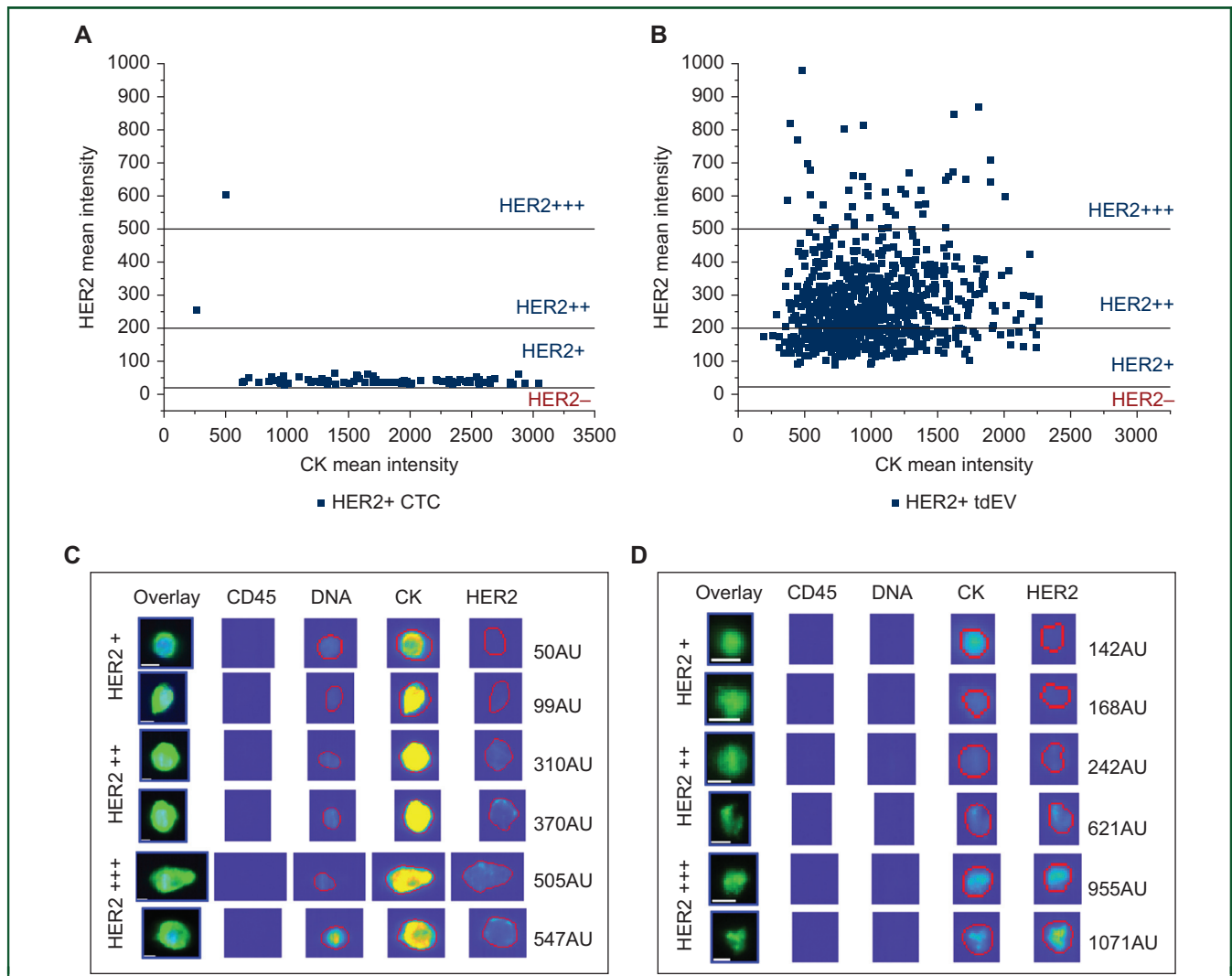


Figure 2. Example of quantification of HER2 expression on circulating tumor cells (CTC) and tumor-derived extracellular vesicles (tdEV) in a single patient sample. The scatter plot of CK versus HER2 expression of CTC (A) and tdEV (B). The thresholds for weakly positive (+), positive (++) or strongly positive (+++) are indicated. Examples of CTC (C) and tdEV (D) that are HER2+, ++, or +++, and their intensity values in arbitrary units (AU). The overlay is a false color image with blue for DAPI, green for CK-PE and red for CD45-APC. The scale bar indicates 6.4 μm. Red contours show the ACCEPT segmentations in each channel.

