Probing single-cell metabolism reveals prognostic value of highly metabolically active circulating stromal cells in prostate cancer

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Despite their important role in metastatic disease, no general method to detect circulating stromal cells (CStCs) exists. Here, we present the Metabolic Assay-Chip (MA-Chip) as a label-free, droplet-based microfluidic approach allowing single-cell extracellular pH measurement for the detection and isolation of highly metabolically active cells (hm-cells) from the tumor microenvironment. Single-cell mRNA-sequencing analysis of the hm-cells from metastatic prostate cancer patients revealed that approximately 10% were canonical EpCAM+ hm-CTCs, 3% were EpCAM− hm-CTCs with up-regulation of prostate-related genes, and 87% were hm-CStCs with profiles characteristic for cancer-associated fibroblasts, mesenchymal stem cells, and endothelial cells. Kaplan-Meier analysis shows that metastatic prostate cancer patients with more than five hm-cells have a significantly poorer survival probability than those with zero to five hm-cells. Thus, prevalence of hm-cells is a prognosticator of poor outcome in prostate cancer, and a potentially predictive and therapy response biomarker for agents cotargeting stromal components and preventing epithelial-to-mesenchymal transition.

INTRODUCTION

In addition to malignant cells, all solid tumors contain a variety of nonmalignant cancer–associated stromal cells, which include endothelial cells (ECs), mesenchymal stem cells (MSCs), cancer-associated fibroblasts (CAFs), pericytes, and immune cells, which can comprise more than 50% of the tumor mass (1–3). These stromal cells are increasingly considered essential for nutrient and growth factor delivery, induction of the epithelial-to-mesenchymal transition (EMT), and tumor growth. Furthermore, their propensity to dissociate from the primary tumor (at a rate of ~1 million cells per day) (4) gives them an important role in metastatic dissemination (2). Both circulating cancer-initiating and cancer-associated cells are present in very low numbers, making their detection and analysis challenging. Liquid biopsies offer a minimally invasive route to gain information about the progress of metastatic disease and the efficacy of treatment (3). However, most diagnostic tools have primarily focused on detection and analysis of circulating tumor cells (CTCs), and relatively few studies have reported the isolation and characterization of circulating stromal cells (CStCs) (3). CStCs exhibit a very high phenotypic diversity and do not have general markers. Size-based, label-free capture methods followed by immunostaining have been the most successful approach in isolation of circulating cancer-associated nonmalignant cells from the blood (3, 5, 6). Although the identification and characterization of CStCs in blood-based biopsy are gaining momentum, it is clear that we lack a thorough understanding of “normal” and “abnormal” levels of CStCs as well as their response to (targeted) treatments, and there is an urgent need for a more general label-free method for their isolation and characterization. One of the hallmarks of cancer is the “aerobic glycolysis" pathway leading to the abnormal production and secretion of lactate and consequently to the acidification of the microenvironment (7, 8). Crucially, cancer-associated metabolic reprogramming also occurs in cancer-associated cells. In particular, stromal metabolic remodeling was reported in CAFs as a result of hypoxic or oxidative stress induced by cancer cells. Cross-talk between CAFs and cancer cells at the tumor microenvironment (TME) triggers anaerobic glycolysis, leading to the production of lactate and pyruvate by CAFs (9, 10). Energy metabolism also plays a key role in stem cell maintenance and differentiation (11). Similarly to CAFs, adipose tissue–derived MSCs secreting lactate exhibited a metabolic coupling with osteosarcoma cells (12). Recent findings support evidence that ECs also rely on the rapid conversion of glucose to lactate (13, 14). Thus, we hypothesize that a liquid biopsy method for measuring single-cell metabolic activity should allow us to detect and isolate a wide range of metabolically active malignant and nonmalignant circulating cancer-associated cells. We previously reported a droplet-based microfluidic method for the label-free CTC detection based on the abnormal metabolism of cancer cells (15). Here, we introduce the MA-Chip (Metabolic Assay-Chip), which exploits a co-flow design for the detection and label-free isolation of highly metabolically active cells (hm-cells), derived from the TME from patients with metastatic prostate cancer. Single-cell transcriptome analysis of isolated hm-cells shows populations of hm-CStCs comprising circulating cancer–associated fibroblasts (cCAFs), circulating MSCs (cMSCs), and circulating ECs (CECs) as well as EpCAM+ and EpCAM− hm-CTCs. We demonstrate that the level of metabolic activity of cancer-associated cells is prognostic and a promising biomarker to study tumor biological mechanisms associated with progression and metastasis formation.

