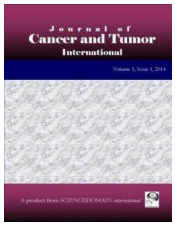

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Circulating Prostate Gland Cells in Benign Prostatic Hyperplasia and Adenocarcinoma in Patients of African Heritage: Diagnosis by Liquid Biopsy: Preliminary Results

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Authors' contributions

This work was carried out in collaboration between all authors. Author MN designed the study, wrote the protocol and wrote the first draft of the manuscript. Author AHL determined PSA levels in plasma and cell cultures and managed the literature search and formatting. Author LM reviewed the manuscript. Author YK performed the histopathological investigations. Authors FBB, BNN and KAM performed and managed the clinical studies. All authors read and approved the final manuscript.

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ABSTRACT

Background: Benign prostatic hyperplasia and adenocarcinoma are two major urological conditions of the human prostate gland. Benign prostatic hyperplasia is due to increase in stromal cells and adenocarcinoma of the prostate is cancer of the epithelial cells. Cellular studies of the

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two conditions have relied on tissue biopsy material of the prostate. Liquid biopsy in the diagnosis and derivation of research materials has been orchestrated.
Aim: To propagate in vitro circulating prostatic cells from patients of African heritage suffering from benign prostatic hyperplasia and adenocarcinoma of the prostate in order to facilitate the diagnosis of the two diseases by a liquid biopsy platform.
Study Design: Hospital – cum - laboratory-based study.
Place and Duration: Kilimanjaro Christian Medical University College, Tumaini University, Moshi, Tanzania. Twenty four months.
Methodology: Eight ml of venous blood from 34 patients was used to culture prostatic cells in plasma before surgery. Plasma from 6 elderly men aged 59-79 years without lower urinary tract symptoms and 12 young men aged 22-35 years were included in the study as corresponding age control groups. Cells were cultured at 37°C in RPMI 1640 medium.
Results: Prostatic epithelial cells were detected in the medium and in the cytoplasm of monocytes of all 34 patients, 6 elderly men without lower urinary tract symptoms and 4 out of 12 young men. Prostatic epithelial cells and acini stained well with Giemsa and H&E stain, and presented with enlarged eccentric nucleoli. Androgen receptor in prostate epithelial cells was detected by an anti-androgen receptor monoclonal antibody. Stromal cells also grew from the plasma of 6 patients. Adenocarcinoma was detected histologically in only 3 of the 34 patients.
Conclusion: The cultivation in vitro of prostatic epithelial cells by a liquid biopsy approach (blood sample) is expected to open a window in attempts at discovering CTC in adenocarcinoma of the prostate.

Keywords: Benign prostatic hyperplasia; circulating tumor cells; prostate adenocarcinoma; liquid biopsy; in vitro cultivation; African heritage.

1. INTRODUCTION

The prostate gland is a fibromuscular glandular organ which surrounds the urethra. It is situated between the neck of the bladder above and the urogenital diaphragm below. Medical or surgical conditions of the prostate gland may lead to enlargement of the gland resulting in excruciating pain and inability to void urine. More remarkable conditions of the gland are due to benign tumor and cancer of the gland.

Many cell types constitute the prostate gland. The human prostatic epithelium has 3 major cell types: secretory epithelial cells, basal cells and neuroendocrine cells [1]. Secretory epithelial and stromal cells have cytoplasmic receptors for testosterone and increase significantly in number during the 2 main diseases of the prostate, Benign Prostatic Hyperplasia (BPH) and adenocarcinoma of the prostate (ACP).

Both BPH and ACP are recognized more commonly in aging men from 50 years of age and occur more commonly in men of African heritage. An increased number of epithelial and stromal cells in the peri-urethral area of the prostrate is characteristic of BPH [2]. The cause of this hyperplasia is unknown, although several contributing factors have been suggested.

The diagnosis of both BPH and ACP is confirmed by examination of a biopsy specimen of the prostate gland. Because the approach of taking a biopsy specimen is a traumatic event there is a need to develop less traumatic approaches to diagnose the two diseases, preferably by liquid biopsy.

Both BPH and ACP are frequently diagnosed at Kilimanjaro Christian Medical Center (KCMC) Moshi Tanzania. Recently it was shown that the top 3 cancer cases from August 2013 to December 2015 according to the Cancer Registry at KCMC were: cervical cancer (363), prostate cancer (268) and breast cancer (157) [3]. Dr. J.W. Arends, visiting pathologist, 2008-2012, examined 85 prostate specimens at KCMC and found that approximately 1-10% consisted of prostate adenocarcinoma (personal communication with the senior author).

