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TITLE: Early Detection of Castration-Resistant Prostate Cancer by
Assessing Interactions Between Circulating Tumor Cells and
Accompanying Immune Cells

PRINCIPAL INVESTIGATOR: Tim H.M. Huang, PhD.

CONTRACTING ORGANIZATION: The University of Texas Health Science Center at San
Antonio

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14. ABSTRACT Prostate cancer is the most frequent diagnosed cancer in men. In majority of 'castration sensitive' patients proliferation of cancer cells depends on supply of androgen and can be attenuated by the androgen deprivation therapy (AOT). Unfortunately, many patients develop "castration resistance" (CR), when the tumor growth and metastatic spread continue despite ADT. For effective second-line therapy that saves lives and improves life quality, the resistance needs to be detected early. To reach the goal of early detection, we test properties of rare cells that are responsible for spreading metastasis. These circulating tumor cells (CTCs) shed from the primary tumor or metastatic lesions are isolated from the standard blood sample and are considered "seeds of metastasis". The majority of CTCs die, however the surviving "aggressive" cells travel with blood, undergo epithelial-to-mesenchymal transition (EMT), extravasate and start secondary tumor growth in distant organs. Our specific aims call for determining the role and interactions with circulating macrophages in survival-promoting mechanical fitness of CTCs. Combining the cell culture studies with profiling of CTCs leads to construction of a predictive model for early CR detection.					
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1. Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men. In majority of “castration sensitive” patients proliferation of cancer cells depends on supply of androgen and can be attenuated by the androgen deprivation therapy (ADT). Unfortunately, many patients develop “castration resistance” (CR), when the tumor growth and metastatic spread continue despite ADT. For effective second-line therapy that saves lives and improves life quality, the resistance needs to be detected early. To reach the goal of early detection, we propose to test properties of rare cells that are responsible for spreading metastasis. These circulating tumor cells (CTCs) shed from the primary tumor or metastatic lesions are isolated from the standard blood sample and are considered “seeds of metastasis”. Majority of CTCs die, however the surviving “aggressive” cells travel with blood, undergo epithelial-to-mesenchymal transition (EMT), extravasate and start secondary tumor growth in distant organs. Since CTCs have to escape from the tumor and then survive in the turbulent blood stream, they have to be mechanically fit. Indeed, we found in retrospective studies that CTCs obtained from CR patients are much softer, deformable and more adhesive than CTCs from CS patients. CTCs are often accompanied by innate immune cells, mostly macrophages. We found that interactions with macrophages of certain polarization may help CTCs to survive. Additionally, we identified a subpopulation of CTCs, tumor-macrophage hybrid (TMH) cells, exhibiting epithelial tumor and macrophage phenotypes. TMH cells were revealed by single-cell proteomics based on cytometry by time of flight (CyTOF) of PBMCs derived from prostate cancer patients. We were able to recapitulate the formation of TMH cells *in vitro* and *in vivo* through incomplete phagocytosis of tumor cells by macrophages. TMH cells were more aggressive than parental tumor cells in proliferation, nanomechanic features and prone to metastasis through immunevasion. We postulate that mechanical and immunochemical profiling of CTCs and co-purifying immune cells (tumor associated circulating cells; TACCs) provides clues about CTCs aggressiveness and the risk of CR. This final report also encompasses months 37-48 of the 1-year no-cost extension (NCE). The extension was granted after the COVID- 19 pandemic conditions significantly slowed down accrual of new patients and collection of follow-up blood samples for our study, both in 2020 and in 2021. The pandemic-related problems in supply chains slowed-down the planned research as well. The NCE was utilized to reach the goals of the project. In short, our specific aims called for determining the role of (1) epithelial-mesenchymal transition and (2) interactions with circulating macrophages in survival-promoting mechanical fitness of CTCs. We

postulate that combining the cell culture studies with profiling of CTCs will lead to (3) construction of predictive model for early CR detection. We reached the goals, as outlined in the Statement of Work (SOW). This is the final report summarizing what we have achieved through the entire project duration, including the extended year.

2. Keywords

androgen deprivation therapy
atomic force microscopy
castration resistance
castration sensitivity
cell adhesion
cell deformation
cell stiffness
CD47
circulating tumor cells
cultured cells
Cytometry by time-of-flight
don't eat me signals
epithelial-mesenchymal transition
gene expression
immune cells
immunostaining
immunoevasion
liquid biopsy
macrophage
macrophage polarization
mass cytometry
mechanical phenotype
metastasis
prostate cancer
protein expression
single-cell profiling
tumor-macrophage hybrid cells

3. Accomplishments

3.1 The major goals for the project, as stated in the approved SOW, with % of completion

Research-Specific Tasks:			
Specific Aim 1: We will determine the role of epithelial to mesenchymal transition in mechanical fitness of CTCs.	Months	Participants	% completion (mo. 1-48)
Major Task 1: Recruit post-castration metastatic PCa patients experiencing biochemical recurrence and starting first line ADT	4-26	Drs. Huang, Liss	100
Major Task 2: Isolate and immunostain TACCs from the samples of blood drawn from the patients right before the start of ADT (70 patients, time t0), enumerate CTCs.	4-28		100
Subtask 1: Perform microfiltration, immunostain the cells. Device for cell isolation: ScreenCell CC ha (ScreenCell)	4-26	Drs. Osmulski, Gaczynska, Chen,	100
Subtask 2: Enumerate the isolated and immunostained CTCs and classify them as EpCAM ⁺ or EMT-CTCs according to surface antigen expression.	4-26	Drs. Gaczynska, Osmulski	100
Major Task 3: Collect multiparameter nanomechanical and morphological data on individual isolated CTCs using PeakForce Quantitative Nanomechanics (PF QNM) AFM imaging.	4-30		100
Subtask 1: Collect the AFM images.	4-26	Drs. Osmulski, Gaczynska	100
Subtask 2: Perform image and data analysis on the collected AFM images.	4-30	Drs. Osmulski, Gaczynska	100
Major Task 4: Perform the gene expression analysis on CTCs.	4-26		100

Major Task 5: Recapitulate EMT in cell culture model	6-24		100
Subtask 1: Culture 22Rv1 and DU145 cells (source: ATCC) according to ATCC recommendations. Induce EMT by treatment with TGF- β . Perform mechanical phenotyping of selected cultured cells.	6-18	Drs. Osmulski, Gaczynska	100
Subtask 2: Perform gene expression analysis of cultured cells that were mechanically phenotyped.	6-18	Dr. Chen	100
Specific Aim 2: We will define the role of CTC-macrophage interactions in mechanical fitness of CTCs.			
Major Task 6: Define the functional composition of macrophage population in TACCs preparations isolated from patients' blood.	1-26		100
Subtask 1: Enumerate and classify non-CTC patient-isolated TACCs according to immunostaining, enumerate CTC-immune cell clusters.	1-24	Drs. Gaczynska, Osmulski	100
Subtask 2: Perform the gene expression analysis on randomly selected cells (up to 30) bearing immune cell markers.	1-32	Dr. Chen	100
Major Task 7: Recapitulate the interactions of model "CTCs" (prostate cancer cell lines) cultured with polarized macrophages.	12-30		100
Subtask 1: Co-culture 22Rv1 and DU145 cells with model macrophages derived from U937 cells (source: ATCC). Determine rates of growth of co-cultured cells, enumerate cell clusters.	12-24	Drs. Gaczynska, Osmulski	100
Subtask 2: Perform mechanical phenotyping of selected cultured cells, free or in clusters.	12-24	Drs. Osmulski, Gaczynska	100
Subtask 3: Perform the gene expression analysis on model CTCs and macrophages.	12-30	Dr. Chen	100
Specific Aim 3. We will construct a model for patients' stratification predicting the risk of castration resistance based on the mechanical fitness of CTCs.			

Major Task 8: Collect mechanical and immunocytochemical properties of TACCs isolated at time t1 (6 months or at failure)	6-30		100
Subtask 1: Perform microfiltration, immunostain the cells, enumerate as for t0.	6-30	Drs. Gaczynska, Osmulski	100
Subtask 2: Perform mechanical profiling of CTCs at time t1, perform image and data analysis on the collected AFM images as for t0.	6-30	Drs. Osmulski, Gaczynska	100
Subtask 3: Compare clinical and biophysical/gene expression data at t1	6-30	Drs. Huang, Liss, Gelfond, Osmulski, Gaczynska	100
Major Task 9: Derive a score for patients' stratification based on the mechanical and immunocytochemical profiling of TACCs	24-36		100
Subtask 1: Develop a mathematical model of EMT and macrophages contribution to the mechanical fitness of CTCs using growth and phenotypic parameters extracted from the cell culture and t0-t1 patients' TACCs data. The model will aid developing the score in Subtask 2.	24-30	Drs. Gelfond, Osmulski	100
Subtask 2: Based on the cumulative mechanical, immunocytochemical and gene expression data construct a risk score for PT stratification and CR prediction.	30-36	Drs. Huang, Gelfond, Liss, Osmulski, Chen, Gaczynska	100

Selected tasks were completed in year 4 (NCE) such as analysis of mechanical and gene expression data, comparison of clinical and biophysical/gene expression data, and model development.

3.2 Specific accomplishments under the Major Tasks listed above:

3.2.1 *Specific Aim 1, Major Task 1*

We planned to accrue a total of 70 metastatic prostate cancer patients starting first-line ADT. We reached the goal fully, with a total of 76 patients accrued (Table 1).

Table 1 presents the total accrual numbers, with information on data collected, relevant for Major Tasks 1-4, 6 and 8, (entire period; 08/15/2018 – 08/14/2022).

Number	Total	Total 1 visit only (t0)	Total 2 visits (t0 and t1)
Patients (PTs) accrued	76	39	37
PT samples with TACCs Collected for Mechanical and Immunochemical Phenotyping	113	39	74
TACCs Retained on Filters (approximately)	18000	5800	12000
PT Samples Mechanically Phenotyped	113	39	74
PT Samples with Mechanical Phenotype Analyzed	113	39	74
CTCs with Mechanical Phenotype Collected	1227	676	551
CTCs with Mechanical Phenotype Analyzed	865	370	495
PT Samples with Immunostaining Performed and Cell Images Collected	113	39	74
Immunostaining Images Collected	4500	1521	3000
PT Samples with TACCs Enumeration Completed	113	39	74
PT Samples with Cells Collected for Gene and Protein Expression Analysis	71	39	32
PT Samples with TACCs analyzed by single-cell transcriptomics	20	20	0
CTCs analyzed by single-cell transcriptomics	273	273	0
Immune cells analyzed by single-cell transcriptomics	143	143	0

3.2.2 Specific Aim 1, Major Task 2

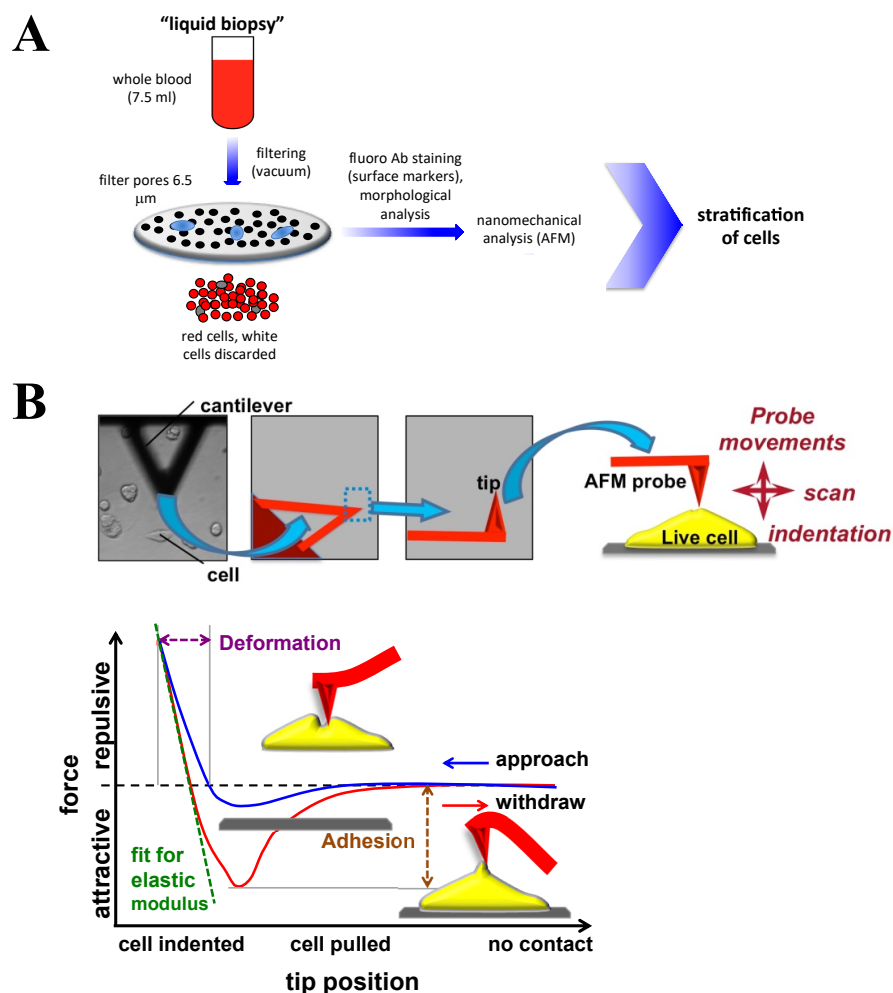


Figure 1 Processing of liquid biopsy samples from prostate cancer patients (as in report period 1).