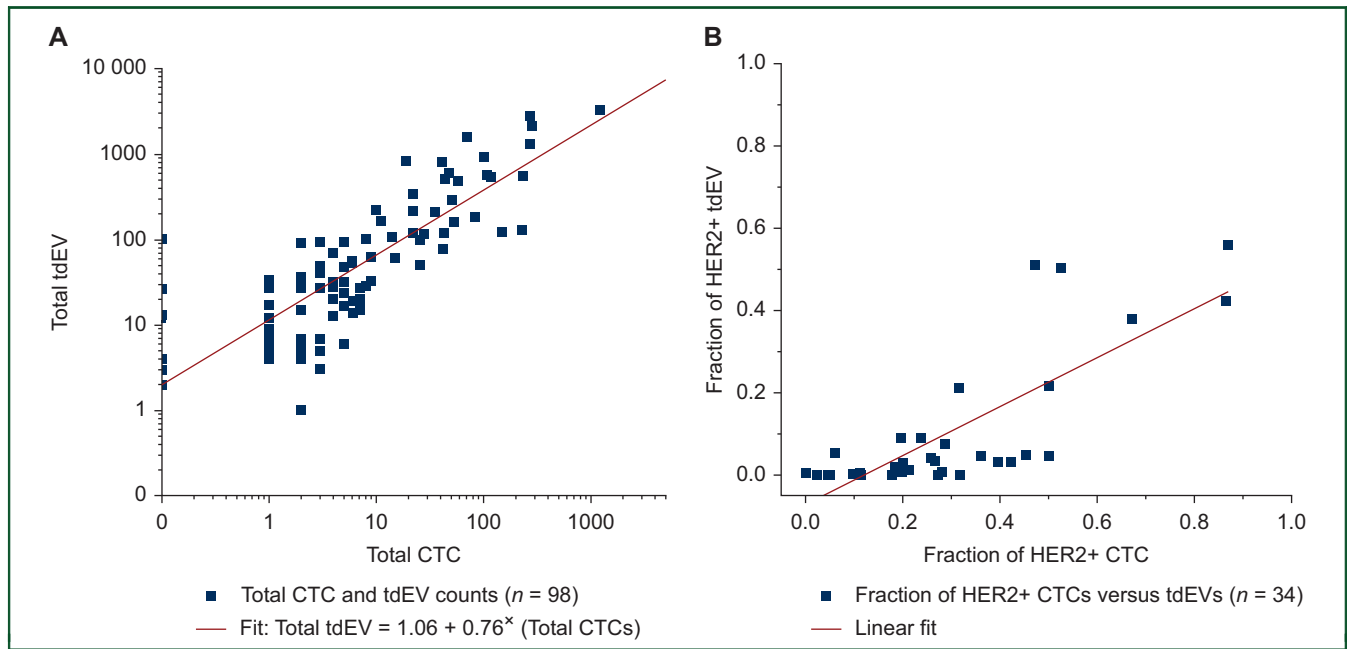


Figure 3. Circulating tumor cells (CTC) and tumor-derived extracellular vesicles (tdEV) correlation. (A) CK+ CTCs and corresponding CK+ tdEV of mBC patients ($n = 98$) show a high correlation ($R^2 = 0.77$) (B) Fraction of CTC and tdEV expressing HER2 in patients with CTC >10 ($n = 34$), showing correlation $R^2 = 0.61$. CK, cytokeratin; mBC, metastatic breast cancer.

where black dots are samples with >10 CTC (35%), blue dots have 1-10 CTC (54%), and purple diamonds have 0 CTC (11%). For the purple diamond samples (no CTC + tdEV/no CTC), no HER2 assessment could be made since that assessment requires the identification of cellular material of tumor origin.

Using a threshold of 10 CTC, HER2 positivity (on CTC) could be reliably determined in 34/98 (35%) breast cancer patients as shown in Figure 4B. Patients with a CTC count of

0 ($n = 11$) could not be analyzed for their HER2 expression, while the remaining patients with CTC count from 1 to 10 ($n = 53$) may have a less reliable HER2 expression due to low CTC frequencies. After the application of BTL, using a threshold based on CTC + tdEV count >10, the assessment of HER2 positivity was increased to 76/98 (78%) of patients (Figure 4A). Importantly, the BTL indicated no correlation with the fraction HER2+ (%) as patients with both low and

