MACQUARIE University
SYDNEY·AUSTRALIA

DEPARTMENT OF PHYSICS AND ASTRONOMY
FACULTY OF SCIENCE AND ENGINERING
MACQUARIE UNIVERSITY

Microfluidics-based isolation of disseminated tumour cells, as the technique for early diagnosis and prognostics of prostate cancer

By Alexey Rzhevskiy, MD, MSc

Principal Supervisor: A/Prof. Andrei Zvyagin
Associate Supervisor: Prof. David Gillatt
Adjunct Supervisor: A/Prof. Majid Ebrahimi Warkiani

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Statement of originality

This work has not previously been submitted for a degree or diploma at any educational or scientific institution. To the best of my knowledge, material presented in this thesis has not been published or written by another person before except where it is referenced in this manuscript.

Collection and analysis of human biological fluids specimens conducted at Macquarie University was performed under an ethical approval provided by Macquarie University Human Research Ethics Committee (HREC No: 5201500707). Collection and analysis of human urine specimens conducted at Sechenov University was performed under an ethical approval provided by Sechenov University Local Ethics Committee (extraction from protocol № 17-19).

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Publications, conference proceedings, awards during candidature

Journal articles

1. Pavlov, A. M., **Rzhevskiy, A. S.**, & Anissimov, Y. G. (2019). Numerical investigation of analytical models of drug flux through microporated skin. *Journal of pharmaceutical sciences*, 108(1), 358-363. doi: https://doi.org/10.1016/j.xphs.2018.11.009
2. **Rzhevskiy, A. S.**, Razavi Bazaz, S., Ding, L., Kapitannikova, A., Sayyadi, N., Campbell, D., ... & Zvyagin, A. V. (2020). Rapid and label-free isolation of tumour cells from the urine of patients with localized prostate cancer using inertial microfluidics. *Cancers*, 12(1), 81. doi: https://doi.org/10.3390/cancers12010081
3. **Rzhevskiy A.**, Popov A., Pavlov C., Anissimov Y., Zvyagin A., Levin Y., Kochba E.. Intradermal injection of lidocaine with a microneedle device to provide rapid local anaesthesia for peripheral intravenous cannulation: A randomised double-blind placebo- controlled clinical trial. *PLOS ONE.* Accepted for publication.
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5. **Alexey S. Rzhevskiy**, Alina Y. Kapitannikova, Denis V. Butnaru, Majid Ebrahimi Warkiani, Evgeniy V. Shpot, Andrei V. Zvyagin. Liquid biopsy in diagnosis and prognosis of non-metastatic prostate cancer. Prepared for submission to *Cancer research*.
6. **Alexey S. Rzhevskiy**, Alina Y. Kapitannikova, Denis V. Butnaru, Evgeniy V. Shpot, Dmitry V. Enikeev, Steven A. Vasilescu, Majid Ebrahimi Warkiani, Andrei V. Zvyagin. Rapid and label-free isolation of tumour cells from the semen of patients with localized prostate cancer using inertial microfluidics. Submitted to *Lab-on-a-chip*.
7. **Alexey S. Rzhevskiy**, Alina Y. Kapitannikova, Denis V. Butnaru, Evgeniy V. Shpot, Majid Ebrahimi Warkiani, Andrei V. Zvyagin. Liquid biopsy via label-free microfluidic isolation of tumour cells from biological fluids in localized prostate cancer. What's better: blood, urine or semen? Prepared for submission to *Cancer science*.

Conference proceedings

1. **Alexey Rzhevskiy,** Sajad Razavi Bazaz, Lin Ding, Alina Kapitannikova, Nima Sayyadi, Douglas Campbell, David Gillatt, Majid Ebrahimi Warkiani, Andrei Zvyagin. Using a spiral microfluidic chip for label-free isolation of prostate cancer cells from urine. Proceedings of the 4 th Advances in Circulating Tumour Cells: "Liquid biopsy: Latest Advances and Future Challenges", Corfu, Greece, October 2019. Poster presentation by Rzhevskiy.

Awards

1. Sydney Vital Research Scholar Award, Australia, 2019. Investigation title: Investigation and comparison of the efficiency of label-free isolation of tumour cells with a microfluidic chip from blood and large volumes of urine collected from prostate cancer patients with diverse forms of the disease including a neuroendocrine form.

Abbreviations

AMACR	Alpha-methylacyl-CoA racemase
AR	Androgen receptor
AR-V7	Androgen receptor splice variant 7
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
CK	Cytokeratin
CD133	Human prominin-1
CD45	Lymphocyte common antigen
CRPC	Castration-resistant prostate cancer
CTC	Circulating tumour cell
ctDNA	Circulating deoxyribonucleic acid
DAPI	4’,6-diamidino-2-phenylindole
d-PCR	Digital polymerase chain reaction
DPBS	Dulbecco's phosphate-buffered saline
DRE	Digital rectal examination
DTCs	Disseminated tumour cells
EDTA	Ethylenediamine tetraacetic acid
EMT	Epithelial-mesenchymal transition
EPCAM	Epithelial cell adhesion molecule
ERG	ETS-related gene
EVs	Extracellular vesicles
FV	First morning void
FDA	Unites States Food and Drug Administration
GPC	Glypican
GS	Gleason score
GSTP1	Glutathione S-transferase p1 gene
HCs	Healthy controls
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICC	Immunocytochemistry

IGFBP3	Insulin-like growth factor binding protein 3
IGFBP7	Insulin-like growth factor binding protein 7
mCRPCa	Metastatic castration-resistant prostate cancer
miR	Particular type of miRNA
miRNA	Micro ribonucleic acid
NA	Not applicable
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline/Tween
PCa	Prostate cancer
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
PTEN	Phosphatase and tensin homolog
PTGS2	Prostaglandin-endoperoxide synthase 2
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SFRP2	Secreted frizzled-related protein 2
SOD2	Superoxide dismutase 2
STR	Short tandem repeat
TNM	Tumour, nodes, metastases
TWEAK	Tumour necrosis factor-like weak inducer of apoptosis
TXNRD1	Thioredoxin reductase 1
TYMS	Thymidylate synthase
WBC	White blood cell

Abstract

Globally, prostate cancer has been the second most frequent cancer occurring in males, being at the same time the second most frequent cancer in terms of mortality. Due to a prolonged progression of the disease, one of the most significant aspects in prostate cancer management is its detection at an early localized stage which allows to perform adequate treatment and decreases the risk of possible cancer-related death. Currently, a screening blood test for prostate specific antigen and 12-core tissue biopsy are necessary diagnostic procedures required to establish the diagnosis of prostate cancer. However, prostate specific antigen level has a relatively low specificity as a prostate cancer biomarker and is often elevated in case of such diseases as benign prostatic hyperplasia and prostatitis. At the same time, tissue biopsy is a highly invasive painful procedure which has a relatively low sensitivity in case of early localized form of prostate cancer and has such possible severe complications as local bleeding and abscesses. Hence, alternative diagnostic and prognostic approaches are highly demanded. One of such approaches, which has been actively investigated during the last decade, is liquid biopsy via isolation of disseminated tumour cells from biological fluids of a human body. Overall, the main goal of the PhD project was to develop liquid biopsy approaches based on the microfluidic label-free isolation of prostate cancer tumour cells from biological fluids of patients with early, non-metastatic form of the disease. In the current work, three biological fluids, including blood, urine, and semen, were analyzed and compared as a source of prostate cancer disseminated tumour cells, using the developed microfluidic techniques. According to the results, biological fluids could have been ranked in terms of the average amount of isolated putative tumour cells in the ascending sequence as follows: blood, urine, and semen. In general, in case of blood, only rare tumour cells could have been isolated from small number of patients; in case of urine, some tumor cells could have been isolated from the majority of patients; in case of semen, large number of the tumor cells could have been isolated from all of the patients. Thus, according to the data presented in Chapter VI, prostate cancer tumour cells could have been isolated from the semen of all 13 (100%) of 13 patients, with the amount of isolated tumor cells varying from 47 to 280.8 units per mL of the semen. Also, strong correlation between the amount of isolated tumour cells and prostate specific antigen serum level was indicated. Consequently, liquid biopsy of prostate cancer via microfluidic enrichment of

disseminated tumour cells from semen is the promising technique, which has a potential for being introduced as a diagnostic and prognostic tool into a clinical practice of managing prostate cancer at its localized form. For this purpose, further validation and optimization of the technique is required.

Chapter I

General introduction

1.1. Thesis structure

In accordance with the Macquarie University "HDR Thesis Preparation, Submission and Examination Policy" this thesis was structured as the thesis by publication. In the first chapter "General Introduction", pros and cons of the existing techniques for early diagnosis and prognostics of prostate cancer (PCa) were discussed. The discussion also shed light on the technique of liquid biopsy via microfluidics label-free isolation of disseminated tumour cells (DTCs) from human biological fluids as perspective alternative to the existing diagnostic techniques which have routinely been used in clinical management of localized PCa. An introductory Chapter II was represented by a comprehensive review of liquid biopsy techniques, which have been developed for the purpose of PCa early diagnosis and prognostics and covered the current place of microfluidics-based isolation of DTCs from biological fluids among these techniques. Next, a study representing the development of microfluidic size-based technique for isolating DTCs from urine of patients with localized PCa, and subsequent evaluation of the technique's diagnostic and prognostic value, were described in Chapter III. Chapter IV represents investigation of the efficiency of microfluidic chip, previously developed by the candidate's adjunct supervisor Dr Majid Warkiani, as a tool for isolating DTCs from blood of patients with localized PCa. Information on the development and testing of the microfluidics-based technology for label-free isolation of PCa DTCs from semen, as the approach for early diagnosis of PCa, was presented in Chapter V. In turn, Chapter VI was followed by an information and discussion on a comparative study involving microfluidics-based isolation and identification of PCa DTCs from all three biological fluids – blood, urine, and semen. Finally, Chapter VII represents general conclusive discussion on the performed investigations and provides outlook on future perspectives of the DTC isolation via microfluidics-based liquid biopsy as the technique for early diagnosis of PCa, its prognosis and monitoring.

1.2. Prostate cancer

The prostate is a walnut-size gland of male reproductive system which is located in front of the rectum, between the bladder and the penis, and is longitudinally crossed by urethra [1]. The gland is composed mainly of glandular and muscular parts and is mainly bearing functions of seminal fluid production and semen ejaculation. A glandular part of the prostate can be divided into three zones: the central zone, the transition zone and the peripheral zone from where prostate carcinoma originate in 70% of cases [2].

According to GLOBOCAN 2018 prostate cancer [3] is one of the most frequent cancers and causes of cancer-related deaths in males worldwide, with its highest prevalence in western countries [4]. In fact, in 2018, 164690 newly diagnosed cases of the disease and 29430 cancer-related deaths were registered in the US, with the lifetime risk of being diagnosed with PCa and of dying from PCa at 11% and 2.5%, respectively. The median age at diagnosis of the disease is of 72 years, while analysis of autopsies from men who died from causes other than PCa demonstrated presence of PCa in 20% and 33% of cases for the age groups of 50-59 and 70-79 years, respectively [5]. In general, the disease begins at its localized form and if untreated progresses with invasion to the nearby tissues, and formation of metastases. Among the newly diagnosed cases of PCa, 79% are represented by a localized form of the disease and 21% by the locally advanced or metastatic forms [6], with the 5-year survival rates at 100% and 29.8% for localized and metastatic form, respectively. Thus, to avoid progression of the disease to its life-threatening stages, detection of PCa at its early localized form has been intensively investigated [7].

During the last decade, limitations of early cancer diagnosis with such conventional diagnostic procedures as prostate-specific antigen (PSA) blood test and tissue biopsy have been actively discussed. Thus, PSA value may vary depending on different disorders which significantly decrease specificity of the test. Furthermore, PSA value is uninformative in identification of PCa aggressiveness. In clinical practice, in case of the PSA value at higher than 4 ng/ml, PCa is further diagnosed with tissue biopsy. However, in only 25% of cases tissue biopsy is positive for PCa, which leads to the risk of unnecessary biopsies and overdiagnosis [8]. At the same time, even though tissue biopsy is highly specific, it is highly invasive, has such possible side effects as local bleeding and abscesses, has low sensitivity

in case of early localized form of PCa, and for better outcome requires assistance with imaging techniques such as MRI [9]. Therefore, even though tissue biopsy is currently a pivotal test in PCa diagnosis, it is far from ideal and is inappropriate for regular screening. Moreover, Gleason score – a quantitative expression of tissue biopsy, and a major parameter in stratification of PCa aggressiveness, has low reproducibility. Therefore, alternative techniques for PCa early diagnosis and prognosis are highly demanded.

As one of such techniques facilitating early diagnosis and prognosis of PCa, liquid biopsy has attracted major attention in the past decade [10]. The technique of liquid biopsy implies collection of biological fluids and their further analysis at cellular or molecular level. In liquid biopsy, standard objects of investigation are tumour cells, circulating DNA, exosomes and exosomal miRNAs. Initially, the technique has been investigated as the patient-friendly and safe diagnostic alternative to the conventional tissue biopsy. Thus, despite tissue biopsy, liquid biopsy is less stressful and harmful to a patient and provides an opportunity to avoid possible side effects common for tissue biopsy [11]. One of the most widely used approaches in the development of liquid-biopsy techniques, especially those which are based on isolation of DTCs, is microfluidics.

1.3. Microfluidics-based liquid biopsy

It was proven that progression-free survival and overall survival are dramatically increased if cancer is detected at an early stage, which allows to timely perform adequate and efficient treatment [12]. Such circulating biomarkers as DTCs, circulating nucleic acids and exosomes are currently considered promising to efficiently replace or substitute the existing biomarkers used in cancer diagnosis, prognostics, and monitoring. In its turn, microfluidics is the inexpensive, convenient and efficient technology for isolating circulating biomarkers, particularly – DTCs [13].

In recent years, rapid development of microelectromechanical systems led to investigation of diverse microfluidics-based devices designated for diagnostics of diverse diseases, including different types of malignant tumours. The microfluidics-based diagnostic devices are characterized by cost-effectiveness, small size, portability and simplicity in manufacturing [14], which made them attractive for investigators in biomedical engineering all around the world. The technology of microfluidics is currently the core for diverse liquid

biopsy techniques. One of such techniques is relying on principles of label-free, or in other terms size-based, microfluidic isolation of DTCs [15]. In this technique, separation of DTCs is based on differences in biophysical characteristics such as size, density, deformability, or dielectric properties, between DTCs and other cells presenting in a biological fluid. Among diverse approaches of microfluidic label-free isolation of DTCs, an inertial size-based approach gained particular interest due to its high separation efficiency, recovery rate and throughput [16]. With this approach, DTCs are isolated in a gentle manner which keeps the isolated DTCs intact and therefore allows further analysis of the DTCs with such techniques as morphological assessment, immunocytochemistry, polymerase chain reaction, etc.

One of the most prominent techniques, based on the microfluidic size-based label-free approach for isolating DTCs from blood, has been previously developed by the candidate's Adjunct Supervisor – Dr Majid Ebrahimi Warkiani [17]. Since the development, the effectiveness of the technique has been proven in numerous studies where DTCs were isolated from blood of patients diagnosed with diverse types of cancer, including head and neck, breast, and lung cancers [18-20]. However, the technique has not been yet tested for isolating DTCs from blood of patients with localized PCa. Thus, in this thesis, a previously developed technique of the microfluidics-based liquid biopsy by Warkiani et al. [21] was taken as a basis for investigating the microfluidics-based label-free liquid biopsy techniques based on isolation of DTCs from urine and semen of patients with localized PCa. Moreover, the liquid biopsy technique by Warkiani et al. has been applied for isolating DTCs from the blood of patients with localized PCa.

1.4. Aims of this thesis

Overall, this thesis is aimed to investigate diagnostic and prognostic potential of liquid biopsy via the microfluidic label-free isolation of DTCs from biological fluids of patients with localized prostate cancer. Accordingly, it was purposed to test the previously developed liquid biopsy technique by Warkiani et al. [17], relying on the microfluidics-based label-free isolation of tumour cells from blood, for isolating PCa DTCs from blood of patients with non-metastatic PCa; and to develop and test novel microfluidic techniques for DTC isolation from urine and semen.

In Chapter II, current status of liquid biopsy as the technique for early diagnosis and

prognostics of PCa, based on isolation and analysis of DTCs, circulating deoxyribonucleic acid (ctDNAs), exosomes and exosomal miRNAs from biological fluids of the human body – blood, urine, and semen, was reviewed. Also, the place of the microfluidics-based label-free isolation of DTCs among the existing liquid biopsy techniques was described. The chapter is in general aimed to highlight a significant relevance of investigations presented in the current thesis.

The aim of the investigations described in Chapter III was to develop a microfluidic chip and a corresponding liquid biopsy technique by means of DTC isolation from urine of patients with localized PCa. The technique was investigated by adopting the previously developed technique of the microfluidics-based liquid biopsy of blood by Warkiani et al. [21]. Also, it was aimed to test the efficiency of immunocytochemical identification of the isolated DTCs by using ant-glypical-1 (anti-GPC-1) antibody as a primary agent.

The aim of the investigations described in Chapter IV was to test efficiency of the microfluidic chip, previously developed for isolating DTCs from blood by the candidate's adjunct supervisor Dr Majid Warkiani, in terms of its capacity for isolating DTCs from blood of patients with localized PCa. Also, potential diagnostic and prognostic value of the technique was described. Due to the promising results which were demonstrated in the studies where the microfluidic chip was successfully applied for isolating DTCs from blood of patients diagnosed with head and neck, lung and breast cancer, a high efficiency of the chip was expected in the current study.

The aim of the investigations described in Chapter V was to develop a microfluidics-based liquid biopsy technique for isolating DTCs from the semen of patients with localized PCa, relying on investigations and findings described in Chapter III and Chapter IV. The isolated DTCs were detected by immunocytochemical labelling with the antibody panel containing such conventional primary antibodies as anti-cytokeratin (anti-CK) and anti-prostate specific membrane antigen (anti-PSMA), and anti-GPC-1 as the antibody with a recently discovered specificity to the PCa tumour cells [22].

The aim of the investigations described in Chapter VI was to compare efficiency of the microfluidic liquid biopsy techniques described in Chapters III-V in terms of isolation of PCa DTCs from all three biologic fluids: blood, urine, and semen. In this study, each patient provided simultaneously all three biological fluids for the analysis and the isolated DTCs were identified by using the same (for urine and semen) or similar (for blood) antibody

panel, therefore direct comparison between three liquid biopsy techniques was performed. Finally, considering the data presented in Chapters III-VI, it was aimed to discuss advantages and disadvantages of all three liquid biopsy techniques and provide possible directions for further investigations in the last chapter - Chapter VII.

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Chapter II

Liquid biopsy in diagnosis and prognosis of non-metastatic prostate cancer

Alexey S. Rzhevskiy [1,2,3,*], Alina Y. Kapitannikova [2], Denis V. Butnaru [3], Evgeniy V. Shpot [3], Majid Ebrahimi Warkiani [4], Andrei V. Zvyagin [1,2]

[1] ARC Centre of Excellence for Nanoscale BioPhotonics, MQ Photonics, Macquarie University, 2109 Sydney, Australia

[2] Institute of Molecular Medicine, Sechenov First Moscow State Medical University, 119991 Moscow, Russia

[3] Institute for Urology and Reproductive Health, Sechenov University, 119991 Moscow, Russia

[4] School of Biomedical Engineering, University of Technology Sydney, 2007 Sydney, Australia

* Correspondence: **alexey.rzhevskiy@hdr.mq.edu.au**

Keywords: prostate cancer, liquid biopsy, circulating tumour cells, circulating DNA, exosomes, miRNA

2.1. Introduction to the chapter

More than a hundred years ago, Australian physician Thomas Ashworth described atypical, presumably - tumour cells presenting in blood of a patient with metastatic form of cancer [1]. Such finding, and further investigations in this research area, led to the establishment of such term as liquid biopsy. Thus, liquid biopsy implied isolation, detection and analysis of tumour cells presenting in biological fluids, predominantly – blood. The technique has been considered a promising minimally or non-invasive alternative to the highly invasive gold standard of diagnostics for most of the malignant tumours - tissue biopsy. However, liquid biopsy has not been actively investigated until recently due to the lack of appropriate technological solutions. To date, liquid biopsy has transformed to a particular subdiscipline in oncology and deals not only with disseminated tumour cells but also with circulating DNAs, exosomes and exosomal miRNAs. Most of the studies describing diverse liquid biopsy approaches, that may potentially be used for early diagnosis and prognostics of non-metastatic PCa, have been published during the last decade. This chapter is represented by a systematic review in the field of liquid biopsy as the tool for diagnosing and prognosing non-metastatic prostate cancer. The main aim of this review was to understand current status and perspectives of PCa liquid biopsy by means of the microfluidics-based label-free isolation of disseminated tumour cells, among other liquid biopsy approaches.

2.2. Author's contribution

The PhD candidate (Alexey Rzhevskiy) conceptualized the study, performed collection of the relevant data, and wrote the manuscript.

Candidate's principal supervisor Dr Andrei Zvyagin participated in conceptualization, design, and revision of the manuscript. Candidate's associate supervisor Dr Evgeniy Shpot, and Dr Denis Butnaru, provided advisory as the specialists in urology. Dr Majid Ebrahimi Warkiani provided advisory as the specialist in liquid biopsy. Alina Kapitannikova assisted with preparing images and literature search.

2.3. Abstract

Background & Aims: In terms of diagnosis and prognostics, prostate cancer at its early form is currently one of the most complicated cases in oncology. To establish the diagnosis and further identify an appropriate treatment strategy, several tests including tissue biopsy and prostate specific antigen (PSA) blood test have to be implemented. The combination of tests is justified by the lack of a highly sensitive, specific, and safe single test. Thus, tissue biopsy is specific but invasive and may have severe side effects, and therefore is inappropriate for screening of the disease. At the same time, PSA blood test which is conventionally used for PCa screening has low specificity and may be elevated in case of noncancerous prostate tumours and inflammatory conditions, including benign prostatic hyperplasia and prostatitis. In this setting, diverse techniques of liquid biopsy have been actively investigated to supplement or replace the existing tests of prostate cancer early diagnosis and prognostics. We preformed a systematic review in the field of advances in diagnosis and prognostics of localized prostate cancer by means of various biomarkers extracted via liquid biopsy, including disseminated tumour cells, exosomes and different types of circulating nucleic acids.

Methods: A systematic review of original articles in the field of liquid biopsy of non-metastatic prostate cancer, published in a period from 1 January 2010 to 31 December 2020, was performed. Thus, diagnostic and prognostic value of diverse liquid biopsy techniques base on isolation and analysis of nucleic acids, exosomes and exosomal miRNAs, and disseminated tumour cells, was examined.