A: isolation of large tumor associated circulating cells (TACCs). A separate filter is used to collect additional TACCs for gene and protein expression analysis. **B:** nanomechanical profiling: a small silicon probe on a cantilever "pokes" the cell with extremely small (nanoNewton-scale) forces to collect "force curves" used to extract precise numerical data on elasticity (reversible change of shape), deformability (reversible and non-reversible, nondestructive change of shape) and adhesiveness.

For all blood samples we collected filters with retained cells and images of immunostained TACCs (*Subtask 1*). The patient's blood samples were processed within two hours from phlebotomy, as planned. Processing included microfiltration with the ScreenCell device retaining all cells that do not pass through 6.5 μm pores randomly distributed in the filters. The filters are formulated for cells' adherence, important to hold cells for AFM profiling. The cells still attached to the filters were subjected to nanomechanical imaging, as presented above in **Figure 1**.

All large cells collected on filters are immunostained and photographed for enumeration and classification. (*Subtask 2*). See **Table 1** for details. Analysis of correlations of enumeration of distinct classes of CTCs and mechanical phenotypes of CTCs is presented below.

3.2.3 Specific Aim 1, Major Task 3

Mechanical phenotypes of single CTCs were collected with atomic force microscopy (AFM) PeakForce Quantitative Nanomechanical mode for all collected samples immediately after microfiltration, as planned (*Subtask 1*), and the data processing and analysis proceeded (*Subtask 2*; see **Table 1** for details). For all analyzed CTCs, a full mechanical phenotype with cell elasticity (=stiffness), deformation and adhesion (**Figure 1**), was collected.

Analysis of the significant portion of the patient-derived data was the core of paper published in August 1, 2021 issue of *Cancer Research*, and highlighted in the journal's issue, with AFM- derived images of scanned CTCs and accompanying immune cells on the *Cancer Research* cover (**Figure 2**). The highlights from the paper relevant for *Task 3* are presented below in **Figures 3 and 4**.



Highlight:

Contacts with Macrophages Promote an Aggressive Nanomechanical Phenotype of Circulating Tumor Cells in Prostate Cancer

Pawel A. Osmulski, Alessandra Cunsolo, Meizhen Chen, Yusheng Qian, Chun-Lin Lin, Chia-Nung Hung, Devalingam Mahalingam, Nameer B. Kirma, Chun-Liang Chen, Josephine A. Taverna, Michael A. Liss, Ian M. Thompson, Tim H.-M. Huang and Maria E. Gaczynska DOI: 10.1158/0008-5472.CAN-20-3595 Published August 2021

About the cover: Circulating tumor cells (CTC) and macrophages interact and form pairs. The interactions increase metastatic potential of CTCs. Atomic force microscopy (AFM) imaging created the pseudo-3D reliefs of CTCs (yellow-orange-red) and macrophages (blue-navy-green), isolated from the blood of prostate cancer patients. Nanomechanical analysis by AFM helped to assess mechanical fitness of CTCs, indicating their capacity to survive and initiate metastasis.

Figure 2 The research performed as a part of this project was described in the recent *Cancer Research* paper and was highlighted in the journal's August 1, 2021 issue. The paper introduced the concept of “mechanical fitness” of CTCs (*Specific Aim 1, Major Task 3*), presented how the fitness is associated with EMT plasticity (*Specific Aim 1, Major Tasks 2 and 5*) and with the presence of circulating macrophages co-purifying with CTCs (see below *Specific Aim 2, Major Tasks 6 and 7*).

Figure 3 presents the classification of CTCs into the categories, and “mechanical fitness chart” based on the classification. CTCs in categories 3 and 4 were exceptionally soft and exceptionally adhesive, respectively, and considered “best fit” to survive and thrive in circulation.

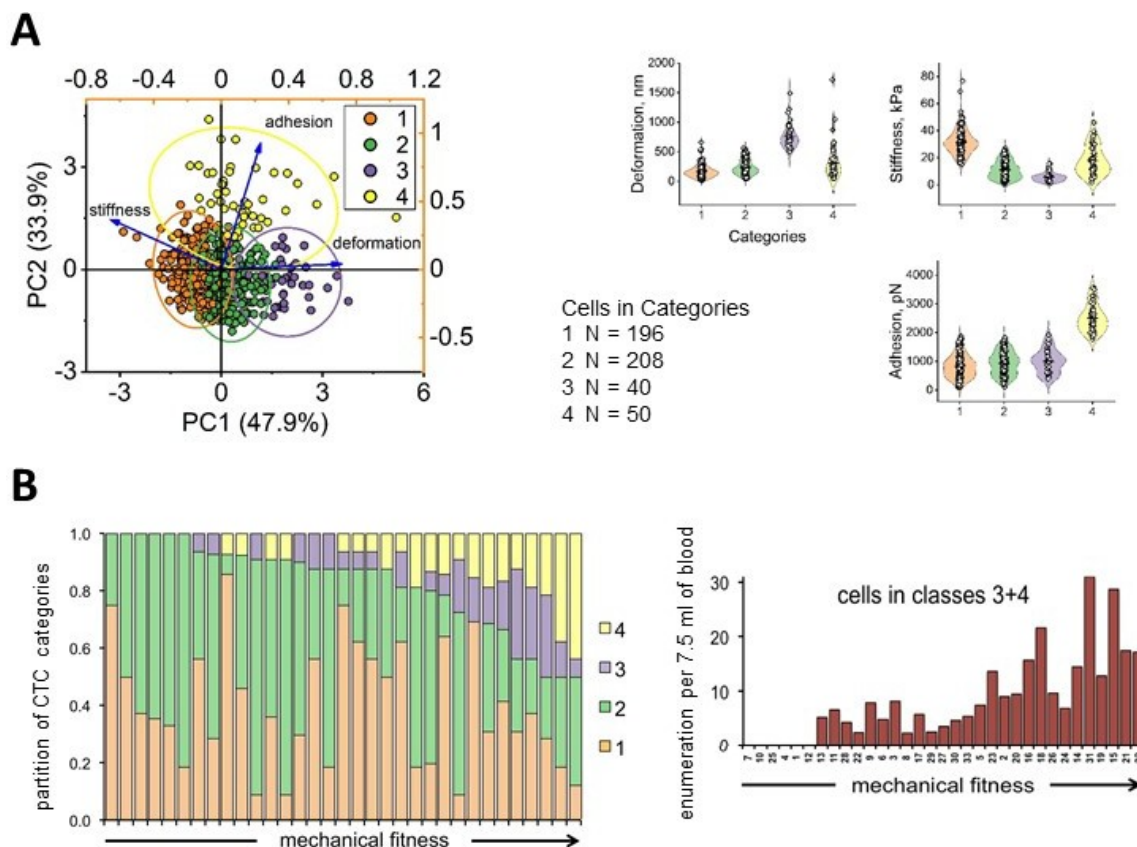


Figure 3 Phenotypic diversity and mechanical fitness of 494 CTCs isolated from 33 blood samples from 23 patients. **A.** Principal component analysis (PCA; left) reveals the presence of four categories of cells with distinct mechanical phenotypes (right). Cell categories were identified with the unsupervised hierarchical clustering. Categories 3 and 4 contain the softest and most adhesive cells, respectively, that we consider the most “mechanically fit” to withstand mechanical stress in the bloodstream, survive and proceed with extravasation and seeding metastasis. **B.** The “CTC fitness chart” of prostate cancer patients. Each column represents data for CTCs isolated from a single blood sample. The samples are ordered according to relative abundance of CTCs belonging to best-fit mechanical categories 4 and 3. Right: enumeration of cells in categories 3 and 4 followed the “fitness axis” of relative abundance of CTCs.

The uniqueness of mechanical fitness as a special feature of CTCs and not tumor cells in general was demonstrated by comparison of mechanical phenotypes of CTCs and “urine prostate cells” (UPCs) shed by prostate tumors to the urine of patients (**Figure 4A**). Both CTCs and UPCs are exposed to damaging fluid shear stress; however only CTCs may act as “seeds of metastasis” while UPCs inevitably perish. We used nanomechanical parameters collected for 122 CTCs (n=10 patients) and 104 UPCs (n=11 patients) positive for prostate-specific markers PSA/PSMA. The patient cohort was comprised of high-risk patients with local disease and patients with low-volume metastatic spread. The data for UPCs were collected in the past, whereas CTCs were collected from the blood of patients (a random subset) accrued for this study. The striking difference between mechanical phenotypes of CTCs and UPCs is apparent in **Figure 4**. With principal component analysis (PCA), we found that CTCs appeared five-fold more adhesive than UPCs. Analysis of stiffness and deformability revealed a remarkable diversity among UPCs: one class of UPCs was not significantly different from CTCs, whereas the other class was almost seven times stiffer than CTCs (**Figure 4B**). We speculate that those stiff UPCs may undergo apoptosis, consistently with published data reporting cell death accompanying increase in stiffness. High softness and low adhesion are known mesenchymal hallmarks of aggressive cancer cells, and the soft, non-adhesive class of UPCs embodied these traits. However, the majority of CTCs retained epithelial adhesion. At the same time the CTCs displayed mesenchymal properties for softness. Consistently, we recognized high adhesion and softness as distinctive “mechanical fitness” features demonstrated by CTCs but not by UPCs. Since CTCs may originate from metastatic sites, not only the primary tumor as assumed for UPCs, we tested if CTCs isolated from patients with confirmed distant spread at the time of blood draw would form a separate class. Two out of ten patients used for this study were metastatic, however phenotypes of their CTCs did not form a separate class in the population of all CTCs (**Figure 4C**).