RESULTS

MA-Chip as a method for detection of hm-cells

Enumeration of EpCAM+ cells in liquid biopsies has been the de facto gold standard for prognostic purposes of CTCs. Here, we provide...
evidence for the clinical utility of the detection of both CTCs and tumor-educated CSCs that are primarily characterized by their high metabolic activity. In addition, we investigate whether EpCAM⁺ or EpCAM⁻ hm-cells carry additional prognostic value. Figure 1 shows an outline of the workflow to enumerate and isolate metabolically active cells based on acidification of the microenvironment; a detailed protocol is in Materials and Methods. Briefly, blood was processed within a couple of hours from withdrawal. First, red blood cells (RBCs) were lysed, and subsequently, white blood cells (WBCs) were negatively depleted based on their high CD45 expression (Fig. 1, A and B). Optional staining was performed to extract information about the number of EpCAM⁺ and CD45⁻ hematopoietic cells based on their high CD45 expression (Fig. 1C). The enriched sample was emulsified (Fig. 1D) together with SNARF-5F (a pH-sensitive ratiometric indicator; emission spectra of SNARF-5F in medium and calibration curve are provided in figs. S1 and S2, respectively). After incubation (Fig. 1E), the water-in-oil emulsion was reinjected into the co-flow sorting device (fig. S3). The content of acidic droplets was selectively released into the aqueous stream by applying an electric pulse across a sorting junction (Fig. 1F), and sorted cells were collected for further downstream cell characterization. For the stained samples, acidic droplets containing EpCAM⁺ or CD45⁻ cells were sorted into the aqueous phase. Additional details of assay validation and comparison of detection for different cancer cell lines (A549, LnCAP, and Sk-BR-3) with WBCs are shown in fig. S4. Recovery efficiency determined experimentally was around 60% (figs. S5 and S6), mainly due to the fact that some cells can be lost during sample preparation (pipetting, centrifugation, and washing steps). Our microfluidic method does not negatively affect cell viability when using phosphate-buffered saline (PBS) buffer for incubation, as shown in fig. S7, with ~90% of both cancer cells and WBCs viable over an incubation period of 3 hours (necessary sorting time). Representative staining images are provided in fig. S8.

**A fourfold increase in the number of hm-cells in metastatic prostate cancer patients**

Blood samples collected from 56 patients with metastatic prostate cancer and 26 healthy donors were processed as outlined above (more details about patients’ characteristics are provided in table S2 and data file S1). All studied patients were metastatic at the time of measurement, and ~70% of them were clearly progressive by prostate-specific antigen or by radiographic imaging (RECIST 1.1 criteria). The count of hm-cells was normalized to 7.5 ml of blood, for ready comparison with the CellSearch platform. We applied an upper threshold of pH 6.5 to identify droplets that contained hm-cells. The number of hm-cells was significantly higher for metastatic prostate cancer patients compared to healthy donor samples (Fig. 2A). The median number of hm-cells in 7.5 ml of blood was 11 hm-cells for metastatic prostate cancer patients and 3 hm-cells for healthy donors. Using a threshold of >3 hm-cells left 37 of 56 (66%) metastatic prostate cancer patients with a median number of 23 hm-cells.

The analysis of the number of droplets containing cells with different pH threshold values of 6.5, 6.8, or 7.0 (comprising both EpCAM⁺ and EpCAM⁻ cells) is shown in Fig. 2B. A full overview of the enumeration of hm-cells for all the metastatic prostate cancer patients and the healthy donors analyzed is available in tables S3 and S4. Additional association between biochemical parameters measured in the blood of metastatic prostate cancer patients and the number of detected hm-cells under pH 6.5 is provided in table S5. No association was found between the number of hm-cells and clinical characteristics including previous lines of treatments.

We also characterized the EpCAM⁺ subpopulation of hm-cells (Fig. 2C). Only 9% of hm-cells (pH <6.5) were EpCAM⁺ (Fig. 2D), 5% of the cells are EpCAM⁺ for pH <6.8 (Fig. 2E), and 6% of the cells are EpCAM⁺ for pH <7 (Fig. 2F). Note that these cells were both EpCAM⁺ and CD45⁻. Flow cytometry independently confirmed that all WBCs had a detectable level of CD45 fluorescence staining after CD45 depletion (figs. S9 and S10). Together, this demonstrated that a large subgroup of EpCAM⁺ hm-cells exists and that not all EpCAM⁺ CTCs were highly metabolically active (pH <6.5) (Fig. 2C).

**The number of hm-cells has prognostic value for overall survival**

Canonical EpCAM⁺ CTCs are associated with poor prognosis, with higher levels showing poorer survival (16, 17). In CellSearch method,
five EpCAM$^+$ CTCs per 7.5 ml of blood were determined as optimal cutoff for metastatic prostate cancer patients (18, 19). To evaluate the clinical relevance of hm-cells, we categorized the patient data into two groups using optimal cutoffs for prognostication purposes: patients with favorable hm-cell count (zero to five hm-cells) and unfavorable hm-cell count (more than five hm-cells). The Kaplan-Meier plot in Fig. A revealed that the presence of hm-cells is associated with a significantly lower survival probability over time ($P = 0.0217$; median survival equals 229 days for patients with more than five hm-cells compared to median not reached for patients with zero to five hm-cells). There is no indication that treatment strongly affects hm-cell counts in individual patients (see fig. S11).