Results: Our final analysis included 48 articles, among which 11 contained information on PCa liquid biopsy by means of isolation and analysis of circulating nucleic acids, 15 contained information on PCa liquid biopsy by means of isolation and analysis of exosomes and exosomal miRNAs, and 22 contained information on PCa liquid biopsy by means of isolation and analysis of disseminated tumour cells. According to the analyzed publications, all of the liquid biopsy approaches have diagnostic and prognostic potential to some extent. However, the results vary between the studies, mostly depending on the liquid biopsy protocol applied.

Conclusions: More data, and standardization of liquid biopsy protocols applied in identifying and analyzing corresponding biomarkers, are required for recommending liquid biopsy in management of non-metastatic PCa.

2.4. Introduction

In clinical practice, prostate cancer (PCa) is currently diagnosed by performing prostate-specific antigen (PSA) blood test and, if PSA serum level is increased, tissue biopsy. However, PSA serum level is a relatively weak indicator of PCa due to its only moderate specificity [2]. Thus, the value of PSA serum level at 4 ng/mL, recommended by most of the clinical guidelines as suspicious for PCa and therefore requiring subsequent performance of tissue biopsy, is often recognized in patients with non-cancerous lesions of the prostate including benign prostatic hyperplasia (BPH) [3] and prostatitis [4]. Further, poor monitoring of PSA level has been thought one of the most significant causes of the advanced form for newly diagnosed PCa [5]. It is also worth noting that in most countries routine PSA screening is recommended for men only in their 55-69 years for avoiding overdiagnosis in other age categories [6], which may significantly increase the risk of missing PCa in men under 55 years. Thus, to increase the probability for diagnosing PCa at its early form, more specific and sensitive alternative or substitution to the PSA blood test is required. Regarding the 12-core tissue biopsy [7] applied in PCa management, even though the test has specificity close to 99%, its sensitivity is relatively low [8] and therefore requires ultrasound or magnetic resonance imaging guidance for better precision, which significantly increase financial cost of the procedure [9]. Approximately, only 40-50% of patients with elevated PSA serum level have the tissue biopsy test positive for PCa [10]. Moreover, outcome of the procedure Is highly dependent on experience of an operator, which compromises its objective diagnostic value. From patient's perspective, tissue biopsy is a highly invasive, painful procedure, having such possible side effects as local hematomas, abscesses and even erectile dysfunction [11].

The most frequent initial tactics for managing localized prostate cancer are active surveillance and watchful waiting [12]. These tactics imply monitoring of PCa after its diagnosis by performing periodic PSA tests and tissue biopsies. In case of cancer progression, the tactics imply proceeding to further actions, the most usual of which is prostatectomy.

However, the tactics of active surveillance and watchful waiting are compromised by a significant genetical heterogeneity of the disease, and variety of forms from indolent localized to rapidly developing castration-resistant [13]. Thus, to increase specificity and sensitivity of PCa detection at an early stage, provide more accurate risk stratification and prognosis of the disease, timely identify the rapidly progressing cases, and to therefore develop more efficient treatment approaches, liquid biopsy via isolation and analysis of circulating biomarkers has been proposed.

To date, liquid biopsy has been considered one of the most promising techniques which could potentially replace or substitute to the existing diagnostic tests – PSA test and tissue biopsy. Liquid biopsy techniques may be stratified in terms of the biomarker of interest, which are: DTCs, exosomes and exosomal miRNAs, and circulating DNA (ctDNA). Thus, liquid biopsy has been termed as sampling of biological fluids containing any of these biomarkers and has been actively investigated as the minimally or non-invasive technique which would first of all replace standard tissue biopsy. As a potential technique for diagnosis and prognosis of malignant tumours, liquid biopsy was discovered in 19th century by Thomas Ashworth [1] who identified presence of DTCs in blood of patients with metastatic form of cancer. However, the technique has not been broadly investigated until recently due to a lack of approaches which would allow efficient isolation, identification and analysis of DTCs. In the past two decades, the development of nano- and micro-technologies led to significant evolution of liquid biopsy via isolation of DTCs from diverse biological fluids, and resulted in discovery of novel liquid biopsy methodologies based on isolation of exosomes and nucleic acids [14] (Figure1).

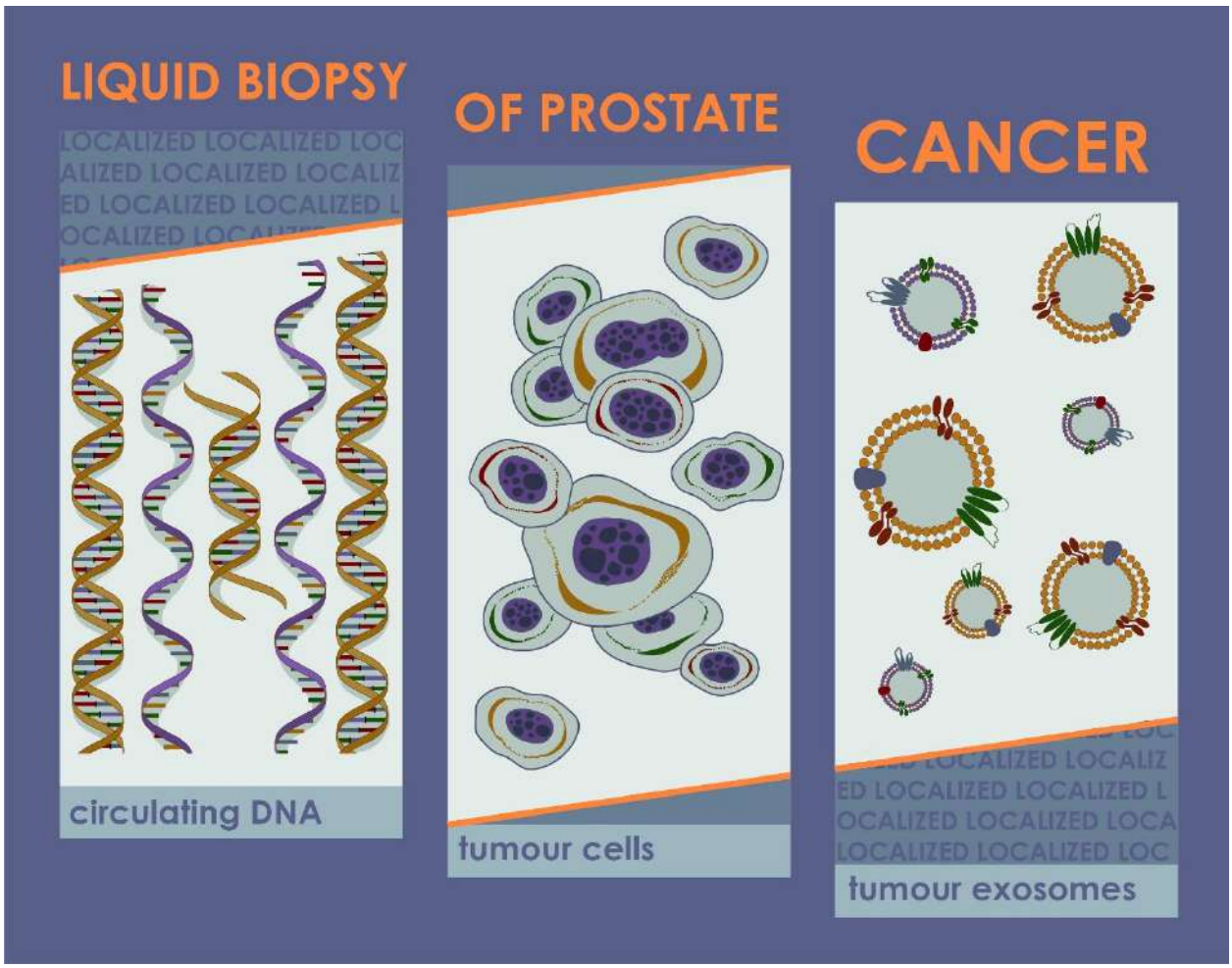

Figure1. Approaches for liquid biopsy of PCa via isolation and identification of circulating biomarkers. The approaches may be stratified into three groups depending on the type of isolated biomarkers, which are: nucleic acids, tumour cells and exosomes. Circulating DNA are the fragments of tumour DNA, which release as a result of cell destruction; tumour cells are the cells which shed into the biological fluids from primary or secondary tumour sites; exosomes are the microvesicles containing diverse biologically active agents which have been considered biomarkers, including exosomal miRNA, and release from tumour cells into the biological fluids as a result of cellular metabolism. In PCa, during the cancer progression, the biomarkers can be mainly found into three biological fluids such as blood, urine and semen.

DTCs are the cells which shed into diverse biological fluids of a human body from primary or secondary tumour sites, and therefore may be detected in cancer patients while undetectable in healthy individuals [15]. The DTCs are vastly heterogeneous, first of all in terms of antigen profile and beared gene mutations [16]. Cell-free ctDNA are the fragments of DNA, which release into the body fluids due to lysis or apoptosis of tumour cells [17]. Exosomes are the extracellular vesicles (EVs) which release from tumour cells during their

functioning. These vesicles are bearing various agents, including miRNA, which may be recognized as tumour biomarkers [18]. The listed biomarkers are identified in such biological fluids of PCa patients as blood, urine and semen [19]. The aim of this systematic review was to compile the existing data within the research field of liquid biopsy as the technique for early diagnosis and prognosis of PCa, to analyse the collected data, and to provide potential directions for investigations which would allow to implement the technique in clinic.

2.5. Materials and methods

The current review was prepared by selecting research articles, written in English, from two data bases: PubMed, and Web of Science. To identify original articles which report on the application of diverse liquid biopsies for diagnosis and prognostics of localized PCa, article search was performed by using such key terms or their combinations as: “localized prostate cancer”, “non-metastatic prostate cancer”, “circulating tumour cells” or CTCs or “circulating cancer cells”, “exosomes” or “extracellular vesicles”, “circulating miRNA(s)”, “circulating DNA(s)”, “circulating RNA(s)”, “liquid biopsy”. Further, a filter for the publication dates from 1 January 2010 to 31 December 2020 was applied. Books, review articles, letters to editor, case studies, studies of PCa animal models or cell lines, and research articles which did not contain data on diagnostic and prognostic potential of liquid biopsy biomarkers in management of localized PCa were excluded from the analysis. Notably, from the research articles chosen for the current review, data for both localized (T1, T2 clinical stages) and locally advanced (T3, T4 clinical stages) forms of PCa was included.

2.6. Results

Abstracts and titles of the articles, which were obtained after initial evaluation, were assessed for inclusion. As a result, 42 studies were considered eligible for being included into the systematic review. Also, 6 more articles were included due to reference tracking. Thus, 48 articles in total were included into the systematic review. Among these 48 articles, 11 were related to the PCa liquid biopsy by means of isolation and analysis of ctDNAs, 15 were related to the PCa liquid biopsy by means of isolation and analysis of exosomes and exosomal miRNAs, and 22 were related to the PCa liquid biopsy by means of DTCs (Figure2).

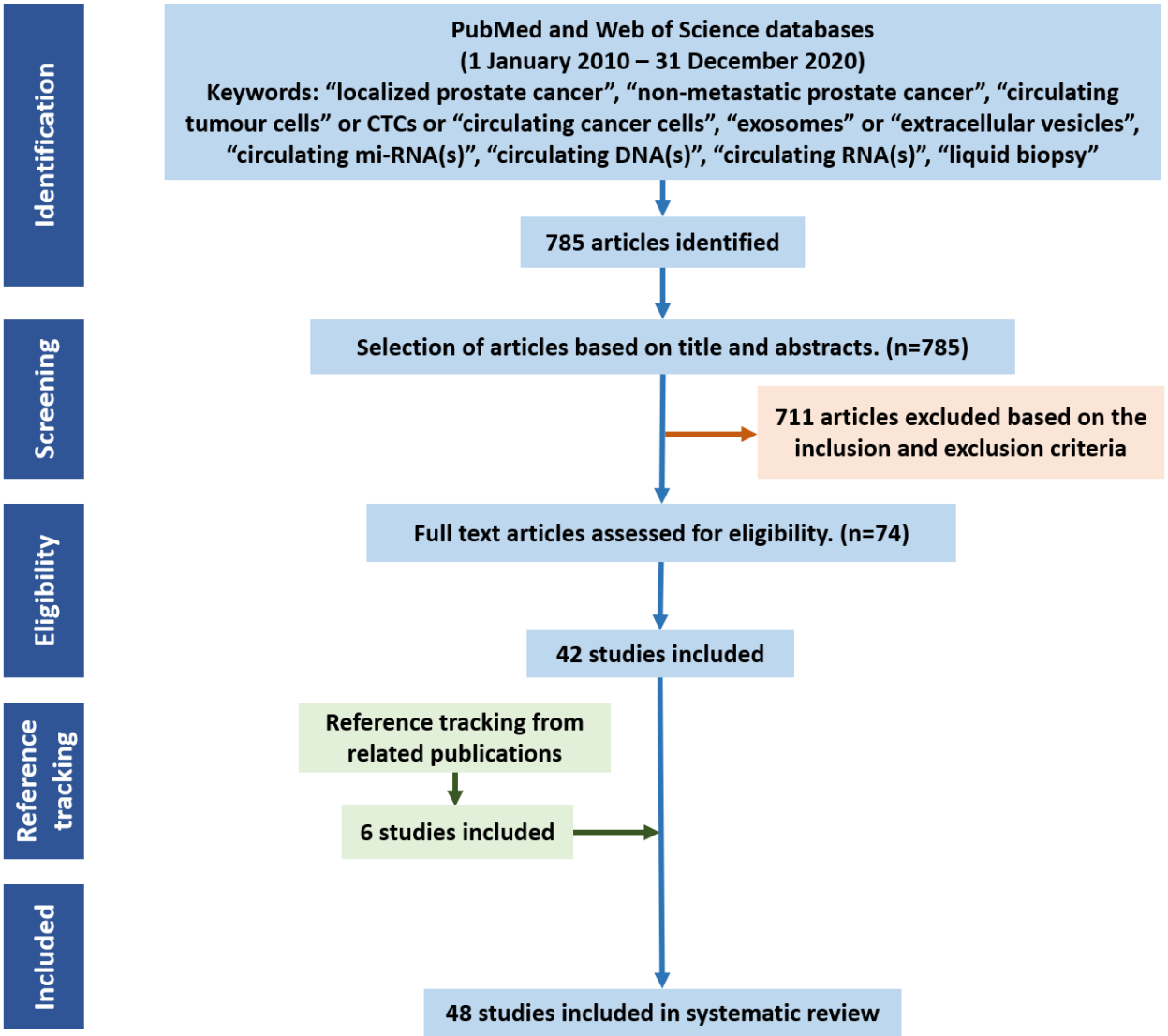

Figure2. The figure demonstrates the process of selecting studies for inclusion into the current systematic review.

2.6.1. Circulating DNAs

For the first time, circulating nucleotides were isolated from human blood in 1948 [20]. Since the early 2000s, they have been actively investigated as potential biomarkers for the diagnosis and monitoring of cancer development [21, 22]. Due to the anatomical features of the male reproductive system, circulating tumour nucleic acids were found not only in blood but also in urine and semen of PCa patients, which provided an opportunity to develop highly accurate non-invasive diagnostic techniques [23]. In PCa, one of the significant issues of ctDNAs as diagnostic and prognostic biomarkers is that the molecular

landscape of the tumour is significantly variable depending on its stage [13]. Thus, it was demonstrated that there is a direct correlation between the stage of the disease, the amount of circulating cell-free DNA in biological fluids, and its variability. In the case of the localized form of PCa, the total amount of cell-free DNA is relatively small [24]. At the initial stages of cancer, the amount of ctDNAs in biological fluids is often insufficient to be determined even by modern, highly sensitive techniques.

Extracellular DNAs are the short fragments of nucleic acids found in most of the biological fluids of the human body, including blood, urine, and semen [25]. The length of the extracellular DNA fragments is approximately 134 to 144 base pairs. It was suggested that they occur due to apoptotic degradation of the cellular DNA [26]. ctDNAs is one of the components of extracellular DNA originating from tumour cells, and its total fraction is mainly dependent on the tumour stage [27]. Previously, the detection and analysis of mutations in small amounts of ctDNA, presenting as one of the fractions of extracellular DNA, posed a severe technical challenge [28]. Although blood has been most widely investigated as the matrix for liquid biopsy, alternative biological fluids have recently gained significant attention [29, 30]. Particularly in the case of urine, PCa biomarkers may be detected earlier, compared to blood [31]. In urine, ctDNAs are exposed to less aggressive effects of damaging agents than those found in blood [32]. In addition, semen is the other perspective source of liquid biopsy biomarkers, which has not been studied thoroughly so far [33].

2.6.1.1. Genetic heterogeneity of PCa

The gradual development of genetic heterogeneity is the distinctive feature of PCa evolution [34]. It is followed by the dominance of subclones which promote metastasis and drug resistance. In the case of PCa, heterogeneity was discovered by pathologists in 1970s: it was found that diverse subtypes of PCa differ in nuclear morphology, cell proliferation, levels of immune cell infiltration, and several other features [35]. In addition to morphological heterogeneity, heterogeneous copy number variations were reported for the primary tumour [36]. Similarly, somatic single-nucleotide polymorphism was demonstrated among both localized and metastatic tumours [37]. Tracking heterogeneity in the

mutational landscape of the tumour made it possible to identify the evolution of clones within individual PCa patients [38].

It was identified that characterization of subclonal alterations in ctDNAs is technically challenging since these alterations are present at lower frequencies compared to the total amount of the prevailing non-tumour extracellular DNA [39]. For instance, mutations within the same patients with PCa were compared in a study by Chae et al. [40]. The results of FoundationONE assay, a tissue-based cancer panel, were compared to the results of Guardant360 ctDNA assay, which is a commercially available test system for cancer diagnostics based on extracellular DNA. It was demonstrated that only 17.1% of the registered alterations were the same for both test systems. In the study by Kuderer et al. [41], similar results were achieved, where only 22% of mutations were recognized by both test systems. It was demonstrated that alterations with an allele frequency of less than 1% in ctDNA were more likely not to be found in tumour tissue. According to the results, some of the alterations found in ctDNA might have been caused by subclones, and therefore could have been found in the tumour tissue more rarely. In the study by Torga et al. [42], blood was collected from 40 PCa patients and analyzed in parallel on the Guardant360 and PlasmaSELECT test systems. According to the results, a significant discrepancy in the data obtained on mutations was demonstrated, with only 30% of patients having the same results on both test systems. Thus, sequencing of tissue samples and serial liquid biopsies need to be followed by each other to identify the mutational landscape in patients with PCa.

2.6.1.2. Circulating tumour DNA in PCa

In early studies, whole-genome sequencing was widely used to characterize extracellular DNAs in PCa patients [43]. With a shallow sequencing depth, following mutations were detected in these studies: AR amplification, ERG fusion, PTEN deletion, MYC amplification [44] (Figure2). Based on the data obtained, the interactions between AR aberrations and clinical data of patients with PCa were identified. According to the results, AR amplification was detected more common in patients who subsequently developed resistance to androgen receptor signaling inhibitors [45]. In addition, AR amplification and mutations found in ctDNA prior to initiation of the therapy were associated with poor clinical outcomes, including poor survival, lower PSA response rates, and shorter time before

tumour progression. Deep sequencing and targeted library enrichment were combined in modern ctDNA research approaches to analyze genes of interest. To further enhance sensitivity and specificity, molecular barcoding technology was developed to distinguish low-frequency mutations from sequencing errors [46]. Currently, there are many cell-free DNA tests available [47]. For instance, the Guardant360 technique performs capture-based enrichment and molecular barcoding followed by targeted sequencing of 73 clinically significant genes [48]. In the study by Odegaard et al. [49], more than 10 000 clinical samples of PCa patients were analyzed using this technique. A sensitivity limitation of 0.02% allelic fraction and a positive predictive value of testing in 92–100% of patients were reported by the authors. Nevertheless, a limited number of studies on localized form of PCa necessitates further research in this direction and verification of the data obtained.

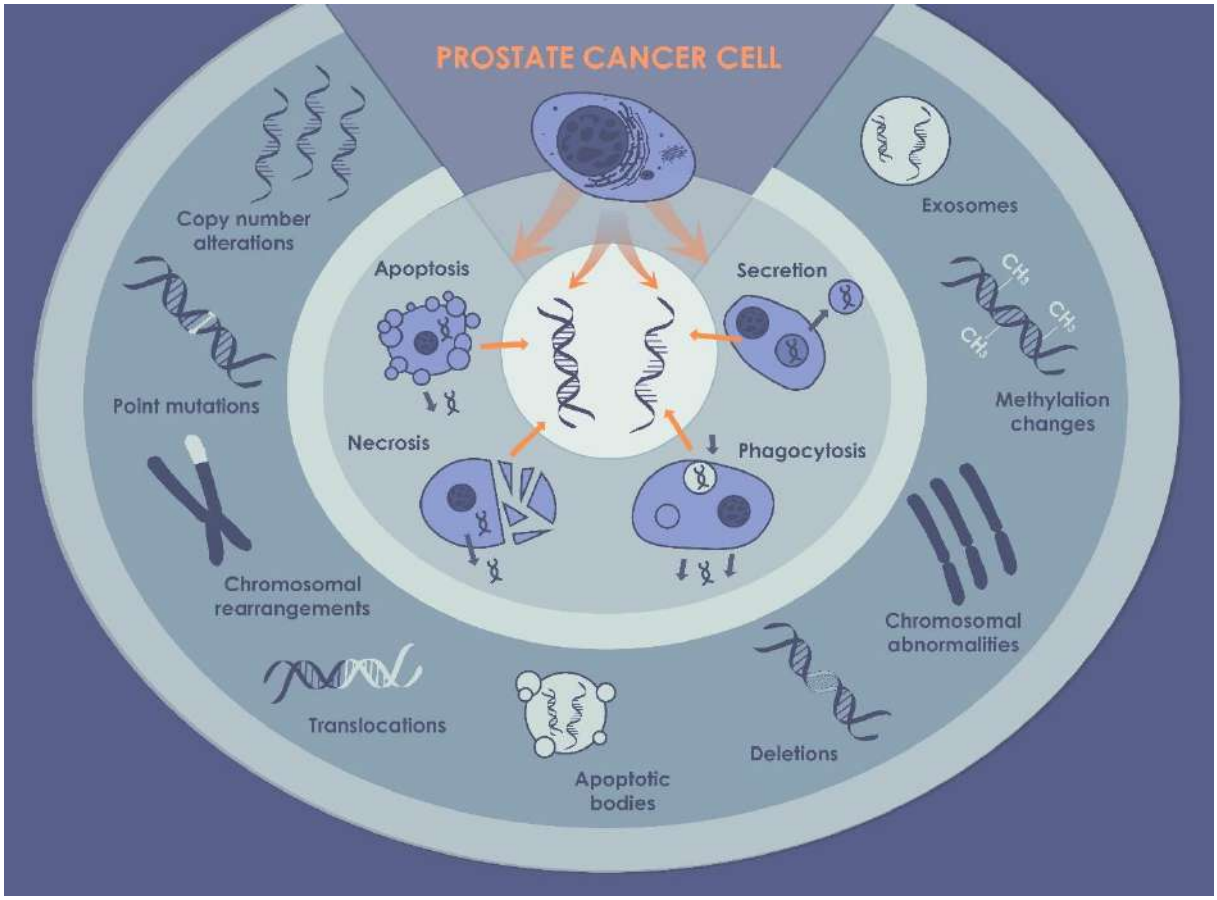

Figure3. ctDNA in PCa. ctDNA, originating from cancer cells, may be detected in biological fluids of patients with non-metastatic PCa, and can be used as a biomarker of the disease.