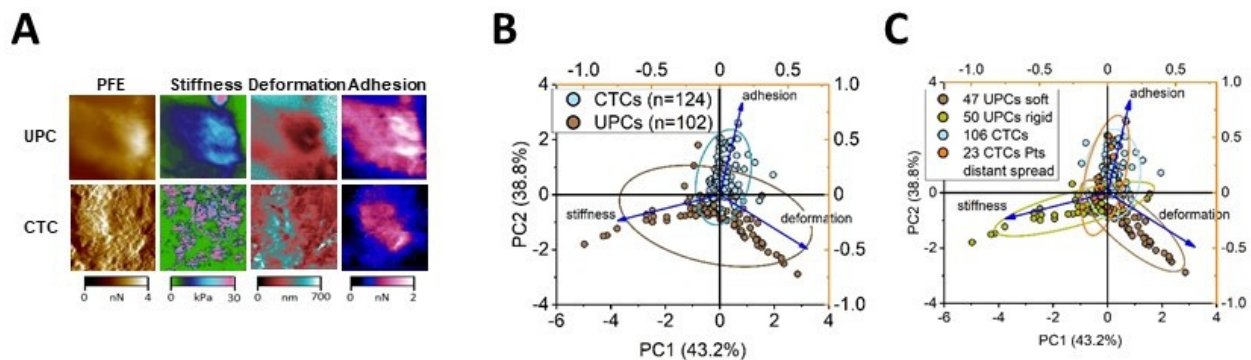


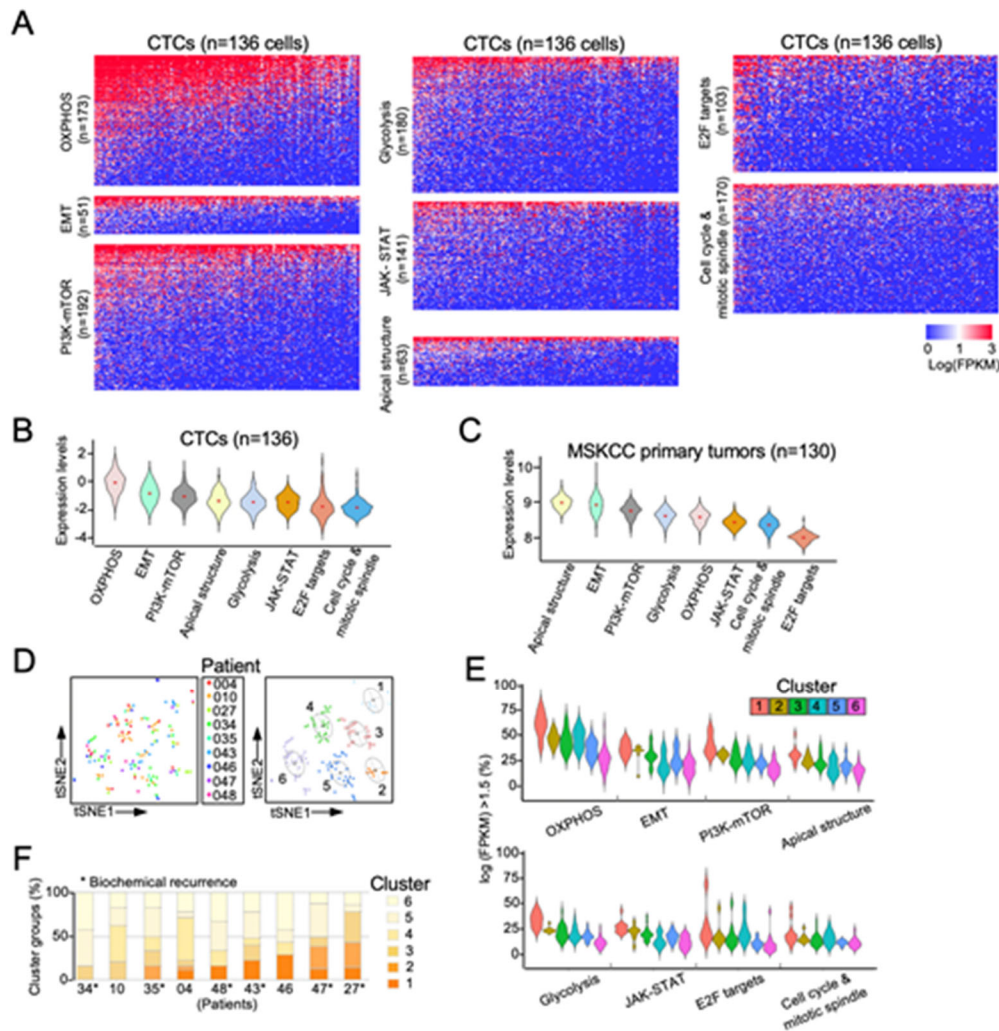
Figure 4 Urine prostate cells (UPCs) and circulating tumor cells (CTCs) present distinct nanomechanical properties. **A.** Comparison of typical examples of mechanical properties between a UPC and CTC. Each row represents the same single cell and its mechanical properties are arranged in columns. The first column: cell topography (peak force error channel; PFE; Bruker). In this example the UPC was more stiff, less deformable and more adhesive than the CTC. Images were false colored using scales covering the data extension; scale 10x10μm. **B and C:** Principal component analysis (PCA) based on the mechanical phenotype of cells separates population of 122 CTCs (n=10 patients) from 104 UPCs (n=11 patients), with CTCs generally more adhesive than UPCs. **C.** CTCs isolated from metastatic patients are colored differently than CTCs from high-risk patients with no distant spread detected at t0.

Major outcomes from this part of the study (*Specific Aim 1, Major Task 3*) are:

1. CTCs are not only detectable in blood, but also can be distinctively aggressive even before distant spread is clinically confirmed.
2. High adhesion is a hallmark property of CTCs, as opposed to tumor cells.
3. CTCs are generally less soft than tumor cells, however invasive CTCs from advanced patients may become softer than CTCs from less-advanced patients.

3.2.4 *Specific Aim 1, Major Task 4*

The CTCs were collected (*Task 2, Subtask 1*) and the gene expression analysis was performed for a subset of collected CTCs, as planned. The outcomes for *Task 4* were presented in the 2019 and 2020 reports. **Figures 5 and 6** below summarize the data.



Major findings for *Specific Aim 1, Major Task 4*:

1. CTCs but not primary tumor cells display high level of OXPHOS (oxidative phosphorylation) markers.
2. Both CTCs and primary tumor cells have upregulated EMT pathway.
3. The OXPHOS markers are especially upregulated in CTCs from castration resistant and/or metastatic patients (a more advanced disease).

3.2.5 *Specific Aim 1, Major Task 5*

Subtask 1: culturing 22Rv1 and DU145 cells, inducing EMT by treatment with TGF- β or EMT-inducing media supplement and performing mechanical phenotyping of selected cultured cells, was completed, as planned. The highlights of data included in the 2020 report are presented below in **Figure 7**. While 22Rv1 and DU145 cells responded to EMT induction with an expected increase

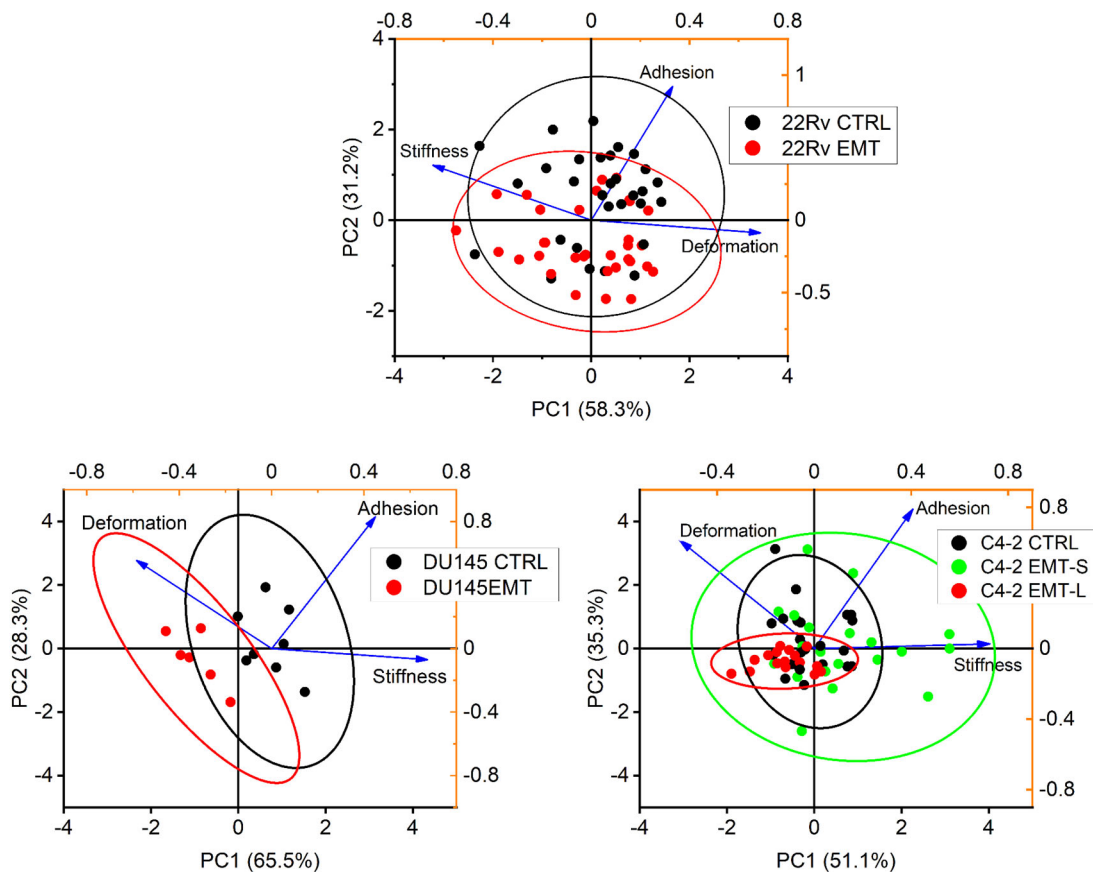


Figure 7 Nanomechanical phenotypes of cultured prostate cancer cells conform to canonical epithelial-to-mesenchymal transition (EMT). 22Rv1 and DU145 cells were induced for 5 days with an EMT-inducing media supplement. C4-2 cells were induced with TGF-beta for one day (short; EMT-S) or one-and a half day (36 hrs. long, EMT-L).

in softness and decrease in adhesion, the C4-2 cells responded in a more diversified way, dependent on the time of exposure to EMT-inducing cytokines.

Expectedly, inducing EMT in “model tumor cells” (cultured prostate cell lines) did not fully reproduce mechanical phenotypes found in patient-isolated CTCs. Therefore, we exposed the model tumor cells to fluid shear stress (FSS) mimicking the conditions experienced by the CTCs in circulation. We used the microfluidic system (IBIDI) that generates highly controlled conditions mimicking fluid flow in veins, arteries or capillaries. We hypothesized that FSS may induce EMT-related changes in the mechanical phenotypes of “model tumor cells”, thus transforming them into “model CTCs”. The data were consistent with our hypothesis. As demonstrated in **Figure 8** (small vein-like/large capillaries-like stress), the model prostate tumor cells, but not benign cells, respond with generally increased adhesion. High adhesion is a hallmark feature of aggressive CTCs. Interestingly, the “model CTCs” displayed improved post-challenge migratory ability, consistent with their putative higher invasiveness (**Figure 8**).

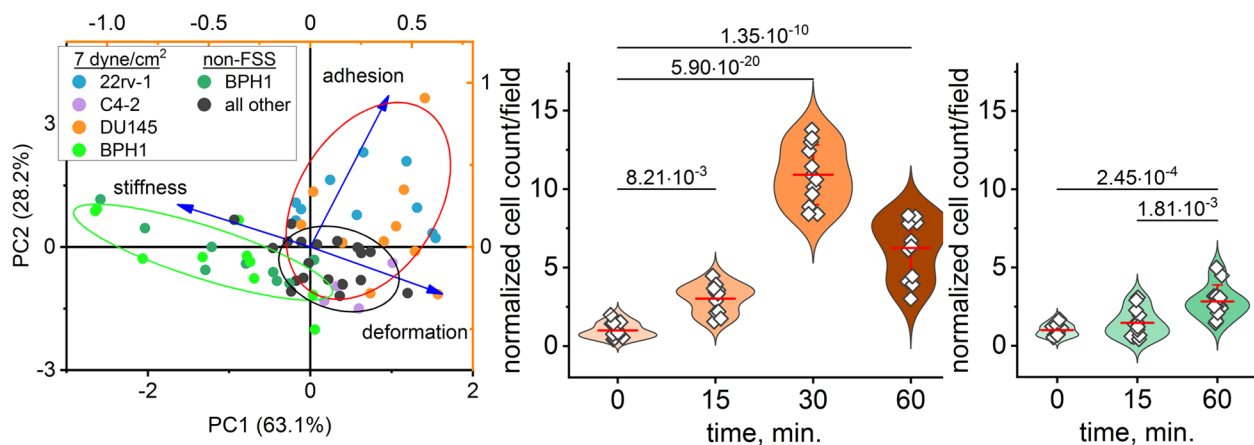


Fig. 8 Model CTCs (but not benign BPH-1 cells) respond to a short vein-like FSS with improved mechanical fitness: higher adhesion and/or soft-ness (left panel: PCA of AFM-derived parameters; Dot=cell) and also with improved post-challenge migratory ability (right - violin plots; TransWell assay, 16 hours post-FSS).