The median size of sorted cells in the group of patients with zero to five hm-cell was higher, and the population was more heterogeneous compared to patients with more than five hm-cells (Fig. 3B). This information might be relevant for future research and development of CStC isolation methods based on size selection. For patient samples that were EpCAM-stained, we also verified whether the number of EpCAM$^+$ hm-CTCs would be a better biomarker for cancer prognostication than the number of hm-cells alone. Therefore, we plotted Kaplan-Meier curves for both EpCAM$^+$ hm-CTCs and EpCAM$^-$ hm-cells at pH <6.5 (fig. S12). On the basis of hm-cell count, the patients were separated into two groups using optimal cutoffs for prognostication purposes: Patients with zero to two EpCAM$^+$ hm-CTCs were associated with more favorable prognosis.

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Fig. 2. Enumeration of the hm-cells for healthy donors and metastatic prostate cancer patients (normalized to 7.5 ml of blood). (A) Comparison of the number of cells acidifying below pH <6.5 for 26 healthy donors (HD) and 56 metastatic prostate cancer patients. The median number of events is shown as a black line: 3 hm-cells for healthy donors and 11 hm-cells for patients. (B) Number of positive events detected for prostate cancer patients determined for droplets containing cells with pH <6.5, 6.8, and 7.0. Median values are 11 hm-cells for pH <6.5, 90 cells for pH <6.8, and 100 cells for pH <7.0. NS, not significant. (C) Number of EpCAM$^+$ cells detected in patient samples established for droplets acidified to pH <6.5, 6.8, and 7.0. Comparison of median between groups (A to C) was performed by Mann-Whitney test ($**P < 0.01$ and $***P < 0.001$). (D to F) Total number of EpCAM$^+$ and EpCAM$^-$ cells for individual prostate cancer patients’ samples encapsulated in droplets acidified to pH <6.5, 6.8, and 7.0, respectively. Plots presented in (A) and (B) were prepared for 56 patients and 26 healthy donors, while plots presented in (C) to (F) were determined for 26 stained patient samples.

Fig. 3. Correlation between hm-cells and survival probability. (A) Kaplan-Meier plot for 54 metastatic prostate cancer patients stratified using a cutoff value of five or less hm-cells (the evolution of the number of patients is provided in the table below the figure). Censored patients are marked with “+” over the curves. Twenty-six patients had zero to five hm-cells, and 28 patients had more than five hm-cells. $P = 0.0217$ was obtained by the log-rank (Mantel-Cox) test. Median survival was 229 days for the patients with more than five hm-cells and was not reached for the patients with zero to five hm-cells. (B) Size evaluation of detected hm-cells for patients with ≤5 (1556 cells) or >5 hm-cells (418 cells) shown as median with interquartile ranges.
compared to patients with more than two EpCAM⁺ hm-CTCs. Similarly, patients with zero to five EpCAM⁺ hm-cells had a more favorable prognosis compared to patients with more than five EpCAM⁺ hm-cells. In the graphs, a trend was seen in both cases, EpCAM⁺ hm-CTCs and EpCAM⁺ hm-cells. However, because of the small patient cohort with limited follow-up, significance was not reached (P = 0.24 for the EpCAM⁺ plots, and P = 0.07 for the EpCAM⁻ plots).

**Single-cell transcriptome analysis of hm-cells**

The molecular signature of the hm-cells isolated by the MA-Chip was evaluated by single-cell mRNA sequencing. We processed five liquid biopsies taken from metastatic prostate cancer patients and isolated hm-cells in single wells (five 96-well plates in total) by limited dilution after sorting using the MA-Chip. For comparison purposes, a plate of patient-derived WBCs was processed in parallel to determine whether WBC contamination was present in the MA-Chip–sorted cells. Subsequently, transcriptomic libraries were prepared according to the modified CEL-Seq2 protocol (section S17 and tables S6 to S13) and sequenced using NextSeq500 (Illumina). Cells with low gene or transcript counts per cell, low percentage of mappable reads, and low sequencing quality scores were excluded from the analysis, resulting in a total of 93 hm-cells and 59 patient-derived WBCs for gene expression analysis. We used t-distributed stochastic neighbor embedding (t-SNE) that readily separated the sequenced cells in two subpopulations (one of which contained all the WBCs and the other containing 80% of the hm-cells) from the six plates analyzed (Fig. 4, A and B) based on differences in the expression profile. The hm-cells were further characterized by looking at differentially expressed genes (DEGs) between the clusters. This unsupervised analysis resulted in a list of 131 DEGs (data file S2) between the two clusters, of which 69 were significantly up-regulated in cluster 0 and 62 were significantly up-regulated in cluster 1 (Fig. 4C). DEGs were then annotated [UniProt, Kyoto Encyclopedia of Genes and Genomes (KEGG) database] on the basis of the main pathways in which they were involved. Twenty DEGs in cluster 0 were specific leukocyte-related genes, and seven of the nine genes with the highest statistical significance (P adjusted value: from 2.79 × 10⁻⁸ to 7.35 × 10⁻⁵, log fold change of the average expression between two clusters: ∼2) were leukocyte-related genes (PTPRC, TRBC2, FCGR3A, CD48, CTSS, GNLY, and FCGR3B). The rest of the significantly up-regulated genes in cluster 0 were pseudogenes (36%), ribosomal proteins regulating genes (13%), noncoding RNA, or nonspecific leukocyte marker genes (expressed in many types of cells). Additional t-SNE plots show WBC-related genes exclusively expressed in the single cells belonging to cluster 0 (PTPRC or CD45, FCGR3A or CD16, TLR4 or CD284, and CD8A) in fig. S13.