2. MATERIALS AND METHODS

2.1 Ethical Consideration and Good Laboratory Practice

Ethical approval to conduct the study was issued by the institutional review board, College Research Ethics and Review Committee (CRERC) of Kilimanjaro Christian Medical University College (KCMUCo), Tumaini

University. Certificate number 548 for research proposal number 557 was issued.

The aim of the study was explained to each patient that we would draw 8 ml of their blood to investigate what cell types are found in their blood. This was explained to all patients in their local language, Kiswahili as per the Belmont report (1979) on Ethical Principles and guidelines section on informed consent. The verbal consent was authenticated by an attending surgeon. For other participants who were not patients we explained the same but informed them that they would be study control group. Participants who agreed to participate gave verbal consent.

2.2 Good Laboratory Practice

We adhered to good laboratory practice in the conduct of this study. Before use, Giemsa stain was filtered through 0.2 µm sterile support membrane (PALL life Sciences: Pall Corporation, 25 Harbor Park Dr. Port Washington NY 11050, USA) in order to eliminate possible contaminating organisms. No antibiotics were used in the monocyte cell culture experiments. Experiments were conducted according to Laboratory biosafety manual (WHO Laboratory Biosafety Manual, 2004 and WHO Good Clinical Laboratort Practice 2009) and materials were handled in a microbiological safety cabinet Bio II (Advance Biosafety, Telstar Technologies, S.L. Spain). During cultivation sterility was tested weekly in common bacteriological media including blood agar, chocolate (heated blood) agar, thioglycollate broth and MacConkey agar. When contamination was detected the culture vessel was discarded.

2.3 Study Population and Characteristics

At the Department of Urology of Kilimanjaro Christian Medical Center (KCMC) referral hospital, we consecutively enrolled 16 participants (59-105 years old, median=65 years) before transurethral resection of the prostate (TURP) because of lower urinary tract symptoms (LUTS). TURP is a surgical procedure that when done reduces the size of the prostate and relieves the patient from the problem of urine retention. Four participants aged 24 to 35 years (median = 26 years) and 6 participants aged 59 to 79 years (median = 66 years) who had not experienced LUTS were included as controls in the first experiment (Table 1). In the second experiment participants consisted of 18 patients aged 52 to 90 years (median 72 years) and 8 participants 22 to 33 years old (median = 28 years) (Table 2).

2.4 Detection of Plasma Prostate Specific Antigen (PSA)

Three (3) ml blood was drawn from each participant into a sterile syringe containing 300 IU heparin (BP) for determination of PSA concentration. The blood was transferred into a 15 ml sterile centrifuge tube and plasma was separated from cells by centrifugation at 1,000 rpm for 15 minutes. Plasma was separated from cells and PSA concentration was measured using the Cobas e411 ECL Analyser according to manufacturer's recommendations. Roche Elecsys® total PSA reagents were used to assay for presence of PSA. Briefly, quality control was performed using Randox® low and high cut-off standards before each sample batch was run as per manufacturer's instructions. Twenty (20) µl of the standards were incubated at room temperature for 20 minutes, and fed into the analyzer through the automated aspiration probe. Fifty (50) µl of test reagent was added and the reaction was run for 18 minutes by a one-step sandwich ELISA assay. Resulting absorbance values were then plotted to determine a detectable range of 0.018 - 50.0 ng/ml (defined by the limit of detection and the maximum of the master/standard curve). Values below the limit of the blank were reported as < 0.010 ng/ml. Values above the measuring range were reported as > 50.0 ng/ml. Values significantly greater than 50.0 ng/ml were then diluted at a ratio of 1:10, 1:20 and 1:50 against reagent diluents (KOH in Tween) and re-run to obtain the corresponding concentration of total PSA in ng/ml. Plasma and culture fluid samples were then incubated at room temperature for 20 minutes and centrifuged at 4000 rpm for 10 minutes to remove particulate matter. Twenty (20) µl of each sample was fed into the analyzer and run alongside the quality control standard reagents. Absorbance was plotted against the standard curve to obtain proportional concentrations of total PSA.