It is known that cancer is accompanied by initial hypomethylation of DNA and further hypermethylation in the promoter regions of tumour suppressor genes, which leads

to inactivation of transcription and translation of the corresponding protein complexes [50]. DNA hypermethylation is one of the earliest and most common aberrations in PCa [51]. In the study by Brikun et al. [52], a panel of 32 epigenetic biomarkers of PCa was developed. Using the methylation-sensitive qPCR, biopsy samples of the prostate gland of 104 patients were analyzed. The prostate tissues were investigated by using 24 well-known DNA methylation biomarkers. According to the results, significant difference was demonstrated between tumour and unaffected tissue in individual patients, and between PCa patients and the control group. In the further study by Brikun et al. [53], ctDNA was isolated from the urine of 94 patients. The expression levels of biomarkers, previously found in tissues, were analyzed, and compared with the data obtained from tissue biopsies. A specificity of the proposed technique was identified at 76%, with sensitivity at 81%. In the other study by the authors [54], a panel of DNA methylation markers suitable for a non-invasive diagnostic test for ctDNAs in urine was identified. The urine was collected after a digital rectal examination (DRE) [48] and/or from first morning void (FV). A specificity at 71% for both DRE and FV, and a sensitivity at 89% for DRE and at 94% for FV, was reported.

In the study by O'Reilly et al. [55], the epiCaPture multi-biomarker panel was tested. Numerical alterations in DNA hypermethylation at the 5 'end of 6 genes (GSTP1, SFRP2, IGFBP3, IGFBP7, and PTGS2) were the targets for the panel. Previously, all these targets were identified as potential PCa biomarkers [56]. More than 450 urine samples of patients with elevated PSA were analyzed. The high diagnostic and prognostic values of the technique were demonstrated. According to the results, a higher degree of DNA methylation correlated with an increased risk of developing an aggressive form of PCa. It was also demonstrated that the specificity of the epiCaPture test was superior to the PSA screening.

2.6.2. Exosomes and miRNAs

Exosomes are small EVs secreted by both normal and cancer cells. Exosomes are found in almost all types of cells and were successfully isolated from blood, urine, saliva, semen, and other body fluids [57]. Exosomes are released into the extracellular space via exocytosis and may carry different contents, such as proteins, different types of RNAs, DNAs, and miRNAs, and functionalize as intercellular messengers transmitting signaling molecules from one cell to another [58] (Figure3). A large body of evidence suggests a critical role of

the tumorigenic exosomes in tumour progression and metastasis. Another important feature of the tumorigenic exosomes is formation of drug resistance by inducing a tumour-friendly microenvironment due to involvement of the exosomes in proliferation, differentiation, and migration of fibroblasts, influence on immune cells, and stimulation of tumour angiogenesis. In its turn, exosomal miRNAs are of particular interest due to their ability to affect cancer progression by reducing apoptosis in a tumour and by increasing proliferation, migration, and adhesion of cancer cells [59-61]. Besides, one of the main factors indicating the critical role of exosomal miRNAs in PCa carcinogenesis is influence on the epithelial-mesenchymal transition, demonstrated by several research groups [62].

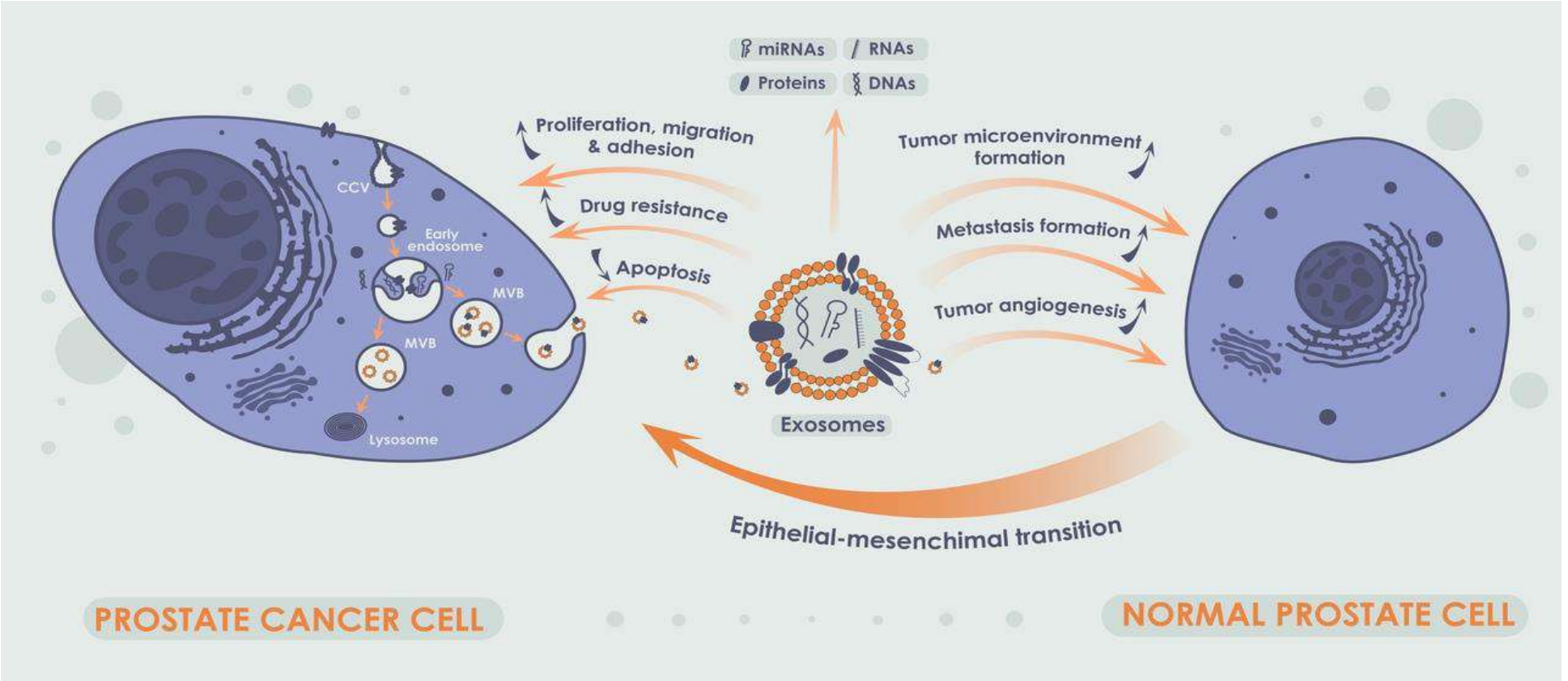

Figure4. Biofabrication of exosomes in cancer cells and their impact on normal prostate cells. A single exosome is formed due to the invagination of the early endosome into the cellular membrane. Exosomes can carry not only miRNAs but also other nucleic acids, as well as a broad spectrum of proteins and lipids, as well as their combinations. Exosomes, acting as intercellular messengers, are capable of influencing tumour progression by stimulating the proliferation and migration of cancer cells, enhancing cell adhesion, and inhibiting apoptosis. Also, exosomes play a crucial role in formation of drug resistance. In addition, exosomes and exosomal miRNA may influence tumour progression by modulating tumour microenvironment, metastasis, and

tumour angiogenesis. Notably, one of the main factors indicating the critical role of exosomal miRNAs in PCa carcinogenesis is its effect on the epithelial-mesenchymal transition.

In recent years, exosomes containing miRNAs have been widely investigated as potential biomarkers of various malignant tumours, due to their functional relationship with tumour progression, including cell growth, differentiation, proliferation, angiogenesis, and apoptosis [63-65]. Extracellular miRNAs have been isolated from various biological fluids, such as blood, urine, saliva, breast milk, and semen [66]. They were found both inside extracellular vesicles, such as exosomes, and in free form [67, 68]. It was identified that extracellular miRNAs influence targeted cells in the same way as intracellular miRNAs. Notably, since exosomes consist of a lipid bilayer, they are stable in various body environments, which makes them a convenient object for being investigated as PCa biomarker. Also, it was demonstrated that PCa cells secrete more exosomes in comparison with normal prostatic cells, which is another argument in favor of investigating techniques for isolating and analyzing exosomes as a perspective approach for PCa liquid biopsy [69].

MiRNAs are short non-coding RNA sequences from 17 to 25 nucleotides in length, regulating gene expression at the post-transcriptional level [70]. Aberrant expression of various specific miRNAs has been observed in various tumours, including breast [71], lung [72], colorectal cancer [73], and prostate cancer [74]. This feature allows not only to distinguish tumour tissue from normal tissue but also to classify tumours according to the tissue origin, and to perform tumour staging [75], which potentially makes miRNAs a biomarker efficient for prognosing the development of oncological diseases [76]. Further, it has been shown that dysregulated miRNAs contribute to tumour initiation and progression by activating a so-called onco-miRNAs or disactivating a so-called tumour-suppressing miRNAs. It was also revealed that one miRNA may have opposite functions, depending on a type of cancer, which indicates the possibility of participation of the same miRNA in different signaling pathways [77-79]. Thus, among all types of exosomes, those which are loaded with miRNAs are considered the most promising for being introduced into a clinical practice as biomarker for early diagnosis and prognosis of PCa. Moreover, it was demonstrated that miRNAs presenting within exosomes have higher diagnostic and prognostic value in comparison with the miRNA-free exosomes [80].

2.6.2.1. Diagnostic value of exosomal miRNAs

Various types of exosomal miRNAs, which may potentially be used as biomarkers in diagnosing PCa at its early form, have been found in blood of patients with PCa. Thus, Bryant et al compared exosomal miRNAs isolated from plasma from patients with PCa (n = 47) and healthy controls (HCs) (n = 28), indicating that both miR-107 and miR-574-3p were upregulated in patients with PCa compared with HCs [81]. The role of miR-141 as an onco-miRNA in PCa was further supported by a more recent study involving patients with localized PCa (n = 20), patients with BPH (n = 20), and HCs (n = 20) [82]. The result of this study confirmed the potential value of miR-141 as a diagnostic biomarker for differentiating localized PCa from BPH. Also, in the study by Endzeliņš et al. [80] , free and exosomal miRNAs isolated from blood plasma were compared in 50 patients with PCa and 22 patients with BPH. As the result, four miRNAs with diagnostic potential were found (miR-375, miR-200c-3p+ miR-21-5p, Let-7a-5p, miR-141). At the same time, in the study by Samsonov et al. [83], no significant differences in miR-141 blood plasma concentration between PCa patients and HCs were identified. According to other results of the study, miR-574-3p, miR-141-5p, and miR-21-5p were identified to be over-expressed in exosomes of 10 PCa patients versus 10 HCs.

It was also proven that the use of a combination of two or more exosomal miRNAs often provides more specific and precise results in PCa diagnostics. According to the study by Foj et al. [84], it was determined that the combination of miR-21 and miR-375 extracted from the urinary exosomes may potentially be more efficient in distinguishing patients with localized PCa from healthy males, compared with the use of each of these miRNAs independently. However, the results were limited by the size of the control group (n = 10) and the localized PCa group (n = 3). Another example of miRNAs combination, with may potentially be highly valuable in PCa diagnostics, is miR-217 and miR-23b-3p, reported by Zhou et al. [85]. In this study, the blood plasma of patients, including those with early PCa and a Gleason score (GS) at < 9, was analyzed for all possible types of the miRNAs. It was demonstrated that 94 different types of miRNAs were expressed at the extent different in PCa patients and HCs. The expression level of 60 of the miRNAs was increased in PCa patients, including the mostly increased miR-217. At the same time, PCa patients had 34 of the miRNAs decreased compared to HCs, including the mostly decreased miR-23b-3p. Further, it was demonstrated by in vitro and in vivo tests, that the increased expression of miR-217 stimulated proliferation and invasion of cancer cells, while increase in the

level of miR-23b-3p suppressed it. Nevertheless, a limited group of patients and a short follow-up period necessitates further research in this direction, and verification of the data obtained.

The usage of ratio of one miRNA to another was also proven promising in diagnostics of localized PCa. Thus, Li et al. [86] conducted a study to elucidate the significance of exosomal miR-125a-5p and miR-141-5p, obtained from plasma, as biomarkers for early diagnosis of PCa. The study included 19 HCs and 31 PCa patients. It was demonstrated for patients with PCa that the expression level of miR-141-5p was in average only slightly increased, while miR-125a-5p level was significantly decreased. It was also demonstrated, that the miR-125a-5p/miR-141-5p ratio was significantly higher in PCa patients compared to HCs. According to the data presented, the miR-125a-5p/miR-141-5p ratio appeared to perform better as an early PCa biomarker than either of the miRNAs taken individually.

It is worth noting that from recent, the attention for alternative sources of exosomal miRNAs, particularly – semen, has been increasing. In the study by Barcelo et al. [87], the expression level of exosomal miRNAs isolated from semen was analyzed to find an appropriate biomarker for early diagnosis of PCa. The study included PCa patients with moderately elevated PSA levels and GS from 6 to 8 points, patients with BPH and HCs. A total amount of 400 different miRNAs was analyzed within all three experimental groups. According to the results, the most valuable diagnostic potential was found in the combination of PSA serum level, miR-142-3p, miR-142-5p, and miR-223-3p, which demonstrated sensitivity at 91.7% and specificity at 42.9%. Moreover, it was found that miR-342-3p may potentially help to distinguish between samples of patients with GS at 6 and 7 and facilitate to TNM staging of PCa.

At the same time, despite promising data on diagnostic potential of exosomal miRNAs, it was indicated that diverse exosome isolation techniques may have a significant impact on the results obtained. Thus, in the study by Mercadacal et al. [88], several different techniques of exosome isolation from semen were tested. Their purifying effectiveness and influence on the analysis of miRNAs was evaluated and compared with the results of the standard ultracentrifugation technique. It was demonstrated that techniques originally developed for exosome isolation from blood and urine were also suitable for semen, though the results could vary depending on the method used.

2.6.2.2. Prognostic value of exosomal miRNAs

Studies of exosomal miRNAs isolated from various biological fluids have revealed several biomarkers with potential value for PCa prognosis. According to the study by Endzeliņš et al. [80], the level of exosomal miR-let-7a-5p could be helpful for staging PCa in patients with GS of ≥ 8 versus ≤ 6. Thus, it was demonstrated by the analysis of exosome samples derived from the patients' blood plasma (26 with GS ≤ 6 and 24 with GS ≥ 8), that the expression of miR-let-7a-5p was significantly lower in patients with a higher GS (> = 8) compared to the patients who had lower GS (≤ 6). In contrast, in the study by Watahiki et al. [89], let-7a demonstrated limited value for indicating the difference between patients with metastatic castration-resistant prostate cancer (mCRPCa) and localized PCa. In addition, in the study by Foj et al. [84], no significant differences in urinary exosome profiles of miR-let-7a-5p were identified between the low-risk and high risk PCa patients. Thus, the role of miR-let-7a-5p as a predictive biomarker of PCa remains controversial and requires further investigation. Also, it was demonstrated that the concentration of miR-1246 in urine has an association with the aggressiveness of PCa. According to the results of the study by Bhagirat et al. [90], the miR-1246 expression level could not only be helpful to differentiate between HCs and PCa patients, but also might serve as a predictor of metastases with specificity at 100% and sensitivity at 75%. In another study by Bhagirat et al. [91], exosomal miR-4287 was investigated in terms of its potential to predict the possibility of metastasis at an early stage of PCa. According to the results of this study, specificity of the proposed method at 88.24% was demonstrated. Further, in the study by Guo et al, the potential of six different exosomal miRNAs to predict the development of castration-resistant prostate cancer (CRPCa) was investigated [92]. It was demonstrated that MiR-423-3p was associated with the development of CRPCa.

The usage of semen as a potential source of exosomal miRNAs has also attracted significant attention. In the study by Ruiz-Plazas et al. [93], semen samples from patients with PCa at various stages including early localized form, were examined to determine the prognostic role of the cytokine tumour necrosis factor-like weak inducer of apoptosis (TWEAK)-regulated exosomal miRNAs. With the miRNA biomarker panel, composed of miR-221-3p, miR-222-3p, and TWEAK, it was possible to classify PCa in terms of its aggressiveness with specificity at 85.7% and sensitivity at 76.9%.

In addition to the studies where single exosomal miRNAs were investigated, it was also identified that a combination of several miRNAs may also provide useful prognostic data. Thus, in the study by Huang et al. [94], a combination of exosomal miR-1290 and miR-375 in urine was investigated in terms of the capacity to predict the overall survival rates of patients with localized PCa. It was demonstrated that patients with high concentrations of both miRNAs had overall mortality rates at around 80%, while patients with average or low concentrations of both miRNAs had a mortality rate at 10% over the same follow-up period. The obtained data necessitates further evaluation of the identified miRNAs in a larger cohort of patients. The overall data on diagnostic and prognostic potential of diverse miRNAs is presented in Table1.

Table1. Diagnostic and prognostic value of miRNAs in patients with localized PCa.

miRNAs	**Biological fluid (volume)**	**Number of PCa patients/ HCs and BPH patients**	**Isolation technique**	**Diagnostic value**	**Prognostic value**	**Ref.**
miR-342-3p	Semen (NA)	40 patients with non-metastatic PCa / 11 HCs and 7 BPH patients	Centrifugation + microfiltration + ultracentrifugation	NA*	Discrimination between GS ≥ 7 and GS = 6	[87]
miR-374b-5p				NA	Discrimination between GS ≥ 7 and GS = 6	
miR-342-3p + miR-374b-5p				NA	Discrimination between GS ≥ 7 and GS = 6	
miR-142-3p + miR-142-5p + miR-223-3p				Discrimination between PCa and BPH	NA	
MiR-375	Blood plasma (400 μl)	50 patients with non-metastatic PCa / 22 BPH patients	Size exclusion chromatography	Discrimination between PCa and BPH	NA	[80]
miR-200c-3p + miR-21-5p				Discrimination between PCa and BPH	NA	
Let-7a-5p				NA	Discrimination between GS ≥ 7 and GS = 6	
let-7c	Urine (30-50 ml)	52 patients with non-metastatic PCa / 10 HCs	Differential centrifugation	Discrimination between low risk, intermediate risk, and high risk PCa and healthy status	Discrimination between GS > 8 and GS = 6	[84]
miR-21				Discrimination between low risk, intermediate risk, and high risk PCa and healthy status	NA	
miR-375				Discrimination between low risk, intermediate risk, and high risk PCa and healthy status	NA	

miR-574-3p	Urine (13-40 ml)	35 patients with non-metastatic PCa / 35 HCs	Differential centrifugation + lectin-based agglutination	Discrimination between PCa and healthy status	NA	[83]
miR-141-5p				Discrimination between PCa and healthy status	NA	
miR-21-5p				Discrimination between PCa and healthy status	NA	
miR-196a-5p	Urine (50-150 ml)	20 patients with non-metastatic PCa / 10 HCs	Differential centrifugation	Discrimination between PCa and healthy status	NA	[95]
miR-501-3p				Discrimination between PCa and healthy status	NA	
miR-217	Blood plasma (0.5 – 1 ml)	10 patients with non-metastatic PCa / 10 HCs	Differential centrifugation + RNeasy Mini Spin kit	Discrimination between PCa and healthy status	NA	[85]
miR-23b-3p				Discrimination between PCa and healthy status	NA	
miRNA-125a-5p	Blood plasma (1 ml)	31 patients with non-metastatic PCa / 20 HCs	Differential centrifugation	Discrimination between PCa and healthy status	NA	[86]
miR-141-5p				Discrimination between PCa and healthy status	NA	
miR-141	Blood serum (400 μL)	20 patients with non-metastatic PCa / 20 HCs and 20 BPH patients	ExoQuick exosome precipitation solution	Discrimination between PCa, BPH and healthy status	NA	[82]
miR-141 + miR-375	Blood plasma (1 ml)	78 patients with non-metastatic PCa / 28 HCs	Qiagen miRNeasy kit	NA	Discrimination between low-risk and high-risk PCa	[81]
miR-107 + miR-574-3p	Urine (5 ml)		mirVana kit	Discrimination between PCa and healthy status	NA	
miR-205	Blood plasma (200 μL)	25 patients with non-metastatic PCa and 25 patients with mCRPCa / NA	Differential centrifugation	NA	Differentiation of localized PCa in terms of aggressiveness	[89]
miR-141				NA	Discrimination between low-risk and high-risk PCa; discrimination between localized PCa and mCRPCa	
miR-151-3p				NA	Discrimination between localized PCa and mCRPCa	
miR-423-3p				NA	Discrimination between localized PCa and mCRPCa	
miR-152				NA	Discrimination between low-risk and high-risk PCa, strong correlation with poor outcome	

miR-375				NA	Discrimination between localized PCa and mCRPCa	
miR-21				NA	Discrimination between docetaxel-resistant and docetaxel-sensitive PCa patients	
miR-141 + miR151-3p + miR-16				NA	Discrimination between localized PCa and mCRPCa	
miR-126				NA	Discrimination between localized PCa and mCRPCa	
miR-1290	Blood plasma (N/A)	48 patients with non-metastatic PCa / NA	Differential centrifugation	NA	Discrimination between low-risk and high-risk PCa; prediction of androgen deprivation therapy failure	[94]
miR-375				NA	Discrimination between low-risk and high-risk PCa; prediction of androgen deprivation therapy failure	
miR-1290 + miR-375				NA	Discrimination between low-risk and high-risk PCa; prediction of androgen deprivation therapy failure	
miR-221-3p	Semen, urine (NA)	97 patients with non-metastatic PCa / NA	Differential centrifugation	NA	Discrimination between low-risk and high-risk PCa	[93]
miR-31-5p				NA	Discrimination between low-risk and high-risk PCa	
miR-222-3p				NA	Discrimination between low-risk and high-risk PCa	
miR-193-3p				NA	Discrimination between low-risk and high-risk PCa	
miR-423-5p				NA	Discrimination between low-risk and high-risk PCa	
miR-221-3p + miR-222-3p + TWEAK				NA	Discrimination between low-risk and high-risk aggressive PCa	
miR-1246	Serum (250µL)	44 patients with non-metastatic PCa / NA	Total exosome isolation reagent	Discrimination between PCa and healthy status	Discrimination between localized and metastatic PCa	[90]
miR-4287	Serum (250µL)	68 patients with non-metastatic PCa / 68 HCs and 68 BPH patients	Total exosome isolation reagent	Discrimination between PCa and healthy status	Discrimination between localized PCa (GS 4 - 6) and metastatic PCa (GS > 7)	[91]
miR-423-3p	Plasma (200µL)	132 patients with non-metastatic	Differential centrifugation	NA	Discrimination between localized PCa and mCRPCa	[92]

		PCa and 66 patients with mCRPCa / NA				

* - not applicable

2.6.3. Disseminated tumour cells

Taking origin at a primary or secondary tumour site, DTCs are the tumour cells which shed into the vessels of circulatory system or ejaculatory ducts of the prostate gland or urethra, depending on the anatomical location of the tumour. DTCs may release passively or as a result of stromal invasion with a subsequent intravasation of the cells, which is followed by translocation of DTCs to distant parts of the body and formation of metastases in case of shedding into the blood vessels. During the process of successful dissemination within a human body and metastasis, DTCs must also overcome a hydrodynamic stress of circulation and evade a host's immune system, as well as adapt to a new microenvironment at a destination, proliferate and colonize [96]. Since the first discovery of DTCs by Thomas Ashworth in 1869 [97], tremendous progress has been achieved in the field of investigating DTCs as a diagnostic and prognostic marker of cancer, including non-metastatic form of PCa. A significant rise of attention to the field has occurred during the last decade, which is primarily associated with the progress in the development of microelectromechanical systems for isolation and detection of DTCs, their qualitative and quantitate analysis.

