Single-Cell Analyses of Prostate Cancer Liquid Biopsies Acquired by Apheresis


Abstract

Purpose: Circulating tumor cells (CTCs) have clinical relevance, but their study has been limited by their low frequency. Experimental Design: We evaluated liquid biopsies by apheresis to increase CTC yield from patients suffering from metastatic prostate cancer, allow precise gene copy-number calls, and study disease heterogeneity.

Results: Apheresis was well tolerated and allowed the separation of large numbers of CTCs; the average CTC yield from 7.5 mL of peripheral blood was 167 CTCs, whereas the average CTC yield per apheresis (mean volume: 59.5 mL) was 12,546 CTCs. Purified single CTCs could be isolated from apheresis product by FACS sorting; copy-number aberration (CNA) profiles of 185 single CTCs from 14 patients revealed the genomic landscape of lethal prostate cancer and identified complex intrapatient, intercell, genomic heterogeneity missed on bulk biopsy analyses.

Conclusions: Apheresis facilitated the capture of large numbers of CTCs noninvasively with minimal morbidity and allowed the deconvolution of intrapatient heterogeneity and clonal evolution. Clin Cancer Res; 24(22): 5635–44. ©2018 AACR.

Introduction

Prostate cancer remains a major cause of male cancer-related deaths (1). Studies elucidating disease biology are restricted by the genomic landscape of both localized and advanced prostate cancer has been recently described but bulk tumor biopsy genomics only provide a snapshot of the disease landscape (3).

Moreover, concerns have been raised regarding the ability of bulk biopsy sequencing to document intratumor heterogeneity and clonal evolution. Serial biopsies are necessary to evaluate changes imposed by therapeutic selective pressures over time, but their acquisition is challenging, invasive, and often not feasible. Less invasive alternatives ("liquid biopsies") could be hugely impactful, allowing serial evaluation, and detecting disease evolution that can influence treatment choices.

Two main forms of liquid biopsy have emerged: circulating plasma cell-free DNA (cfDNA) and circulating tumor cell (CTC) analyses. Although measuring cfDNA concentrations has utility (4), limitations in qualitative analyses deconvoluting intrapatient heterogeneity and accurate calling of copy-number aberrations (CNAs), especially deletions, have been acknowledged (5). CTCs, shed from solid tumors (6) and found in the peripheral blood (PB) of patients with both nonmetastatic (5%–24%) and metastatic (26%–49%) disease (7, 8), can allow the early detection of disease dissemination, prognostication, and benefit from therapy (9, 10). Indeed, CTC evaluation may be superior to radiologic assessment in determining response to treatment and outcome (11–13).

CTC studies can allow noninvasive, serial, tumor genomic characterization during treatment, but a major challenge to this has been their detection in significant numbers to enable genomic, transcriptomic, and protein analyses. To overcome these limitations, apheresis has been suggested to increase CTC yield (14). Apheresis allows processing of the whole blood volume by centrifugation, separating blood components (e.g., red cells,
Translational Relevance

Apheresis is well tolerated and is a noninvasive alternative to tumor tissue biopsies, substantially increasing circulating tumor cell yields and allowing the study of tumor evolution and intrapatient heterogeneity during treatment. Serial, repeated, apheresis can interrogate disease evolution, drive key therapeutic decisions, and transform prostate cancer drug development.

platelets, and leukocytes) based on density. Apheresis has a therapeutic role in the management of hemolytic disorders and is well tolerated with few safety concerns (15). Previous studies have suggested that CTCs can be collected from apheresis product from patients with and without metastases (14, 16, 17). CTCs can have a similar density to mononuclear cells and apheresis can increase CTC separation from a larger volume of processed blood. We hypothesized that apheresis, followed by CTC enrichment methods, could allow the safe acquisition of large numbers of viable and intact purified CTC populations from patients with advanced prostate cancer, permitting a true liquid biopsy and tumor molecular characterization.

Materials and Methods

Patient selection and clinical assessment

Eligible patients had histologically confirmed mCRPC. Additional eligibility criteria included detectable PB CTCs (CellSearch), good bilateral antecubital fossa venous access, and no coagulopathy. Clinical assessments included medical history and physical examination, full blood count, biochemical tests, and coagulation. Safety assessments were done during apheresis and after 30 days. All patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki, with the ethics committee of the Royal Marsden and The Institute of Cancer Research approving the study.