2.5 Plasma Monocyte Cultures

The tissue culture technique developed for culturing in monocytes a rickettsial parasite of dogs, *Ehrlichia canis,* was adapted in this study [4]. Briefly, 8 ml blood was drawn from each participant into a syringe containing heparin as described above. The sheath of the needle was replaced and the syringe was gently twirled to mix the contents. The syringe was supported

vertically by a stand, needle facing up. After 60 to 90 minutes when red blood cells had settled, the sheath on the needle was used to bend the needle at a 90^0 angle and 0.5 ml plasma was delivered into Leighton tubes containing coverslips (Bellco Glass, Inc. Vineland, NJ, USA) by applying gentle pressure on the plunger from below. The Leighton tubes were inspected to ascertain that the coverslip was fully submerged in the plasma. Plasma was also delivered into plastic culture vessels (Orange Scientific, Graignette Business Park, Avenue du Commerce 32, B-1420, Braine-l/ Alleud, Belgium). Leighton tubes and plastic culture vessels containing plasma were incubated at 37°C for 3 days at which time 0.5 ml HEPES-buffered RPMI 1640 with L-glutamine (GIBCO, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum was added (GIBCO Thermo Fisher Scientific); this is referred to as complete medium. No antibiotics were included in the culture medium.

2.6 Monocyte Cultures from Prostatic Chips

Two prostatic chips each from 2 patients with BPH were obtained during TURP. They were placed in 5 ml complete medium and transported to the lab where they were placed in petri dishes containing 5 ml complete medium. One chip from each patient was used to prepare impression smears and the other to prepare epithelial and stromal cells. Cells were teased out of the chips using 2 apposing scalpels. A half (0.5) ml cell suspension was placed into a Leighton tube containing a coverslip. A three (3) ml cell suspension was delivered into tissue culture flasks. Cultures were incubated at 37°C and subsequently handled similar to monocyte cultures from plasma.

2.7 Growth Rate of Prostatic Epithelial Cells

Two (2) ml medium containing prostatic epithelial cells from 2 patients with BPH were used to determine the rate of growth of epithelial cells. After centrifugation at 1500 rpm for 15 minutes the medium was decanted and 4 ml complete medium was added. Cell concentration per ml culture medium was determined in the Neubauer – improved counting chamber (Laud – Konigshofen, Germany). Cell counts at time zero and at every 24 hour interval up to 96 hours were determined and used to plot a growth curve at log2 for the 2 patients.

2.8 Staining Prostatic Epithelial Cells

Plasma smears of prostatic cells were made, air-dried and fixed in either absolute methyl alcohol or cold (4°C) acetone then stored at minus 20°C. When needed, smears were retrieved from the freezer and allowed to air dry. Cells were stained with Giemsa stain (GS) or hematoxylin and eosin (H&E) stain. The coverslip in the Leighton tube was removed, rinsed in tap water and allowed to air dry at room temperature and handled similar to smears as described above.

Sub-culturing of prostatic cells growing in suspension was by transferring 0.5 ml culture fluid into an Orange Scientific plastic tissue culture flask containing 2.5 ml complete medium. Incubation at 37°C was continued for 7 to 10 days when another subculture was made; this continued up to 90 to 100 days before cultivation was terminated.

2.9 Detection of Androgen Receptor

Anti-mouse primary antibody to androgen receptor and goat anti-mouse secondary antibody conjugated to horseradish peroxidase and chromogen diaminobenzidine (DAB) (Molecular Probes Invitrogen Detection Technologies 29851, Eugene OR 97402, USA) were used to detect the epithelial cell androgen receptors according to manufacturer's instructions. Briefly, slides containing prostatic epithelial cells, stromal cells grown in suspension and monocytes grown on coverslips were fixed in cold (4°C) acetone and stored at minus 20°C, where on retrieval they were allowed to dry at room temperature. After drying, endogenous peroxidase activity in the cells was quenched by incubation in 1-3% H_2O_2 for 1 hour at room temperature. This was followed by application of 1% blocking reagent solution for 1 hour at 37°C. Specimens were then labeled with primary antibody (mouse anti - human androgen receptor monoclonal antibody AR 441 (Thermo Fisher Scientific Inc. Rockford IL. 61105 USA) in 1% blocking buffer for 1 hour at room temperature. Specimens were then rinsed three times in phosphate buffered saline (PBS) pH 7.2 and allowed to air dry. Secondary antibody, a goat anti - mouse IgG (H+L) HRP conjugate (Thermo Fisher Scientific Inc. Rockford IL. 61105, USA) was applied and allowed to react for 1 hour after which specimens were rinsed 3 times in PBS and

allowed to air dry at room temperature. The chromogenic immune-detector, DAB substrate was applied and allowed to react for 3 minutes, during which time the brownish color developed in a positive test was visualized under the microscope at 400X magnification and photographed.