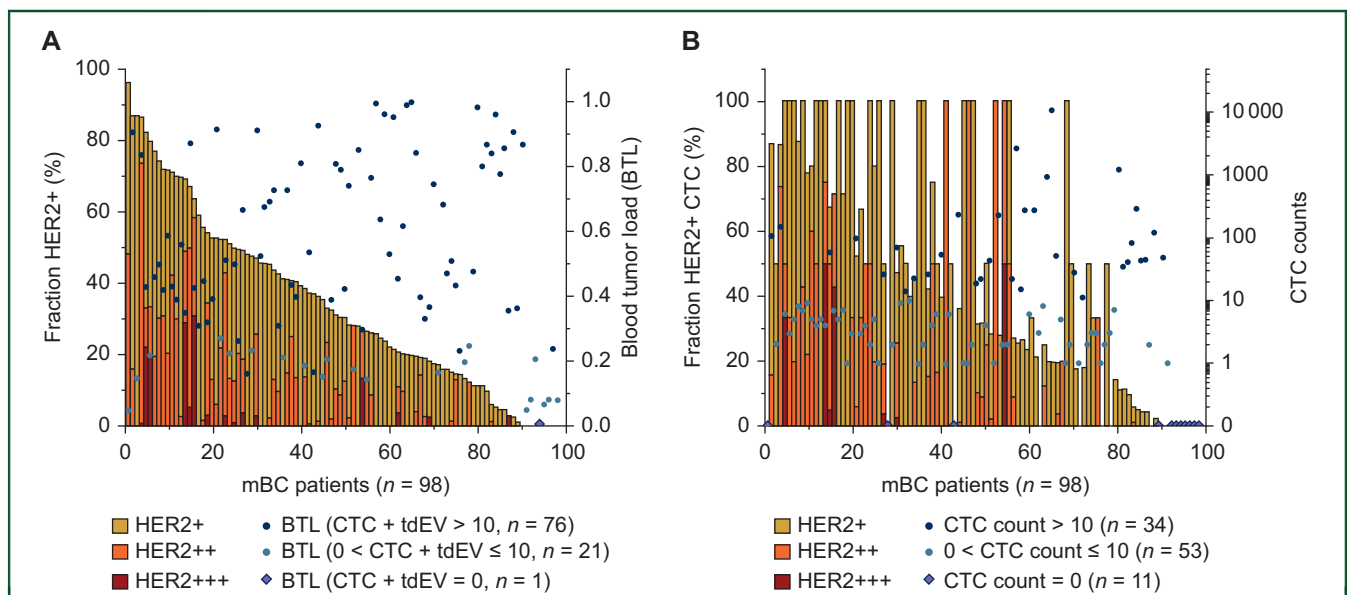


Figure 4. HER2 expression in 98 metastatic breast cancer (mBC) patients. (A) Bars show the Fraction HER2+ expression sorted by decreasing blood tumor load (BTL) HER2+ fraction. The fraction HER2+ is indicated in yellow, fraction HER2++ in orange, and fraction HER2+++ in red per patient sample. Markers show the BTL values corresponding to each HER2 assessment (secondary vertical axis) and differentiate between a BTL based on a circulating tumor cells (CTC) + tumor-derived extracellular vesicles (tdEV) count >10 (black dots, $n = 76$), 1-10 (blue dots, $n = 21$), and 0 (purple diamond, $n = 1$). (B) Bars show the fraction of HER2+ CTC sorted by decreasing BTL HER2+ fraction. Markers show the CTC counts corresponding to each HER2 assessment (secondary vertical axis) and differentiate between CTC of >10 (black dots, $n = 23$), 1-10 (blue dots, $n = 64$), and 0 (purple diamonds, $n = 11$).

high BTL showed varying expression levels (+, ++, +++) and fractions HER2+ (%).

Association of CTC, tdEV, and BTL with Overall Survival in CRPC patients

CTC and tdEV counts from blood samples of CRPC patients ($n = 157$) were obtained from the CellSearch images at baseline and first follow-up, with the first follow-up at 2-5 weeks after the initiation of chemotherapy (mean 3.8 weeks). Figure 5A shows the correlation of the CTC and tdEV counts at baseline (black squares, $R^2 = 0.73$) and follow-up (red dots, $R^2 = 0.75$). The ACCEPT and manually identified CTCs correlated well with $R^2 = 0.77$ and 0.58 for baseline and follow-up timepoints respectively (Supplementary Figure S1B, available at <https://doi.org/10.1016/j.esmooop>.

2025.105302). The BTL was computed from the CTC and tdEV counts for each sample and is shown at baseline and first follow-up in Figure 5B. BTL at baseline was between 0.03 and 0.99 with a median of 0.58 and BTL at follow-up was between 0.00 and 0.97 with a median of 0.45 indicating an overall decrease in tumor load following treatment.