Quantitatively, DTCs may potentially provide an opportunity to determine presence of PCa, its stage and response to treatment. Initially, most of the studies in the field of liquid biopsy were related to enumeration of DTCs detected in blood samples, which still attracts significant attention. The technique of detection and enumeration of DTCs was considered prospective in PCa diagnosis and prognostics although a clear evidence has not been reached in this context so far. Potentially, DTCs may be the precursors of metastases and their detection at an early stage of cancer may be a reliable marker facilitating to a right decision between active surveillance and surgery in case of localized form of PCa [98]. In diagnosis and prognostics of localized PCa, DTCs have been primarily investigated as a parameter for pathological risk stratification of the disease. It is anticipated that, as a diagnostic and prognostic parameter, quantity of DTCs may successfully substitute other conventional parameters such as PSA blood level, and GS. Also, a correlation between the amount of DTCs and other clinico-pathological parameters is of a great interest.

Qualitatively, isolated DTCs are an object for gene analysis, biomarker assay, and cultivation with a subsequent drug testing and identification of possible drug resistance mechanisms, and therefore are a suitable option for personalized medicine (Figure4). However, despite an outstanding diagnostic and prognostic potential of DTCs, several issues have to be resolved prior to introducing the technology as a part of medical guidelines. Thus, a common diameter of a single DTC is in average higher compared to the width of capillaries' bores [99] and only the smallest and elastic DTCs may release into a systemic circulation, which may be a cause of a relatively low number of DTCs in blood at an early stage of tumour development [100]. Also, it is known that diverse cancers have different typical localizations of metastases. Hence, difference in a number of DTCs collected from blood vessels at different sites of a human body is inferred, and a conventional minimally invasive blood sampling from cubital vein may not be appropriate in all cases. Further, most of the existing techniques of DTC detection are at their early stage of development and require significant improvement. Particularly, CellSearch® (Menarini Silicon Biosystems Inc, USA) - the only FDA cleared DTC test, is based on immunofluorescent labelling with non-specific epithelial markers [101]. Thus, benign epithelial cells from different organs and noncancerous tumour components, which may also present in systemic circulation, may be recognized as DTCs and therefore be a cause of false positive results in CellSearch tests. In addition, a heterogeneity of DTCs in terms of antigen expression between individuals, and even within a particular individual, should also be considered. Hence, the development of assays composed of multiple markers with an affinity to a particular type of DTCs is highly demanded, which would significantly increase specificity and sensitivity of the corresponding tests. As an alternative to DTC detection via labelling with specific antibodies, profiling of DTCs in terms of specific mutations is considered. Lastly, cultivation of isolated DTCs is highly challenging so the usage of DTCs as a tool for personalized drug testing and investigation of drug resistance mechanisms is promising but technically very problematic [102]. At the same time, even though more novel techniques of liquid biopsy via isolation and analysis of exosomes or ctDNA are thought to be highly efficient in diagnosis and prognosis of malignant lesions, the cost-effectiveness of liquid biopsy via isolation of DTCs justifies its no less attractiveness.

The techniques of DTC isolation and detection may be in general stratified into two groups: label-free and affinity-based. The affinity-based approaches imply isolation of DTCs by means of adhesion of the DTCs to specific antibodies onto adhesive surface of a liquid-

biopsy tool. At the same time, the label-free group includes the techniques which allow to remain the isolated DTCs intact, which may be further subjected to a versatile analysis.

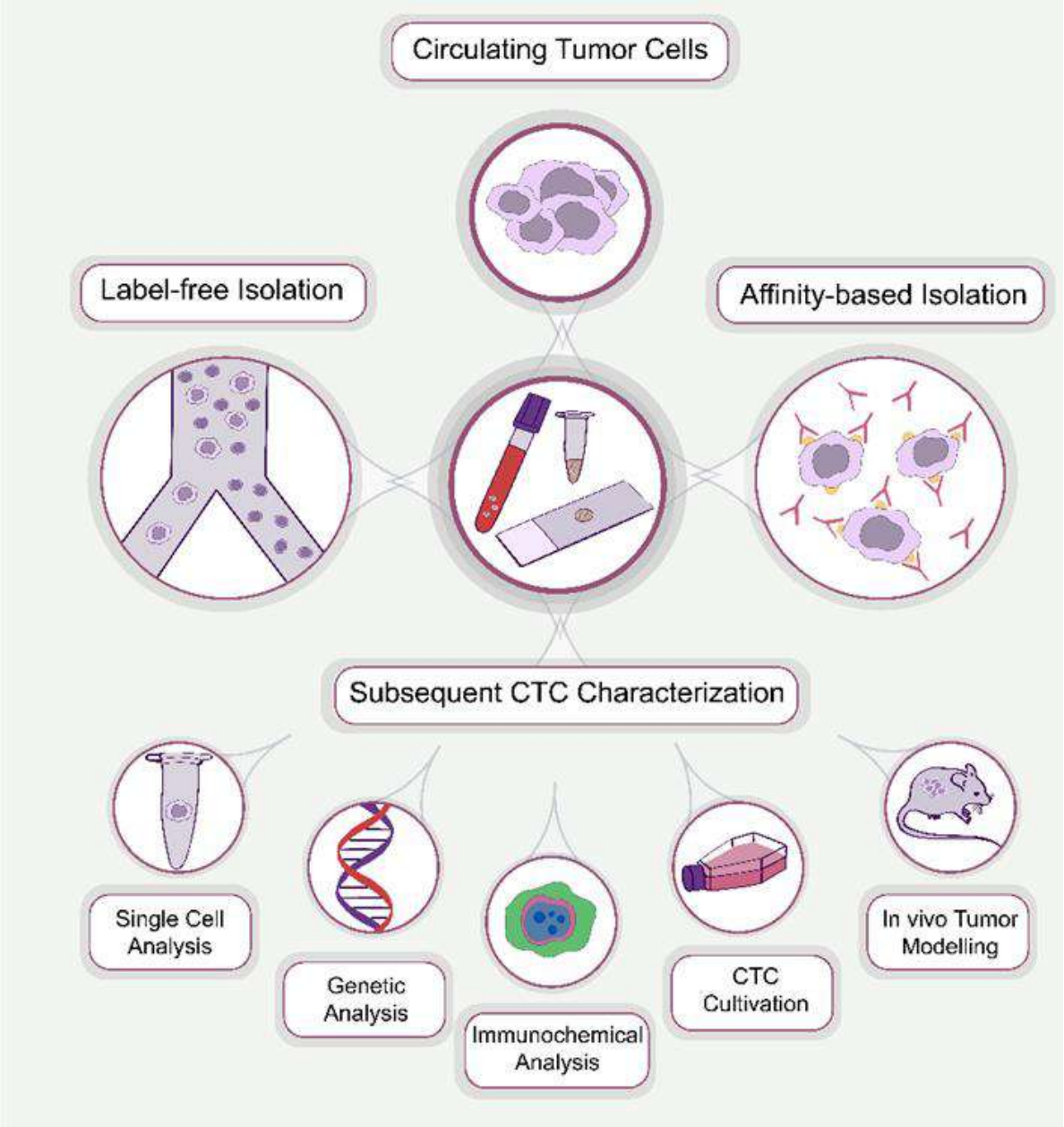

Figure5. Methods of DTC processing. DTCs are the tumour cells which present in biological fluids from where they can be isolated via so-called liquid biopsy techniques, which are principally based on two different approaches: the affinity-based approach and the label-free approach. The affinity-based approach is commonly associated with the usage of antibodies, presenting on adhesive surface, specific to antigens which are carried by DTCs. After adhesion, DTCs are subjected to a variety of analytical techniques from simple counting to antigen profiling, genetic, or single cell analysis. In case of the label-free approach, isolated DTCs are subjected to all the techniques intrinsic to the affinity-based approach, and, in

addition, to cultivation and in vivo tumour modelling. The approach is commonly based on filtration, or microfluidics which utilizes the inherent Dean vortex flows which occur in nonlinear microchannels under continuous fluid flow. These flows result in different distribution of particles/cells, mostly depending on their size and weight, within a microchannel. Thus, the combination of properly selected spatial parameters of the microchannel and the right speed of fluid flow result in separating healthy and tumour cells at a bifurcation of the microchannel.

2.6.3.1. Label-free isolation

Currently, potential of the label-free techniques for isolating tumour cells from blood of PCa patients has been highlighted in few studies. In the study by Giesing et al. (2010) [103], DTCs were obtained by means of filtration and then detected via RT-PCR analysis for antioxidant genes. As a result, with the described technique, DTCs could have been detected in 42 (32.5%) of 129 patients with localized PCa. Further, in the study by Kolostova et al. [104] where DTCs were isolated from the blood with MetaCell filtration device, 28 (52%) of 55 patients were positive for DTCs and no correlation between the presence of DTCs and GS or T stage. In addition to the techniques of cell filtration, microfluidics-based techniques of label-free isolation of DTCs have attracted significant attention. Thus, in the study by Todenhofer et al. (2016) [105], microfluidic isolation of DTCs from blood followed by immunostaining with the anti-epithelial cell adhesion molecule (anti-EPCAM) antibody was successful in 25 (50%) of 50 patients, with the amount of isolated DTCs varying from only few to few hundred DTCs among the patients. However, no correlation was observed between the DTC detection and clinical parameters of PCa. Considering the results of similar studies, in which DTCs were subjected to the size-based isolation and subsequent immunocytochemical detection, a vast antigen heterogeneity of the DTCs can be inferred [106, 107]. Particularly, in the study by Renier et al. [106], it was determined that some of the isolated DTCs did not express cytokeratin (CK) while expressing mesenchymal markers such as Vimentin and N-cad, which may signify the involvement of PCa DTCs into the epithelial-mesenchymal transition [108]. In was also demonstrated that liquid biopsy via the size-based microfluidic isolation of DTCs followed by their gene analysis is valuable in terms of prognosis of the disease. Thus, in the study by Miyamoto et al. [109],

isolated from blood DTCs were investigated via whole transcriptome amplification, which allowed to predict dissemination of the DTCs to seminal vesicles and lymph nodes In a cohort of patients at 34 subjects. Another approach for label-free isolation of PCa DTCs, gel centrifugation, was proposed by Murray et al. [110]. Notably, in this study, blood was collected from patients at 3 months post-radiotherapy. According to the results of the study, in intermediate-risk PCa participants DTCs were identified at approximately two times more often than in the low-risk participants. Further, it was determined that DTC positive participants had a worse prognosis and shorter time period until biochemical recurrence, and after 15 years of follow-up had shorter survival rate.

Considering the inconsistent results of diverse studies where blood was used as a source of DTCs, it can be inferred that investigation of other biological fluids in terms of DTC isolation is highly demanded. Thus, in the past few years, DTC isolation from urine has attracted significant attention. In the study by Nickens et al. [111], urine samples collected from PCa patients were filtered by using Swinney (Sterilitech Corporation, Kent, WA) filtration device, and the cell sediments were further immunocytochemically labelled with anti-ERG, anti-AMACR and anti-PSA antibodies. According to the results, such approach demonstrated moderate sensitivity and specificity at 64% (16 of 25 patients) and 68.8% (22 of 32 HCs), respectively. Further, Campbell et al. [112] investigated urine sediments which were labelled with the MIL-38 monoclonal antibody against the GPC-1 antigen, which has recently been proven specific for several types of cancers including PCa [113]. The study included 41 patients with localized PCa and 37 patients with benign prostate hyperplasia as a control group. As a result, the technique demonstrated sensitivity and specificity at 71% and 76%, respectively, with the highest specificity at 89% for patients with PSA serum level at ≥4 ng/ml. Later, in the study by Rzhevskiy et al. [114], the same anti-GPC-1 antibody MIL-38 was applied for detecting PCa tumour cells isolated from large volumes (30-100 mL) of urine with a microfluidic chip. Among 14 patients, 12 (86%) contained GPC-1+ putative DTCs in their urine, while urine samples of 11 (79%) of 14 HCs were free from GPC-1+ cells. The amount of putative tumour cells varied from 4 to 194 units in patients and was up to 7 units in HCs. Notably, moderate positive correlation was identified between the amount of GPC-1+ cells per mL of urine and GS.

2.6.3.2. Affinity-based isolation

In the majority of studies utilizing affinity-based approach for DTC isolation, the most well know liquid biopsy platform CellSearch was exploited. The technique is based on capturing DTCs by using the adhesion to EpCAM, with further defining DTCs among the captured cells as the cells positive for DAPI and CK, and negative for CD45. However, such concept is first of all specific to all disseminated epithelial cells rather than DTCs in particular, which may be the cause for compromised specificity of the technique for detecting PCa DTCs. Thus, in the study by Davis et al. [115], where CellSearch-based isolation of PCa DTCs was first reported, 4 of 20 (20%), HCs were identified positive for DTCs. However, it is worth noting that increased amount of blood at 22.5 mL, instead of the standard portion at 7.5 mL, was analyzed in the study. This hypothetically might has decreased the specificity of the technique. At the same time, in the study by Thalgott et al. (2015) where blood was sampled at the amount of 20 mL, neither of the HCs was identified positive for DTCs. Further, in other studies, where HCs providing blood at the amount of 7,5 mL, all the donors were negative for DTCs [116, 117]. In its turn, a significantly variable data was reported on the sensitivity of the technique. Thus, depending on the study, DTCs could have been found in blood of the PCa patients with the probability from 0% to 73% [116-123], with the amount of the tumour cells in patients positive for DTCs varying from 0 to 13 units per mL of the blood. Nevertheless, it is difficult to perform direct comparison between the results obtained in diverse studies due to the difference in cutoff value of the putative DTCs at which a particular patient or healthy volunteer was considered positive for DTCs. Thus, in the study by Pal et al. [120] the threshold was set at 1 DTC per 22.5 mL of blood, while in most of the studies the threshold was set at 1 DTC per 7.5 mL. Finally, in neither of the CellSearch studies, a strong correlation between the amount of isolated DTCs and PSA serum level, or GS score, or PFS, or OS, was identified.

Considering the imperfections of the CellSearch system in terms of DTC isolation and the inconsistency of the results between different studies, alternative affinity-based approaches have been developed in recent years. As two of such approaches, CellCollector and EPISOT were applied in the study by Kuske et al. (2016) [124], and compared with the CellSearch. In case of CellCollector, DTCs are captured by the EPCAM-based adhesion *in vivo* to the antibody-coated wire introduced into a peripheral vein of the human body. At the same time, EPISOT assay is the EPCAM-independent technique which is based on the

negative depletion of leucocytes with further identification of DTCs as the PSA-positive cells. In this study positivity for DTCs was considered at ≥1 of the tumour cells per 7.5 mL. According to the results of the study, DTCs were identified in blood samples of 58.7%, 54.9% and 37% by the EPISOT, CellCollector and CellSearch assays, respectively. Also, significant correlation between the amount of isolated DTCs and PSA serum level, or clinical tumour stage, was indicated only in case of EPISOT while in case of CellSearch and CellCollector no significant correlations were observed. Another EPCAM-dependent approach based on the microfluidic enrichment of DTCs was tested in the study by Stott et al. [125]. In this study, putative DTCs were identified in blood of 8 out of 17 HCs at the amount of up to 14 units, which was set as a cutoff value in case of patients. Thus, DTCs were identified in blood of 8 (42%) out of 19 patients, at a relatively high amount of up to 222 DTCs per mL with the median value at 95 DTCs. Further, among the 8 patients identified positive for DTCs, 6 patients had significant decline in the amount of DTCs isolated at 24 hours post-prostatectomy. Further, in the study by Russo et al. [126] the immune-magnetic isolation relying on EpCAM and anti-mucin1 antigens was utilized. To further detect the isolated DTCs, the immune-magnetic enrichment was followed by the application of several kinds of AdnaTest: AdnaTest ProstateCancer which detects the overexpression of PSA, PSMA and EGFR mRNAs, AdnaTest StemCell which evaluates expression of transcripts specific for cancer stem cells and AdnaTest EMT detecting epithelial-mesenchymal transition in the isolated DTCs. Also, isolated DTCs were assessed by PCR for AR, c-met, c-kit and thymidylate synthase (TYMS). According to the results of the study, AdnaTest ProstateCancer was positive in 3 (16.7%) of 18 patients with the low-risk clinically localized PCa and 2 (8.0%) of 25 patients with the high-risk clinically localized PCa. The AdnaTest StemCell was positive in 5 (27.8%) of 18 and 4 (16.0%) of 25 patients with the low-risk and high-risk localized PCa, respectively. At the same time, AdnaTest EMT was positive in neither of the cases of localized PCa. Moreover, expression of TYMS, AR, c-met and c-kit by the isolated DTCs was a rare event.

In addition to the EPCAM-dependent alternatives to the CellSearch system, several EPCAM-independent approaches have also been tested. Thus, in the study by Puche-Sanz et al. [127] where the cytokeratin-based immune-magnetic technique was applied, DTCs at the small amounts ranging from 1 to 4 units were detected in 16 (18.6%) of 86 patients. Notably, despite a relatively low amount of DTC positive patients and small numbers of isolated DTCs, a significant correlation was observed between the presence of DTCs in blood

and expression of AR in PCa tissue. It is worth noting that in the study by Garcia et al. [128], assessment of AR-V7 splice variant in blood plasma through a capillary nano-immunoassay was proposed as the technique for identifying DTCs. The data from studies where DTCs were investigated as the diagnostic and prognostic marker of non-metastatic PCa is summarized in Table2.

Table2. Circulating tumour cells in patients with non-metastatic PCa.

Type of DTC isolation	DTC isolation technique (label-free or affinity-based)	Biological fluid (volume)	Number of PCa patients / HCs and BPH patients	Cut-off amount of DTCs for DTC+ patients	Percentage of DTC+ patients / healthy volunteers	Min-max amount of DTCs per mL in patients	Correlation between the amount or presence of DTCs and clinico-pathological parameters of PCa	Ref.
Label-free	Filtration + RT PCR for PSA and antioxidant genes	Blood (NA)	129 patients with non-metastatic PCa / NA	NA	32.5% / NA	NA	Strong correlation between the expression of SOD2 or TXNRD1, and tumour size or GS	[103]
	Filtration + CK dependent ICC*	Blood (8 mL)	55 patients with non-metastatic PCa / NA	≥ 1	52% / NA	NA	No correlation between the presence of DTCs and GS or T stage. No correlation between the DTC count and response to treatment	[104]
	Microfluidics + EPCAM. CK dependent ICC	Blood (2 mL)	50 patients with non-metastatic PCa / NA	≥ 1	50% / NA	0.5-208.5	No correlation between the presence of DTCs and the PSA serum level, age, GS, T stage, or N stage. No correlation between the number of DTCs and GS, T stage, or N stage	[105]
	Filtration + CK, AR dependent ICC	Blood (3 mL)	41 patients with non-metastatic PCa / NA	≥ 1	100% / NA	NA	NA	[107]
	Microfluidics + droplet digital PCR (dd-PCR)	Blood (20 mL)	34 patients with non-metastatic PCa / 34 HCs	NA	NA / NA	NA	d-PCR** for 8 PCa-specific genes allowed to predict dissemination of DTCs to seminal vesicles and lymph nodes	[109]
	Filtration + ERG, AMACR, PSA dependent ICC	Urine (NA)	25 patients with non-metastatic / 32 HCs	≥ 1	64% / 21.2%	NA	NA	[111]
	Sedimentation + GPC-1 dependent ICC	Urine (50-100 mL)	41 patients with non-metastatic PCa / 47 HCs and 37 BPH patients	≥ 1	71% / 30% and 24%	NA	NA	[112]
	Gel centrifugation +	Blood (8 mL)	241 patients with non-metastatic PCa at 3	≥ 1	47.8% / NA	NA	NA	[110]

	PSA dependent ICC		months post-radiotherapy / NA					
	Microfluidics + GPC-1 dependent ICC	Urine (30-100 mL)	14 patients with non-metastatic PCa / 14 HCs	> 8	79% / 21%	0.1 – 4.9	Moderate correlation between the amount of DTCs and GS, or PSA serum level	[114]
Affinity-based	Microfluidics + EPCAM dependent ICC	Blood (20 mL)	19 patients with non-metastatic PCa / 17 HCs	≥ 14 mL^{-1}	42% / 47%	38-222	Poor correlation between the amount of DTCs and PSA serum level	[125]
	CellSearch	Blood (7.5 mL)	26 patients with non-metastatic PCa / 7 HCs	≥ 1	73% / 0%	NA	No correlation between the number of DTCs and GS, or PSA serum level, or T stage	[116]
	CellSearch	Blood (7.5 mL)	10 patients with non-metastatic PCa / NA	≥ 1	10% / NA	0.0 – 0.1	NA	[118]
	CellSearch	Blood (7.5 mL)	20 patients with non-metastatic PCa / 15 HCs	≥ 1	5% / 0%	0.0 – 0.1	NA	[117]
	CellSearch	Blood (7.5 mL)	36 / NA	≥ 1	14% / NA	0.0 – 0.4	NA	[119]
	CellSearch	Blood (22.5 mL)	32 patients with non-metastatic PCa / 5 HCs	≥ 1	45% / 0%	0.0 – 0.1	No correlation between DTC amount and biochemical recurrence of PCa	[120]
	CellSearch	Blood (20.0 mL)	15 patients with non-metastatic PCa / 15 HCs	≥ 1	20% / 0%	0.0 – 0.2	No correlation between DTC detection and GS, PSA serum level, T and N stages, positive surgical margin	[121]
	CellSearch	Blood (7.5 mL)	152 patients with non-metastatic PCa / NA	≥ 1	11% / NA	0.13 – 13.3	Weak correlation between DTC detection and GS, PSA serum level, or T - stage	[122]
	CellSearch EPISPOT CellCollector	Blood (7.5 mL)	86 patients with non-metastatic PCa / NA	≥ 1	37% / NA 54.9% / NA 58.7% / NA	0.13 – 1.3 0.13 – 1.6 0.13 – 1.7	Significant correlation between DTC detection and PSA serum values, or clinical tumour stage. No correlations in case of CellSearch and CellCollector	[124]
	CellSearch	Blood (7.5 mL)	59 patients with non-metastatic PCa / NA	≥ 1	0% / NA	NA	NA	[123]
	Assessment of AR-V7 through a capillary nano-immunoassay	Blood (10 mL)	16 patients with non-metastatic PCa / 11 HCs	NA	18.7% / 0%	NA	Significant correlation between presence of AR-V7 and expression of CD133 antigen	[128]
	Multi-CK dependent immune-magnetic collection	Blood (10 mL)	86 patients with non-metastatic PCa / 17 HCs	≥ 1	18.6% / 0%	0 – 0.4	NA	[127]

	AdnaTest Prostate-Cancer	Blood (5 mL)	47 patients with non-metastatic PCa / NA	NA	25.5% / NA	NA	NA	[106]

* - immunocytochemistry

** - digital polymerase chain reaction

2.7. Discussion

In this systematic review, 48 studies investigating ctDNAs, exosomes and exosomal miRNAs, and DTCs as biomarkers for early diagnosis, prognostics, and treatment response monitoring in patients with non-metastatic PCa, were included. It was identified that liquid biopsy by means of isolation and analysis of the listed biomarkers has a potential to substitute or to some extent replace the existing conventional tests applied in PCa management such as PSA blood test and tissue biopsy. Therefore, ctDNAs, exosomes and exosomal miRNAs, and DTCs, are the promising targets for being investigated as PCa biomarkers.