Apheresis (method and CTC detection)

Apheresis was performed using a Spectra Optia Apheresis System (Terumo, BCT). Patients were connected to this via two peripheral venous catheters in each cubital vein. Whole blood was anticoagulated before entering the rotating centrifuge. Heavier blood elements including erythrocytes migrated to the outside of the channel, plasma to the center, and theuffy coat (including mononuclear cells and CTCs) to the middle. The mononuclear cell layer was removed and the remaining blood cells and plasma were constantly returned to the patient to the contralateral arm. The buffy coat was removed and the remaining blood cells and plasma were constantly returned to the patient to the contralateral arm. Granulocyte-colony-stimulating factor was not used. Blood was anticoagulated with citrate dextrose solution A (two to four 500-mL infusion bags were required for each procedure).

CTC enumeration using CellSearch platform

CTC counts were determined in 7.5 mL of PB drawn immediately before, and after, the apheresis; an aliquot of apheresis product containing 200 × 10⁶ white blood cell (WBC) was transferred to a CellSave preservative tube (Menarini, Silicon Biosystems) and mixed with CellSearch dilution buffer to a final volume of 8 mL. All samples were processed within 96 hours, and CTC counts were determined by CellSearch (Menarini, Silicon Biosystems). Briefly, cells were subjected to immunomagnetic capture using anti-EpCAM antibodies and stained with antibodies specific for cytokeratin 8, 18, and 19 (CK-PE), CD45 (CD45-APC), and nuclei acid dye (DAPI). Cells were defined as CTCs when positive for cytokeratin and DAPI and negative for CD45. Images were captured using the CellTracks Analyzer II (Menarini, Silicon Biosystems) and manually examined to determine the presence of CTC. CellSearch cartridges were stored in the dark at 4°C before further analyses.

Single-cell isolation and amplification

CellSearch cartridge contents were transferred into fresh Eppendorf tubes, washed twice with 150 µL of phosphate-buffered saline, and FACS sorted (FACSAria III; Becton, Dickinson and Company) to single CTCs (DAPI+, CK+, CD45−) or WBC (DAPI+, CD45+, CK−). Sorted single CTC or WBC were whole-genome amplified (WGA) using AmpII1 (Menarini, Silicon Biosystems) according to the manufacturer’s instructions with minor modifications. Cells were lysed, digested for 30 minutes, adaptor ligated for 3 hours, and PCR amplified. The WGA DNA was purified (MiniElute PCR Purification Kit; Qiagen), quantified using Qubit (Invitrogen), and stored at −20°C.

DNA from biopsies

DNA from formalin-fixed paraffin-embedded (FFPE) biopsies was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen), quantified using Qubit (Invitrogen), and evaluated by Illumina FFPE QC kit. Whole-genome amplification was carried out on 10 ng of tumor DNA using WGA2 (Sigma-Aldrich). WGA DNA was purified (MiniElute PCR Purification Kit; Qiagen), quantified (Qubit; Invitrogen), and stored at −20°C.

Array comparative genomic hybridization (aCGH)

Five hundred nanograms of amplified single CTC DNA was fluorescently labeled with Cy5, and WBC reference DNA labeled with Cy3 (SureTag Complete DNA Labeling Kit; Agilent Technologies). Labeled DNA was purified and hybridized utilizing the Agilent SurePrint G3 Human array CGH Microarray Kit, 4 × 180K. Slides were scanned and ratios of CTC/WBC determined using CytoGenomics Software v 4.0.3.12 (Agilent Technologies). Log₂ ratios of aCGH segments were matched with gene coordinates to assign per-gene values. Copy states of genes were classified by the assigned log₂ ratio values. Log₂ ratio values < −0.25 were categorized as losses; those > 0.25 as gains; and those in between as unchanged. Amplifications were defined as smoothed log₂ ratio values ≥ 1.2 and homozygous deletions as the segment log₂ ratio values ≤ −1.2. Per-sample CNA burden was calculated as the proportion of the human genome (3,000 megabase pairs) affected. Unsupervised hierarchical clustering was performed using R (v3.4) with Ward method and the Euclidean distances of unique copy-number changes. When clustering samples from multiple tissue types, X-chromosome genes were excluded (aside from the AR gene and 10 genes on either side) due to different reference X-chromosome ploidies (as a female reference was used). Per-patient functional diversity was derived from cluster dendrograms of CTC samples by calculating the sum of connecting branches in a dendrogram (from the R package vegan v2.4.4) and divided by the number of samples.
Fluorescence in situ hybridization (FISH) analysis