Subtask 2. As demonstrated in **Figure 9**, mesenchymal markers vimentin and N-cadherin were up-regulated in DU145 cells even after a short exposure to FSS. To the contrary, cytokeratins (epithelial markers; cytokeratin 19 and cytokeratin 8/18) were down-regulated under the same conditions, as compared to non-stressed DU145 cells.

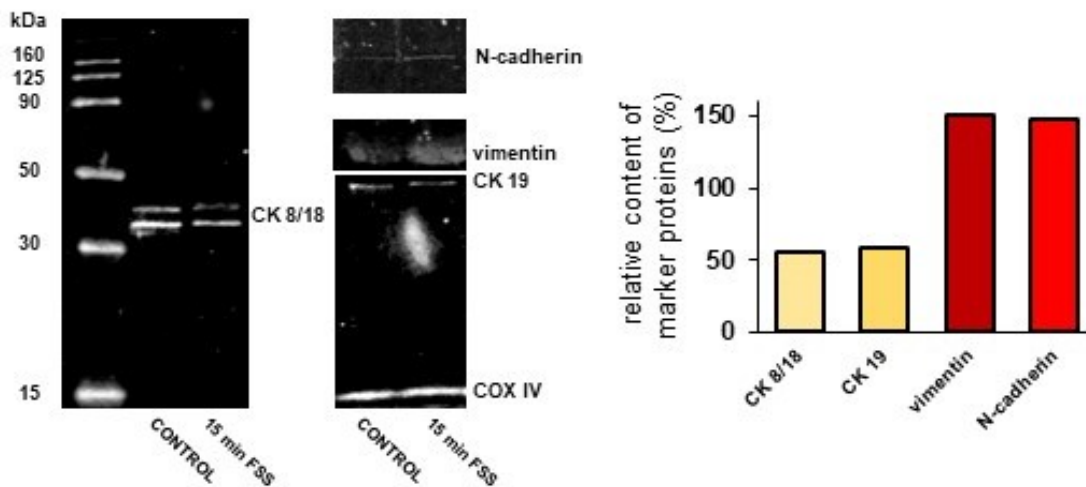


Figure 9 Short-time fluid shear stress (FSS) challenge induces EMT-like changes in DU145 cells. We tested a 15 min challenge under conditions imitating blood flow in small veins or large capillaries. The viability of cells remained at 90% for control (floating, not stressed cells) and stressed cultures. **Left.** Proteins from lysates prepared from control and stressed cells were separated by SDS-PAGE (denaturing polyacrylamide gel electrophoresis), transferred to nitrocellulose membrane (Western blotting) and probed with specific antibodies recognizing mesenchymal and epithelial marker proteins, and COX IV (cytochrome c oxidase subunit 4; a loading control). Protein bands were visualized with Odyssey Infrared Imaging System (Li-Cor). **Right.** Relative contents of protein markers are presented (control circulated without FSS: 100%), adjusted for loading (COX IV used as loading control).

Highlights of the research performed in *Specific Aim 1, Major Task 5*:

1. Treatment of static culture of prostate cancer cells with EMT-inducing cytokines leads to expected (albeit cell line-dependent) changes in mechanical phenotypes and EMT marker make-up of these “model tumor cells”, however it does not reproduce phenotypes of patient-isolated CTCs.
2. Exposing “model tumor cells” to circulation-like mechanical cues (fluid shear stress; FSS) leads to CTC-like elevated adhesiveness (elevated mechanical fitness) and to induction of selected EMT markers, indicating epithelial-mesenchymal plasticity (EMP; “hybrid EMT”) in these “model CTCs”.
3. Importantly, high mechanical fitness in “model CTCs” is accompanied by gaining invasiveness in the post-FSS static culture.

3.2.6 Specific Aim 2, Major Task 6

Subtask 1: The immunostaining-based composition of non-CTC, immune cells population in TACCs was determined for all patients' samples, as stated in **Table 1**.

The data collected and analyzed for this *Task* were included in the published *Cancer Research* paper. In short, a presumed unique relationship between CTCs and co-isolating immune cells was demonstrated by our comparison between populations of tumor cells shed to urine and shed to blood (tumor-associated circulating cells; TACCs; **Figure 10**). Both blood retentates and urine sediments contained EpCAM⁺ cells but also numerous EpCAM⁻ cells positive for pan-leukocyte marker CD45 (leukocyte common antigen; **Figure 10A**). Morphometric analysis of a random sample of light/fluorescent microscopy images of these cells revealed that EpCAM⁺ cells were predictably large in both blood and urine preparations. Footprints of these cells corresponded to squares with average lengths of 24 μm (44 CTCs; range of lengths 10 μm – 32 μm) or 21 μm (51 UPCs; range of lengths 8 μm – 30 μm), well within morphological parameters reported for CTCs. Population of CTCs with larger footprints was more numerous than a similar population in UPCs (**Figure 10B**). This may reflect the high content of CTC pairs in blood retentates (cells retained on the filter; **Figure 1**), consistent with high adhesiveness of CTCs as compared to UPCs. Such cell pairs are customarily counted as single objects in FDA-approved diagnostic/prognostic enumerations of CTCs. In turn, footprints of the immune cells found in urine sediments were rather small (average diameter 10 μm , range 6 μm – 18 μm ; **Figure 10B**) corresponding to typical leukocytes with expected diameters ranging from 7 μm to 15 μm . Instead, blood-derived EpCAM⁻CD45⁺ cells had an average diameter of 23 μm , (range: 16 μm – 25 μm ; **Figure 10B**). The observed difference in size distribution could not be attributed solely to the expected enrichment of blood retentates in large cells, as partitions of tumor-to-immune cells were very similar, close to 1:1, in preparations recovered from the two types of liquid biopsies (see caption to **Figure 10**). However, large immune cells were absent in urine. Those large cells are macrophages.

Further, we classified the circulating macrophages as M1-like (CD80⁺CD163⁻), intermediate ("TAM-like"; CD80⁺CD163⁺), and M2-like (CD80⁻CD163⁺). The "intermediate" class proved to be especially interesting, as their enumeration most closely followed mechanical fitness of CTCs (**Figures 11, 12**). These data positioned enumeration of macrophages as a good candidate for biomarker parameter.

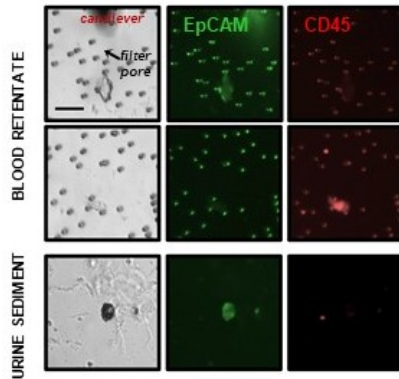
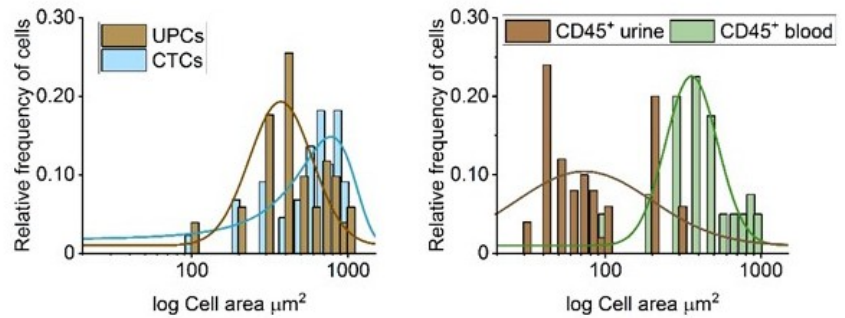
A**B**

Figure 10 Tumor cells and immune cells are isolated from urine and blood liquid biopsies collected from patients with aggressive prostate cancer, however large immune cells are present only in blood. A. Cells from blood (two top rows) were isolated by microfiltration, cells from urine were sedimented; next they were stained with anti-EpCAM and anti-CD45 (pan-leukocyte) fluorescently labelled antibodies. The filter pores and the AFM cantilever may reflect glow from the labels as an artifact. **B.** Morphometric analysis of footprints (area) of: Left - CTCs and UPCs (EpCAM⁺CD45⁻; 44 and 40 cells, respectively; $p < 0.0002$ Kolmogorov-Smirnov test) and Right – EpCAM⁻CD45⁺ cells captured in blood filtrates (51 cells) and in urine sediments (50 cells) of 21 prostate cancer patients; $p < 0.0001$ Kolmogorov-Smirnov.

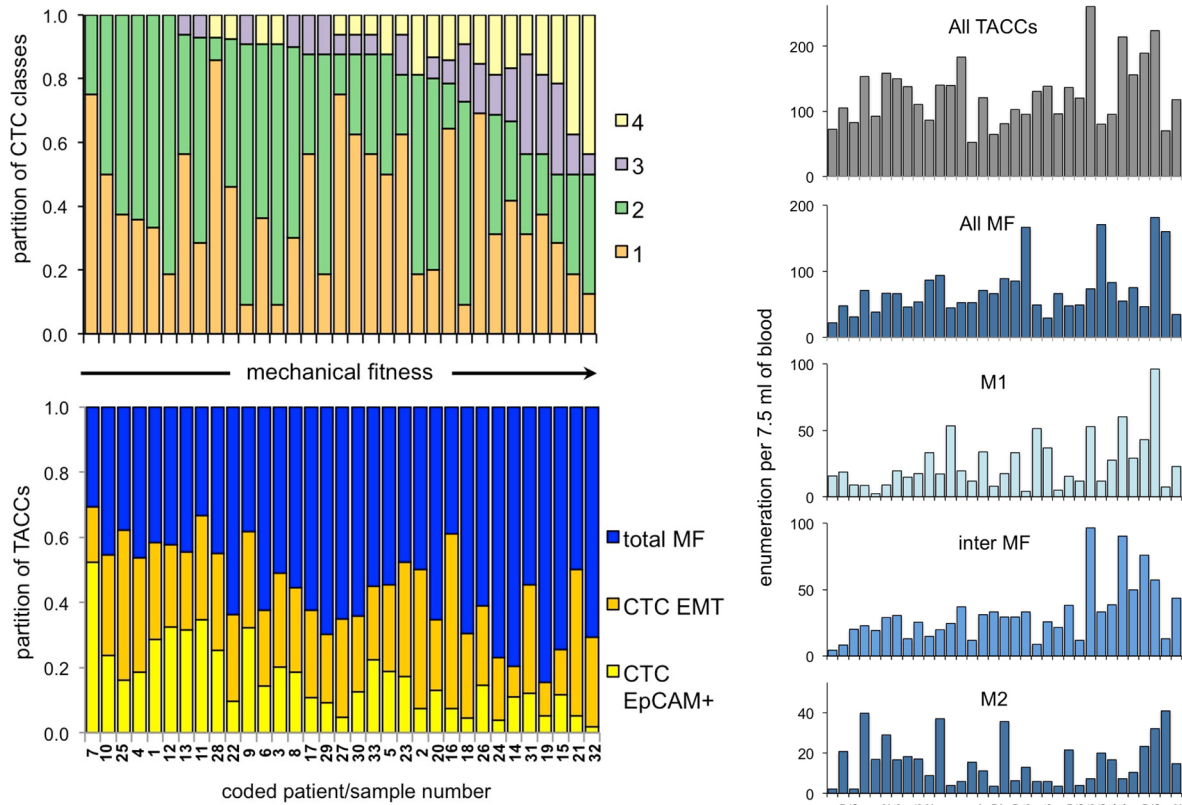


Figure 11 Abundance of macrophages, especially of intermediate and M1 –like phenotypes, increases along the “CTC fitness axis”. The 33 samples are ordered according to relative abundance of well-fit CTCs (see Figure 3). Left-top: the “CTC fitness chart” is copied from Figure 4 as a reference. Left-bottom: relative abundance of distinct TACCs. Right: enumeration of all TACCs, and distinct classes of macrophages.