According to the results of the studies included in this systematic review, all the investigated biomarkers, such as ctDNAs, exosomal miRNAs, and PCa DTCs, demonstrated potential clinical utility. Thus, several types of miRNAs (Table1) demonstrated high diagnostic and prognostic value. In addition, it was identified that grouping of two or more miRNAs into the panels could improve sensitivity and specificity in early detection of PCa. Also, in several studies, additional conventional parameters such as PSA serum level were added to the miRNA-based biomarker panel, which could increase diagnostic and prognostic accuracy of the panel. Notably, in addition to blood serum, urine and semen were successfully investigated as the sources of exosomal miRNAs in several studies. In contrast, comparatively moderate sensitivity was demonstrated by ctDNAs as biomarkers for detecting non-metastatic PCa. Even though several promising ctDNA-based biomarker panels were developed, only few of them were proven effective. However, ctDNA-based biomarker panels are the least investigated among other types of liquid biopsy biomarkers, and therefore further investigations are required in this field. Regarding DTCs, either weak or moderate sensitivity, specificity, and prognostic value, were demonstrated in the studies where blood was used as the source of DTCs. At the same time, significantly higher

diagnostic and prognostic values were identified in rare studies where urine was chosen as the DTC source.

However, although liquid biopsy of PCa by means of isolation and detection of ctDNAs, or exosomal miRNAs, or DTCs, is a perspective diagnostic and prognostic technique, it has a number of limitations which have to be considered. The major of the limitations is the inconsistency in protocols, applied for isolation and analysis of the liquid biopsy biomarkers, which leads to inconsistency in the results obtained in different studies. Thus, due to such inconsistency, effectiveness of that or another liquid biopsy approach cannot be reliably compared with alternatives. Therefore, standardization of the liquid biopsy protocols is highly relevant. According to the results of the studies included in the current review, liquid biopsy by means of DTC isolation and analysis was the mostly standardized compared to other liquid biopsy approaches based on the analysis of ctDNAs and miRNAs. Notably, even though CellSearch platform did not demonstrate high capacity in terms of isolating DTCs from blood of patients with non-metastatic PCa, it has already been approved by FDA for clinical use. Also, few other platforms for DTC isolation from blood are currently on track for receiving the FDA approval. In case of miRNAs, as well as in case of ctDNAs, the data is limited and does not provide sufficient information to conclude on the diagnostic or prognostic value of any type of miRNA or ctDNA. Thus, optimization and standardization of methodologies in liquid biopsy of PCa is highly demanded before it could be introduced into a clinical practice. Also, while most of the liquid biopsy studies were based on isolation of the biomarkers from blood, such biological fluids as urine and semen are of a great interest for being investigated as the sources of the biomarkers.

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Chapter III

Rapid and label-free isolation of tumour cells from the urine of patients with localized prostate cancer using inertial microfluidics

Alexey S. Rzhevskiy [1,2,3,*] , Sajad Razavi Bazaz [4] , Lin Ding [4] , Alina Kapitannikova [1,2] , Nima Sayyadi [1,5] , Douglas Campbell [6] , Bradley Walsh [6] , David Gillatt [3] , Majid Ebrahimi Warkiani [2,4,*] and Andrei V. Zvyagin [1,2,*]

[1] ARC Centre of Excellence for Nanoscale BioPhotonics, MQ Photonics, Macquarie University, 2109 Sydney, Australia; a.kapitannikova@gmail.com (A.K.); nima.sayyadi@mq.edu.au (N.S.)

[2] Institute of Molecular Medicine, Sechenov First Moscow State Medical University, 119991 Moscow, Russia

[3] Department of Clinical Medicine, Faculty of Medicine and Health Sciences, Macquarie University, 2109 Sydney, Australia; david.gillatt@mq.edu.au

[4] School of Biomedical Engineering, University of Technology Sydney, 2007 Sydney, Australia; Sajad.RazaviBazaz@student.uts.edu.au (S.R.B.); Lin.Ding@student.uts.edu.au (L.D.)

[5] Department of Molecular Sciences, Macquarie University, 2109 Sydney, Australia

[6] Minomic International Ltd., 2113 Sydney, Australia; douglas.campbell@minomic.com (D.C.); Brad.Walsh@minomic.com (B.W.)

* Correspondence: **alexey.rzhevskiy@hdr.mq.edu.au (A.S.R.)**; majid.warkiani@uts.edu.au (M.E.W.); andrei.zvyagin@mq.edu.au (A.V.Z.); Tel.: +61-457-248-832 (A.S.R.); Tel.: +61-424-100-396 (M.E.W.); Tel.: +61-298-507-760 (A.V.Z.)

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3.1. Introduction to the chapter

This chapter describes the development of a microfluidic chip, and corresponding technique for isolation and immunocytochemical identification of PCa tumour cells from urine of patients with localized PCa, which was the candidate's first experience in the field of PCa liquid biopsy. Among three biological fluids of interest – urine, blood and semen, the advantage of urine as the source of PCa DTCs is non-invasiveness at collection, possibility to collect large volumes of the fluid and therefore highest accessibility in comparison with other biological fluids. Further, the promising results signifying presence of PCa tumour cells in urine of patients with localized PCa were recently published by the industrial partner Minomic International Ltd [1]. Thus, it was hypothesized that microfluidic isolation and subsequent identification of the isolated cells from urine may potentially be an efficient technique for diagnosis of PCa at an early stage. The immunocytochemical identification of the isolated tumour cells was performed with MIL-38 antibody which has an affinity to the GPC-1 antigen, specific for PCa tumour cells according to the recent data [2]. The antibody was kindly provided by Minomic International Ltd. Potentially, the developed technique can be modified for isolation and identification of tumour cells from other urologic cancers, including bladder and renal cancer. The clinical samples for this work were kindly provided by patients of Macquarie University Hospital.

3.2. Author's contribution

The PhD candidate (Alexey Rzhevskiy) participated in developing a design of the study, manufacturing of the investigated microfluidic chip designated for isolating DTCs from urine, collecting urine samples from the patients, performing laboratory analysis of the urine samples by isolating DTCs with the developed microfluidic chip, depositing of the isolated DTCs onto the glass slides with further immunocytochemical labelling of the DTCs, microscopy of the glass slides, collecting and interpretating the data, and manuscript writing.

Candidate's principal supervisor Dr Andrei Zvyagin conceptualized and supervised the study and reviewed the manuscript. Associate Supervisor Dr David Gillatt provided advice as the specialist in urology, and organized collection of urine samples from the

patients. Adjunct supervisor Dr Majid Warkiani provided advice as the specialist in liquid biopsy. Sajad Razavi Bazaz assisted in designing the microfluidic chip and preparing figures. Lin Ding assisted in manufacturing of the chips. Dr Bradley Walsh and Dr Douglas Campbell provided general advice and reviewed the manuscript. Alina Kapitannikova assisted in microscopy of the isolated putative tumour cells. Dr Nima Sayyadi assisted in developing the protocol for immunocytochemistry.

3.3. Abstract

During the last decade, isolation of circulating tumour cells via blood liquid biopsy of prostate cancer [3] has attracted significant attention as an alternative, or substitute, to conventional diagnostic tests. However, it was previously determined that localized forms of PCa shed a small number of cancer cells into the bloodstream, and a large volume of blood is required just for a single test, which is impractical. To address this issue, urine has been used as an alternative to blood for liquid biopsy as a truly non-invasive, patient-friendly test. To this end we developed a spiral microfluidic chip capable of isolating PCa cells from the urine of PCa patients. Potential clinical utility of the chip was demonstrated using anti-Glypican-1 (GPC-1) antibody as a model of the primary antibody in immunofluorescent assay for identification and detection of the collected tumour cells. The microchannel device was first evaluated using DU-145 cells in a diluted Dulbecco's phosphate-buffered saline sample, where it demonstrated > 85 (±6) % efficiency. The microchannel proved to be functional in at least 79 % of cases for capturing GPC-1+ putative tumour cells from the urine of patients with localized PCa. More importantly, a correlation was found between the amount of the captured GPC-1+ cells and crucial diagnostic and prognostic parameter of localized PCa - Gleason score. Thus, the technique demonstrated promise for further assessment of its diagnostic value in PCa detection, diagnosis, and prognosis.

3.4. Introduction

According to GLOBOCAN 2018, prostate cancer [3] is the second most frequent cancer as well as the second leading cause of the cancer deaths in males worldwide [4]. Statistical reports show that only a minor decrease in cancer-related mortality has been achieved during the past decade. This was primarily due to a lack of efficient techniques for detection and prognosis of PCa, as well as monitoring the treatment outcomes [5].

Currently, the gold standard for PCa detection is a costly multistage process, which conventionally includes the prostate-specific antigen (PSA) blood test and subsequent tissue biopsy. However, these diagnostic techniques have significant limitations. For instance, the PSA test is sensitive to diverse disorders, including benign prostatic hyperplasia and prostatitis; therefore, its specificity is relatively low [6]. On the other hand, the tissue biopsy is a highly specific yet invasive examination with possible adverse side effects as local bleeding and infectious complications including drug resistant bacterial strains. These shortcomings call for development of cost-effective and reliable alternatives to the existing diagnostic tests for PCa, which would first of all reduce the number of unnecessary tissue biopsies. Liquid biopsy via isolation of circulating tumour cells (CTCs) is a promising diagnostic tool capable of supplementing state-of-the-art PCa diagnostics [7]. However, standard liquid biopsy of the blood samples from PCa patients suffers from low CTC yield, especially in the case of the localized forms of cancer, implying a requirement for providing a large volume of blood for PCa detection. This requirement makes the feasibility of the standard liquid biopsy questionable. Urine represents an obvious and natural choice of the biological fluid to yield diagnostically relevant amounts of cancer cells from patients with diverse urologic cancers including PCa [8, 9]. Since a prostate gland is anatomically connected to the lower part of the urinary tract in males, diverse cells can be shed from the gland into the urinary tract, including tumour cells, which can be concomitantly washed out in the process of urination. The major advantages of the tumour cell isolation via liquid biopsy of the urine in comparison with the isolation of CTCs from the blood, as a truly non-invasive method, is a lack of limitation in the sample volume. Compared with blood, urine can be readily collected, stored, and transported. The urine collection process has enviable patient compliance and does not require a skilled medical professional.

Recently, isolation of tumour cells from biological samples via microfluidic technology has gained significant attention. Among the existing microfluidic modalities, inertial microfluidics based on a spiral microchannel device configuration has experienced a massive growth as exemplified by the separation of CTCs and circulating fetal trophoblasts [10-13], identification of bacterial spoilage from beers [14], blood fractionation [15], enrichment of circulating head and neck tumour cells [16], and separation of microalgae [17]. The technique is attractive for its cost-effectiveness and high throughput. The technology exploits inertial lift and Dean drag forces exerted on cells and particles flowing through the spiral microchannel. The interplay of these two forces causes lateral migration of cells and their focusing at equilibrium positions. As the amount of these two forces is directly related to the cell size, the cell eventually equilibrates at a lateral position, which precisely corresponds to its diameter. As a result, the larger cells (or particles, or both) tend to focus at regions near the inner wall, whereas the smaller cells (or particles, or both) tend to move away from it.

In this communication, we report on the development of a microfluidic chip capable of collecting PCa tumour cells from the urine using the principles of cell sorting employing inertial microfluidics. Isolated tumour cells were assayed via immunocytochemistry with monoclonal mouse anti-glypican-1 (anti-GPC-1) [18] antibody MIL-38 (Minomic International Ltd, Australia) previously reported to be highly specific and sensitive in the detection of PCa [19]. MIL-38 was used as a model primary antibody. The GPC-1 is a heparan sulphate proteoglycan which is found attached to the cancer cell surface and which has recently received significant attention as a biomarker for PCa, especially in terms of evaluating its aggressiveness and growth [2]. Furthermore, to showcase the versatility of the developed microchannel in terms of diagnosis and prognosis of localized PCa, correlations between the number of isolated tumour cells and conventional diagnostic parameters of PCa such as the level of prostate-specific antigen (PSA) in blood and Gleason score (GS) were analyzed.

3.5. Materials and Methods

3.5.1. Device design and fabrication

A spiral microchannel device for the isolation of PCa cells from urine samples was designed, fabricated, and tested. This device represented an adaptation of the microfluidic technology previously developed for the isolation of CTCs from peripheral blood [11], [20]. Briefly, polydimethylsiloxane (PDMS) pre-polymer with the curing agent (Sylgard 184, DowsilCorp., Midland, MI, USA) were first mixed at the ratio of 10:1 and then was degassed in a vacuum chamber. This mixture was poured onto an aluminum mold with subsequent baking in a laboratory oven for 2 hours at 70 °C. The mold can be fabricated via a milling process [21] or 3D printing technology [22]; in this study, we used a milling process. Furthermore, the cured PDMS was peeled from the mold, and holes with 1.5 mm diameter were pierced with a Uni-Core™ Puncher (Sigma-Aldrich Co. LLC. SG) at the sites designated for the inlet and outlets of the chip. Eventually, the PDMS microchannel was irreversibly bonded to another layer of PDMS using an oxygen plasma machine (Harrick Plasma, USA).

3.5.2. Experimental setup and procedure

The efficiency of the spiral microfluidic chip for capturing PCa tumour cells was evaluated by spiking a predetermined number of DU-145 cells (ATCC HTB-81) (approximately 1000) into 50 mL of Dulbecco's phosphate-buffered saline (DPBS). The solution was injected into the chip using a peristaltic pump (Baoding Shenchen Precision Pump Co., Ltd, China) at a 1.7-mL/min flow rate. When the volume of DPBS in the sample tube decreased to approximately 5 mL, 5 mL of DPBS was added, and the solution was further processed until its volume decreased to 1 ml. The enriched cells were further deposited onto an adhesive glass slide (ThermoFisher Scientific, Australia) using a Thermo Scientific™ Cytospin™ 4 Cytocentrifuge (ThermoFisher Scientific, Australia) and then air-dried. To determine if there were cells in the waste outlet, DPBS in the waste tube was centrifuged, and cell pellets were deposited onto 4 adhesive glass slides with Cytospin™ 4 Cytocentrifuge for further quantitation. Deposited cells on slides were fixed with ice-cold acetone for 3 min at -20°C and mounted with a Fluoroshield medium containing DAPI

(Prolong Gold Antifade Reagent, ThermoFisher Scientific, Australia) covered with a coverslip and counted using a fluorescence microscope (Zeiss Axio Imager Z2 Upright Microscope).

3.5.3. Ethics statement and clinical samples preparation

A part of this study that includes collection and analysis of clinical samples from patients with localized PCa was conducted under an ethical approval provided by Macquarie University Human Research Ethics Committee [HREC No: 5201500707), Australia. All patients and healthy volunteers provided written informed consent for collection of the samples and provision of their clinical data. The samples were collected and analyzed in a non-blinded manner, 14 midstream urine samples from healthy volunteers under 30 years of age and 14 midstream urine samples from patients with localized PCa were acquired. The midstream specimen of urine was chosen for analysis to avoid possible channel occlusion with debris which may present in the first pass specimen. The volume of urine collected from patients and healthy volunteers varied from 30 to 100 mL. Prior to sample processing, amount of cells per mL of urine was identified with an automated cell counter (TC20™ Automated Cell Counter, Bio-Rad, Australia). After the sample collection, samples were processed through the spiral microchannel on the same day with the peristaltic pump at the optimum flow rate of 1.7 mL/min. During the sample processing, once the volume reduced to approximately 5 mL, 5 mL of DPBS was added, and the processing was continued until the volume of solution decreased to approximately 1 mL. Afterwards, cells from the solution were sedimented with Cytospin™ 4 Cytocentrifuge onto four adhesive glass slides. The glass slides were then air-dried and fixed with ice-cold acetone for 3 min at -20°C and processed either immediately or stored at 4°C in a dry chamber and analyzed within a week. The schematic representation of the sample processing is illustrated in Figure1.

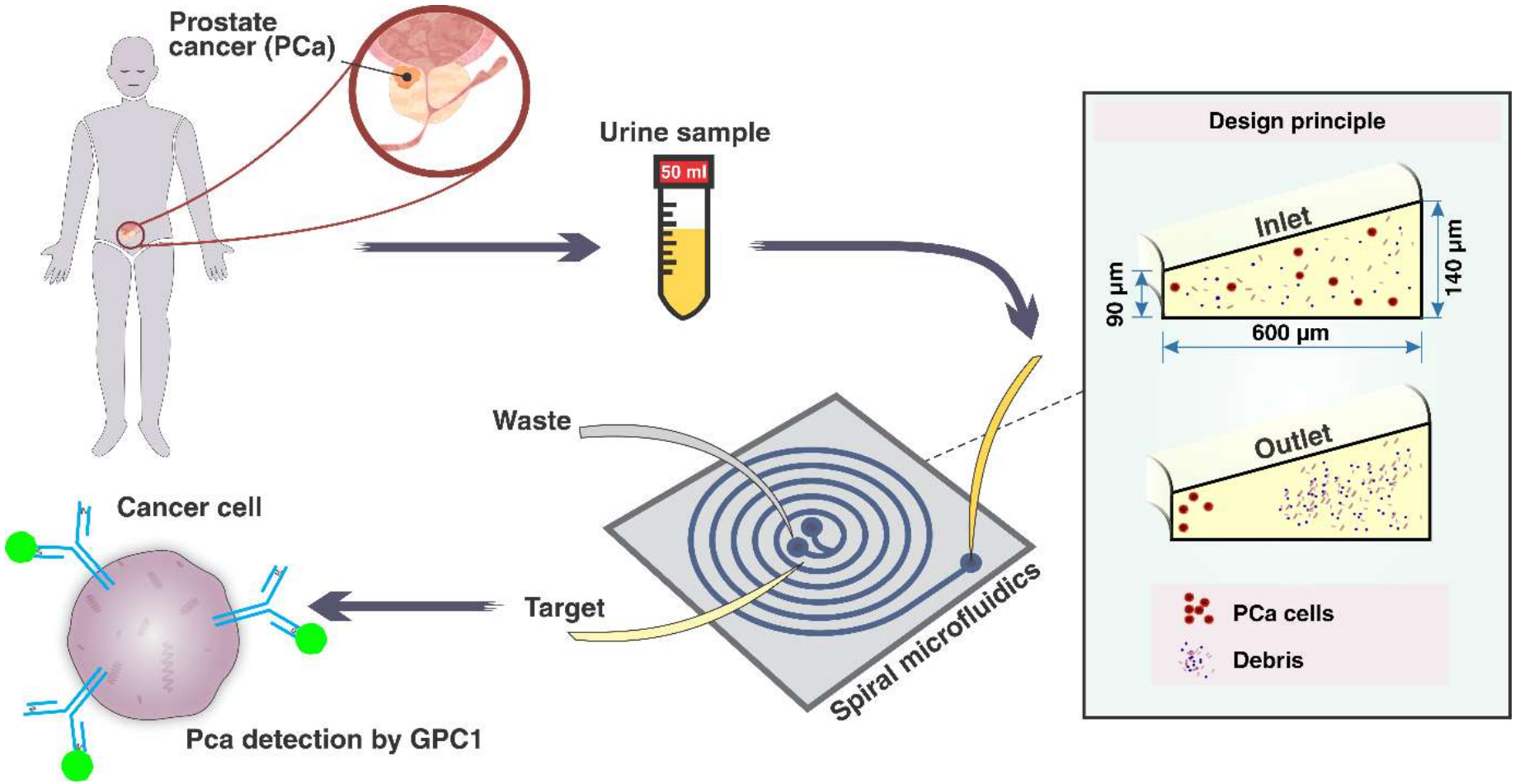

Figure1. A schematic representation of the workflow for PCa cell detection from the urine sample employing a spiral microfluidic chip. First, PCa cells shed from the prostate gland into the urethra are collected into a container in the process of urination. Then, the collected PCa cells present in urine are isolated via processing through the spiral microchannel. Finally, the collected cells are labelled with fluorescent antibodies, i.e., anti-GPC-1 and immunoassayed under a microscope.