We carried out univariate Cox regression with CTC, tdEV, or BTL as a continuous variable to predict overall survival. Figure 5C shows the continuous HR, for the overall survival of the patients ($n = 157$). HR 1.86, 95% CI 1.54-2.26 for CTC at baseline, HR 2.59, 95% CI 1.96-3.42 for tdEV at baseline and HR 3.37, 95% CI 2.33-4.88 for BTL at baseline. The Cox regression at follow-up similarly shows that elevated CTC, tdEV and BTL levels are associated with decreased survival, as shown with HR (95% CI) of 2.12 (1.69-2.64) for CTC, 2.62

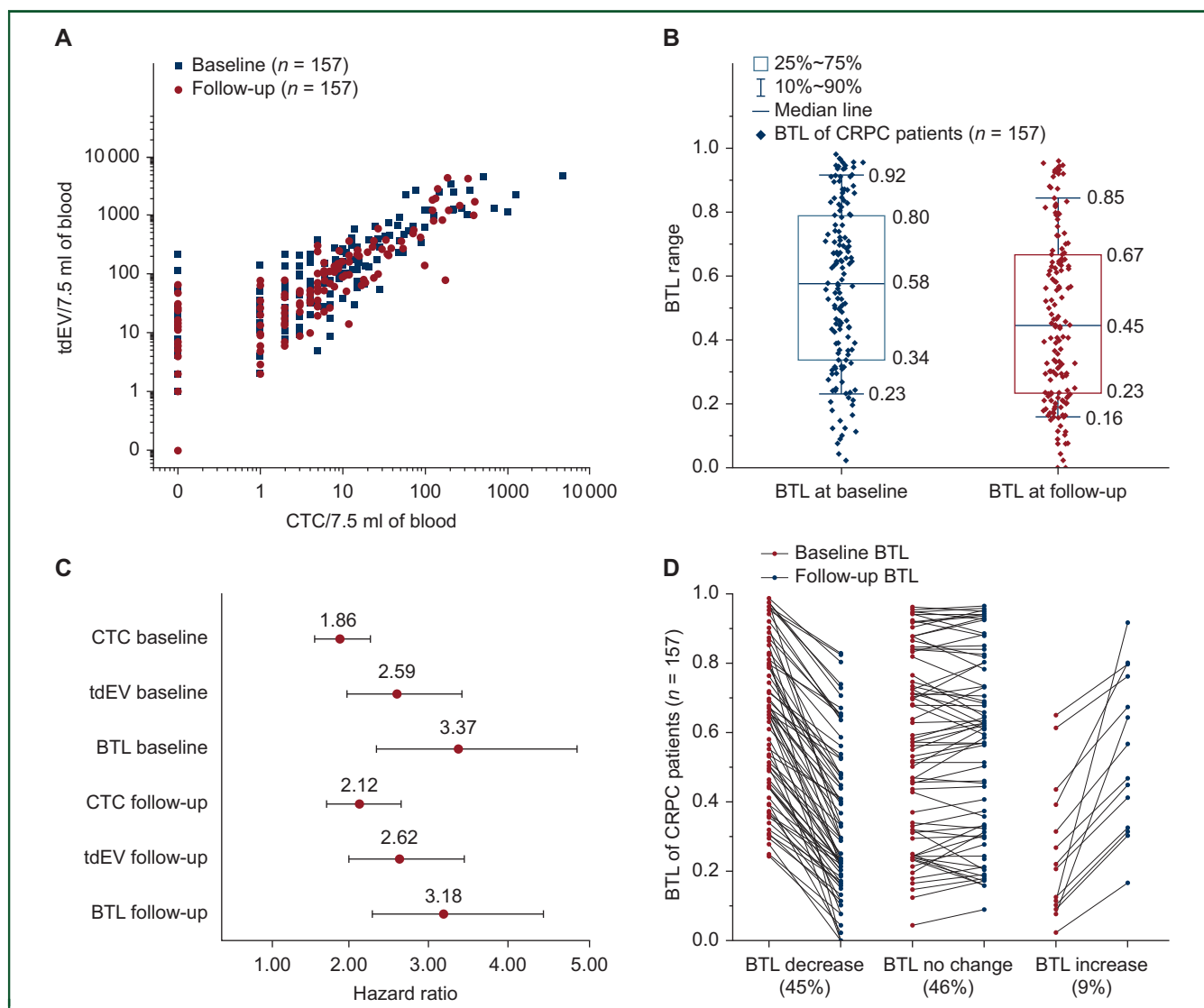


Figure 5. Blood tumor load (BTL) in 157 castration-resistant prostate cancer (CRPC) patients. (A) circulating tumor cells (CTC) and tumor-derived extracellular vesicles (tdEV) counts before treatment ($R^2 = 0.73$) and at first follow-up ($R^2 = 0.75$). (B) Box plot of BTL values at baseline and follow-up. The box shows the 25-75 percentile, the mid-line shows the median, and the whiskers represent the 10-90 percentile. The BTL at follow-up was significantly lower as compared with the baseline BTL ($P < 0.001$). (C) Cox hazard ratio for overall survival as a function of the log-transformed CTC and tdEV, and BTL at baseline and first follow-up. Hazard ratios were scaled to the interquartile range. (D) BTL response of the 157 CRPC patients, 45% showed a decrease in BTL, 46% an insignificant change and 9% showed an increase in BTL.