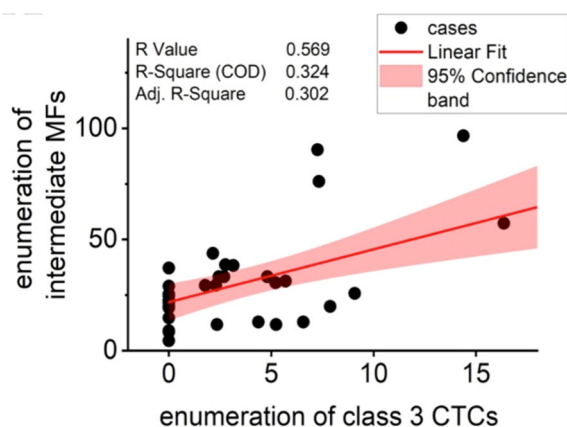


Figure 12 Abundance of well-fit CTCs positively correlates with enumeration of macrophages, especially those with TAM-like “intermediate” markers, positive for both M1 (CD80⁺) and M2 (CD163⁺). Correlation between class 3 CTCs (well-fit deformable and non-stiff (soft) cells; see Figure 3) and intermediate macrophages.

Subtask 2 Proteomic analysis of the circulating immune cells yielded data that formed a core of manuscript now under review in *Nature Communications*. This part of the study was finalized in months 37-48. In short, using our previously described microfiltration method, we detected putative tumor-macrophage hybrid (TMH) cells in PBMCs from prostate cancer patients. Based on the finding, we sought to determine the frequency of TMH cells in PBMCs of 16 advanced prostate cancer patients using CyTOF (**Figure 13A**, next page). Single-cell profiling of ~2.8 million PBMCs identified ten main subpopulations based on the expression of different cell-surface and oncogenic markers (**Figure 13B-C**). CTCs were identified as one minor subpopulation (n=1081 or 0.038%), positive for the epithelial marker EpCAM and three prostate cancer markers but negative for CD45 (Fig. 6D). Another minor subpopulation (n=8213 or 0.285%) contained fusion cells also positive for epithelial, prostate cancer, and cell-surface markers specific to macrophages, MDSCs, NKT cells, or NK cells, respectively (i.e., TMH, T-MDSC-H, T-NKT-H, and T-NK-H in **Figure 13C**). In line with the low androgen response expression in the xenograft TMH tumor (not shown, see manuscript), TMH cells in PBMCs had lower expression of prostate cancer markers than CTCs and other types of fusion cells (**Figure 13D**). In addition, TMH cells appeared the most common (75%) among the four fusion cell types analyzed (**Figure 13E**). Consistent with the mouse PBMC finding, TMH cells were present 5.6X more often than CTCs in the blood of prostate cancer patients. Then, we compared TMH/CTC ratios in patients with biochemical recurrence (BCR)-only *versus* those later having castration resistance and/or metastasis (CR/MET). The results show that CR/MET patients had higher ratios than BCR-only patients, suggesting TMH cells as a new prognosticator of patient outcome ($p < 0.01$; **Figure 13F**). In addition to overexpressing prostate cancer markers, TMH cells expressed M1-like CD86, M2-like CD163, and mesenchymal markers vimentin and fibronectin like those of monocytes or macrophages (**Figure 13G**). Compared to these immune cells, a higher proportion (38%) of TMH cells displayed elevated CD86 and CD163, suggesting a dual M1/M2 phenotype (**Figure 13H**).

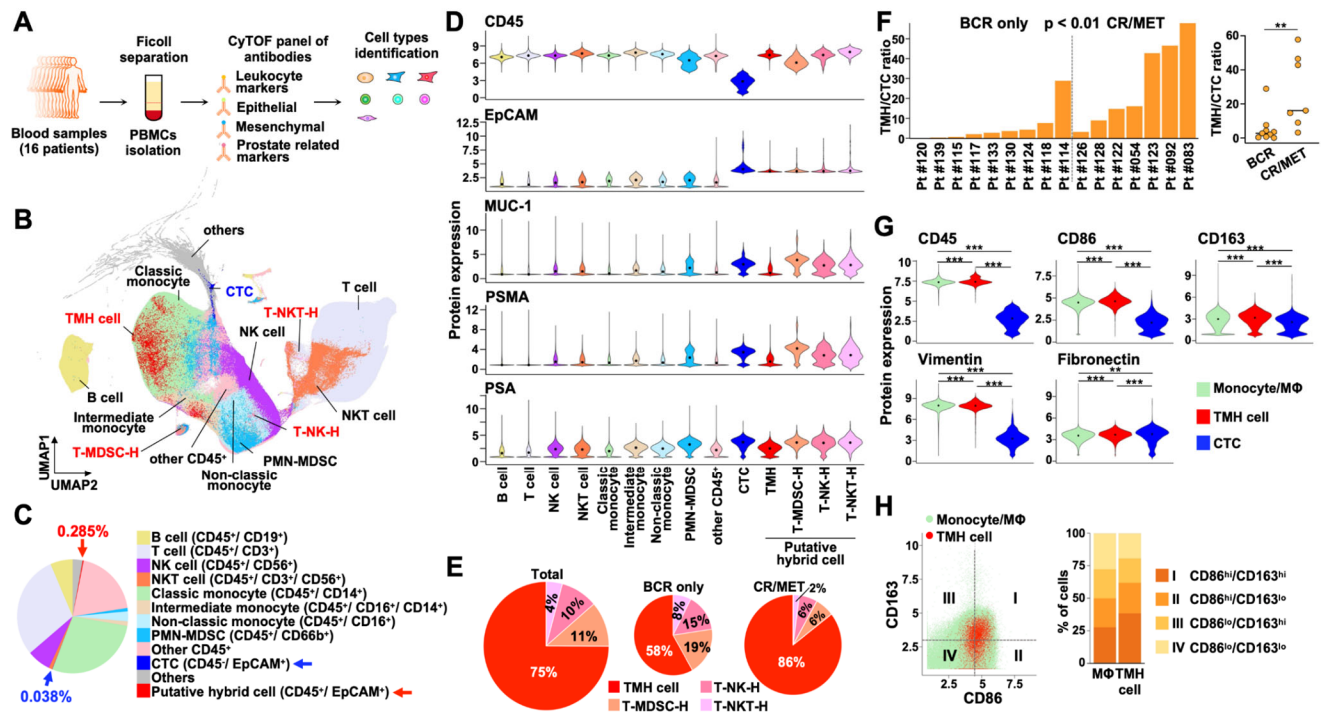


Figure 13. CyTOF profiling identifies rare fusion cells in PBMCs of prostate cancer patients. **A**, Schematic CyTOF diagram to distinguish different cell types in PBMCs. **B-C**, UMAP and pie chart of 12 cell types, including circulating tumor cells (CTCs; blue arrow) and four putative hybrid cell subtypes based on their respective surface markers (red arrow). **D**, Expression levels of immune marker CD45, epithelial marker EpCAM, and MUC-1, PSMA, and PSA, known to be highly expressed in prostate cancer. **E**, Pie charts of 4 putative hybrid cells in all patients, patients with biochemical recurrence (BCR)-only, or patients with subsequent castration resistance and/or metastasis (CR/MET). **F**, Higher TMH/CTC ratios in CR/MET patients relative to BCR-only patients. **G**, Higher expression levels of macrophage-related markers in monocytes/macrophages and TMH cells relative to CTCs. **H**, Scatter plot and bar graph depicted ~38% of TMH cells displaying high levels of M1 CD86 and M2 CD163 markers. In contrast, macrophages showed a lower proportion of this dual M1/M2 phenotype. ** $p < 0.01$, *** $p < 0.001$.

Highlights of the research performed in *Specific Aim 2, Major Task 6*:

1. Diverse macrophages, likely derived from TAMs (tumor associated macrophages) co-isolate with CTCs. Enumeration of macrophages, especially those of mixed TAM-like markers (intermediate macrophages), positively correlates with mechanical fitness of CTCs.
2. We found an abundant population of TACCs with mixed CTC-immune markers: the tumor-macrophage hybrids (TMH). The cells are especially abundant in castration resistant and/or metastatic patients.
3. Abundance of intermediate macrophages and TMHs may have the biomarker value for early detection of castration resistance.

3.2.7 Specific Aim 2, Major Task 7

Subtasks 1 - 3 Recapitulation of the interactions between model CTCs (prostate cancer cell lines) and model macrophages was completed ahead of schedule in the 2020 reporting period. The data were included in the 2021 *Cancer Research* paper. **Figures 14** (*Subtasks 1-2*) and **15** (*Subtask 3*) present highlights of the data. New in the final year of the research was *in cellulo* recapitulation of TMH formation and analysis of model hybrid cells, included in the manuscript under review in *Nature Communications* and presented in **Figure 16**.

In short, to determine if TMH cells can be formed *in vitro*, we incubated macrophages and prostate cancer cells (2:1 ratio) in a co-culture model. Before the co-culture, U937-derived macrophages were engineered to contain a lenti-mCherry-hygromycin B construct while prostate cancer cells were tagged with a lenti-EGFP-luciferase-puromycin vector (**Figure 16A**). Note: additional primary macrophages will later be used in the study. The dual fluorescent tags were used to distinguish cancer cells undergoing complete or incomplete phagocytosis (**Figure 16B**). Incomplete phagocytosis could lead to a subset of fusion cells stably exhibiting both mCherry/EGFP fluorescent tags in co-culture. These putative TMH cells also expressed epithelial and macrophage markers (**Figure 16C-F**).

Highlights of the research performed in *Specific Aim 2, Major Task 7*:

- 1. Co-culture with model macrophages have a profound effect on mechanical fitness of cultured prostate cancer cells. Apparently, interactions with macrophages improve mechanical fitness of model tumor cells, with elevated adhesion as the most pronounced feature.**
- 2. Co-culture with model macrophages induces epithelial-mesenchymal plasticity (EMP) in model tumor cells. The plasticity manifests as partial reversal of EMT, promoting mechanical fitness.**
- 3. The fusion between cancer cells and macrophages can be recapitulated in cell culture. This opens the door for research on the elusive TMH cells, most likely present both in the tumor and in circulation.**

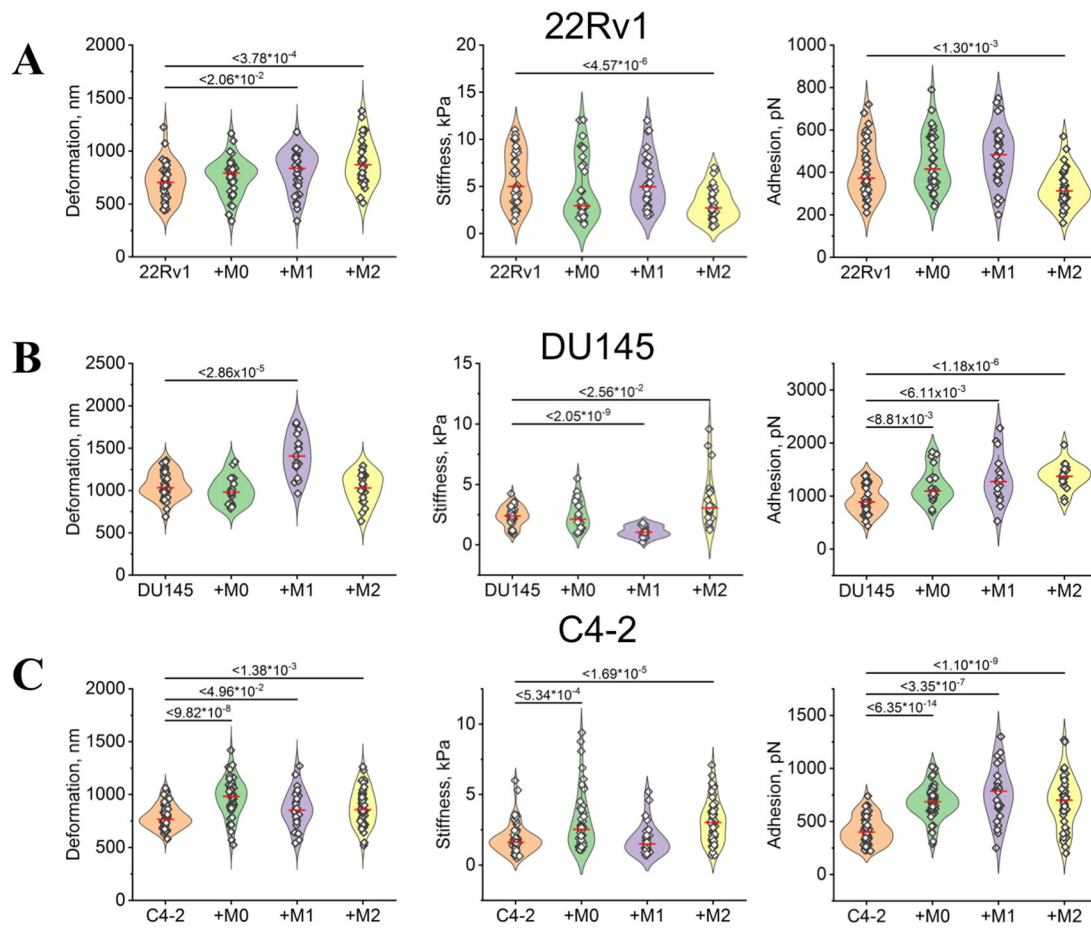


Figure 14 Nanomechanical parameters of human cultured prostate cancer cells specifically respond to co-culture with model macrophages. **A.** Nanomechanical parameters of 22Rv1 cells, control and co-cultured with model macrophages of distinct polarization. **B.** DU145 cells and **D.** C4-2 cells.