2.10 Characterization of Cells by Histopaque Medium

Cultured cells in 6 ml complete medium from 2 patients with BPH on day 60 of cultivation were each layered on 6 ml Histopaque® density 1.077+ 0.001 g/ml in a 15 ml sterile centrifuge tube and then centrifuged at 900x g for 20 minutes. Three separate bands of cells were formed from each patient's monocyte culture. Bands were harvested and each band was transferred into a sterile tube. Ten (10) ml complete medium was added and cells were washed in 2 changes of the medium. At the end of the second centrifugation the medium was decanted. Smears made from the resulting pellet were fixed and stained with H&E and GS then examined under the light microscope for cell identification.

3. RESULTS

Cultures grew with minimal bacterial or fungal contamination in the 100 days of cultivation and when contamination was detected, the culture was discarded. Overall, only prostatic acini (not epithelial cells) were detected in plasma smears and prostatic chip impression smears stained by GS. Fibroblast cells were also present in prostatic chip impression smears.

The rate of growth of epithelial cells, i.e. the doubling time interval, was estimated to be 30 hours (Fig. 1).

3.1 Plasma and Monocyte Culture PSA

Table 1 summarizes plasma and monocyte culture PSA concentrations in 16 patients and 10 control participants in the first experiment. Plasma PSA concentrations above 5.0 ng/ml were detected in 9 patients. The highest reading was from a patient with BPH (12.2 ng/ml in patient 1) followed by 11.0 ng/ml in a patient with ACP (patient 15). The rest of the patients had plasma PSA concentrations less than 5.0 ng/ml. Note that plasma PSA concentration above 5.0ng/ml is suspicious of a presence of BPH or ACP. Monocyte culture PSA concentrations higher than 5.0 ng/ml were detected in 6 patients consisting both the ACP patient and 5 BPH patients. In both control groups (under 35 years and the 59-79 year group) monocyte culture and plasma PSA levels were less than 5.0 ng/ml.

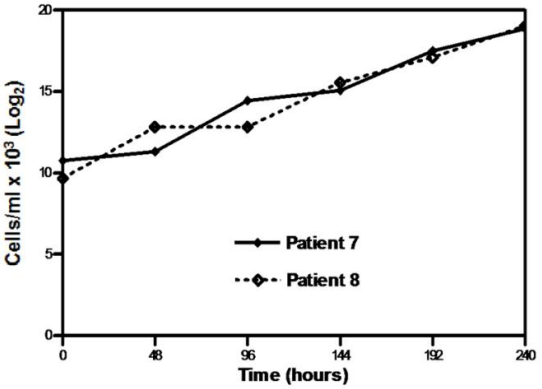

Fig. 1. Growth curve of prostatic epithelial cells from 2 patients suffering from BPH
The doubling time interval was estimated to be 30 hours

Table 2 summarizes the plasma and monocyte culture PSA levels of 18 patients and 8 control participants included in the second experiment. Markedly high plasma PSA concentrations were detected in 6 patients, the highest reading being 30.9ng/ml in patient 2 with BPH. Correspondingly high concentrations were also noted in the monocyte culture media of two patients, both with ACP (patients 7 and 8). In the 8 control participants aged 22 to 34 years, PSA concentrations in both plasma and monocyte culture media were unremarkable.

Fig. 2 shows monocytes devoid of epithelial cells from a young man aged 32 years, while Fig. 3 presents monocytes from a patient with BPH which contain epithelial cells; A, B and C represent possible progressive loss of monocyte cytoplasm integrity and death induced by PSA. Giemsa stain, original magnification 40X.

3.2 Detection of Androgen Receptor

Presence of androgen receptor on epithelial cells and acini growing in suspension (Fig. 4) and in monocyte cytoplasm (Fig. 5) was detected in all 18 patients by the anti-androgen receptor monoclonal antibody. The androgen receptor was also detected in monocyte cytoplasm and epithelial cells that grew in culture in 4 out of 8 control participants aged 22 to 34 years.

3.3 Cell Fractionation

Fractionation of cells from patients 7 and 12 with BPH in Histopaque medium yielded 3 bands of cells. In patient 7 the first band consisted of individual epithelial cells (Fig. 6), the second band consisted of epithelial cells supported by a mesenchymal layer (Fig. 7), and band 3 consisted of a mixture of band 1 and 2. Fractionation of cells from patient 12 with BPH yielded fibroblast-like cells (Fig. 8) as the first band, and individual epithelial cells as the second band. Fig. 9 is histology of a prostate chip from patient 5 with ACP.