(1.99-3.45) for tdEV, and 3.18 (2.27-4.45) for BTL, respectively.

A change in CTC number could be informative on the effectiveness of therapy. However, the number of CTC is frequently low (1-10) inhibiting observation of a significant change. As the BTL is based on a larger number than CTC, the number of samples for which a significant change in BTL may be observed becomes larger. Using a shot noise model, we simulated the required change for CTC and BTL to reach 90% significance, see [Supplementary Information](#) and [Figure S2](#), available at <https://doi.org/10.1016/j.esmoop.2025.105302>. From their CTC at baseline, a significant reduction in level was possible for 110 of 157 patients and observed for 37 patients. From their BTL at baseline, a significant reduction in level was possible for 151 of 157 patients and observed for 71 patients. To assess whether the therapy led to a durable reduction of CTC/BTL, longer-term follow-up would be needed. A significant decrease in BTL after treatment was defined as a decrease less than -0.125 (i.e. $\Delta BTL < -0.125$), an insignificant change in BTL as a change between ≥ -0.125 and ≤ 0.125 , and a significant increase in BTL as a significant increase in BTL as an increase greater than 0.125 (i.e. $\Delta BTL > 0.125$). [Figure 5D](#) indicates that in 45% of patients a decrease in BTL was observed, in 46% of patients an insignificant or no change was observed and in 9% of patients, an increase in BTL was observed. Although a decrease in BTL indicates a response to therapy the objective of therapy should be a BTL of 0, so the ΔBTL can be large but when the BTL value does not reach zero, further improvement in therapy may be warranted.

DISCUSSION

Rapid advancements in the diagnosis, monitoring and treatment of cancer patients call for efficient biomarkers that can determine response to therapy accurately. The spread of cancer through metastasis is the major cause of death in cancer patients. As metastases are initiated by CTC, they play an essential biological role in cancer progression. It is not surprising that the presence and load of CTC are strongly associated with poor survival. Although the role of tdEV is not yet fully understood,¹⁵⁻¹⁷ their presence and load are still strongly associated with poor overall survival. While CTC are clinically well-established biomarkers, the ability to quantify and assess their expression and response to treatment relies heavily on their frequencies.^{18,19} Hence, in patients with low frequencies, CTC alone may not suffice as an effective biomarker. In this study, we present for the first time a way to combine CTC and tdEV into a single biomarker called BTL. The BTL is studied to evaluate the treatment target expression of HER2 in mBC patients and evaluate therapy response in mCRPC patients.

Clinical decisions on treating mBC remain dependent on the expression of HER2.^{20,21} Drugs targeting HER2 significantly improved the prognosis of patients with HER2-positive breast cancer defined by an immunohistochemical

score of 3+ and/or an *in situ* hybridization gene amplification.²²⁻²⁶ Traditional HER2-targeting therapies are effective in HER2-positive breast cancer and comprise 15%-20% of all breast cancer patients, whereas in patients considered HER2-negative, these treatments are not effective.^{24,25} Recently introduced novel anti-HER2 agents, such as antibody-drug conjugates are shown to be effective in patients with HER2 1+/2+ expression as evaluated by immunohistochemistry with no HER2 gene amplification.²⁶⁻²⁸ These HER2-low patients represent 50% of all breast cancer patients, but how accurate is this classification? As the cell composition of the tumors is heterogeneous and changes over time due to exposure to treatment, real-time information on the tumor composition is essential. CTC pose the opportunity for a real-time quantitative HER2 assessment. However, the low frequency of CTC prevents a reliable assessment in most mBC patients, as indicated by the poor correlation between HER2-expressing CTC and tdEV in patients with at least 1 CTC ($R^2 = 0.19$). By evaluating HER2 assessment on the BTL, we were able to increase the percentage of assessable patients with a reliable HER2 assessment from 35% to 78% and only in one patient was no assessment possible ([Figure 4A](#) and [B](#)). Moreover, the degree of HER2 positivity could be assessed as illustrated for the 98 patients in [Figure 4A](#). Expression levels were divided into low (HER2+), medium (HER2++), and high (HER2+++). Here, we used a minimum number of 10 CTC and or tdEV to assign the HER2 status of a patient. This number is merely based on stochastic noise^{29,30} to assure a level of certainty to assess a patient's HER2 status.

The actual HER2 expression threshold as well as HER2 fraction needed for a patient to respond to anti-HER2 agents can only be evaluated and determined in dedicated trials where such agents are being administered. With this information, the effectiveness of HER2 agents can be improved, and informed decisions can be made as to whether or not a patient can benefit from the therapy.