FISH was performed by FFPE hybridization as previously described (18). Briefly 3- to 4-μm FFPE sections were deparaffinized, heat pretreated, pepsin digested and hybridized with FISH probe hybridization mix overnight at 37°C. FISH probes used were BRCA2/CEN13q (Abnova), RB1 (Abbott Laboratories), PTEN (10q23)/SE 10, MYC (8q24)/SE 8 (Leica Microsystems), and a custom-made AR/CEPX probe (Menarini, Silicon Biosystems). Stringency washes were performed on all slides; for AR, where the probe was indirectly labeled, a secondary incubation with anti-digoxigenin–fluorescein antibody (Roche Diagnostics) was done. Slides were digitally imaged (BioView Ltd.), and a pathologist (DNR) evaluated a minimum of 100 tumor cells; the ratios between probes of interest and reference probes were recorded. Amplification was reported if the ratio was >2; heterozygous loss and homozygous deletion if at least one of three of the cells showed loss of one copy, or loss of all copies, of the tested probe, respectively.

Organoid culture

For CTC enrichment, 1 mL of single-cell suspension was immunomagnetically separated with EasySep Epcam-positive selection (Stem Cell Technologies), and the selected fraction was used for organoid culture (negative fraction cultured as a control). Isolated cells were seeded in 3D using growth factor-reduced Matrigel (Corning) in spheroid-forming suspension in ultra-low attachment surface-coated microplates (Nunclon Sphera, Thermo Fisher Scientific) utilizing previously described growth media.

Figure 1.
Overview of methodology, CTC counts, and the overall genomic analyses: A, Methodology workflow of the study; B, CTC counts from 7.5 mL of PB taken preapheresis and postapheresis and compared with inferred harvested CTC counts in the total volume of apheresis product. C, The top plot represents the frequency of the genomic aberrations found in 185 single CTCs harvested by apheresis from 14 mCRPC patients; the middle plot represents the frequency of genomic aberrations from 150 mCRPC exomes (SU2C/PCF cohort), and the lower plot represents the frequency of genomic aberrations from available tissue biopsies from 12 of 14 patients. Chromosomes are shown across the x-axis, whereas the y-axis represents the frequency of gains, losses, amplification, and homozygous deletions. Gains are depicted in light pink, losses are depicted in light blue, amplification in dark red, and homozygous/deep deletions are in dark blue. *, aCGH of tissue biopsies were performed using female reference DNA (Agilent).
conditions (19). Organoids were passaged after 4 to 6 weeks and cells collected manually for molecular studies by dissociation with TrypLE (Sigma-Aldrich) for 5 minutes at 37°C.

**Next-generation sequencing**

Whole-exome sequencing (WES) was performed using Kapa HyperPlus library prep kits and the Agilent SureSelectXT V6 target enrichment system. Paired-end sequencing was performed using the NextSeq 500 (2 × 150 cycles; Illumina). FASTQ files were generated from the sequencer’s output using Illumina bcl2fastq2 software (v.2.17.1.14, Illumina) with the default chastity filter to select sequence reads for subsequent analysis. All sequencing reads were aligned to the human genome reference sequence (GRCh37) using the BWA (v. 0.7.12) MEM algorithm, with indels being realigned using the Stampy (v.1.0.28) package. Picard tools (v.2.1.0) were used to remove PCR duplicates and to calculate sequencing metrics for QC check. The Genome Analysis Toolkit
(GATK, v. 3.5-0) was then applied to realign local indels, recalibrate base scores, and identify point mutations and small insertions and deletions. Somatic point mutations and indels were called using MuTect2 by comparing tumor DNA to germline control, and copy-number estimation was obtained through modified ASCAT2 package.

**Results**

**Patient characteristics**

From November 2015 to July 2017, 14 eligible mCRPC patients with detectable CTCs by CellSearch were enrolled (median age, 70.4 years; range, 60–77); time from prostate cancer diagnosis to procedure ranged from 2–11.6 years (mean, 6.2 years; median, 3.9 years). Median PSA level at apheresis was 506 ng/mL (range, 41–6089 ng/mL); all 14 (100%) had metastatic bone disease. Prior to apheresis, patients had received 1 to 5 lines of systemic therapy for CRPC (Supplementary Table S1; Supplementary Fig. S1a). At apheresis, none of the subjects was receiving active treatment other than androgen deprivation.