3.5.4. Immunofluorescence staining

All incubations were performed in a humidified chamber. Cells were fixed on the surface of dry glass slides, rehydrated in DPBS for 3 min, followed by blocking with 1% Bovine Serum Albumin (BSA) solution in DPBS for 1 h at room temperature. Cells were then incubated for 2 h at room temperature with MIL-38 anti-GPC-1 mouse primary antibody diluted in blocking solution (1% BSA) at 10 µg/mL. At this step, one of the 4 slides was incubated with the blocking agent instead of the secondary antibody and was used as a secondary antibody alone control to monitor the non-specific binding of second antibody. Then, the glass slides were washed with DPBS 3 times for 5 min and air-dried. Then, cells were incubated for 1 h with goat-anti-mouse Alexa Fluor 488 (Abcam, Australia) diluted in the blocking solution at 4 µg/mL and washed with DPBS 3 times for 5 min after the incubation. Finally, cells were mounted with a Fluoroshield medium containing DAPI, covered with a coverslip, and observed under a fluorescence microscope.

3.5.5. Cell enumeration and data analysis

To identify a quantitative gold standard for enumeration of GPC-1+ putative tumour cells collected from the urine samples, approximately 1000 of DU-145 cells were added to a healthy urine sample, processed with the chip, immunostained as described above, captured under the fluorescence microscope, and the intensity of fluorescence signal from the cells was measured with ImageJ software. Then, a mean (±SD) for the signal intensity was calculated, and the intensity at the value equal to mean-SD was chosen as the threshold. Above this threshold, cells from the urine samples of PCa patients or healthy volunteers were registered as putative tumour cells and counted. Thus, in the case of cells isolated from the urine sample of PCa patients, during observation under the microscope, images of suspicious cells were captured, and the cells were analyzed with ImageJ software for signal intensity. GPC-1+ cells, whose signal intensity was higher than the mean-SD (determined using DU-145 cells), were registered as putative tumour cells and counted. Finally, to evaluate potential diagnostic and prognostic value of the technique, correlations between the amount of GPC-1+ cells collected from the patients and the levels of conventional PCa markers such as the blood PSA level and GS were measured. To further evaluate the

applicability of the developed microfluidic device, correlations between the amount of collected GPC-1+ cells, the volume of the urine sample and total amount of cells in urine sample (mL-1) were measured. The correlations were calculated using Microsoft Excel 2016 software.

3.6. Results

3.6.1. Spiral microfluidic device

In this study, we report a novel, efficient, and non-invasive method for isolation of tumour cells from urine samples. This is the first time that detection of PCa cells using the urine samples based on inertial microfluidics in a spiral channel has been reported.

Prostate cancer cells experience two major inertial lift and Dean drag forces inside the spiral channel. These forces can be calculated by Equations (1) and (2).

$$F_L = \rho \left(\frac{U_{max}}{D_h}\right)^2 C_L a^4 \quad (1)$$

$$F_d = 5.4 \times 10^{-4} \pi \mu De^{1.63} a \,, \quad (2)$$

where U_{max} indicates the maximum fluid velocity, ρ is the fluid density, μ denotes the dynamic viscosity of the fluid, C_L is the lift force coefficient, D_h represents the channel hydraulic diameter, a is the particle diameter, and De is Dean number. Considering these equations, a particle (or cell) of the diameter a experiences F_L and F_d different uniquely determined by the a [14]. As schematically shown in Figure1, the larger cells (prostate cancer cells) are focused at the inner wall (where its height is purpose-designed to be smaller than that of the outer wall), whereas the smaller cells and debris drift to the outer wall.

Generally, the ratio of particle diameter to the height of channel, as well as the hydraulic diameter of microchannel play a critical role in the particle focusing. The channel cross-section designed in this study was carefully analyzed to focus PCa cells (~15-20 µm) featuring a trapezoidal cross-section with the base of 600 µm, inner wall of 90 µm, and outer wall of 140 µm. The bifurcation at the outlet of the channel was placed at a location 350 µm to the inner wall. Upon the optimisation of the channel flow rate, DU-145 cells in DPBS solution were introduced from the inlet via a peristaltic pump. The results demonstrated

that the flow rate of 1.7 mL/min was optimal where 85 (±6) % of cells were collected through the target outlet (Figure2).

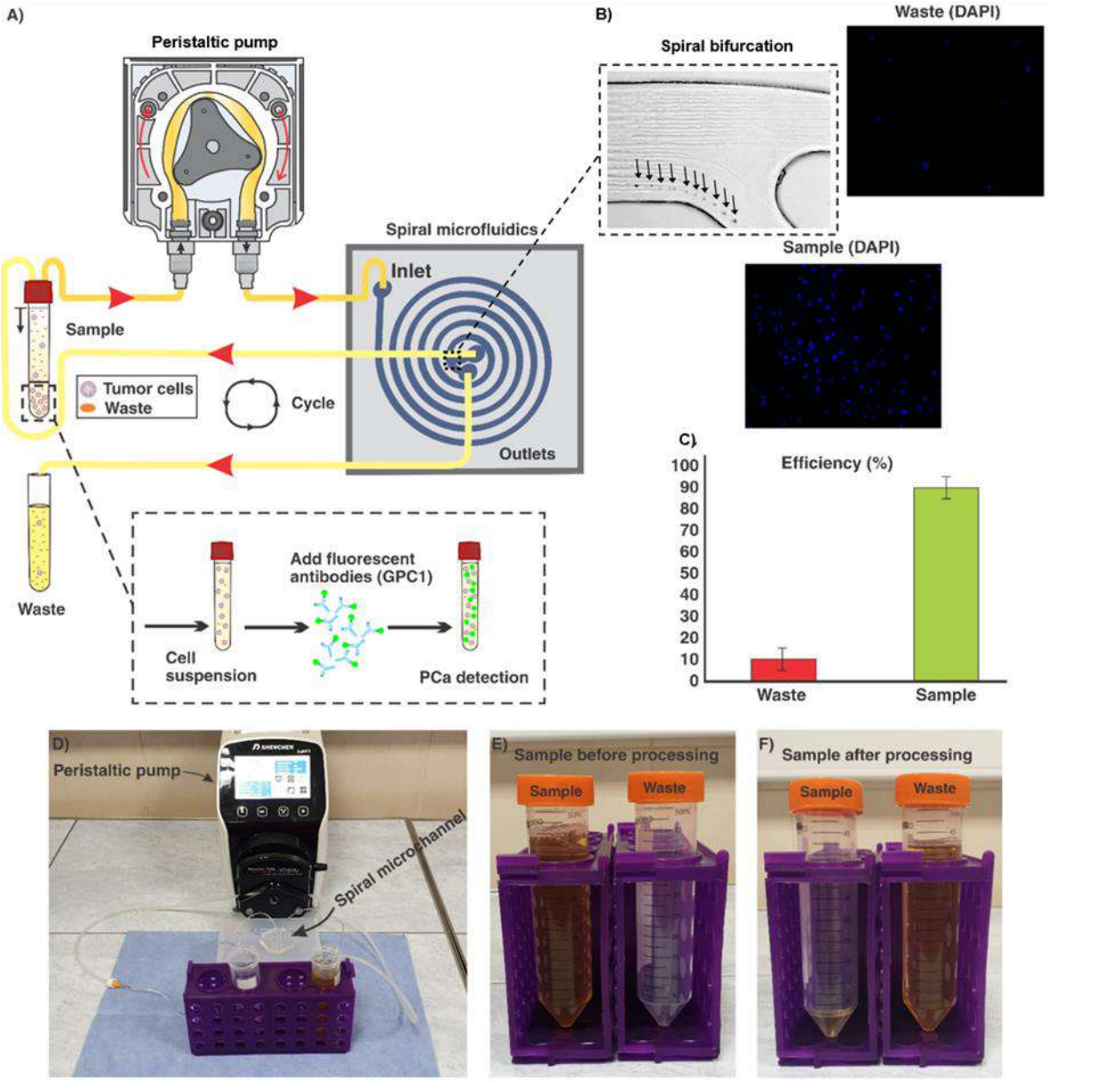

Figure2. A) Schematic representation of the processing of a urine sample containing PCa cells through the spiral microfluidic chip. Samples are introduced via a peristaltic pump and then recycled until 1 mL of the urine sample remains in the sample tube. Then, PCa assaying is implemented by the use of fluorescent antibodies. B) Illustration of the bifurcation of the spiral, target (sample) and waste outlets. The results show that most of cells were collected through the target outlet. The flow rate of 1.7 mL/min was selected as the optimum flow rate for the separation efficiency of PCa cells. C) 85 (±6) % of cells were collected through the sample outlet of the chip. D) Experimental setup used in this study. E) State of urine sample

before processing, and F) after processing, when about 1 mL of sample remains in the tube. The remaining 1-mL contains most of PCa cells and is subsequently analyzed as described.

3.6.2. Collection of GPC-1+ cells from urine

Midstream urine samples in volumes ranging from 30 to 100 mL were collected from 14 patients with localized PCa and processed using our spiral microfluidic chip. GPC-1+ cells exhibiting the fluorescence signal >1781 arbitrary units, according to the mean (2069)-SD (288) arbitrary units measured from DU-145 cells with ImageJ. were registered as putative tumour cells. Mostly, these cells exhibited round nuclei and a high ratio of nuclear to cytoplasmic size. We note that these cells were located in groups or clusters (Figure3 A and B). Putative tumour cells were detected in 12 out of 14 patients (86%). The total amount of detected cells (n) varied from 4 to 194 among the samples and patients, with the median value of 22. In the case of healthy volunteers, 11 samples out of 14 (79%) were negative in terms of GPC-1+ cells. It is worthy to note that the urine samples of the 3 healthy volunteers registered as GPC-1+ positive, contained only <8 cells. Thus, in case of PCa patients, only those patients whose urine samples contained n > 8 were considered positive with confidence. Accordingly, the number of such positive patients was 11 out of 14 (79%).

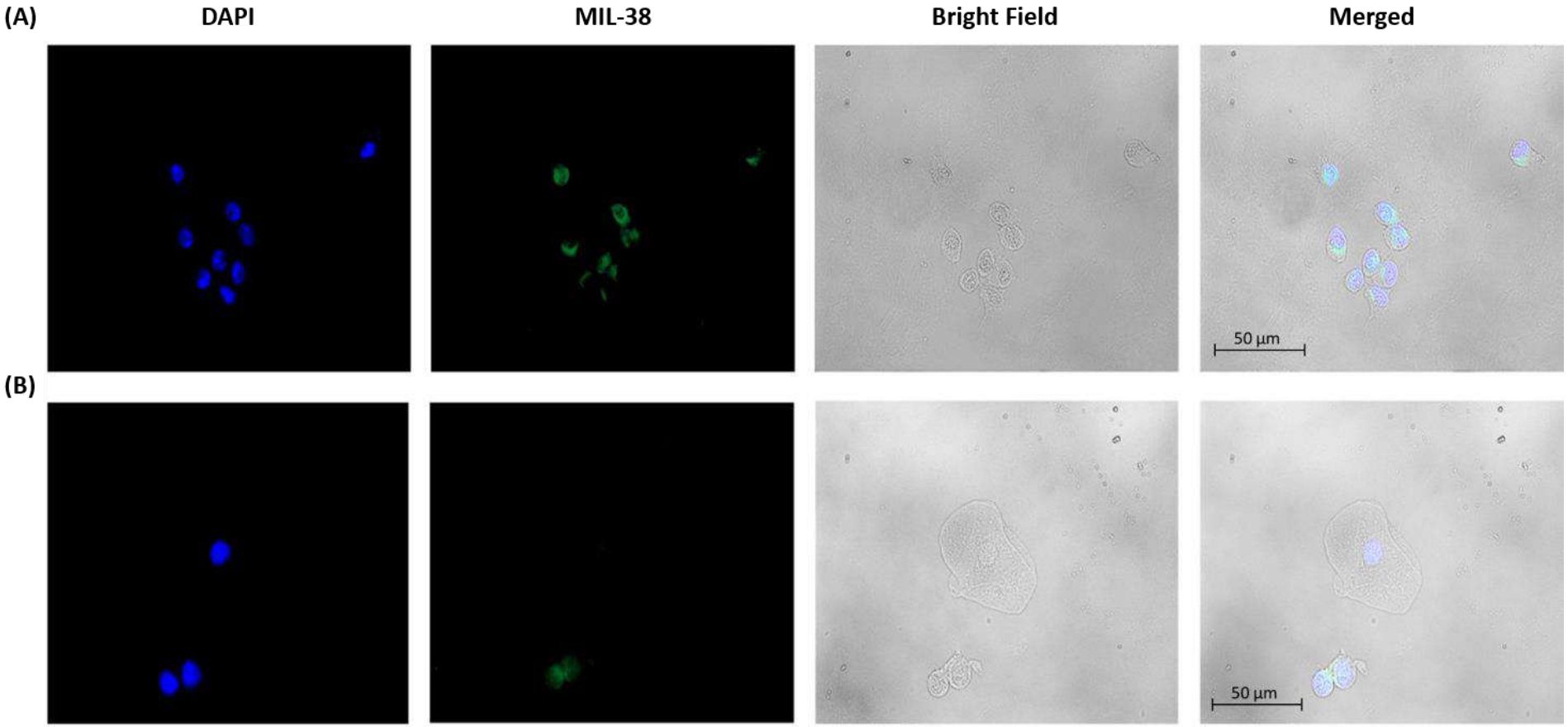

Figure3. Numerous GPC-1+ cells (A), two GPC-1+ cells and one GPC-1- squamous epithelial cell (B) isolated from the samples of PCa patients. The GPC-1+ putative tumour cells typically featured a high ratio of the nucleus to cytoplasm size and were prone to grouping or clustering.

Table1. The number of GPC-1+ cells detected in the urine samples and relevant clinical diagnostic test results. The number of cells with high GPC-1+ expression (n) isolated from the patients' and urine samples varied from 4 to 194 units with the median value of 22. n was < 8 in the urine samples of the healthy volunteers, i.e., 11 (79%) out of 14 healthy volunteers were registered PCa-negative in terms of the n-number. The number of patients positive with confidence for GPC-1+ cells (n > 8) was 11 out of 14 (79%).

Patient number	*n*	Urine sample volume (mL)	N	Blood PSA level (ng/mL)	Total GS
1	23	90	1.6×10^4	5.4	6
2	21	30	8.1×10^3	8.7	7
3	37	100	1.3×10^4	7	8
4	16	60	6.3×10^4	4.4	6
5	4	60	4.4×10^3	11	8
6	10	40	5.2×10^4	4.9	7
7	37	30	1.5×10^4	7.2	7
8	12	90	8.3×10^4	3.9	7
9	194	40	3.6×10^4	7.9	8
10	42	50	7.4×10^4	11.6	6
11	0	40	1.9×10^4	1.3	6
12	37	30	9.7×10^3	11	7
13	11	50	1.5×10^4	5.5	6
14	0	70	2.7×10^3	5.6	6

n - number of GPC-1+ cells; N - total number of cells in the analyzed sample

Low negative correlation between the amount of GPC-1+ cells and the volume of urine sample (r = -0.18, where r is correlation coefficient) and negligible positive correlation between the amount of GPC-1+ cells and the total number of cells per mL of the initial urine sample (r = 0.02) were identified. At the same time, moderate positive correlation between the volume of the urine sample and the total number of cells (mL^{-1}) (r = 0.32) was identified. Among all 28 samples analyzed, 1 (4%) sample was discarded due to the high concentration of non-cellular elements (i.e., urine crystals).

3.6.3. Analysis of potential diagnostic applicability of the method

To evaluate the flexibility of the method proposed in this study for early detection and diagnosis of PCa, correlations between the amount of isolated putative tumour cells and conventional diagnostic parameters of PCa were analyzed. Thus, correlations between the amount of GPC-1+ cells (n) and PSA level, or total GS were recognised (Figure4). Furthermore, correlations between the ratio of n/V (where V is volume of urine sample), or the ratio n/N (where N is a total number of cells in urine sample), and PSA level, or GS, were also identified. As a result, low positive correlations were identified between n and PSA level ($r = 0.27$) as well as n/V and PSA level ($r = 0.30$), moderately positive correlations between n and GS ($r = 0.48$), and between n/V and GS ($r = 0.46$), and moderately positive correlations between n/N and PSA level ($r = 0.47$) and GS ($r = 0.61$). At the same, it is worth noting that there also was a moderately positive correlation between PSA serum level and GS ($r = 0.40$).

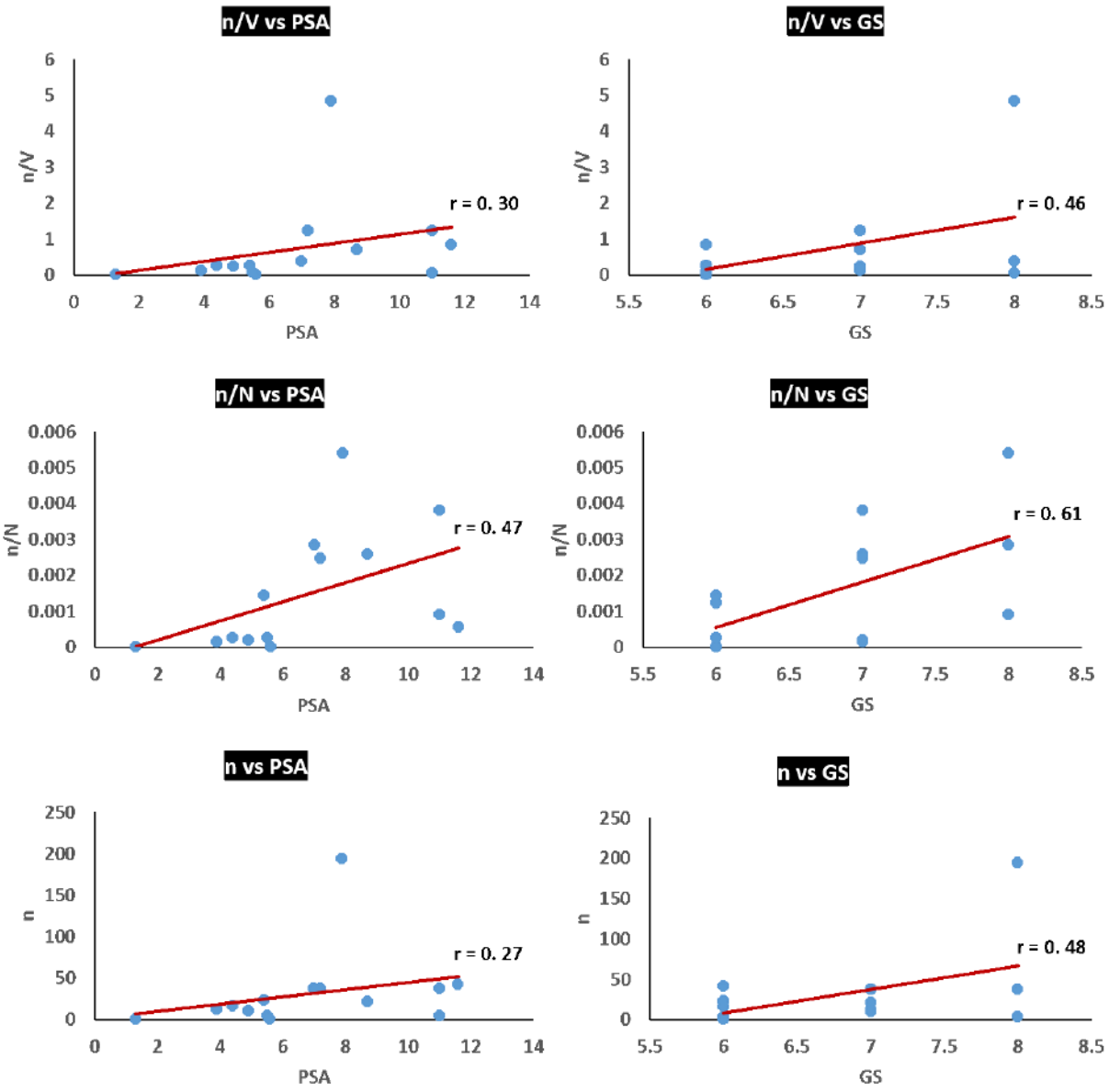

Figure4. Correlations between the amount of GPC-1+ cells (n), n/V (V, volume of urine sample), n/N (N, total number of cells in urine sample) and conventional clinicopathological parameters of PCa – PSA level and GS. The lowest correlation was identified between n and PSA level, and the highest correlation was identified between n/N and GS.

3.7. Discussion

PSA screening is the most common method of early detection of PCa. A prostate biopsy remains the only definitive diagnostic test for the presence of PCa. However, the high prevalence of false positive PSA tests gives rise to a large number of un-necessary prostate

biopsies. There remains a high clinical need for tests better able to guide a decision to proceed to prostate biopsy. This provides an opportunity to choose the right time for surgical intervention in the case of the parameter increments [23]. However, the specificity of PSA screening is not high, and a GS is obtained via an invasive tissue biopsy causing significant discomfort to a patient. In recent years, early diagnosis, and prognosis of PCa by means of CTC isolation via liquid biopsy of blood has been broadly investigated. However, progress has been minimal and the technique is not efficient due to the low number of CTCs in a standard sample of 7.5 mL of peripheral blood [24]. As an alternative, in this study, we developed and investigated a high-throughput microfluidic chip for enrichment of PCa tumour cells derived from the urine.

In the pilot enrichment of DU-145 cells from DPBS, the chip demonstrated 85 (±6) % efficiency in capturing cells at the optimum flow rate i.e., 1.7 mL/min. For urine sample analysis, 12 out of 14 PCa patient samples (86 %) were positive, and 11 out of 14 patients (79 %) were positive with confidence in terms of cells with the high level of GPC-1$^+$ expression – the cells which were registered as putative tumour cells in the current study. At the same time, 11 out of 14 healthy volunteers (78 %) were identified as PCa-negative in terms of GPC-1$^+$ cells. Such results corresponded to the specificity and sensitivity of anti-GPC-1 primary antibody MIL-38 used as a model primary antibody for the detection of putative tumour cells [19]. A median amount of GPC-1$^+$ cells of 22 units, captured from patient samples, identifies urine as a preferable medium for liquid biopsy of localized PCa. Furthermore, low negative correlation (r = -0.18) identified between the amount of captured GPC-1$^+$ cells (n) and the volume of the urine (V) suggested that the major amount of n were released in the first stream of the urine – a reasonable assumption considering the anatomical connection of the prostate gland to urethra [25]. This assumption is corroborated by moderately positive correlation between the total number of cells in urine samples (N) and V (r = 0.32) alongside a positive correlation between N and n (r = 0.02). This seems to indicate that other cells normally present in urine are gradually released during urination.