The nature of hm-cells belonging to cluster 1 was diverse. The significantly up-regulated genes in cluster 1 were classified using pathway analysis (Fig. 4D), revealing an enrichment in positive regulators for 14 pathways compared to WBCs. A full overview of the classification of the DEGs between the two clusters is available in table S14. Of particular interest was that cluster 1 displayed a marked enrichment in (i) positive regulators of the extracellular matrix (ECM) organization, (ii) oncogenic mitogen-activated protein kinase signaling and angiogenesis, (iii) activation of the cell cycle, and (iv) cell metabolism. These pathways were already reported to be involved in cancer progression (20–23). Analysis of KEGG pathways was performed to determine the most statistically significant pathways characterizing each cluster (Fig. 4E). Cluster 0 (WBC cluster) showed up-regulation of genes related to the natural killer–mediated toxicity, FcγR-mediated phagocytosis, and phagosome, while cluster 1 (hm-cells cluster) was enriched for genes related to focal adhesion, platelet activation, ECM receptor interaction, and chemokine signaling pathways.

**hm-cells comprise subpopulations of CStCs, together with EpCAM⁺ and EpCAM⁻ CTCs**

Because a proportion of hm-cells would be derived from sites of metastatic prostate cancer, we checked the expression of prostate...
cancer–related genes (AR, ERG, HOXB2, KLK3, PCA3, and TMPRSS2). Figure 5A shows the total expression of prostate cancer–related genes, which is up-regulated for a fraction (3%) of hm-cells in cluster 1. To further characterize metabolically active EpCAM− cells, we checked the expression of mesenchymal-, endothelial-, and fibroblast-related genes and displayed them over the t-SNE plots (Fig. 5, B, C, and E, respectively). The choice of genes is provided in Materials and Methods. The MSC-related genes (ANPEP, CD24, CD9, ITGB1, and TFRC) are indicative of the presence of cMSCs. Up-regulation of endothelial-related genes (CD34, IGFBP7, MCAM, and VWF) found within hm-cells from cluster 1 indicated the occurrence of CECs. Another important observation was that EPCAM (epithelial cell adhesion molecule), which is often used to detect CTCs, was only expressed in a minority of hm-cells (10%) that formed a subcluster within cluster 1 (visually evident in Fig. 5D). This is in line with the number of stained EpCAM+ hm-CTCs detected by the MA-Chip. EPCAM expression did not overlap with prostate-related gene expression, indicating the presence of EpCAM+ and EpCAM− CTCs. Expression of fibroblast-related marker genes (EGF, FGF2, FGFR1, FN1, PDGFB, PDGFC, and TGFB1) strongly suggests the presence of cCAFs (24). It appeared that hm-cells with up-regulation of fibroblast-related genes detected by our platform show a good correlation with the transcriptome landscape of vascular CAFs reported by Bartoschek et al. (25) (Fig. 5F).

Platelet-related genes (ITGA2B, ITGB3, PF4, and SELP) were principally expressed in cluster 1 (fig. S14), which suggests that CStCs are covered by platelets in the bloodstream, as has also been reported in the literature for CTCs (26). An important open question is related to the EMT and, in particular, to the characteristics of CTCs in the bloodstream (27, 28). Therefore, the expression of four additional mesenchymal-related genes (MYLK, GNG11, SERPINE1, and MYL9) was superimposed on the two clusters in the t-SNE plots (fig. S15). Last, more characteristics that have been reported in the literature were evaluated, such as capabilities of the cells for invasion and EMT (fig. S16), evasion of apoptosis (fig. S17), migration and intravasation (fig. S18), extravasation and organ colonization (fig. S19), and chemoresistance (fig. S20). The expression of all these genes was up-regulated in cluster 1 (hm-cells) compared to cluster 0 (WBCs), consistent with a role in metastasis for hm-cells comprising hm-CStCs and hm-CTCs.