To measure BTL as a response biomarker, we compared BTL scores of CRPC patients ($n = 157$) at baseline and first follow-up (2-5 weeks) ([Figure 5B](#)). Several approaches have been conducted to evaluate how to best interpret CTC changes for evaluating treatment response.³¹⁻³⁴ The low number of CTC in most patients, however, prevents an accurate assessment of treatment response. The addition of tdEV provides a much higher number with which to evaluate response, such that decreases and increases can be more reliably measured. How can a patient's status with both tdEV and CTC counts be interpreted? We solved this by combining CTC and tdEV numbers in one value expressed as BTL. At baseline and first follow-up, BTL improved the prediction of favorable survival in patients, compared to CTC and tdEV alone ([Figure 5C](#)). To observe the effect of treatment on the BTL, patients were divided into those with a decrease in BTL (45% of patients), suggesting a response to therapy; those with no change in BTL (46% of patients), suggesting no response to therapy; and an increase in BTL (9% of patients), suggesting progression under treatment. Although a change in BTL can be indicative of response to

therapy, the best outcome is persistently low BTL regardless of the decrease in BTL, similar to when CTC remains no longer detectable post-treatment.^{31,33}

Combining biomarkers to simplify and improve its clinical usage has been explored and used for prostate-specific antigen (PSA). The prostate health index, for example, combines the results of total PSA, free PSA, and proPSA.³⁵ In the 4Kscore test, the results of total PSA, free PSA, and intact PSA are combined with human kallikrein 2 to improve the identification of aggressive prostate cancer.³⁶ The Iso-PSA test uses different forms of PSA proteins to help determine whether they came from cancer cells.³⁷ In metastatic prostate cancer, CTC and tdEV have been proven superior to PSA,^{7,19,38,39} suggesting a better performance of BTL in this disease setting. For non-metastatic prostate cancer, the utility of CTC, tdEV or BTL is not yet established and will need to be explored. A change in BTL over time, may serve a similar role to PSA velocity,⁴⁰ and maybe useful in identifying patients with more aggressive disease.

Although one can use 1 CTC or 1 CTC + tdEV for BTL, to determine marker expression this assessment surely will not be accurate. Therefore, we used a minimum of 10 CTC or 10 CTC + tdEV to report the HER2 expression. Understanding patient response to treatment targets will provide a basis to determine the accuracy needed for assessment of the HER2 fraction of the BTL. Additionally, increasing the number of CTC and tdEV can improve the accuracy. This can be achieved by increasing the number of CTC and tdEV detected in the images through the use of StarDist image segmentation⁴¹ and improving the accuracy and predictive value of CTC and tdEV through the implementation of deep learning algorithms.^{42,43} The BTL equation used in this study may also need further optimization for different applications such as finding the presence of clinically undetected metastatic disease.^{44,45} For early detection of disease, the sensitivity and specificity of the biomarkers is of utmost importance. For CTC and tdEV, sensitivity and specificity can be increased by probing a large blood volume such as leukapheresis.^{13,46,47} Alternatively, other biomarkers can be added to the BTL equation. The inclusion of PSA in the BTL equation may lead to a therapy management tool with better specificity and sensitivity than PSA, CTC or tdEV on their own. The presence of cell-free DNA may also aid BTL, as the low copy numbers for CTC and tdEV in the sample volume tested is a limitation.⁴⁸ The most straightforward manner to combine multiple biomarkers is to scale each biomarker using a function suitable for that biomarker and then divide by the number of biomarkers. This will ensure high risk corresponding to BTL of 1, and low risk to BTL of 0. For example, a scaling function for ctDNA could be a threshold function like $(z\text{-score} > 5) \times 1$. BTL thus offers an opportunity to combine various liquid-biopsy-obtained biomarkers that can improve and individualize treatment options. In support of this, efforts are required to further evaluate and validate the proposed biomarker.

Although we demonstrate that BTL shows promise for guidance of therapy in patients treated for metastatic breast and prostate cancer, the size of the study population

is relatively low and will need to be validated in larger patient cohorts and expanded to other cancer types. Thresholds used to define a response to therapy based on BTL can be obtained from existing studies, provided that both the CellSearch image data and clinical response data are available. Establishing criteria to determine whether a patient will respond to a targeted therapy such as HER2, requires prospective studies. In these studies, patients will undergo HER2-targeted treatment, and the response to this therapy can be evaluated by using both BTL and clinical evaluation. By correlating the response outcome to the BTL HER2 expression, it would be possible to determine the optimal BTL HER2 threshold for identifying patients who should and should not receive this treatment.

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DISCLOSURE

FAWC, AN, LT are listed as inventors on a patent application for BTL filed by MSB. All other authors have declared no conflicts of interest.