The low positive correlation between n/V and PSA level (r = 0.30) suggests that larger volumes of urine do not contain proportionally greater n, and the first stream of urine is sufficient to collect the majority of PCa cells. Therefore, it can be concluded that ≤30 mL of the voided urine is likely to be optimal for rapid isolation of tumour cells using our spiral

microfluidic chip. The low positive correlation between n and PSA level (r = 0.27) can be explained by the relatively low specificity of the PSA level increment as a marker for the PCa progression and aggressiveness [26]. However, the moderately high correlation between n/N and PSA level (r = 0.47) is noteworthy. The highest positive correlation in this study was achieved between n/N and GS (r = 0.61) and can be explained by the high specificity of GS as a marker of the progression and aggressiveness of localized PCa [27]. This assumption is corroborated by the moderate correlation between n and GS (r = 0.48) and between n/V and GS (r = 0.46).

The reported technique lends itself to some straightforward improvements. Fine-tuning of the chip by including an additional outlet to remove large waste elements will enable handling of a greater variety of urine samples, including samples containing a large amount of urine crystals, which we had to discard. The use of more specific and sensitive PCa antibodies or their combination will improve the immunocytochemistry assaying and increase the sensitivity and specificity of our technique.

3.8. Conclusion

It is for the first time to the best of our knowledge, when we introduce here a spiral microfluidic chip capable of rapid and label-free isolation of tumour cells from urine. The spiral microchannel used in this study had a trapezoidal cross-section with width of 600 µm, inner wall of 90 µm and outer wall of 140 µm. The microchannel was first tested using a spiked cancer cell line, proving its high efficiency: separation of ca 86% of cancer cells at the optimum flow rate of 1.7 mL/min. Secondly, ≥ 79% of the analyzed clinical samples from urine of patients with localized PCa were positive for GPC-1$^+$ putative tumour cells. Thirdly, moderate correlations were observed between the ratio of GPC-1$^+$ cells (n) to the total number of isolated cells (N) and PSA; n and GS; and n/V (V, urine volume) and GS. These results demonstrate promise of the spiral inertial microfluidic technique in terms of diagnosis and prognosis of localized PCa by liquid biopsy of urine, paving the way for inexpensive rapid, non-invasive diagnosis, as well as screening and monitoring therapeutic outcomes of PCa and other urology cancers.

3.9. References

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Chapter IV

Label-free isolation of tumour cells from the blood of patients with nonx-metastatic prostate cancer using inertial microfluidics

4.1. Introduction to the chapter

In liquid biopsy, blood is in general the most commonly used biological fluid. Even though urine as a source of DTCs has such advantage as non-invasiveness at collection and is potentially more attractive for being explored due to a higher scientific novelty, it was also essential to examine blood for performing comparison with the urine in terms of the efficacy of microfluidic label-free isolation of DTCs. Thus, in this chapter, a study on isolation and analysis of DTCs from blood of patients with localized PCa was described. In the described study, the microfluidic chip, previously developed by the candidate's adjunct supervisor Dr Majid Ebrahimi Warkiani [1] and successfully tested for isolating DTCs from blood of patients with diverse types of cancer [2-7], was applied. The applied chip was a prototype for its modified version, developed for DTC isolation from urine, which was described in Chapter III. Also, based on the results of the study described in the current chapter, potential diagnostic value of blood liquid biopsy via the label-free microfluidic isolation of DTCs was discussed.

4.2. Author's contribution

The PhD candidate (Alexey Rzhevskiy) conceptualized the study and designed the experiments. He also conducted the experiments, including fabrication of the microfluidic chips, development of immunofluorescence assay, microscopy, and imaging of the DTCs, image analysis, clinical data collection and interpretation. Candidate's principal supervisor Dr Andrei Zvyagin and adjunct supervisor Dr Majid Ebrahimi Warkiani were involved in the conceptualization, design, and supervision of the current study.

4.3. Introduction

DTCs presenting in blood are commonly termed as circulating tumour cells (CTCs), which imply tumour cells invading the blood stream by detaching from the primary or secondary tumour sites [8]. Being the crucial actors of metastasis, CTCs are potentially the universal biomarker for early diagnosis, prognostics, personalized treatment and follow up [9]. As a biomarker, after being isolated, CTCs are subjected to identification and may be followed by quantification, antigen profiling and single cell analysis [10]. Successful isolation of CTCs from blood of metastatic PCa patients with diverse techniques, including the mostly investigated liquid biopsy technique of CellSearch (Menarini Silicon Biosystems Inc, USA), was described in numerous research articles [11]. However, the value of CTCs in management of localized PCa remains unclear. Depending on the technique applied for CTC isolation, the published results are highly variable.

The CellSearch technique utilizes the affinity-based capturing of CTCs via their adherence to epithelial cell adhesion molecule (EPCAM), with definition of the captured CTCs as nucleated ($DAPI^+$) cells of epithelial and non-haematogenic origin (CK^+ and $CD45^-$). According to the data presented in different studies, detection of CTCs in blood was successful in 0% to 73% from total number of patients involved [12-19]. Such discrepancy between the results can be explained by the fact that the affinity of CellSearch technique is specified for non-specific capturing all circulating epithelial cells, most of which but not all are CTCs. Therefore, with this technique, up to 20% of healthy donors may be identified as false-positive for CTCs [16]. Further, the amount of blood analyzed in diverse studies varied from 7,5 mL to 22.5 mL, depending on the study [16, 17]. Also, no significant correlation between the number of isolated CTCs and standard diagnostic parameters of PCa such as preoperative PSA serum level, GS, overall survival, progression-free survival, or tumour stage was observed in the CellSearch studies.

Another affinity-based techniques, which were also tested in terms of the efficiency for CTC isolation from blood of patients with localized PCa, are: EPISPOT assay [20] based on removal of white blood cells (WBCs) from lysed blood containing WBCs and CTCs, and CellCollector [21] based on EPCAM-dependent adhesion of CTCs to a specifically designed venous catheter. Thus, in the study by Kuske et al. [19] where a comparison between the

CellSearch, EPISOT and CellCollecor techniques was performed, CTCs were detected in 37%, 58,7% and 54,9% of patients, respectively. However, a cumulative result calculated from all three techniques of CTC isolation showed that only 21.5% (23/107) of patients had ≥5 CTCs per 7.5 ml of blood, which is a very small amount considering a relatively low specificity of the affinity-based CTC isolation techniques. Also, significant correlation between the number of isolated CTCs and PSA serum levels, or clinical tumour stage, was observed only in case of using the EPISPOT assay. The EPCAM-dependent affinity-based microfluidic enrichment by Stott et al. [22] also demonstrated a relatively low sensitivity at 42% (8 of 19 patients). However, more positive results with the usage of affinity-based techniques of CTC isolation from blood have also been reported. Thus, an immune-magnetic enrichment by Fizazi et al. [23] demonstrated sensitivity at 70%, without false positive results from healthy donors.

The other approach for CTC isolation from blood of patients with localized PCa, which has been widely investigated, is the label-free or so-called size-based approach. In the current study, the technique for label-free microfluidic isolation of CTCs from blood, previously developed by the candidate's (Alexey Rzhevskiy) adjunct supervisor Dr Majid Ebrahimi Warkiani, was applied [24]. The technique has already been successfully tested for isolating CTCs from blood of patients with melanoma, lung cancer, head and neck cancer, breast cancer, and was also adopted for isolating fetal trophoblasts for prenatal testing, blood plasma separation, malaria detection and separation of water from algae [3-6, 25-31]. The technique was also a prototype for the microfluidic technique of label-free isolation of PCa DTCs from urine described in Chapter III. The technique utilizes the principles of inertial cell separation, relying on the tendence of cells with different sizes to different trajectory of movement in a liquid stream within a specifically designed curvilinear microchannel. The technique is represented by the spiral microfluidic channel with one inlet for receiving lysed blood and two outlets, where one outlet is designated for separating WBCs and another outlet for isolating CTCs (Figure1).

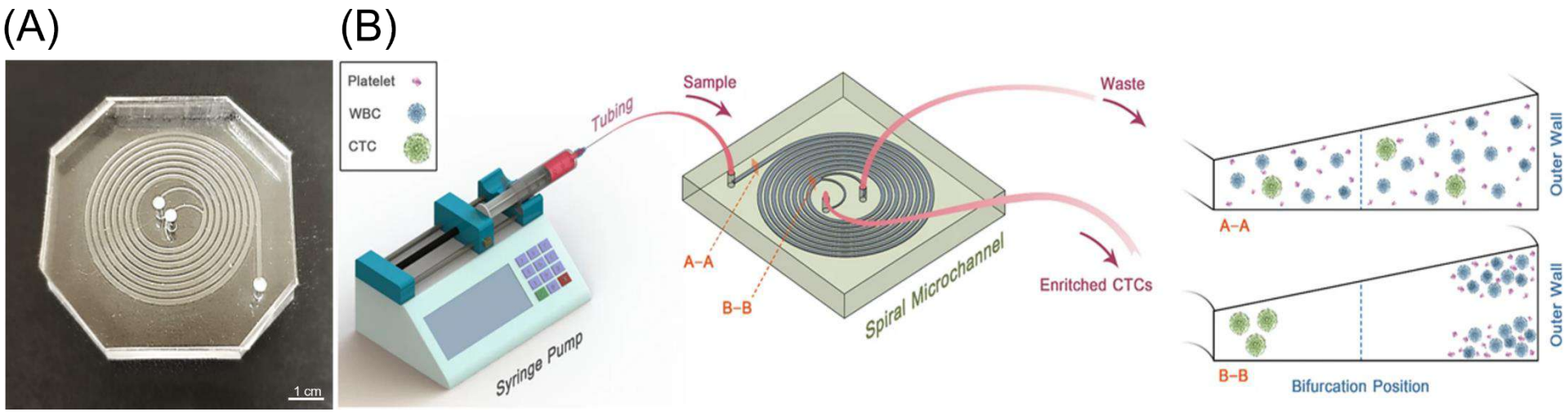

Figure1. Photograph of the spiral microfluidic chip (A) for CTC isolation from blood, and schematic representation of its functioning (B) [3]. The microfluidic chip is composed of PDMS and contains a curvilinear microchannel with a trapezoidal cross section. The microfluidic chip has one inlet to the microchannel, one bifurcation near its ending and therefore two outlets. Using a syringe pump, a preliminary lysed blood is delivered into the microchannel through the inlet, with a continuous flow. At this stage, the delivered blood comprises a mixture of WBCs, platelets and CTCs (A-A). Being processed through the microchannel with a continuous fluid flow, at the bifurcation site, the initial blood sample is devided into two fractions: waste fraction which release through one outlet of the chip and contains most of the WBCs and platelets, and targeted fraction which release through another outlet of the chip and contains most of the CTCs. The separation of cells is based on the size-based approach, which utilizes the inertial lift forces and Dean vortex forces that occur into the curvilinear microchannel under a continuous fluid flow. These forces determine the focusing of large CTCs near the inner wall of the microchannel, while smaller WBCs and platelets focus near the outer wall. This provides

separation of the larger and smaller cells at the bifurcation site. The described microfluidic chip and subsequent techniqe allows to separate > 99.9% of WBCs and platelets presenting in the initial blood sample and enrich > 80% of CTCs of diverse non-small cell carcinomas.

4.4. Materials and Methods

4.4.1. Device design and fabrication

The design of the microfluidic chip and its fabrication process was similar to those described in section "3.4.1. Device design and fabrication". Thus, as well as the chip, which was designed for isolating DTCs from urine, the chip designed for isolating CTCs from blood was also composed of two PDMS parts. One of the parts was flat while another part was performed via the micromolding process and contained the spiral microchannel, one hole for the inlet and two holes for the outlets. The flat PDMS part was bounded with the PDMS part containing microchannel by using the oxygen plasma pretreatment. The main difference between the process of chip fabrication described in section "3.4.1. Device design and fabrication", and current section, was the usage of different mold for fabricating the PDMS part containing microchannel. The mold was different in terms of the dimensions of the microchannel, designed for the most efficient separation of CTCs from WBCs presenting in lysed blood. Due to a very high ratio of the amount of WBCs to the amount of CTCs presenting in the blood sample (millions of WBCs to one CTC), the microchannel was first of all designed to separate as much of WBCs as possible (> 99.9%) and enrich sufficient amount of CTCs (> 80%). Therefore, the cut-off diameter of cell separation at the bifurcation site of the microchannel was set at 13 µm. To reach the desired principle of cell separation, the dimensions of the trapezoidal cross-section was as follows: base at 500 µm, inner wall at 80 µm, outer wall at 120 µm, and bifurcation point at 300 µm.

4.4.2. Cell culture

DU145, PC3, and LNCaP are the patient-derived cell lines, considered standard PCa cell lines used in laboratory research of PCa. Cell lines were cultured under standard conditions in a humidified incubator (Panasonic, South Korea) at 37 °C, in 5% CO2. DU-145 and PC-3 cell lines were cultured in RPMI-1640 medium (Thermofisher Scientific, USA) with Glutamax supplement (Gibco, USA), 10% heat-inactivated, 0.22 µm filtered fetal bovine serum (Life Technologies, Inc), and 1% Penicillin-Streptomycin (Life Technologies, Inc). For

LNCaP cell line, an RPMI-1640 medium (Thermofisher Scientific, USA) supplemented with 10% heat-inactivated, 0.22 µm filtered fetal bovine serum (Life Technologies, Inc), 1% insulin-transferrin (Paneco, Russia), 1% HEPES pH 7.2-7.5 (Paneco, Russia), 1% nonessential amino acid solution (Gibco, USA), 1% gentamycin (Paneco, Russia). T-25 and T-75 filtered flasks (Corning, USA) were used for subculturing of the cell lines. Cell dissociation was performed using 0.25% (w/v) Trypsin-0.53 mM EDTA solution (Thermofisher Scientific, USA). A subcultivation ratio was 1 to 6. Cell culture medium was renewed every 2 to 3 days. Before any further management, cell lines were STR profiled for authenticity and were confirmed negative for mycoplasma infection by PCR.

4.4.3. Experimental setup and procedure

The efficiency of the microfluidic chip for isolating PCa DTCs was evaluated by spiking a predetermined amount of DU-145 (or PC3, or LNCaP) cells into 7.5 mL of healthy donor's blood. The blood with the added DU-145 (or PC3, or LNCaP) cells (approximately 1000 cells) was incubated with the red blood cell lysis (RBC) buffer, centrifuged, and the liquid part was replaced with DPBS. The solution was then processed through the spiral microchannel using a syringe pump (Baoding Shenchen Precision Pump Co., Ltd, China) at the flow rate of 1.7-mL/min. After processing, the targeted liquid fraction was processed through the microchannel one more time. Cells in the targeted fraction were fixed with 4% paraformaldehyde (PFA) (ThermoFisher Scientific, USA). The targeted fraction was then divided into four parts and deposited onto four glass slides (ThermoFisher Scientific, USA) using a Thermo Scientific™ Cytospin™ 4 Cytocentrifuge (ThermoFisher Scientific, USA) and air-dried. The deposited cells were labelled with the mouse monoclonal primary antibody to pan-Cytokeratin (anti-CK) (Abcam, Cambridge, UK) and goat anti-mouse IgG H&L Alexa Fluor 488 (Abcam, Cambridge, UK) secondary antibody. The cells were mounted with a ProLong Gold Anti-Fade Mountant (Abcam, Cambridge, UK) containing DAPI, covered with a coverslip and counted under the fluorescence microscope (Zeiss Axio Imager Z2 Upright Microscope). Also, the amount of DU-145 (or PC3, or LNCaP) cells, same to the amount added to the blood sample, was deposited onto the glass slide, and labelled with the anti-CK+Alexa Fluor 488 antibodies, then mounted with with a ProLong Gold Anti-Fade Mountant (Abcam, Cambridge, UK) containing DAPI, covered with a coverslip and counted under the

fluorescence microscope (Zeiss Axio Imager Z2 Upright Microscope). The experiment was repeated five times for each cell line (DU-145, PC3, LNCaP), and the efficiency of the tumour cell enrichment was expressed as the ratio of the amount of tumour cells isolated from PCa blood mock (healthy blood + cultured tumour cells) to the initial amount of tumour cells added. The corresponding value was expressed as the percentage ± SD.

4.4.4. Clinical samples

A part of this study that includes collection and analysis of blood samples from patients with localized PCa and healthy volunteers was conducted under ethical approval provided by Sechenov University Local Ethics Committee (extraction from protocol № 17-19), Russia. The samples were collected at Sechenov University Clinical Hospital № 2. All patients and healthy volunteers provided written informed consent for collection of the samples and provision of their clinical data. The samples were collected and analyzed in a non-blinded manner, samples of venous blood were obtained from 8 subjects of both groups - patients with localized PCa and healthy volunteers. The group of healthy volunteers included healthy males at the age from 18 to 30 years. The blood was collected from cubital or antecubital vein into the 9 mL VACUETTE K2 EDTA tube (Greiner Bio-One, Austria). After blood collection, to avoid temperature drops, the tubes were transported to laboratory in a thermal container. At the laboratory, the blood was transferred to the 15 mL Corning Costar centrifuge tubes (SigmaAldrich, USA) incubated for 10 minutes with warm (37 °C) RBC buffer (BioLegend, USA) at the ratio of 1 (blood): 3 (buffer) and centrifuged for 5 minutes at 1000 rpm. After centrifugation, the supernatant was removed using a mechanical pipette, and the sedimented cell pellet was resuspended in 9 mL of PBS at room temperature. To remove the residual RBC lysis buffer and lysed RBCs, centrifugation and resuspension with PBS was repeated once again. Then, the sample was processed through the spiral microchannel with the syringe pump (Baoding Shenchen Precision Pump Co., Ltd, China) at the optimum flow rate of 1.7 mL/min. After the first processing, targeted fraction containing most of the DTCs was processed through the microchannel again for better purification from WBCs. After, the sedimented cells were fixed with 4% paraformaldehyde (PFA) (ThermoFisher Scientific, USA) for 10 minutes. After fixation, the cell sediment was washed from the residual PFA with PBS.

Afterwards, cells from the solution were sedimented with Cytospin™ 4 Cytocentrifuge onto four adhesive glass slides.

4.4.5. Immunofluorescence staining

The method of sequential labelling, which implies a sequential application of primary and secondary antibodies, was used for more efficient and specific antibody labelling of the isolated DTCS. In the current study, antibody panel contained three primary and three secondary antibodies. The primary antibodies were: rabbit polyclonal recombinant Anti-PSMA antibody (Abcam, Cambridge, UK), mouse monoclonal antibody to pan-Cytokeratin (Abcam, Cambridge, UK) and rabbit polyclonal antibody to CD45 (Abcam, Cambridge, UK). The secondary antibodies were: goat anti-rabbit IgG H&L Alexa Fluor 568 (Abcam, Cambridge, UK), goat anti-mouse IgG H&L Alexa Fluor 488 (Abcam, Cambridge, UK), goat anti-rabbit IgG H&L Alexa Fluor 647 (Abcam, Cambridge, UK). All incubations were performed in a humidified chamber. The cells onto the glass slides were permeabilized with 0.2% PBS solution of Tween-20 (ThermoFisher Scientific, USA) detergent for 10 minutes at room temperature. After permeabilization, the glass slides were washed with DPBS 3 times for 5 minutes and air-dried. Further, glass slides were incubated with 3% solution of BSA in PBST (0, 05% solution of Tween-20 in PBS) for 1 hour at room temperature for minimization of unspecific binding of the antibodies. As the next step, the BSA+PBST solution was gently washed from the glass slide, and the solution of first primary antibody in PBST with 1% BSA was added onto the glass slides and incubated overnight at 4 °C. After, the glass slides were gently washed with DPBS 3 times for 5 minutes, air-dried and incubated with the 3% BSA PBST solution of secondary antibody for 1 hour. After, the glass slides were washed with DPBS 3 times for 5 minutes and air-dried. The incubations with other two primary and secondary antibodies were performed as described above. Finally, cells were mounted with ProLong Gold Anti-Fade Mountant (Abcam, Cambridge, UK) containing DAPI, covered with a coverslip.

4.4.6. Cell enumeration and data analysis

The glass slides were examined with scanning laser microscope Zeiss LSM 880 and Zen Black software (ver 3.1). For the detection of 4 dyes, a 405 nm diode laser with a power of 30 mV (for detecting the DAPI dye signal), a multilinear argon laser with a power of 25 mV (Alexa Fluor 488), a 561 nm diode-pumped solid-state laser (DPSS) with a power of 20 mW (Alexa Fluor 568), and a He-Ne laser 633 nm with a power of 5 mW (Alexa Fluor 647) were used. A fluorescent unit with a metal-halogen lamp was used to focus on the area of the cells. An LD LCI Plan-Apochromat 40x / 1.4 objective at 0.7 magnification was used to scan the samples. The frame size was 2048 x 2048 pixels, the field of each scan was 350 x 350 µm. Each image was saved separately in czi format without any additional processing.

To identify a quantitative gold standard for enumeration of CTCs isolated from blood of PCa patients, approximately 1000 of PC3, or DU-145 cells were added to a healthy blood sample, processed through the chip, immunostained as described above, captured under the laser scanning microscope Zeiss LSM800 (ZeissAG, Germany) using Zen Black software. The intensity of fluorescence signal from the cells was measured with Fiji ImageJ software. After, a mean (±SD) value of the signal intensity was measured from the PC3 and DU-145 cells from each of the channels (Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647), and the value equal to mean-SD was indicated as a threshold. Thus, the putative tumour cells isolated from the patients' blood, and expressing CK and PSMA above the consequent thresholds while expressing CD45 below the consequent threshold were considered as CTCs (CK+, PSMA+, CD45-) and counted. Also, in cell counting, the presence of a cell nucleus (DAPI +) was also taken as an essential criterion. In addition, the size of the cell (>13 µm), the area of the nucleus (mean – SD) and the area of the cytoplasm (mean – SD) were also set as the additional threshold parameters. Cells meeting all the criteria mentioned above were registered as CTCs. Also, to evaluate potential diagnostic and prognostic value of the technique, it was planned to estimate a correlation between the amounts of CTCs enriched from patients' blood, and PSA serum levels and Gleason score (GS) were estimated.

4.5. Results

4.5.1. Spiral microfluidic device

The pilot experiment with the mock of PCa patient's blood sample, represented by the healthy blood sample with an addition of designated amount of DU-145 (or LNCaP, or PC3) cells, demonstrated a potentially high efficiency of the microfluidic chip in terms of the enrichment of PCa DTCs. Thus, according to the results, it was possible to collect 84 (±4) % of DU-145 cells, 80 (±6) % of PC3 cells and 78 (±9) % of LNCaP cells. The obtained values corresponded to the anticipated value of CTC isolation efficiency by the applied microfluidic chip at around 80%, which was reported in the previous study by Wakiani et al. [7].