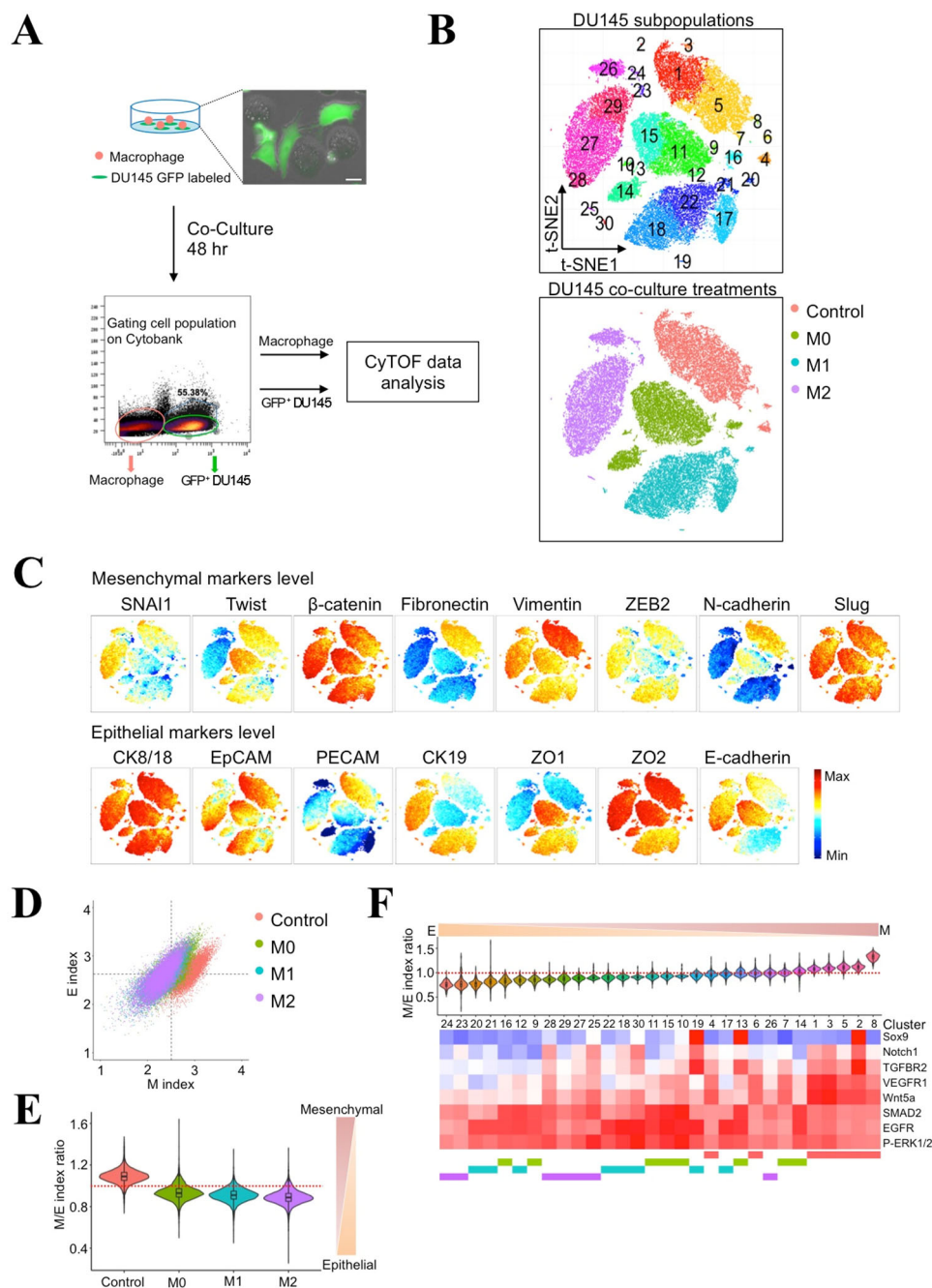


Figure 15 Subpopulations of prostate cancer DU145 cells co-cultured with model macrophages (U937) display hybrid EMT. **A.** GFP-DU145 were separated from macrophages in Cytobank and subjected to CyTOF analysis with epithelial/mesenchymal panel of 16 antibodies. **B.** t-SNE projections of treatments of DU145 cells. Each of four treatment-populations was further divided into subpopulations. **C.** Profiles of selected epithelial and mesenchymal markers under distinct co-culture conditions. **D.** Scatter plot of Mesenchymal (M) and Epithelial (E) indices amount treatments. **E.** Violin plots of M/E ratio. **F.** Heat map of oncogenic signal components of each cluster aligned based on M/E ratio order.

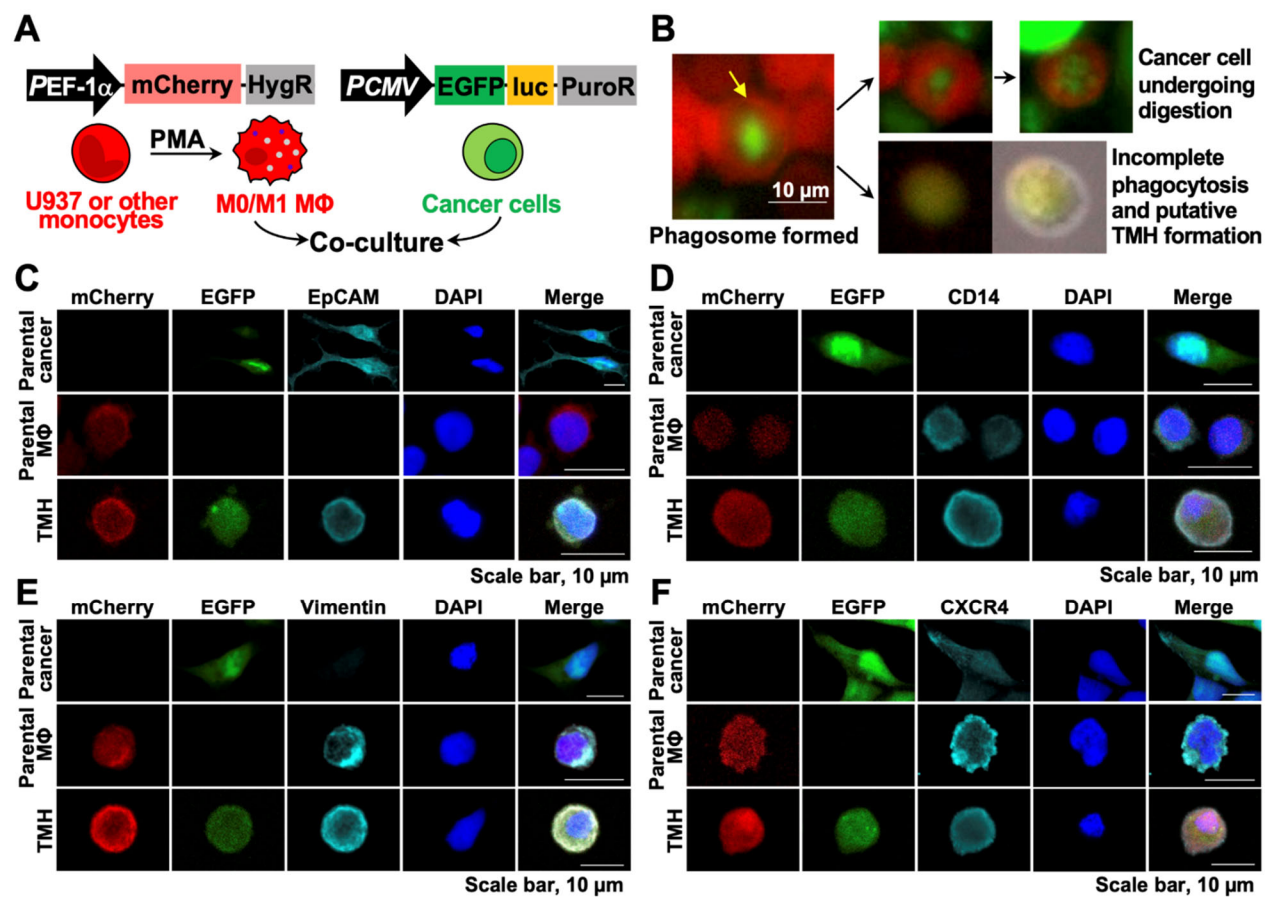


Figure 16. Identification of TMH cells with dual fluorescent tags. A-B, Development of dual fluorescent vectors to identify TMH cells in a co-culture model. These cells were co-cultured in suspension in ultra-low attachment dishes for up to a month. C-F, Immunofluorescence analysis of TMH cells, parental macrophages, and cancer cells. Epithelial cancer cell markers (i.e., EGFP and EpCAM) and macrophage markers (i.e., mCherry, CD14, vimentin, and CXCR4) were both expressed in TMH cells. Note: CXCR4 is also expressed in prostate cancer cells.

3.2.8 Specific Aim 3, Major Task 8

Subtasks 1 and 2 As noted in the previous reports, we relied on the data for t0 patients' visits, with t1 visits available for about half of patients (37 cases; **Table 1**). Some of the t0-t1 comparisons were presented in the previous reports and included in the 2021 *Cancer Research* paper. The comparisons confirmed the unique significance of t0 samples for potential biomarking.

Subtask 3 We are currently finalizing construction of the risk-predicting model for patients' stratification.

Highly encouraging results of test cluster analysis on the biophysical data are included in the 2021 *Cancer Research* paper. The analysis revealed that patients that remained CS for up to a year after t0 tended to cluster together (**Figure 17**). Also, our presented before cluster analysis of mechanical and enumeration parameters separated cases according to a metastatic spread status. With more patient data available we refined our analysis (*Subtask 3*).

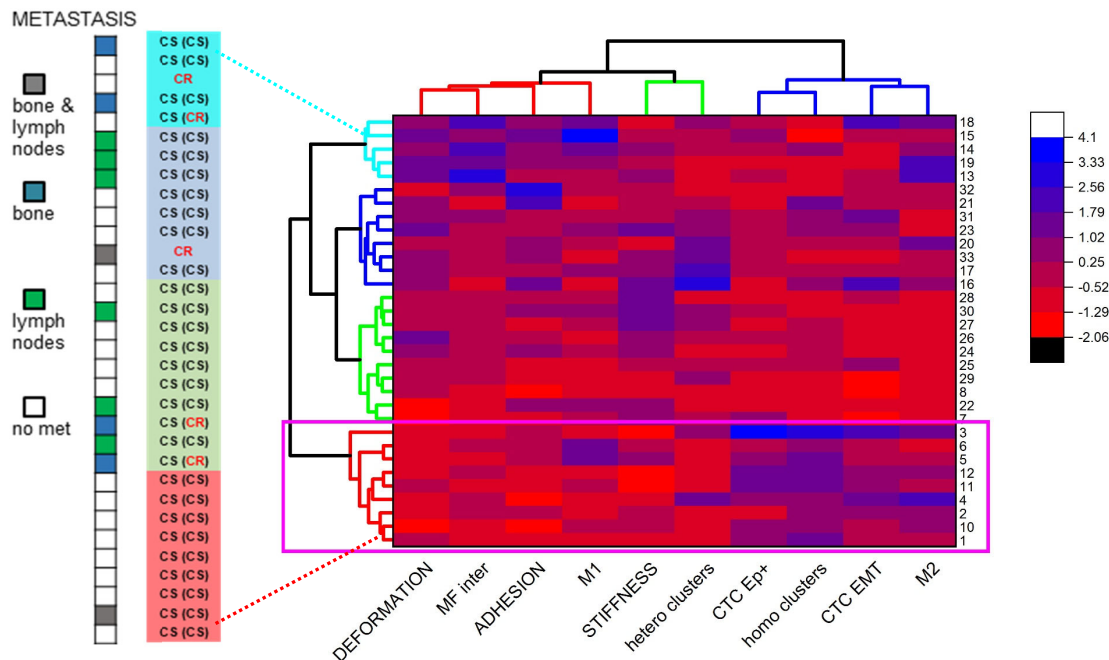


Figure 17 Cluster analysis of mechanical and enumeration parameters of TACCs distinguishes four groups of patients' samples. The pink-framed cluster consists of t0 samples, with a sole exception of sample No. 2. Other clusters have a mixed t0 and t1 representation. In the No. 2 case the pseudo-t0 blood sample was obtained only 3 months after the start of ADT. The panel on the left presents the status of sensitivity to ADT in the clusters. The status in parentheses indicates a clinical update at least 6 months after the blood sample was obtained and the first-time ADT or other therapy was initiated. The far-left strip presents the status of metastasis. Coded identity labels for patients/samples are shown on the right.