Table 1. Histopathology of prostate chips, PSA in plasma and monocyte culture medium

Serial no	Age (yrs)	Pathological diagnosis	Plasma PSA (ng/ml)	Monocyte culture medium (ng/ml) (day tested)
1	78	BPH	12.2	6.4*(37)
2	63	BPH	8.6	2.4*(45)
3	59	BPH	ND	ND
4	74	BPH	10.4	8.8*(28)
5	80	Moderately differentiated adeno-carcinoma. Gleason grade 10	8.4	6.4 (13)
6	80	BPH	3.8	3.0 (21)
7	105	BPH	ND	ND
8	65	ND	6.4	8.8* (28)
9	65	BPH	0.9	6.4 (13)
10	66	ND	3.8	3.0 (14)
11	77	BPH	6.6	1.8 (25)
12	80	BPH	5.8	0.6 (30)
13	61	BPH	ND	ND
14	65	BPH	5.2	1.6 (30)
15	64	Moderately differentiated adeno-carcinoma. Gleason grade 8	11.0	5.6 (14)
16	61	BPH	4.2	2.8 (20)
Control 1: 4 participants aged 24, 26, 35, 35 years who have not had LUTS				
Serial number	**Age**	**Pathological diagnosis**	**Plasma PSA (ng/ml)**	**Monocyte culture medium PSA (ng/ml)(day tested)**
1	24	NA	1.2	1.2 (14)
2	26	NA	0.9	1.6 (20)
3	35	NA	ND	0.7 (18)
4	35	NA	0.6	0.4 (26)
Control 2: 6 participants aged 59 to 79 years who have not had LUTS				
Serial number	**Age (years)**	**Pathological diagnosis**	**Plasma PSA (ng/ml)**	**Monocyte culture medium PSA (ng/ml) (day tested)**
1	59	NA	3.8	0.4(20)
2	59	NA	ND	0.6 (18)
3	66	NA	2.0	ND
4	72	NA	1.8	0.8 (21)
5	77	NA	4.8	0.2 (10)
6	79	NA	ND	1.0 (26)

** Monocyte culture derived from prostate chip. BPH = benign prostatic hyperplasia. ND = Not done. LUTS = lower urinary tract syndrome. NA = not applicable*

Table 2. Plasma PSA, monocyte culture medium PSA, androgen receptor and pathological diagnosis in BPH and ACP Patients

1. Experiment serial number	Age (years)	Plasma PSA (ng/ml)	Monocyte culture medium PSA (ng/ml)/day tested	Androgen receptor monoclonal antibody detection	Pathological diagnosis
1	70	ND	1.53 (10)	+	Basal cell hyperplasia, chronic inflammation
2	73	30.9	2.0 (10)	+	Hyperplasia. No sign of malignancy
3	81	4.2	3.37 (15)	+	ND
4	61	4.1	ND	+	ND
5	64	4.8	1.1 (21)	+	ND
6	84	15.4	3.45 (15)	+	Chronic inflammation and hyperplasia
7	62	15.0	38.54 (21)	+	Glandular and stromal hyperplasia. No sign of malignancy
8	70	14.3	41.59 (21)	+	Moderate to poorly differentiated adenocarcinoma Gleason grade 9
9	90	4.3	0.82 (15)	+	BPH
10	74	17.15	ND	+	ND
11	52	3.85	4.5 (21)	+	Extensive fibromuscular hyperplasia
12	71	1.8	ND	+	Glandular and fibromuscular hyperplasia
13	63	4.6	6.32 (30)	+	ND
14	77	5.0	ND	+	BPH
15	63	13.75	ND	+	BPH
16	70	4.5	3.6 (15)	+	BPH
17	83	6.0	5.4 (21)	+	BPH
18	89	ND	9.2 (15)	+	BPH
Control					
Serial number	**Age (years)**	**Plasma PSA (ng/ml)**	**Monocyte culture medium PSA (ng/ml/ day tested)**	**Androgen receptor monoclonal antibody detection**	
1	22	0.265	0.23 (10)	-	NA
2	34	0.897	0.27 (10)	+	NA
3	28	0.187	0.20 (15)	+	NA
4	28	0.332	0.33 (21)	-	NA
5	23	ND	0.07 (10)	+	NA
6	27	0.08	0.12 (15)	+	NA
7	33	0.182	0.18(21)	-	NA
8	26	0.118	0.13 (10)	-	NA