REFERENCES

1. Lone SN, Nisar S, Masoodi T, et al. Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments. *Mol Cancer*. 2022;21(1):79.
2. Armakolas A, Kotsari M, Koskinas J. Liquid biopsies, novel approaches and future directions. *Cancers (Basel)*. 2023;15(5):1579.
3. Dathathri E, Isebia KT, Abali F, et al. Liquid biopsy based circulating biomarkers in metastatic prostate cancer. *Front Oncol*. 2022;12:863472.
4. Asif S, Teply BA. Biomarkers for treatment response in advanced prostate cancer. *Cancers (Basel)*. 2021;13(22):5723.
5. Bodaghi A, Fattahi N, Ramazani A. Biomarkers: promising and valuable tools towards diagnosis, prognosis and treatment of Covid-19 and other diseases. *Heliyon*. 2023;9(2):e13323.
6. Pierga J-Y, Hajage D, Bachelot T, et al. High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. *Ann Oncol*. 2012;23(3):618-624.
7. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res*. 2008;14(19):6302-6309.
8. Allard WJ, Matera J, Miller MC, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res*. 2004;10(20):6897-6904.
9. Ligthart ST, Bidard F-C, Decraene C, et al. Unbiased quantitative assessment of Her-2 expression of circulating tumor cells in patients with metastatic and non-metastatic breast cancer. *Ann Oncol*. 2013;24(5):1231-1238.
10. Nanou A, Zeune LL, Bidard F-C, Pierga J-Y, Terstappen LWMM. HER2 expression on tumor-derived extracellular vesicles and circulating tumor cells in metastatic breast cancer. *Breast Cancer Res*. 2020;22(1):86.