Figure 18, presents extended analysis of cells isolated from the blood of high risk/low-volume metastatic castration sensitive prostate cancer patients starting or undergoing androgen deprivation therapy (ADT). Hierarchical Cluster Analysis (HCA) of mechanical and immuno cytochemical parameters identified three groups of patients. Clinical updates collected after the analysis indicated that the risk of developing castration resistance (CR) within 4-20 months from t0 sampling differed greatly between the groups. In one group ("green") all patients remained CS for at least 34-45 months (no CR). The other two groups ("navy" and "red") show a low and high CR development risk within 4-20 months from sampling (two-sample proportion test: exact $p=0.249$). Not all data are included in this draft HCA, so we predict that the full analysis will reach statistical significance. Interestingly, enumeration of EpCAM "canonical" CTCs was associated

with long term CS, in contrast to enumeration of CTC-EMT and certain classes of MΦs. This underlines the shortcoming of EpCAM-based methods of CTC isolation, including the sole FDA-approved method, that neglect EpCAM-negative CTCs and accompanying immune cells. Although the risk prediction did not reach statistical significance, power analysis indicates that it can be prudently tested with the number of patients that we intend to accrue and analyze.

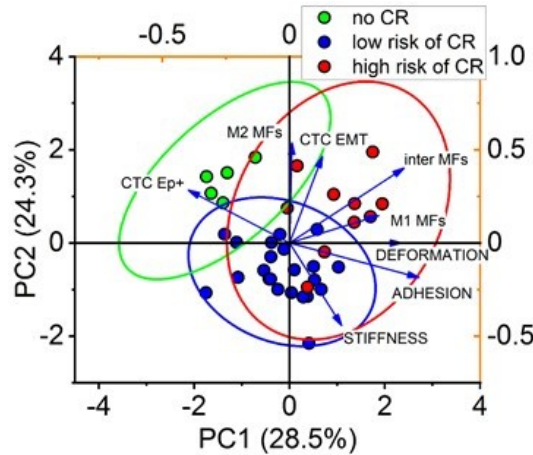


Figure 18 Predictive potential of nanomechanical phenotyping combined with immunocytochemical analysis of TACCs. Principal component analysis (PCA) of adhesion, deformation and stiffness of CTCs, as well as enumeration of EpCAM⁺ CTCs, EMT-undergoing EpCAM⁻ CTCs and three classes of macrophages, is shown at *right*; each dot represents data of a single patient.

The most unexpected finding of our research to-date was that enumeration of M1-like CD80⁺CD163⁻ macrophages associated with a high mechanical fitness of CTCs (reported in 2020 and in the 2021 *Cancer Research* paper; **Figure 17**) and with a high risk of CR (**Figure 18**). Our starting hypothesis was that pro-inflammatory M1-like macrophages would act as “predators” attacking CTCs, in contrast to M2-like anti-inflammatory protective “chaperones” of CTCs. One explanation, discussed in the *Cancer Research* paper, is that macrophages co-purifying with CTCs, including M1-like macrophages, are derived from diverse and pro-cancer tumor-associated macrophages (TAMs). In fact, the CD80⁺CD163⁺ macrophages are frequent among TAMs and we detect them in great numbers among TACCs. We envision that CTCs leaving the tumor are taking a “mini- tumor microenvironment”, most notably TAMs, with them. Another possibility worth considering is that M1-like macrophages are recruited from the blood, however fail to destroy CTCs and are ultimately “hijacked” by CTCs as chaperones. Our co-culture experiments with model tumor cells and model M1-type or M2-type macrophages conformed that macrophages of both polarizations are capable of supporting mechanical fitness of tumor cells (*Major Task 7*). To learn more about the potential CTC-macrophages associations, we attempted to mathematically model the dynamic interactions between these cells. As demonstrated in **Figure 19**, even if M1-like macrophages are “predators” for CTCs, the former may never exterminate the latter, ultimately allowing for metastasis. Moreover, M1-like macrophages may be recruited by CTCs to support their survival (**Figure 19 - rightmost**).

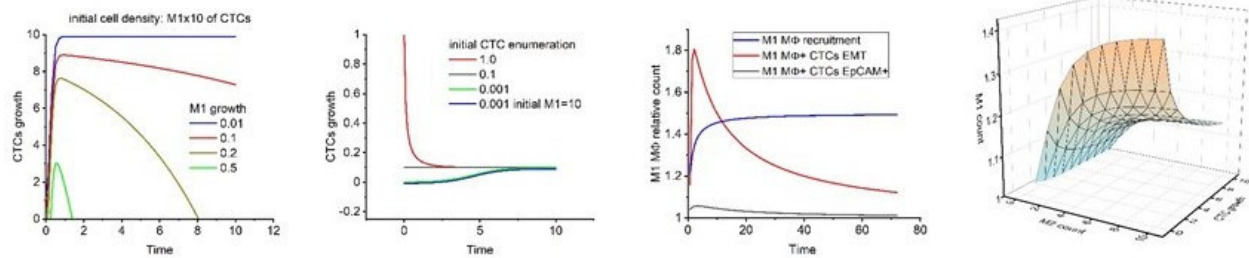


Figure 19 Assessing dynamics of nanomechanical phenotypes of model prostate cancer cells co-cultured with model macrophages. The Gompertz model of cell cancer growth modified by the presence of M1 (anti-tumor) and M2 (pro tumor) macrophages. From left to right: content of CTCs constant, content of M1 constant, model with recruitment of macrophages by CTCs, model of cells' growth with CTCs, M1, and M2 macrophages present.

Highlights of the research performed in *Specific Aim 3, Major Task 8*:

1. Mechanical phenotyping supplemented with enumeration of distinct classes of TACCs can provide a biomarker for early detection of castration resistance in patients starting ADT.
2. Adhesion of CTCs emerges as the strongest parameter correlating with early castration resistance.
3. Among enumeration parameters, intermediate macrophages and EMT-CTCs provide the strongest indicators of resistance.
4. Enumeration of EpCAM⁺ CTCs, used as the FDA-approved indicator of disease progression, may be misleading, as the EpCAM⁻ CTCs (EMT-CTCs) rather than EpCAM⁺ CTCs are most abundant in the high-risk patients.
5. High abundance of circulating macrophages is a generally strong indicator of poor prognosis. Apparently, even M1-like macrophages are not capable to efficiently eliminate CTCs and instead may be recruited by CTCs to support their survival.

3.3 Opportunities for training and professional development provided by the project:

Dr. Chia-Nung Hung, Postdoctoral Fellow, contributed mass cytometry proteomic profiling of cells for the project. He mastered the method and is a co-author of the 2021 *Cancer Research* paper. He is a second-author of the manuscript under review in *Nature Communications*.

Yusheng Qian, M.Sc., a senior PhD Graduate Student in Molecular Medicine is continuing his work on the project. He is a co-author of the *Cancer Research* paper. The project will constitute a core of his PhD thesis. He is extending the work into a unique AFM-based characterization of single cell-to-cell interactions. He presented the work in multiple meetings and is currently finishing preparation of a first-author manuscript describing the use of the method for model CTCs.

3.4 Dissemination of the results to communities of interest:

Listed below are posters and seminars presented during all reporting periods, starting from the most recent:

Seminar presentation: Seminar series of the Cancer Development and Progression Program in Mays Cancer Center, UT Health SA.” The tale of macrophages and well trained circulating tumor cells in prostate and lung cancers.” 05/19/2022 Presenter: Gaczynska.

Seminar presentation: Seminar series of the Cancer Development and Progression Program in Mays Cancer Center, UT Health SA.” The aberrant signaling pathways of advanced prostate cancer.” 05/19/2022 Presenter: Gaczynska.

Poster Presentation: Pawel A. Osmulski, Alessandra Cunsolo, Yusheng Qian, Meizhen Chen, Chun-Lin Lin, Chia-Nung Hung, Josephine Taverna, Michael Liss, Devalingam Mahalingam, Daniel DeArmond, Nameer Kirma, Chun-Liang Chen, Ian M. Thompson, Tim H.-. Huang, Maria Gaczynska “Macrophages connection: aggressive nanomechanical phenotypes of circulating tumor cells in prostate and lung cancers.” UT Health SA Long School of Medicine Research Day. 04/18/2022. Presenter: Osmulski.

Poster presentation: Pawel A. Osmulski, Alessandra Cunsolo, Yusheng Qian, Meizhen Chen, Chun-Lin Lin, Chia-Nung Hung, Josephine Taverna, Michael Liss, Devalingam Mahalingam, Daniel DeArmond, Nameer Kirma, Chun-Liang Chen, Ian M. Thompson, Tim H.-. Huang, Maria Gaczynska “Macrophages connection: aggressive nanomechanical phenotypes of circulating tumor cells in prostate cancer.” 66th Annual Biophysical Society Meeting, February 2022. Presenter: Osmulski.

Poster presentation: Yusheng Qian, Shelby N. Waddle, Pawel Osmulski, Maria E. Gaczynska "Cells in motion: model of circulating tumor cells in prostate cancer". 66th Annual Biophysical Society Meeting, February 2022. Presenter: Qian.

Invited seminar presentation: Multimodal analyses reveal aberrant signaling pathways of advanced prostate cancer. Invited distinguished speaker talk. Center for Cancer Research and Therapeutic Development, Clark Atlanta University, Atlanta, Georgia. February 2022. Presenter: Chun-Liang Chen.

Departmental seminar: Aberrant signaling pathways of advanced prostate cancer. Seminar of the Department of Molecular Medicine, UTHSCSA. Chen CL, November, 2021. Presenter: Chun-Liang Chen

Seminar presentation: Seminar for the Cancer Systems Biology Consortium/Physical Sciences in Oncology Network Cell and Tissue Mechanics special interest group, virtual. "Contacts with Macrophages Promote an Aggressive Nanomechanical Phenotype of Circulating Tumor Cells in Prostate Cancer." 10/14/2021 Presenter: Gaczynska.

Poster presentation: Pawel A. Osmulski (presenter), Alessandra Cunsolo, Yusheng Qian, Meizhen Chen, Chun-Lin Lin, Chia-Nung Hung, Devalingam Mahalingam, Nameer Kirma, Chun-Liang Chen, Josephine Taverna, Michael Liss, Ian M. Thompson, Tim H.-. Huang, Maria Gaczynska (May 2021) Aggressive mechanical phenotype of circulating tumor cells in prostate cancer is supported by contacts with macrophages. The Robert A. Clark Frontiers of Translational Science Research Day (San Antonio, TX)

Conference presentation: Modeling biophysical dynamics of receptor tyrosine kinases in castration resistant prostate cancer. NCI Cancer Systems Biology Consortium U54 site visit conference. 3/2021. Presenter: Chun-Liang Chen

Poster presentation: Pawel A. Osmulski (presenter), Alessandra Cunsolo, Yusheng Qian, Meizhen Chen, Chun-Lin Lin, Chia-Nung Hung, Devalingam Mahalingam, Nameer Kirma, Chun-Liang Chen, Josephine Taverna, Michael Liss, Ian M. Thompson, Tim H.-. Huang, Maria Gaczynska (February 2021) Macrophages support the aggressive mechanical phenotype of circulating tumor cells in prostate cancer. 65th Annual Biophysical Society Meeting, Biophysical Society, virtual.