ND = Not done, PSA = Prostate specific antigen, BPH = benign prostatic hyperplasia, ACP = Adenocarcinoma of the prostate

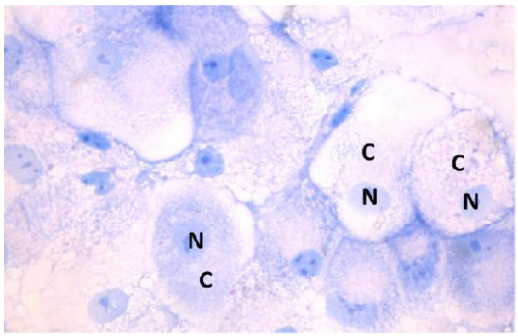

Fig. 2. Twelve monocytes from a day 21 plasma culture of a 32 year-old participant

Monocytes have capacity to adhere to substrates including glass and when doing so stretch out the cytoplasm. In this case note that the cytoplasm (C) of each monocyte has spread out on the coverslip, and the nucleus (N) is clearly visible. No prostatic epithelial cells are detectable in the cytoplasm after staining with GS or H&E stain or anti-androgen receptor monoclonal antibody. Giemsa stain: Original magnification, 40X

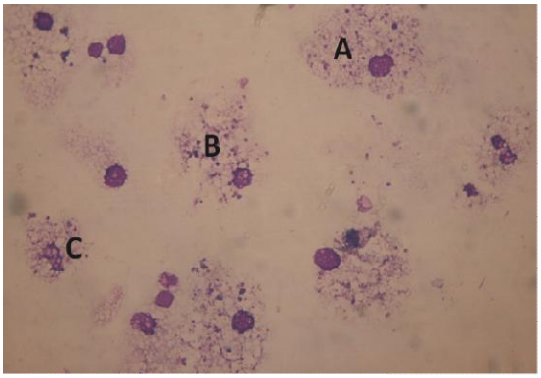

Fig. 3. Giemsa - stained monocyte culture of a patient aged 74 years with BPH

Monocyte cytoplasm is stretched out. This makes it possible to detect easily intracytoplasmic inclusions. In this Figure, sixteen monocytes each containing prostatic epithelial cells appearing as dots are shown. The cytoplasm of each monocyte appears to have formed strands. We hypothesize that the PSA released by the epithelial cells is deleterious to the monocyte cytoplasm as depicted sequentially in cells A, B, and C. Original magnification, 40X

4. DISCUSSION

Through a liquid biopsy approach (blood sample that provided the plasma) this study has demonstrated for the first time 6 intriguing phenomena that: (i) epithelial and stromal cells from patients with BPH and ACP circulate in blood (Figs. 3, 4 and 6); (ii) epithelial cells and stromal cells were present in plasma of all persons above 50 years who had neither BPH nor ACP; (iii) some young men aged 22 to 35 years have epithelial and stromal cells circulating in their plasma as well, without accompanying LUTS; (iv) prostate epithelial cells are found in the cytoplasm of monocytes and appear to exert deleterious and lethal effects probably due to the liquefying property of the acidic PSA that they secrete (Fig. 3); (v) when prostatic epithelial cells are cultured in RPMI 1640 medium, they produce detectable amount of PSA as they do in blood (Table 1 and Table 2); (vi) the mesenchyme upon which the epithelial cells are supported was detected (Fig. 7). This observation mimics the *in vivo* situation whereby a high number of epithelial cells are found supported by a mesenchyme-connective tissue cells (Fig. 9). Detection of a large number of epithelial cells on a mesenchyme-connective tissue is the determining observation for ACP on pathological examination of prostatic chips obtained after TURP (Fig. 9). It is intriguing that the adenocacinoma can be detected in a monocyte cell culture (compare Fig. 7 (culture) with Fig. 9 (histopathology). Our findings have implications in the pathogenesis of ACP (and BPH) particularly as regards the mode and route of metastasis and homing of prostate cancer cells at different sites in the body [5-6]. Additionally, circulating tumor cells are extremely few [7-12].