11. Coumans FAW, Doggen CJM, Attard G, de Bono JS, Terstappen LWMM. All circulating EpCAM+CK+CD45- objects predict overall survival in castration-resistant prostate cancer. *Ann Oncol*. 2010;21(9):1851-1857.
12. Nanou A, Miller MC, Zeune LL, et al. Tumour-derived extracellular vesicles in blood of metastatic cancer patients associate with overall survival. *Br J Cancer*. 2020;122(6):801-811.
13. Coumans FAW, Ligthart ST, Uhr JW, Terstappen LWMM. Challenges in the enumeration and phenotyping of CTC. *Clin Cancer Res*. 2012;18(20):5711-5718.
14. Davidson-Pilon C. Lifelines: survival analysis in Python. *J Open Source Softw*. 2019;4(40):1317.
15. Bandini S, Ulivi P, Rossi T. Extracellular vesicles, circulating tumor cells, and immune checkpoint inhibitors: hints and promises. *Cells*. 2024;13(4):337.
16. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. *Cancer Cell*. 2016;30(6):836-848.
17. Kalluri R, McAndrews KM. The role of extracellular vesicles in cancer. *Cell*. 2023;186(8):1610-1626.
18. Isebia KT, Dathathri E, Verschoor N, et al. Characterizing circulating tumor cells and tumor-derived extracellular vesicles in metastatic castration-naïve and castration-resistant prostate cancer patients. *Cancers (Basel)*. 2022;14(18):4404.
19. Nanou A, Coumans FAW, van Dalum G, et al. Circulating tumor cells, tumor-derived extracellular vesicles and plasma cytokeratins in castration-resistant prostate cancer patients. *Oncotarget*. 2018;9(27):19283-19293.
20. Dieci MV, Miglietta F, Griguolo G, Guarneri V. Biomarkers for HER2-positive metastatic breast cancer: beyond hormone receptors. *Cancer Treat Rev*. 2020;88:102064.
21. Swain SM, Shastry M, Hamilton E. Targeting HER2-positive breast cancer: advances and future directions. *Nat Rev Drug Discov*. 2023;22(2):101-126.
22. Martínez-Sáez O, Prat A. Current and future management of HER2-positive metastatic breast cancer. *JCO Oncol Pract*. 2021;17(10):594-604.
23. Wolff AC, Hammond MEH, Allison KH, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. *J Clin Oncol*. 2018;36(20):2105-2122.
24. Fehrenbacher L, Cecchini RS, Geyer CE Jr, et al. NSABP B-47/NRG oncology phase III randomized trial comparing adjuvant chemotherapy with or without trastuzumab in high-risk invasive breast cancer negative for HER2 by FISH and with IHC 1+ or 2. *J Clin Oncol*. 2020;38(5):444-453.
25. Burris HA 3rd, Rugo HS, Vukelja SJ, et al. Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy. *J Clin Oncol*. 2011;29(4):398-405.
26. Tarantino P, Hamilton E, Tolaney SM, et al. HER2-low breast cancer: pathological and clinical landscape. *J Clin Oncol*. 2020;38(17):1951-1962.
27. Modi S, Jacot W, Yamashita T, et al. Trastuzumab deruxtecan in previously treated HER2-low advanced breast cancer. *N Engl J Med*. 2022;387(1):9-20.
28. Nicolò E, Boscolo Bielo L, Curigliano G, Tarantino P. The HER2-low revolution in breast oncology: steps forward and emerging challenges. *Ther Adv Med Oncol*. 2023;15:17588359231152842.
29. Tibbe AGJ, Miller MC, Terstappen LWMM. Statistical considerations for enumeration of circulating tumor cells. *Cytometry A*. 2007;71A(3):154-162.
30. Zeune LL, van Dalum G, Decraene C, et al. Quantifying HER-2 expression on circulating tumor cells by ACCEPT. *PLOS One*. 2017;12(10):e0186562.
31. Lorente D, Olmos D, Mateo J, et al. Decline in circulating tumor cell count and treatment outcome in advanced prostate cancer. *Eur Urol*. 2016;70(6):985-992.
32. Lorente D, Ravi P, Mehra N, et al. Interrogating metastatic prostate cancer treatment switch decisions: a multi-institutional survey. *Eur Urol Focus*. 2018;4(2):235-244.
33. Coumans FAW, Ligthart ST, Terstappen LWMM. Interpretation of changes in circulating tumor cell counts. *Transl Oncol*. 2012;5(6):486-491.
34. Lorente D, Olmos D, Mateo J, et al. Circulating tumour cell increase as a biomarker of disease progression in metastatic castration-resistant prostate cancer patients with low baseline CTC counts. *Ann Oncol*. 2018;29(7):1554-1560.
35. Loeb S, Catalona WJ. The Prostate Health Index: a new test for the detection of prostate cancer. *Ther Adv Urol*. 2014;6(2):74-77.
36. Punnen S, Pavan N, Parekh DJ. Finding the wolf in sheep's clothing: the 4Kscore is a novel blood test that can accurately identify the risk of aggressive prostate cancer. *Rev Urol*. 2015;17(1):3-13.
37. Klein EA, Partin A, Lotan Y, et al. Clinical validation of IsoPSA, a single parameter, structure-focused assay for improved detection of prostate cancer: a prospective, multicenter study. *Urol Oncol*. 2022;40(9):408.e9-408.e18.
38. Heller G, McCormack R, Kheoh T, et al. Circulating tumor cell number as a response measure of prolonged survival for metastatic castration-resistant prostate cancer: a comparison with prostate-specific antigen across five randomized phase III clinical trials. *J Clin Oncol*. 2018;36(6):572-580.
39. Goldkorn A, Tangen C, Plets M, et al. Circulating tumor cell count and overall survival in patients with metastatic hormone-sensitive prostate cancer. *JAMA Netw Open*. 2024;7(10):e2437871-e2437871.
40. Vickers AJ, Wolters T, Savage CJ, et al. Prostate-specific antigen velocity for early detection of prostate cancer: result from a large, representative, population-based cohort. *Eur Urol*. 2009;56(5):753-760.
41. Stevens M, Nanou A, Terstappen LWMM, Driemel C, Stoecklein NH, Coumans FAW. StarDist image segmentation improves circulating tumor cell detection. *Cancers (Basel)*. 2022;14(12):2916.
42. Zeune LL, Boink YE, van Dalum G, et al. Deep learning of circulating tumour cells. *Nat Mach Intell*. 2020;2(2):124-133.
43. Nanou A, Stoecklein NH, Doerr D, Driemel C, Terstappen LWMM, Coumans FAW. Training an automated circulating tumor cell classifier when the true classification is uncertain. *PNAS Nexus*. 2024;3:1-8.
44. Janni WJ, Rack B, Terstappen LWMM, et al. Pooled analysis of the prognostic relevance of circulating tumor cells in primary breast cancer. *Clin Cancer Res*. 2016;22(10):2583-2593.
45. van Dalum G, Stam G-J, Scholten LFA, et al. Importance of circulating tumor cells in newly diagnosed colorectal cancer. *Int J Oncol*. 2015;46(3):1361-1368.
46. Coumans FAW, Terstappen LWMM. Detection and characterization of circulating tumor cells by the CellSearch approach. *Methods Mol Biol*. 2015;1347:263-278.
47. Stoecklein NH, Fluegen G, Guglielmi R, et al. Ultra-sensitive CTC-based liquid biopsy for pancreatic cancer enabled by large blood volume analysis. *Mol Cancer*. 2023;22(1):181.
48. Liu H, Gao Y, Vafaei S, Gu X, Zhong X. The prognostic value of plasma cell-free DNA concentration in the prostate cancer: a systematic review and meta-analysis. *Front Oncol*. 2021;11:599602.