The apheresis workflow is depicted in Fig. 1A. Each apheresis procedure lasted between 90 and 160 minutes; apheresis product volume ranged from 40 to 100 mL (Supplementary Table S2). Apheresis was well tolerated with no related adverse events recorded during the procedure or in the 30-day follow-up. Neutrophil and lymphocyte counts did not change significantly following apheresis (Supplementary Fig. S1b).

**Figure 3.**

Intrapatient CTC genomic heterogeneity. **A,** Individual CTC genome plots of patient P09 show very homogeneous CTCs similar to a metastatic bone biopsy. **B,** Heat map depicting CNA of 25 CTCs (gray bars) and two tumor biopsies (black bars) from patient P13 showing two different subclones, readily visualized by focusing on chromosome 5q, and an additional group of highly heterogeneous CTCs (far left). **C,** FISH analysis of treatment-naive prostatectomy tissue and a bone mCRPC biopsy from patient P13 using probes for 5p11 (red) and 5q21.1 (green). **D,** A schematic diagram showing the percentage of cells with copy-number alterations on 5q21.1 with disease progression from the time of the prostatectomy until apheresis in patient P13.
CTC counts
The mean CTC count taken before and after apheresis was 167 and 193, per 7.5 mL of PB, respectively. Surprisingly, the CTC count did not decrease significantly following apheresis ($P = 0.48$). The average inferred CTC harvest from an apheresis (mean volume = 59.5 mL) was 12,546, with apheresis yielding a 90-fold average increased yield ($P < 0.001$; Fig. 1B; Supplementary Table S2).

Single CTC genomic profiling
To validate the serial WGA and array CGH that we performed on single CTCs, we first used normal male and female DNA (aCGH verified by Agilent), as well as single WBC-amplified DNA and showed that there was no bias amplifications or deletions (Supplementary Fig. S2a and S2b). Extracted single CTC DNA from a patient with known tumor biopsy CNAs was then evaluated, confirming robust CNA calling. WGA of 1 μL of serially diluted samples (starting DNA templates: 10 ng/μL, 1 ng/μL, 0.1 ng/μL, and 0.03 ng/μL) showed no amplification bias with consistent calling of gains and losses at all dilutions (Supplementary Fig. S2c).

We then analyzed 205 single CTC aCGH genomic profiles for CNAs from the apheresis products of 14 patients with 185 CTC (90%) showing complex genomic copy change profiles and 20 (10%) cells having relatively flat genomic copy-number profiles. Surprisingly, only two of the evaluated 14 patients had cell populations with both flat and cancer-like aCGH profiles, suggesting that these sorted cells could be associated with specific tumor subtypes or induced by some treatments. We then aggregated the aCGH copy-number profiles of all the individual CTCs and showed that the overall profile matched that previously reported for advanced prostate cancer whole biopsy exomes (ref. 20; Fig. 1C). Details for individual CTCs per patient are shown in Supplementary Table S3.

Tumor biopsies (treatment-naïve diagnostic biopsies and/or metastatic biopsies) were available for 12 of these 14 patients; these samples were also evaluated. Copy-number traces of single CTCs and matching, same patient, biopsies showed broadly similar genomic profiles (Fig. 1C; Supplementary Fig. S3) and again matched that of publicly available data (20). Differences were frequently observed between treatment-naïve biopsies and castration-resistant CTCs including AR gain (X chromosome), MYC gain (8q), and RB1 loss (chromosome 13), likely reflecting tumor evolution under treatment-selective pressures (Supplementary Fig. S4). High concordance between single CTC genomic profiles and contemporaneous, same patient, metastatic biopsies was seen, although intrapatient genomic heterogeneity was discernible from the single CTC analyses but not the bulk biopsy analyses.

CTC diversity
Overall, the genomic analyses of 185 single CTCs from 14 patients (Fig. 2A) revealed that some patients had highly homogenous CTC CNA traces (Fig. 2A, left), while others had highly diverse single CTC CNA traces (Fig. 2A, right) with many lethal PCs displaying intercell heterogeneity. This may be related to disease phenotypes or acquired treatment resistance mechanisms (AR and MYC gain at chromosomes X and 8q, respectively; BRCA2/BRCA1 locus loss at chromosome 13). There was no significant correlation between median percentage genome alteration and intrapatient, intercell, diversity (Fig. 2B), suggesting that this was due to true clonal diversity rather than aberration accumulation. Despite this, the unsupervised hierarchical clustering of all the CNA data from individual CTCs and same patient biopsies indicated that most samples from one patient clustered together (Supplementary Fig. S3).