Last, we demonstrated how the Warburg phenotype could be maintained in cancer-associated cells (i.e., CAF) along the complex experimental setup from blood collection to pH detection in droplets. We cultured CAFs (derived from the tumor of the prostate cancer patient) under hypoxic conditions for 24 hours and subsequently measured the metabolic activity of the cells after 0, 0.5, 1, 2, and 4 hours under normoxic conditions (figs. S21 and S22). Our results.

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![Fig. 5. t-SNE plots with the cumulative expression of known genes for hm-cells compared to WBCs.](http://advances.sciencemag.org/)
 indicat that CAFs that have adopted the Warburg phenotype in the TME and circulate in the blood can be detected using our metabolic assay even hours after the “source” of the phenotype (i.e., there is no longer a TME or paracrine signaling when the cells circulate or are processed in our assay) has disappeared.

**DISCUSSION**

While the diagnosis of cancer progression and metastatic spread has focused on the detection and characterization of malignant cells, there is a growing awareness of the importance of nonmalignant cancer–associated stromal cells. It is now becoming well recognized that these cells play an important role in the priming, seeding, and growth of the metastatic niche. However, only a limited number of studies have focused on CStCs (3) and even less is known about the characteristics of such cells present in the bloodstream. Nonmalignant cancer–associated cells constitute a highly diverse and heterogeneous class of cells, and the lack of common biomarkers greatly limits the development of assays for their isolation and characterization. Here, we exploit the fact that even nonmalignant stromal cells acquire the aberrant metabolism of cancer cells while in the TME (9–11) and that this molecular signature can be picked up at the single-cell level in blood-based biopsies.

Our MA-Chip technology allows the detection of hm-cells by single-cell extracellular pH measurements in droplet microfluidics. Blood samples of metastatic prostate cancer patients were treated to remove RBCs and to deplete WBCs, and the remaining cells were subsequently encapsulated in picoliter droplets in the presence of an acid-sensitive fluorescent dye. The enumeration of acid-positive events (droplet pH below 6.5) revealed that the number of hm-cells was significantly higher on average for prostate cancer patients (11 hm-cells per 7.5 ml of blood) in comparison to healthy donors (three hm-cells). Additional immunostaining for CD45 and EpCAM revealed that only 10% of detected hm-cells are EpCAM+ CTCs, indicating that the majority of circulating hm-cells are hm-CStCs. Kaplan-Meier analysis showed that patients with zero to five hm-cells have a significantly better survival probability than patients with more than five hm-cells. This clear correlation further supports the growing realization that nonmalignant cancer–associated cells are actively involved in disease progression. These absolute numbers of cells are comparable to other reports in the literature. For example, a study focused on the detection of fibroblast-like cells found that more than five cells per 7.5 ml of blood are associated with poor prognosis in metastatic prostate cancer patients (29). For CECs, more than 40 cells per milliliter corresponded to lower progression-free survival in metastatic colorectal cancer patients (30).

It should be noted though that these comparisons are only indicative of the rare nature of these cells; detection based on size or physical properties versus immunostaining versus metabolic activity will inevitably capture a slightly different (but probably overlapping) subset of rare cells in blood-based biopsies. The MA-Chip not only detects hm-cells but also can sort acid-positive droplets for further analysis. Downstream single-cell mRNA analysis allowed us to compare the expression profile of patient-derived WBCs and hm-cells isolated by the MA-chip. Differential gene expression analysis discriminated the WBCs from all other cells and revealed that EpCAM+ hm-cells (canonical CTCs) formed a subcluster within the other hm-cells. Further analysis of the most prominently expressed genes for metabolically active EpCAM+ cells allowed us to assign these as EpCAM+ CTCs due to the expression of prostate cancer–related genes: AR, ERG, HOXB2, KLK3, PCA3, and TMPRSS2; cCAFs based on up-regulation of EGF, FGFR2, FGFR1, FN1, PDGFB, PDGFC, and TGFβ1; cMSC that exhibited higher expression of ANPEP, CD24, CD9, ITGB1, and TFRC; and CECs with up-regulation of CD34, IGFBP7, MCAM, and VWF.

The aberrant cancer-like metabolism thus seems to be a general biomarker for detection of not only cancer cells but also CStCs (cCAFs, cMSCs, and CECs) derived from the TME. The detection and isolation method provided by the MA-Chip method has significant potential in retrieving a much wider set of circulating nonmalignant tumor–associated cells than possible when using specific markers. Because of the CD45 depletion step in our protocol, cancer-associated macrophage-like cells or cancer-associated neutrophils are mostly removed before encapsulation of cells in droplets. Furthermore, although acidification of the TME is one of the hallmarks of cancer (7, 8), CStCs, WBCs, and cells of the immune system also do secrete lactic acid under certain conditions (9–14, 31). Therefore, our method may be best suited for assessing prognosis and monitoring the efficacy of treatment or disease progression, and not for, for example, large-scale screening studies to detect early-stage disease. We also note that our current MA-Chip prototype is relatively slow, with sorting rates in the range of 200 to 300 Hz. Here, we used CD45 depletion to remove excess WBCs (and thus shorten experiment time), but positive enrichment using size-based technologies (commercially available from a.o. Creatv MicroTech, Parsortix) is an interesting alternative. Increasing the sorting speed could also offer a promising option to analyze bigger volumes of sample, allowing organoid culture and longitudinal analyses of CStCs and CTCs, for example, in the context of clinical trials with agents specifically targeting cells within the TME.