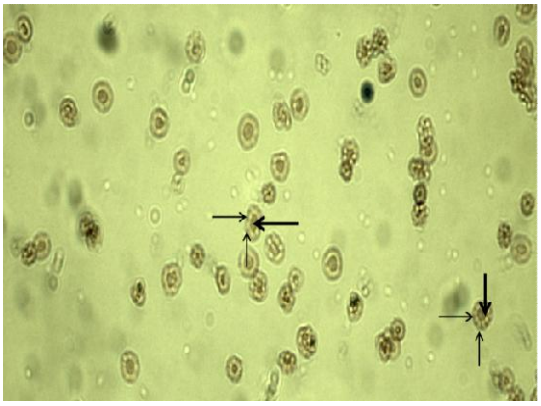

Fig. 4. Fifty prostatic acini that grew in suspension for 17 days from a patient with ACP

Acini consist of groups of peripherally arranged epithelial cells (thin arrows) ranging from 10 to 20 with a common duct (thick arrow) for collection of PSA they secrete. (Reference 1. R. Veltri and R. Rodriguez: The prostate forms acini and collecting ducts by arborization into the urethra). Giemsa stained, original magnification, 40X

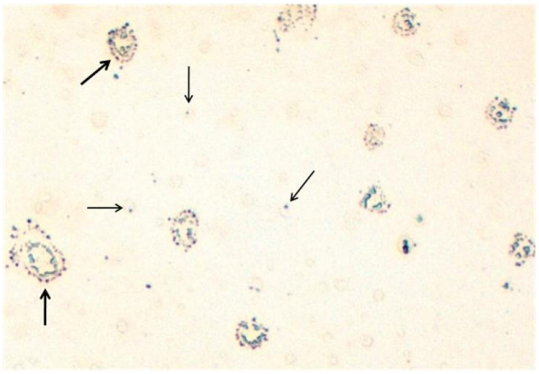

Fig. 5. Recognition of intracellular androgen receptors as brown dots (thick arrows) in epithelial cells inside 10 monocytes recognized by anti-androgen receptor monoclonal antibody

Few extracellular epithelial cells (thin arrows) were also detected. Original magnification, 40X

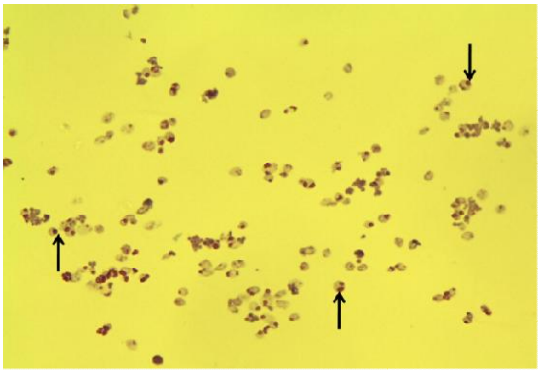

Fig. 6. One hundred and thirty Individual epithelial cells (with enlarged nucleoli) that grew in suspension for 14 days from the plasma of a patient with BPH`

H&E stain: Original magnification, 40X

The amount of PSA in the blood is termed 'Total' PSA. Total amount of PSA is free PSA plus PSA bound to other proteins, for-example alpha-1-antichymotrypsin. PSA could also be enveloped by alpha-2 macroglobulin, in which form it is not detectable by immunoassays. There is evidence that suggests a lower proportion of free PSA could be associated with more aggressive cancer, hence more clinically recognizable ACP [13-14]. Our study evaluated 'Total' PSA [15]. We did not study and compare clinical severity of either BPH or ACP in terms of free plasma PSA levels. Nevertheless Table1 shows elevated plasma PSA levels in ACP patient 5 (8.4 .ng/ml) and patient 15, (11.0 ng/ml). Similar elevated plasma PSA levels for 2 ACP patients are shown in Table 2 (15 ng/ml in patient 7 with corresponding monocyte culture media PSA level of 38.54 ng/ml and 14.3 ng/ml plasma PSA with corresponding high monocyte culture PSA of 41.59ng/ml for patent 8). Elevated plasma PSA in BPH patients was detected in patients 1, 2, and 4 (12.2 ng/ml, 8.6 ng/ml, 10.4 ng/ml, respectively (Table 1) and in patients 17 and 18 (5.4 ng/ml) and 9.2 ng/ml), respectively (Table 2). These intriguing findings call for more studies that will determine the clinical significance and severity of elevated plasma PSA vis-a-vis clinical severity of patients with BPH and ACP.