Poster presentation: Yusheng Qian (presenter), Alessandra Cunsolo, Meizhen Chen, Chia-Nung Hung, Nameer B. Kirma, Michael Liss, Tim H. Huang, Pawel A. Osmulski, Maria E. Gaczynska (February 2021) Strategies of mechanical adaptation of CTCs to blood circulation. 65th Annual Biophysical Society Meeting, Biophysical Society, virtual.

Poster presentation: Lin C-L (presenter), Xi T, Hung C-N, Osmulski PA, Chou C-W, Chen M, Wang C-M, Mitsuya K, Kirma N, Gaczynska ME, Chen C-L, Huang TH-M. Abstract 2766: "Amplification-associated upregulation of genes involved in oxidative

phosphorylation for disseminated prostate cancer cells.” on the America Association for Cancer Research Annual Meeting, Atlanta, GA, March 2019

Poster presentation “Toxic relationship? Modelling Dynamics of Interactions between Circulating Tumor Cells and Macrophages in Metastatic Prostate Cancer” on the 10th Annual Frontiers of Translational Science Research Day, San Antonio, TX, May 2019, presenter: Dr. Osmulski (see Products). The event is attended by scientists from research institutions in San Antonio area, including UT Health, Mays Cancer Center, University of Texas at San Antonio, Southwest Research Institute, Southwest Foundation for Biomedical Research and Brooke Army Medical Center.

Poster presentation “Nano-forces in the cellular world: probing interactions between model CTCs and macrophages by atomic force microscopy” on the Annual Molecular Medicine Departmental Retreat (August 2019). Presenter: Yusheng Qian.

Seminar presentation Capabilities of the AFM technique on the Genome Repair Focus Group (UT Health SA, July 2019). Presentation included examples of CTC and model CTC analysis by single-cell AFM and elicited great interest in the technique by colleagues from UT Health. Presenters: Drs. Gaczynska and Osmulski.

Seminar presentation “From molecules to cells: the power of atomic force microscopy.”, Department of Biochemistry and Structural Biology, UT Health SA, September 2019. Presenters: Drs. Gaczynska and Osmulski.

4. Impact

4.1 Impact on the development of the principal discipline of the project.

The major findings are highlighted in the Accomplishment section, separate for each Major Task. In short:

We confirmed and refined our initial finding that patient isolated CTCs and model prostate cancer cells display unique epithelial-mesenchymal plasticity, with high adhesiveness (epithelial trait) confirmed as the major nanomechanical parameter distinguishing aggressive tumor cells from aggressive CTCs. We propose that invasive tumor-residing cells already advanced on the EMT axis may reverse to regain high adhesion, crucial for survival-promoting clustering in circulation. At the same time, we observe that CTCs of patients with rapid metastatic spread are poorly adhesive, most likely indicating their regained advanced EMT status and high invasiveness.

Significance: the finding broadens the knowledge about the diversity of cancer-related epithelial-to-mesenchymal transition. The concept of epithelial-mesenchymal plasticity is now gaining increased interest as critical for our understanding of cancer cells transition to

invasiveness. Importantly, the knowledge will impact development of anti-metastasis therapies, with targeting adhesiveness of CTCs as an attractive goal not considered before.

We found that mechanical properties of prostate cancer model cell lines cultured under static conditions only partially represent properties of CTCs. Moreover, their culture in the presence of macrophages still not completely reproduce properties of CTCs. Therefore, we initiated studies on properties of the model cells exposed to fluid shear stress (FSS) since the static conditions do not mimic flow of cells through blood vessels. Our data based on viability and mechanical properties of the exposed cells indicate that the model cell lines are characterized by unique set of responses to the FSS that strongly depends on their metastatic potential. It seems plausible that FSS as a selective force shows capability to at least substantially increase the metastatic capabilities in certain cell lines considered as low invasive. We also collected data that point at other than EMT processes responsible for CTC survival in blood stream.

Significance: our initial findings stress the importance to further develop of cell model more realistically reproducing natural history of CTCs. It also shows that data collected based on the static culture only are insufficient to develop complete understanding of the mechanism controlling spread of metastasis.

-We confirmed association of mechanical fitness of patient-isolated CTCs with abundance of macrophages with markers of TAMs. We speculate that macrophages that co-purify with CTCs are in fact TAMs that escaped the tumor, possibly in clusters with CTC. Importantly, abundance macrophages with M1-like features and mixed M1/M2 phenotypes is especially strongly associated with high mechanical fitness and poor prognosis. We hypothesize that the M1-like macrophages could be recruited from diverse TAM population or from the blood monocytes. We will further explore this notions.

Significance: Enumeration of macrophages emerges as a predictive marker, alone or together with mechanical phenotype and enumeration of CTCs. Targeting macrophages emerges as a viable anti-metastatic strategy. The findings also point at diversity of TAMs and at critical involvement of immune cells in metastasis.

We initiated research on a unique class of TACCs, the tumor-macrophage hybrids (TMHs). We developed methods to generate and characterize model TMHs in cell culture.

Significance: We postulate that TMHs may constitute especially invasive members of TACCs abundant in metastatic patients. These findings are an excellent starting point to further studies on these cells, elusive and rarely studied but likely very important for metastatic spread.

4.2 Impact on other disciplines.

Nothing to Report

4.3 Impact on technology transfer.

Nothing to Report

4.4 Impact on society beyond science and technology.

Nothing to Report

5. Changes/problems

5.1 Changes in approach and reasons for change.

There were no significant changes in approach. Minor changes and additions have been discussed in annual reports.

5.2 Actual or anticipated problems or delays.

The project required a one-year NCE. The delay is caused by COVID-19 pandemic related slowdown in patients' accrual. All tasks have been completed now.

5.3 Changes that had a significant impact on expenditures.

None

5.4 Significant changes in use or care of human subjects/vertebrate animals/biohazards/select agents.

None

6. Products

Papers and published Abstracts are listed below. Posters and Seminars are listed above in **3.4 Dissemination** section.

Paper published (highlighted with a cover):

Osmulski PA, Cunsolo A, Chen M, Qian Y, Lin CL, Hung CN, Mahalingam D, Kirma NB, Chen CL, Taverna JA, Liss MA, Thompson IM, Huang TH, Gaczynska ME. (2021) Contacts with Macrophages Promote an Aggressive Nanomechanical Phenotype of Circulating Tumor Cells in Prostate Cancer. *Cancer Res.* 2021 Aug 1;81(15):4110-4123. doi: 10.1158/0008-5472.CAN-20-3595. PMID: 34045187.

Paper under review (*Nature Communications*):

Chih-Wei Chou, Chia-Nung Hung, Cheryl Hsiang-Ling Chiu, Xi Tan, Meizhen Chen, Moawiz Saeed, Michael A. Liss, Chiou-Miin Wang, Zhao Lai, Nathaniel Alvarez, Pawel Osmulski, Maria E. Gaczynska, Li-Ling Lin, Veronica Ortega, Nameer B. Kirma, Kexin Xu, Zhijie Liu, Josephine A. Taverna, Gopalrao V. N. Velagaleti, Chun-Liang Chen, Zhao Zhang, and Tim Hui-Ming Huang. Phagocytosis-initiated hybrid tumor cells acquire a c-Myc-mediated quasi-polarization state for immunoevasion and distant dissemination. (see appendices)

Abstracts published:

Biophysical Journal 121(3):418a February 2022. DOI: 10.1016/j.bpj.2021.11.682

“Macrophages connection: aggressive nanomechanical phenotypes of circulating tumor cells in prostate cancer” Pawel A. Osmulski, Alessandra Cunsolo, Yusheng Qian, Meizhen Chen, Chun-Lin Lin, Chia-Nung Hung, Josephine Taverna, Michael Liss, Devalingam Mahalingam, Daniel DeArmond, Nameer Kirma, Chun-Liang Chen, Ian M. Thompson, Tim H.-. Huang, Maria Gaczynska

Biophysical Journal 121(3):418a February 2022. DOI: [10.1016/j.bpj.2021.11.673](https://doi.org/10.1016/j.bpj.2021.11.673)

"Cells in motion: model of circulating tumor cells in prostate cancer". Yusheng Qian, Shelby N. Waddle, Pawel Osmulski, Maria E. Gaczynska

Biophysical Journal 2021 vol 120 (3) p. 64a-65a DOI: 10.1016/j.bpj.2020.11.619

“Macrophages support the aggressive mechanical phenotype of circulating tumor cells in prostate cancer.” Pawel A. Osmulski, Alessandra Cunsolo, Yusheng Qian, Meizhen Chen, Chun-

Lin Lin, Chia-Nung Hung, Devalingam Mahalingam, Nameer Kirma, Chun-Liang Chen, Josephine Taverna, Michael Liss, Ian M. Thompson, Tim H.-. Huang, Maria Gaczynska.

Biophysical Journal 2021 vol 120 (3) p. 67a DOI: 10.1016/j.bpj.2020.11.631

“Strategies of mechanical adaptation of CTCs to blood circulation.” Yusheng Qian, Alessandra Cunsolo, Meizhen Chen, Chia-Nung Hung, Nameer B. Kirma, Michael Liss, Tim H. Huang, Pawel A. Osmulski, Maria Gaczynska.

Cancer Res [Internet]. 2019 Jul 1;79(13 Supplement):2766 LP-2766. Available from: http://cancerres.aacrjournals.org/content/79/13_Supplement/2766.abstract Lin C-L, Xi T, Hung C-N, Osmulski PA, Chou C-W, Chen M, Wang C-M, Mitsuya K, Kirma N, Gaczynska ME, Chen C-L, Huang TH-M. Abstract 2766: Amplification-associated upregulation of genes involved in oxidative phosphorylation for disseminated prostate cancer cells.

7.Participants

7.1 Individuals working on the project

Name	Tim H. Huang
Project Role	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-5985-9176
Nearest person month worked:	0.96 cal. mo.
Contribution to Project:	Oversight and coordination of the project
Funding Support:	No Change

Name	Maria Gaczynska
Project Role	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-9033-5706
Nearest person month worked:	3 cal. mo.
Contribution to Project:	Collection and analysis of immunofluorescence images of TACCs, design and oversight of co-culture experiments.
Funding Support:	No Change

Name	Pawel A.Osmulski
Project Role	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-5359-9200
Nearest person month worked:	1.8 cal. mo.
Contribution to Project:	Collection and analysis of mechanical phenotypes of patient isolated and model CTCs, design of co-culture experiments.
Funding Support:	No Change

Name	Chun-Liang Chen
Project Role	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-6774-9003
Nearest person month worked:	1.2 cal. mo.
Contribution to Project:	Collection of cells for single-cell gene expression analysis, single cell gene expression analysis of selected CTCs.
Funding Support:	No Change

Name	Michael Liss
Project Role	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-6978-1026
Nearest person month worked:	.36 cal. mo.

Contribution to Project:	Coordination of patients' accrual, collection of clinical data.
Funding Support:	No Change

Name	Byeongyeob Choi
Project Role	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	.6 cal. mo.
Contribution to Project:	Processing of data for statistical analysis
Funding Support:	No Change

Name	Meizhen Chen
Project Role	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	.6 cal. mo.
Contribution to Project:	Co-culture experiments with model CTCs and macrophages.
Funding Support:	No Change

Name	Chia-Nung Hung
Project Role	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3 cal. mo.
Contribution to Project:	CyTOF analysis of model CTCs and macrophages, collection of cells for single-cell gene expression analysis, single cell gene expression analysis of selected CTCs, model CTCs and model macrophages.
Funding Support:	No Change

Name	Yusheng Qian
Project Role	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 cal. mo.
Contribution to Project:	Co-culture experiments with model CTCs and macrophages, collection and analysis of mechanical phenotypes of model CTCs.
Funding Support:	No Change

1.1 Changes in the active other support of PD/PI and key personnel

Nothing to report

1.2 Other organizations involved as partners

Nothing to report

2. Special Reporting Requirements

Nothing to report

3. Appendice