Intrapatient heterogeneity and tumor evolution
As depicted in Fig. 2A (far left patients), the minority of patients had highly homogeneous CTC, including P09 (Fig. 3A); his contemporaneous mCRPC biopsy had a virtually identical CNA profile to these CTCs. Most evaluated patients had heterogeneous CTC CNA profiles that gross biopsy genomic analyses could fail to identify. To further interrogate this intrapatient heterogeneity, we studied additional cells in patient P13, who had heterogeneous CTCs, with CNA data suggesting distinct groups of cells (Fig. 3B). Some CTCs clustered with his diagnostic prostatectomy sample, while others clustered with the mCRPC bone biopsy, with a breakpoint in the PIK3R1 locus including most of chromosome 5q (Fig. 3C). A third group of cells was also apparent, displaying more complex genomic aberrations.

FISH analyses of the 5q21.1 locus were then performed on both the hormone-sensitive prostate cancer sample and the metastasis and revealed the presence of distinct copy-number aberrant cells, with 5q21.1 being either gained, normal, or lost in a mixed cell population. Overall, these analyses indicated that these three copy states were equally common in the prostatectomy. Over time and following treatment, the proportion of tumor cells with 5q copy gain increased as shown in the mCRPC biopsy and apheresis CTCs and was confirmed by tissue FISH analyses (Fig. 3C and D).

We then studied patient P03 because his CTC CNA profiles were also highly heterogeneous, and multiple tumor
samples taken at different time points were available, including a transurethral resection of the prostate (TURP) with four geographically and morphologically distinct regions (A, B, C, and D) that were microdissected (Fig. 4A). aCGH genomic profiles of these regions identified intrapatient heterogeneity (Fig. 4B). Homozygous deletion of BRCA2 and 8q gain was present in all four regions; however, loss of chromosome 18 was present only in areas A and D, whereas gain of 7q was present in only areas A and C. The CNA profile of a lymph node (LN) biopsy acquired from this patient 6 years later, following treatment with docetaxel P (75 mg/m²), enzalutamide (160 mg), and cabazitaxel (25 mg/m²), enzalutamide, and cabazitaxel, identified the BRCA2 homozygous deletion and 8q gain, as well as previously undetected AR amplification and 17q gain (Fig. 4B).

In patient P03, we performed WES of the microdissected TURP regions. This identified truncal patogenic mutations of SPOP (p.Trp131Cys) and FOXA1 (p.His168del), with intrapatient heterogeneity of other mutations indicating that regions A and C had similar mutation profiles when compared with regions B and D of the TURP, with the later LN biopsy WES identifying a mixture of these cell populations (Fig. 4C). Single CTC analyses acquired at a later time point by apheresis also detected this heterogeneity, delineating this cancer’s evolution as depicted by unsupervised hierarchical clustering of 13 CTCs, four microdissected TURP areas, the gross biopsy, and the LN biopsy (Fig. 4D and E). Figure 4D highlights key genomic differences in commonly altered pathways in these samples with heterogeneous PTEN and BRCA2 loss in different subclones. FISH analysis of TURP tissue using MYC and BRCA2 probes revealed that some TURP tumor cells had concurrent MYC amplification and BRCA2 homzygous deletion (Fig. 4F), while others had MYC amplification but no BRCA2 loss, indicating that the latter was probably subclonal and occurred later, as indicated by the single CTC analyses (Fig. 4E).

The apheresis from patient P05 also revealed heterogeneous CTCs: we successfully generated organoid cultures from these (Supplementary Fig. S5a and S5b) utilizing previously described methods (21). The CNA profile of these organoids clustered with this patient’s CTCs with two genomically divergent subclones in culture (Supplementary Fig. S5c) with both subclones detectable in the CTC analyses (Supplementary Fig. S5c and S5d), indicating that CTC-derived organoid culture can recapitulate this diversity.

Discussion

Liquid biopsy by apheresis is noninvasive and well tolerated, increasing CTC yield 100-fold from mCRPC patients. Apheresis did not significantly affect blood CTC counts, suggesting constant replenishment or inefficient capture. Apheresis facilitated the interrogation of tumor genomes, interpatient genomic heterogeneity, and the dissection of prostate cancer evolution. We show for the first time that the genomic landscape of prostate cancer CTCs captured by apheresis mirrors that of mCRPC biopsy exomes, validating these CTC capture methods (20). Copy-number traces of individual CTCs frequently closely resembled same patient biopsies, with evidence for CTC CNA events evolving over time due to therapeutic pressures (including gains in MYC and AR). Critically, subclonal CNA events not discernible from bulk biopsy analyses were easily detected by singe CTC analyses dissecting disease clonal evolution.