The MA-Chip–based assay can be easily combined with various types of downstream analysis, i.e., DNA or RNA sequencing (bulk or single-cell) or immunostaining. Subtyping using genomic, transcriptomic, or proteomic biomarkers would allow a much deeper understanding of the clinically relevant hm-CStCs detected and isolated in this study. This is especially relevant as CStCs are highly heterogeneous, and their populations likely change during disease progression and in response to treatment. Future studies should include single-cell RNA sequencing on isolated hm-cells from patients during various stages of treatment. Such studies might shed further light on the role CStCs play in disease progression and potentially offer previously unidentified targets for personalized therapies.

We report here the first study on the enumeration, isolation, and characterization of clinically relevant CStCs based on their aberrant metabolism, a promising general biomarker for cancer-associated cells disseminating from the TME. The correlation between the number of hm-cells detected by the MA-Chip with overall patient survival probability suggests that the analysis of liquid biopsy should be expanded to interrogate the entire population of cancer-initiating and cancer-associated cells.

**MATERIALS AND METHODS**

**Study design**

A total of 82 participants including 56 patients with metastatic prostate cancer (all patients were castration resistant) and 26 healthy donors were recruited. Blood was typically withdrawn when the patient was progressing biochemically or radiographically but before
the initiation of a new line of therapy. Two patients were not included in the Kaplan-Meier analysis due to lack of the date of the last follow-up.

**Clinical samples**

Blood collection for this study was approved by the medical ethics review committee under a biomarker protocol at the Radboud University Medical Center (CMO 2016-2793). The samples from metastatic prostate cancer patients were collected between October 2017 and November 2018 at the Radboud University Medical Center. Baseline characteristics were prospectively collected, and survival was updated until April 2019. Written informed consent was obtained from all patients according to good clinical practice for the use of their biomaterials as approved by the institutional review boards and local committees on research involving human subjects (ethics commission of the Radboud University Medical Center, Nijmegen, project number NL60249.091.16).

**Sample preparation**

Fresh whole blood (3 to 6 ml) was taken with a venous puncture and collected in K$_2$-EDTA Vacutainer tubes (Becton Dickinson). A sample processing was started no longer than 30 min after a blood draw. RBCs were lysed by osmotic shock using BD Pharm Lyse Lysing Buffer (BD Biosciences, USA) following the manufacturer’s protocol. Cells were then spun down, the supernatant was removed, and the pellet was resuspended in PBS (20012019, Thermo Fisher Scientific, USA) supplemented with 0.5% (w/v) bovine serum albumin (BSA) and 2 mM EDTA (final pH 7.4 adjusted by titration with NaOH solution). Tumor cells were subsequently enriched with CD45 and GlycophorinA depletion microbeads (Miltenyi Biotec, Germany) following the manufacturer’s instructions. Briefly, 30 µl of CD45 microbeads and 10 µl of GlycophorinA microbeads were added to 150 µl of cell suspension and incubated for 15 min at 4°C. Then, 2 ml of PBS (with BSA and EDTA) was used to wash the walls of falcon, and the sample was spun down to remove the supernatant. The pellet was gently resuspended in 500 µl of PBS (supplemented with 0.5% BSA and 2 mM EDTA) and loaded through LD column (Miltenyi Biotec, Germany), according to the protocol attached by the manufacturer. In the case of staining, the enriched sample was incubated with Brilliant Stain Buffer (the buffer mixed with the sample in the ratio 1:2; 563794, BD Horizon, USA), EpCAM BV421 mouse anti-human (1:100 dilution; 563180, BD Horizon), and CD45 BV480 mouse anti-human (1:100 dilution; 566156, BD Horizon). Last, the sample was resuspended in solution composed of Joklik’s modified EMEM with BSA (w/v), 2 mM EDTA (final pH 7.4 adjusted by addition of NaOH solution) mixed with 15% (v/v) OptiPrep Density Gradient (Sigma-Aldrich, USA), and 11 µM SNARF-5F (Thermo Fisher Scientific, USA) before emulsification. All solutions were filtered through sterile polyvinylidene difluoride filters (pore size, 0.22 µm) before use. PBS and Eagle’s MEM (MEM)–based medium were stored on ice before cell resuspension and washing steps.