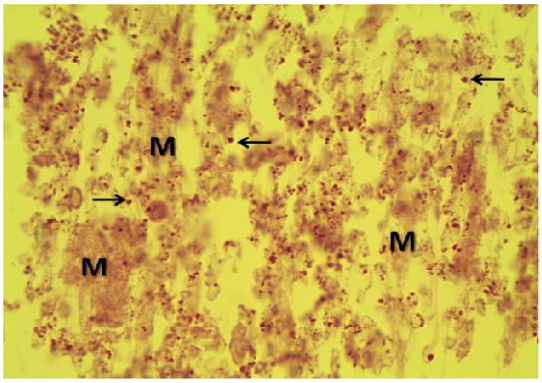

Fig. 7. A monocyte cell culture on day 21 of cultivation from an ACP patient 5, Table 1

There is possibly a mesenchyme layer (M) on which the acini/epithelial cells (arrows) are situated). H&E stain: Original magnification, 40X

Liquid biopsy (LB) is a terminology that has gained popularity in the search of a technique that would minimize invasive and painful procedures during the collection of specimens for diagnosis or research [7-8]. Obtaining specimens by tissue biopsy causes considerable discomfort to the patient and can create surgical complications including bleeding, spread and dissemination of the tumor. Therefore, the diagnosis of ACP by liquid biopsy as described here could circumvent the disadvantages associated with the practice of obtaining specimen by tissue biopsy. Furthermore, liquid biopsy as stated by Crowley can provide an almost complete picture of both the genetic and epigenetic profile of a normal and cancerous situation since it allows the isolation and characterization of living cells [5]. Therefore there is a high probability that the liquid biopsy cell culture technique we have developed will aid and facilitate the search for CTC in cancer of the prostate.

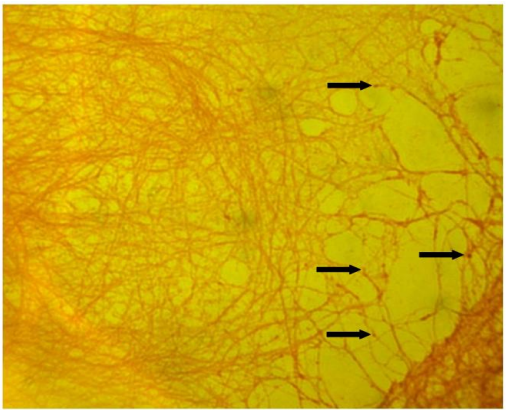

Fig. 8. Fibroblast-like cells that grew in the plasma of 5 patients with BPH on day 35 of cultivation

Nuclei of fibroblast cells are shown (arrows). H&E stain: Original magnification 40X

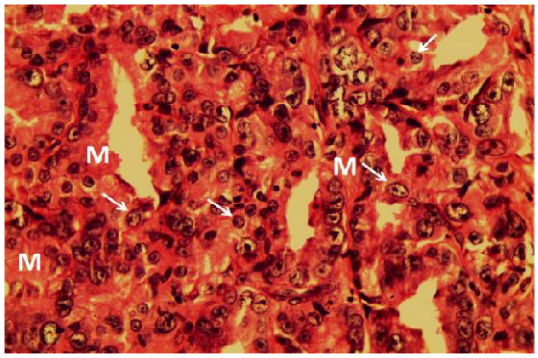

Fig. 9. Histopathological features of a prostatic chip from patient 15 with a Gleason grade 10 ACP consisting of discrete well formed acini (arrows) and possibly mesenchyme (M)

H&E stain: Original magnification, 40X

Friedlander and Fong [16] commented on CTC as a biomarker in castration -resistant prostate cancer. They stated that although CTC were first observed more than 100 years ago from patients suffering from cancer only recently has the clinical and research potential of these cells been widely applied. CTC can be considered as prognostic or predictive biomarkers in many malignancies, including ACP. Clinically CTC could be used to estimate total burden of disease progression. As a research tool CTC could be used to personalized treatment of a person's disease. The CellSearch platform (Veridex, Raritan NJ) has been used more often than other equipment and provides CTC counts obtained at specific time point that are then compared to a specific clinical manifestation of the disease at a later stage. Specific antibodies to prostate epithelial cells, for example EpCAm, can be used to identify CTC. We anticipate that use of modern tools and reagents used for detection of CTC will readily apply in the liquid biopsy technique described.

5. CONCLUSION

The search for CTC in prostate cancer research and diagnosis has intensified [10-12]. Researchers have used different methods in an attempt to grow CTC. A technique that is very manipulative may impact harmful shearing forces to CTC. We speculate that CTC are very delicate and therefore, should be isolated live and able to survive propagation under different conditions to be of use in research and diagnosis. This report may likely open a window toward the identification and propagation of prostate CTC by use of special equipment and specific markers [15].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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