Yields of evaluable single cells decrease significantly through our experimental procedures; stringent settings in FACS sorting to allow isolation of only pure single cells results in a 60% to 80% retention rate of CTCs from CellSearch cartridges. DNA from approximately another 20% of these cells fails quality control after whole-genome amplification. Therefore, in order to end up with sufficient CTCs for genomic analyses, a high number of cells are required, making the concentrated apheresis product a much more efficient source than PB.

Surprisingly, we identified by unsupervised clustering varying degrees of intrapatient heterogeneity with some patients having highly homogeneous single CTCs but most having intrapatient CTC genomic diversity. Some CTCs resembled diagnosis biopsies, with others genomically mirroring metastases. We envision that the dynamic analyses of these clones by serial, repeated, apheresis before, during, and after treatment will not only dissect disease evolution but also help guide therapeutic switch decisions. Such heterogeneity remains difficult to identify from circulating free DNA, with the analyses of CTCs captured by apheresis, allowing a more precise evaluation of emerging clones/subclones. Early identification of resistant clones can be utilized to reverse treatment failure, guiding drug combination administration or the serial utilization of drugs not tolerated when administered together. We propose that serial, multiple, apheresis procedures should now be embedded in drug trials to analyze tumor clones/subclone eradication/evolution during therapy to further evaluate this strategy, while also generating estimates of CTC counts for monitoring response to therapy (22).

Further work is also now needed to explore the clinical implications of this diversity in intrapatient heterogeneity, evaluating whether distinct genomic subtypes of advanced prostate cancer display different levels of single CTC diversity. Moreover, further optimization of methodology generating successful organoid growth from apheresis products, along with subsequent molecular and functional analyses to confirm that these CTC-derived organoids can model mCRPC ex vivo, may also support the future study of drug testing in CTC organoid cultures.

We acknowledge the limitations of the data presented, particularly with regard to the limited cohort size and the fact that all the patients were treated at one tertiary cancer center, making it difficult to draw broader clinical conclusions. In order for apheresis to have widespread utility, it needs to be easily accessible, with high-throughput CTC isolation from patients with other cancer types and with lower burden disease (23). Moreover, improved methods to enhance CTC mobilization and yield through chemokine axis manipulation are warranted with such procedures potentially having therapeutic utility in patients with lower burden disease.

Moving forward, studies are needed to identify the optimal number of individual CTCs from one patient to sufficiently interrogate heterogeneity yet minimize cost. Low-coverage whole-genome next-generation sequencing with barcoding of DNA from each CTC may allow this, as well as exploration of single-cell RNA sequencing to better understand resistance mechanisms. Direct comparison of CTCs acquired by apheresis with both CTCs and cfDNA from PB, as well as with single cells dissociated from tissue should be pursued. Finally, studies to
evaluate the large numbers of immune cells in the apheresis product from these patients are also merited.

In conclusion, we have demonstrated that the analyses of single CTCs captured by apheresis permits the identification of intra-patient tumor genomic heterogeneity previously missed by bulk biopsy analyses, providing previously undescribed detail on different mCRPC subclones. Although the study of biopsies remains a gold standard, the challenges of acquiring serial biopsies and disaggregating these to single-cell suspensions to study disease evolution remain. We now posit that successfully and safely improving CTC yield for genomic analyses by apheresis is highly advantageous and has major potential implications for more precise cancer care.

Disclosure of Potential Conflicts of Interest

M. S. Fontes reports receiving speakers bureau honoraria from Janssen Pharmaceutical Companies and AstraZeneca do Brasil Ltda., and is a consultant/advisory board member for Janssen Pharmaceutical Companies. N. Mehra reports receiving speakers bureau honoraria from Janssen; Astellas; Bristol, Myers, Squibb; and MSD, and is a consultant/advisory board member for Janssen. D. Bianchini reports receiving speakers bureau honoraria from Janssen. R.P.L. Neves is a consultant/advisory board member for Janssen. R. Pereira, C. Berton, R. Risnaes, A. Sharp, J. Goodall, S. Carreira, D. Bianchini, P. Rescigno, Z. Zafeiriou, J. Hunt, D. Moloney, J. Sweeneyhuis, J.S. de Bono

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