**Microfluidic device fabrication for emulsification and sorting**

Microfluidic devices were produced by using photo and soft lithography. Silicon wafers were spin-coated with a uniform layer of SU-8-2025 photoresist (MicroChem Co., USA), soft-baked, ultraviolet-exposed through transparency mask (JD Photo-Tools, UK), baked again post-exposure, developed, and hard-baked according to the manufacturer’s protocol (MicroChem Co., USA). After production, the height of the structures was measured by Dektak profilometer. The emulsification devices had the height of 25 µm, and the sorting devices had the height of 30 µm. These wafers were used as masters for the polydimethylsiloxane (PDMS) devices. PDMS prepolymer and cross-linking agent were mixed at a 10:1 ratio (w/w) and poured on the master bearing the microchannel structure. The PDMS was further degassed (for 30 min) and cured at 65°C for at least 2 hours. Then, the PDMS replica was cut out from the master, and the inlets and outlets were punched using a biopsy puncher of 1 mm inner diameter (pfn medical, USA). Thereafter, the replica and a glass slide were carefully washed first with a mixture of soap and water and then with ethanol. The clean replica was bonded to the glass slide after oxygen plasma treatment (Femto, Diener electronic). The channels of emulsification device were rendered hydrophobic by silanization with 5% 1H,1H,2H,2H-perfluorooctyltriethoxysilane (Sigma-Aldrich, USA) in FC-40 (Sigma-Aldrich, USA). In the case of sorting devices, two phases were run parallelly to modify channels: (i) 5% solution of 1H,1H,2H,2H-perfluorooctyltriethoxysilane (Sigma-Aldrich, USA) in FC-40 (v/v) to modify oil phase channel and (ii) 1% (v/v) 11-bromoundecyldimethylchlorosilane (Fluorochem, UK) in cyclohexane to modify water phase channel. During modification, these two phases were run in a co-flow manner. Then, the chip was flushed with HFE-7500 oil 3M (Fluorochem, UK). The emulsification and sorting devices were incubated at 95°C overnight. Electrodes in a sorting chip were prepared immediately before analysis. Small rods (2 to 3 mm) of indium (51%), bismuth (32.5%), and tin (16.5%) alloy (Indium Corporation of America, IPN 51962) were introduced into punched holes, and a device was placed on the hot plate. Once alloy started to melt, we placed tinned copper wires (Rowan Cable Products Ltd., TCW21 1230994) in the channels to provide the contact. The electrodes in the sorting chip were observed under the microscope whether they completely filled channels.

**Droplet production and incubation**

A sample, cells resuspended in Joklik’s modified EMEM with BSA, EDTA, OptiPrep, and SNARF-5F, was emulsified immediately after preparation. Monodisperse water-in-oil droplets were generated by using the emulsification device described above with 20-µm-wide T-junction. The composition of the continuous phase was 2.5% (w/w) of RAN (RAN Biotechnologies, USA) in HFE-7500. The flow rate for the continuous phase was 700 µl/hour, and the flow rate for the dispersed phase was 300 µl/hour, which allowed the production of 30-pl droplets. Emulsification step was performed at 4°C to decrease cell metabolism and prevent the secretion of lactate acid in the bulk. The collected emulsion was incubated in 37°C in the thermal block for 30 min. After the incubation step, the sample was immediately placed on ice.

**Detection and sorting**

A sorting chip with three inlets (water phase, droplet, and oil spacer) and three electrodes embedded in PDMS was used for droplet re-injection and sorting process. The water phase was composed of 50 mM tris (15567027, Thermo Fisher Scientific, USA) and 150 mM NaCl (S3014, Sigma-Aldrich, USA) in nuclease-free water (Ambion, Thermo Fisher Scientific, USA). The oil phase consisted of 0.5% (w/w) of RAN in HFE-7500 oil. The emulsion was loaded to the tubing with a previously made air spacer to avoid spreading droplets in an
HFE-7500–loaded syringe. The tubing with emulsion was wrapped around the ice pack to prevent the increase of temperature and further secretion of lactic acid by the cells. The flow rates of the water phase, oil spacer, and emulsion were ~3000, 300, and 30 μl/hour, respectively. Solutions were pumped using neMESYS (CETONI GmbH, Germany) syringe pumps. First, the water phase was run through the chip. As soon as the flow was stabilized and the water phase was not dripping into the oil channel, droplets were introduced into the channel, flowing one by one. A modified microscope setup for detection of acidic droplets is described elsewhere (15). An inverted microscope (Olympus IX71, Japan) was used to detect droplets with acidified pH. Expanded (2×) laser beam (408 Argon-ion Cyonics) was focused down with a cylindrical lens across the microfluidic channel. The fluorescence signal was collected with a 40× objective (Olympus LUCPlanFLN, 40×/0.60), and four fluorescence bands (450, 525, 580, and 630 nm) were acquired using a cRIO-9024 acquisition system (National Instruments) at a scan rate of 10 Hz. In-house written LabVIEW software was used to control the acquisition system to detect all data points of droplets over a given threshold and provides trigger pulse for image capture. The voltage generation setup consisted of a 33220A arbitrary waveform generator (Agilent, USA) and high-voltage power amplifier (TREK model 2220, TREK Inc., USA). The function generator was used to generate sine voltage waveform of the electric field, typically at 3.5 V (Vpp), 5 kHz (output five cycles) that was amplified by a factor of 200. Further details can be found in the Supplementary Materials (fig. S23). The count of detected hm-cells was normalized to 7.5 ml of blood for convenient comparison with the CellSearch method.

**Statistical analysis**

Pearson’s correlation (r) was tested for associations between hm-cells and other continuous baseline prognostic variables using SPSS software platform, and the t test was used for differences between hm-cells and dichotomous variables.

**Survival probability plot**

Median overall survival (OS) was estimated using the Kaplan–Meier method, and multivariable Cox proportional hazard models were tested for associations with OS. The Kaplan–Meier survival plot was obtained using GraphPad Prism 8 (version 8.0.1). Data were censored at the date of the last follow-up when death had not occurred. Log-rank (Mantel-Cox) test was used to compare the survival curves. The optimal cutoff for prognostication purposes was evaluated based on c-statistics. P < 0.05 was considered significant. The Kaplan–Meier plot shown in Fig. 3A is based on data for 54 of 56 patients due to missing clinical information.

**Library preparation for single-cell mRNA sequencing**

The cells after sorting were collected in four to five sterile 1.5-ml low-binding Eppendorf tubes and kept at 4°C. The cell suspension obtained after sorting was concentrated by centrifugation (5 min, 500 relative centrifugal force, 4°C), pooled together, and subsequently centrifuged, yielding in 100 μl. Single cells were distributed in 96-well plates with barcoding primers, by limited dilution, by pipetting 1 μl of cell suspension solution in each well. To avoid doublets in wells, we used λ = 0.5 to 1 cell per well. If more than 96 cells were sorted out, then an additional plate was used for barcoding of the mRNA material. The plates with the cells and barcoding primers were stored at −80°C until further processing. The library preparation was based on CEL-Seq2 (32). A detailed protocol is provided in section S17. Prepared libraries were sequenced with NextSeq500 (targeting 10 million reads per sample).

**Single-cell mRNA data processing**

From the FASTQ files generated by the Illumina sequencer, transcriptome count tables were generated using the published CEL-Seq2 pipeline (32) with some minor modifications. To allow compatibility with the pipeline, read1 and read2 were swapped. Briefly, first, the reads were demultiplexed to be assigned to each to a specific cell. Second, they were aligned to the known human genome using Bowtie. Last, UMIss (Unique Molecular Identifiers) for each cell were counted and a count table was generated. Further processing of the data was performed in R software using Seurat V3 package (33). All cells from the different plates analyzed were merged in one Seurat object. Quality check excluded cells with less than 200 genes per cell and with more than 5000 genes per cell detected from sequencing. The count of transcripts per cells was normalized using Seurat in the form of reads per million.

**Differential expression and pathways analysis**

Single cells were devised in two clusters in an unsupervised manner using t-SNE in Seurat package. The single-cell counts were normalized and scaled to remove unwanted sources of variation. Analysis was run on 13 significant principal components, and clusters were generated from Seurat package setting the resolution variable to 0.5. Last, we identified the DEGs between the clusters in unbiased way using Wilcoxon rank sum test. Only genes detected in a minimum fraction of 30% of cells in one of the two populations and that showed, on average, at least 0.25-fold difference (log scale) between the two groups of cells were tested. Gene set overrepresentation analysis was performed using UniProt database and KEGG.

**t-SNE plots with the collective expression of known genes**

The cumulative expression of genes with known functions or characteristics of a cell type was plotted over the t-SNE. For the MSCs, we chose the genes presented as positive cell markers on LifeMap Discovery. For the fibroblasts, we used genes related to ECM components (FN1 or fibronectin1), growth factors, and cytokines (EGF, FGFR2, PDGFβ, PDGFC, and TGFβ1) and related to receptors and other membrane-bound proteins (FGFR1), which were expressed in our dataset and were mostly used to identify CAFs in literature (24). Last, for the endothelial genes, we summed up the most frequently used markers in the various CEC assays (34), when expressed in our dataset.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/40/eaaz3849/DC1

View/request a protocol for this paper from Bio-protocol.

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Competing interests: A. P. and W. T. S. H. are inventors on a patent related to this work filed by Università degli Studi di Udine (Italy) and Stichting Katholieke Universiteit (The Netherlands) (no. PCT/IB2014/067057, filed on 18 December 2014, published on 1 May 2018). All other authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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