4.5.2. Collection of CTCs from blood

Whole blood samples at the amount of 7.5 mL were collected from 12 patients with localized PCa and processed using the microfluidic chip. The isolated cells were considered CTCs if they were >13 µm cell cytoplasm area at more than 241 μm^2 (mean at 273 μm^2 - SD at 32 μm^2) and area of the cell nucleus at more than 88 μm^2 (mean at 92 μm^2 – SD at 4 μm^2). The mean fluorescence signals measured with ImageJ from PC3, and DU-145 cells were (1872) ± SD (254) and (1341) ± SD (146) arbitrary units, for anti-PSMA+AlexaFluor568 and pan-CK+AlexaFluor488, respectively. The mean fluorescence signals measured with Fiji ImageJ from healthy blood WBCs was (735) ± SD (56) arbitrary units for anti-CD45+AlexaFluor647. Thus, the isolated cells were considered CTCs in case of exhibiting the fluorescence signal >1618 arbitrary units (PSMA+) for PSMA+AlexaFluor568, >1495 arbitrary units (CK+) for panCK+AlexaFluor488, and < 791 arbitrary units (CD45-) for anti-CD45+AlexaFluor647. As well as in case of the enrichment of DTCs from urine, most of such cells had round nucleus and exhibited a high ratio of nuclear to cytoplasmic size. Thus, CTCs (Figure2) were detected in 3 of 12 patients (25%) (Table1). The total amount of detected CTCs (n) varied from 0 to 3 units among the patients, with the median value at 0 units and the mean value at 0.5 units. In case of healthy volunteers, 0 samples of 12 were positive

(0.0%) in terms of CTCs (CK+, PSMA+, CD45- cells). Thus, the number of CTCs at 0 units was set as a threshold, and the patients who had CTCs in their blood sample above this threshold were considered positive with confidence. Therefore, the number of patients positive for CTCs with confidence was 3 of 12 (25%).

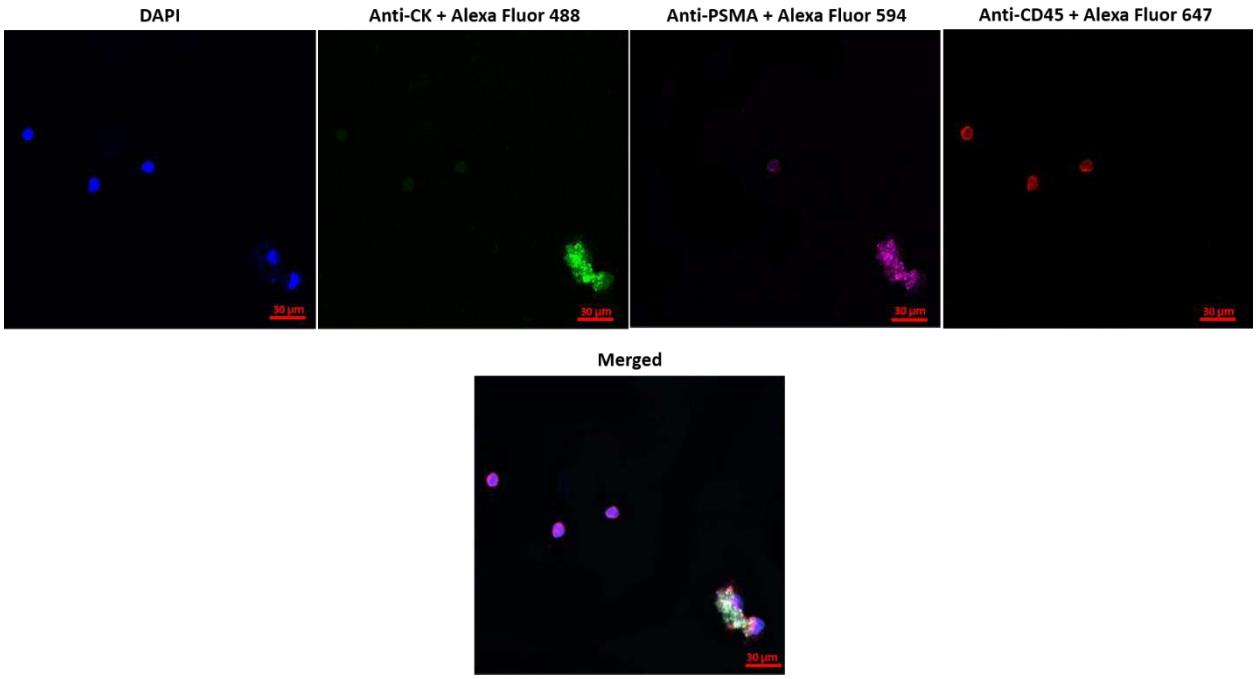

Figure2. CTCs isolated from blood with the microfluidic chip and identified via immunocytochemistry. On the image, three smaller lymphocytes (CK-, PSMA-, CD45+) and two bigger CTCs (CK+, PSMA+, CD45-) can be observed.

Table1. Amounts of the isolated CTCs and PCa characteristics of the patients.

Patient number	n*	TNM stage	Gleason score	PSA level
1	0	T2cNoMx	6	7,4
2	1	T1cN0M0	7	8,2
3	2	T1cN0M0	7	11
4	0	T1cN0M0	7	8,3
5	0	T1cN0M0	6	5,3
6	3	T3bN0M0	7	6,8
7	0	T2bN0M0	8	5,1
8	0	T3bN0M0	7	1,6
9	0	T1cN0M0	6	4.1
10	0	T1cN0M0	6	5.6
11	0	T2cNoM9	7	4.8

12	0	T1cN0M0	7	6.3

* – total number of CTCs in the blood sample at 7.5 mL.

4.5.3. Analysis of potential diagnostic applicability of the method

Due to the absence or marginal amounts of CK+, PSMA+, CD45- cells identified in the patients' blood samples, the investigation of blood as the potential source of CTCs in patients with localized PCa was stopped after bloods samples were collected from 12 patients. In this setting, calculation of the correlation between the amounts of CTCs and PSA serum level or GS score was considered unreasonable.

4.6. Discussion

According to the results of the conducted study, the technique of label-free isolation of CTCs from blood of patients with localized PCa by means of the microfluidic chip followed by the identification of isolated CTCs with CK, PSMA, CD45 dependent immunocytochemistry (ICC) did not demonstrate high potential as the tool for liquid biopsy of PCa at its early stages. Thus, only 3 patients of 12 were positive for CTCs, with only a negligible number of CTCs in their blood samples at 1, 2 and 3 units. This result is not promising for further investigation of the technique in terms of its diagnostic and prognostic value in non-metastatic PCa. At the same time, considering the absence of putative CTCs in the blood samples from healthy volunteers, the technique may be successfully applied and is worth to be investigated for liquid biopsy of metastatic PCa.

In general, despite the positive results from the studies where the described microfluidic chip was successfully applied for isolating CTCs from blood of patients with breast [6], head and neck [3], and lung cancers[4], the results obtained in the current study were expectable. Particularly, sensitivity of the label-free technique for detecting non-metastatic prostate cancer reported in the studies by Giesing et al. [32], Kolostova et al. [33], Todenhofer et al. [34], and Murray et al. [35], were 32.5%, 52%, 50% and 47.8%, respectively. At the same time, Awe et al. [36] reported a sensitivity at 100% for the CTCs isolation technique based on filtration with further detection of the CTCs via the ICC for CK

and androgen receptor. However, no specificity was reported for this technique, and therefore no positive conclusion regarding its probable clinical applicability could has been made. Also, it is worth noting that neither of the affinity-based techniques demonstrated an outstanding diagnostic potential (Table2, Chapter II).

4.7. Conclusion

Overall, the described microfluidic technique of label-free isolation of PCa DTCs from blood did not demonstrate high potential for being used in early diagnostics of PCa. The obtained results are minor in comparison with the results presented in Chapter III, where the microfluidic technique for isolating PCa DTCs from urine of patients with localized PCa was described. However, direct comparison between these two techniques cannot be performed due to a high difference between the ICC protocols applied for the identification of isolated DTCs. Therefore, the study in which each patient provides both biological fluids for the analysis, and the same or similar ICC protocol is applied for detecting the isolated DTCs, should be conducted to directly compare the described techniques for isolating DTCs from blood, and urine.

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Chapter V

Rapid and label-free isolation of tumour cells from the semen of patients with localized prostate cancer using inertial microfluidics

Alexey S. Rzhevskiy [1,2,3,*], Alina Y. Kapitannikova [2], Denis V. Butnaru [3], Evgeniy V. Shpot [3], Dmitry V. Enikeev [3], Steven A. Vasilescu [4], Majid Ebrahimi Warkiani [4], Andrei V. Zvyagin[1,2]

[1] ARC Centre of Excellence for Nanoscale BioPhotonics, MQ Photonics, Macquarie University, 2109 Sydney, Australia

[2] Institute of Molecular Medicine, Sechenov First Moscow State Medical University, 119991 Moscow, Russia

[3] Institute for Urology and Reproductive Health, Sechenov University, 119991 Moscow, Russia

[4] School of Biomedical Engineering, University of Technology Sydney, 2007 Sydney, Australia

* Correspondence: **alexey.rzhevskiy@hdr.mq.edu.au**

Keywords: prostate cancer, liquid biopsy, circulating tumour cells, microfluidics

5.1. Introduction to the chapter

According to the results presented in Chapter III, liquid biopsy via microfluidic label-free isolation of PCa putative DTCs (GPC-1+ cells) from urine demonstrated a relatively high specificity and sensitivity at around 80%. At the same time, as reported in Chapter IV, microfluidic label-free isolation of putative DTCs from blood (CK+, PSMA+, CD45-) did not demonstrate significant output. In case of the urine, the presence of putative DTCs in the samples of PCa patients was hypothetically due to the anatomical junction between the prostate and urethra. Thus, if DTCs primarily present in prostatic ducts, such junction may provide shedding of DTCs into urethra. This may further lead to the release of DTCs during urination. Therefore, the third possible biological fluid of interest, which may potentially contain PCa DTCs, is prostatic fluid or semen. In this chapter, a study on the development and testing of microfluidic label-free technique designed for isolating DTCs from semen of PCa patients is described. In the described study, to increase a specificity of the developed technique, the isolated putative DTCs were immunocytochemically identified by using the antibody panel containing multiple primary antibodies specific to PCa, including anti-CK, anti-PSMA and anti-GPC-1.

5.2. Author's contribution

The PhD candidate (Alexey Rzhevskiy) conceptualized the study, designed, and conducted the experiments including isolation of DTCs with the microfluidic chip with further identification of the isolated DTCs via ICC, performed collection of the relevant clinical data, analyzed, and interpreted the data obtained in the study, and wrote the manuscript.

Candidate's principal supervisor Dr Andrei Zvyagin supervised the study and reviewed the manuscript. The adjunct supervisor Dr Majid Ebrahimi Warkiani participated in the conceptualization, design, and supervision of the study, provided advisory as the specialist in microfluidics, and reviewed the manuscript. Alina Kapitannikova participated in the development of ICC protocol and microscopy of the DTCs. Steven Vasilescu participated in the development of the protocol for isolating DTCs with the microfluidic chip. Dr Denis

Butnaru and Dr Evgeniy Shpot provided advisory as the specialist in urology and reviewed the manuscript. Dr Dmitry Enikeev organized collection of the clinical samples and reviewed the manuscript.

5.3. Abstract

Early diagnosis and prognostics of prostate cancer [1] has been a significant issue in oncology. In clinical practice, prostate specific antigen (PSA) blood test and tissue biopsy have been the conventional diagnostic tools. However, these tools have significant disadvantages, such as compromised specificity in case of PSA blood test and serious side effects in case of tissue biopsy. In this setting, liquid biopsy by means of isolation and analysis of tumour cells from biological fluids, mostly blood, has been widely investigated as an alternative to the existing conventional diagnostic techniques. However, in case of localized form of PCa, isolation of PCa tumour cells from blood is barely effective due to a relatively low number of the tumour cells per mL. Thus, development of the techniques for isolating PCa tumour cells from other biological fluid, instead of blood, is highly relevant. In the current study, we developed the technique of microfluidics-based label-free isolation of PCa tumour cells from semen. According to the results of the study, the technique demonstrated capacity to separate approximately 99% of sperm cells and isolate more than 80% of PCa tumour cells presenting in the semen. It was possible to isolate high amounts of PCa tumour cells from the patients' semen samples, varying from tens to hundreds of units per mL of the semen. Importantly, the tumour cells could have been isolated from the semen of all 15 patients involved. At the same time, abnormal cells could have been identified in semen samples from 2 of 15 healthy volunteers, at marginal amounts of 1.2 and 3 units per mL. Further, the amounts of isolated PCa tumour cells correlated with such standard prognostic parameter, used in PCa prognosis, as Gleason score. Overall, the proposed technique is highly promising for being used in diagnosis and prognosis of localized PCa.

5.4. Introduction

For the past decade, isolation of disseminated tumour cells (DTCs) via liquid biopsy of blood attracted significant attention as the method for diagnosis and prognostics of cancers, including prostate cancer (PCa) [1]. However, in case of localized form of PCa, the number of DTCs per mL of blood is low and their isolation with the existing techniques is barely effective. Therefore, isolation of PCa DTCs from blood is considered inefficient for early detection of the disease [2]. At the same time, in prostate cancer, DTCs which release from a primary tumour may enter not only blood stream but also prostatic ducts. The fact of possible DTC release into the prostatic ducts may hypothetically be supported by the evidence on presence of DTCs into urine, which was described In the previous study by Rzhevskiy et al. [3] and other studies [4, 5]. Also, the increase of PCa biomarkers in urine due to digital rectal examination, including the increase of prostate cancer antigen 3 [6], signifies that the primary origin of the biomarkers presenting In urine may be the prostatic fluid [7]. In its turn, semen, which contains high amount of prostatic fluid as the component of the seminal fluid, may be a logic alternative to blood and urine as a potential source of PCa DTCs.

In 1996, Gardiner et al. described presence of PCa DTCs in ejaculate specimens of PCa patients [8]. In this study, to identify PCa DTCs, the ejaculate specimens were centrifuged to form a pellet. Further, smears were made from the formed pellet, and microscopically examined in terms of presence of the tumour cells. With this technique, of patients who had prostatic carcinoma confirmed with histopathology of the transrectal ultrasonography – guided prostatic biopsies, 75% were considered positive for atypical prostatic cells in the semen. However, since semen contains tens of millions of sperm cells in 1 mL, such technique of finding small amount of PCa DTCs in ejaculate smears has low efficiency and is inconvenient. In other study, flow cytometry was proposed as the technique for detection of prostate cancer cells in semen [9]. However, this technique is in general known to compromise viability and to lose rare cells. Further, in contrast to the flow cytometry, inertial microfluidics provides isolation of DTCs in the intact form which allows a vast spectrum of possible investigations including single cell analysis, antigen profiling, genetic analysis, and cell cultivation with subsequent usage of the cultivated tumour cells at *in vivo* models.

To our belief, inertial size-based microfluidics is one of the most promising approaches for being adopted to isolating PCa DTCs from the semen [10]. The approach has already been successfully applied for isolating DTCs from blood [11] and urine [3]. However, it should be noted that semen normally contains high amount of sperm cells at hundreds of millions per mL. Further, sperm cells have an unusual non-round shape represented by a prolonged head and long tail [12]. Even though the size of a sperm cell head is at 5 µm in average, which is few times lower that the size of an average DTC and is therefore convenient for the size-based microfluidic separation, a very long tail at around 50 µm may significantly alter a fluid-flow movement of sperm cells. Further, semen has much higher viscosity in comparison with urine and blood [13], which overall may determine a principally different approach of the DTC isolation via inertial size-based microfluidics. At the same time, compared to blood and urine, semen is much more homogeneous in terms of cell types presenting in it. Such cellular homogeneity facilitates to the development of an appropriate technique for the microfluidic DTC enrichment.

In this chapter, the development of microfluidic technique for isolating DTCs from semen of PCa patients was described. To our knowledge, this is the first report on isolating PCa DTCs from semen by means of inertial microfluidics. It is anticipated that this technique may supplement existing techniques, which are used in PCa diagnosis such as PSA blood test and tissue biopsy. Further, the technique may be efficient in diagnosis and prognostics of localized form of PCa. The antigen profiling of the isolated DTCs via immunocytochemical antibody labelling may facilitate to stratifying the disease by its aggressiveness, and therefore may be used for monitoring of the disease during active surveillance and watchful waiting, which are two the most common approaches in management of localized PCa. It is also anticipated that the technique may be applicable for isolating DTCs originating from adenocarcinoma and testicular cancer, as well as for purifying semen from epithelial cells and large debris with the purpose of its further preservation.

5.5. Materials and Methods

5.5.1. Microfluidic device and its fabrication

Due to a very high concentration of sperm cells in semen, the principle of cell separation was first of all based on isolating as much of sperm cells as possible and secondly on the enrichment of the highest possible amount of DTCs. Thus, Isolation of DTCs from semen was performed with the microfluidic chip previously developed by Warkiani et al. [11, 14-16], which was initially designated for isolating DTCs from blood. Such choice was also determined by a high separation threshold of the chip at 13 μm, which is convenient for the case of DTC enrichment from the biological fluid containing high amount of one type of small cells (sperm cells in case of semen) with the average size significantly lower than 13 μm.

The microfluidic chip was composed of two polydimethylsiloxane (PDMS) parts. Both parts were crafted via the micromolding process. One part was flat, while other part contained spiral microfluidic channel. The part containing the microchannel had 1.5 mm holes, at the sites designated for one inlet and two outlets, pierced with a Uni-Core™ Puncher (Sigma-Aldrich Co. LLC. SG). Surfaces of the two PDMS parts were attached after pretreatment of the surfaces into the oxygen plasma machine (Harrick Plasma, USA).

5.5.2. Cell culture

DU145, PC3, and LNCaP cell lines are considered standard PCa cell lines used in therapeutic research and were kindly gifted by Dr. Olga Burova (Laboratory for Experimental Diagnostics and Biotherapy of Cancer, National Medical Research Center of Oncology, Moscow). Cells were cultured under standard conditions in a humidified incubator (Panasonic, South Korea) at 37 °C, in 5% CO2. Du-145 and PC-3 cell lines were cultured in RPMI-1640 medium (Thermofisher Scientific, USA) with Glutamax supplement (Gibco, USA), 10% heat-inactivated, 0.22 μm filtered fetal bovine serum (Life Technologies, Inc), and 1% Penicillin-Streptomycin (Life Technologies, Inc). For LNCaP cell line, an RPMI-1640 medium (Thermofisher Scientific, USA) supplemented with 10% heat-inactivated, 0.22 μm filtered fetal bovine serum (Life Technologies, Inc), 1% insulin-transferrin (Paneco, Russia), 1% HEPES pH 7.2-7.5 (Paneco, Russia), 1% nonessential amino acid solution (Gibco, USA), 1% gentamycin (Paneco, Russia). T-25 and T-75 filtered flasks (Corning, USA) were used for subculturing of the cell lines. Cell dissociation was performed using 0.25% (w/v) Trypsin-

0.53 mM EDTA solution (Thermofisher Scientific, USA). A subcultivation ratio was 1 to 6. Cell culture medium was renewed every 2 to 3 days. Before any further management, cell lines were STR profiled for authenticity.

5.5.3. Spiking experiments

The efficiency of the microfluidic chip for isolating PCa DTCs from semen was evaluated by spiking a predetermined amount of DU-145 (or LNCaP, or C3) cells at approximately 1000 units were added into a standard portion (2-3 mL) of healthy male's semen collected into a sterile plastic container. Further, the healthy semen sample spiked with DU-145 (or LNCaP, or C3) cells was incubated for 30 minutes in a humidified incubator (Panasonic, South Korea) at 37 °C for liquefaction of the sample. After incubation, the sample was transferred to a 15 mL tube and centrifuged at 1000 rpm. Then, the supernatant was replaced with 5 mL of DPBS, and the solution was carefully resuspended. As the next step, the cells were fixed with 4% PFA (ThermoFisher Scientific, USA) solution in DPBS for 10 minutes. After fixation, the solution was centrifuged at 1000 rpm and the supernatant was replaced with 5 mL of DPBS. Then, the solution was processed through the microfluidic chip using a syringe pump (Baoding Shenchen Precision Pump Co., Ltd, Baoding, China) at the flow rate of 1.7 mL/min. As the result of first processing, the solution was divided into a targeted and waste fractions, where targeted fraction contained most of sperm cells while waste fraction contained most of DU-145 (or LNCaP, or C3) cells. For better purification of the targeted fraction from sperm cells, targeted fraction was also processed through the chip. After second processing, targeted fraction was centrifuged onto four adhesive glass slides (ThermoFisher Scientific, Australia) using a Thermo Scientific™ Cytospin™ 4 Cytocentrifuge (ThermoFisher Scientific, Australia) and air-dried. Further, the following antibodies were applied onto the glass slides: monoclonal anti-CK (Abcam, Cambridge, UK) primary antibody and anti-mouse IgG H&L Alexa Fluor 488 (Abcam, Cambridge, UK) secondary antibody. After incubations with the primary and secondary antibodies, the cells were mounted with a ProLong Gold Anti-Fade Mountant (Abcam, Cambridge, UK) containing DAPI, covered with a coverslip and counted under the fluorescence microscope (Zeiss Axio Imager Z2 Upright Microscope). Also, the amount of DU-145 (or PC3, or LNCaP)

cells, same to the amount added to the semen sample, was deposited onto the glass slide, and labelled with the anti-CK+AlexaFluor488 antibodies, then mounted with a ProLong Gold Anti-Fade Mountant (Abcam, Cambridge, UK) containing DAPI, covered with a coverslip and counted under the fluorescence microscope (Zeiss Axio Imager Z2 Upright Microscope). The experiments were repeated 5 times and the average recovery rate, expressed in percentage±SD, was identified. Also, a set of the experiments, technically similar to the experiments described above but where targeted fraction was collected after the first processing through the chip, was conducted.

5.5.4. Clinical samples

A part of this study that includes collection and processing of the semen samples from patients and healthy volunteers, and also collection and analysis of relevant clinical data, was performed under the ethical approval provided by Sechenov University Local Ethics Committee (extraction from protocol № 17-19). The samples were collected at Sechenov University Clinical Hospital № 2, Moscow, Russia. All patients and healthy volunteers provided written informed consent for collection of the samples and provision of their clinical data. The samples were collected and analyzed in a non-blinded manner, 15 patients with diagnosed PCa and 15 healthy volunteers provided semen samples. Each participant provided a portion of semen at the amount varied from 0,5 mL to 3 mL, each semen sample was collected into a sterile container. After semen collection, to avoid temperature drops, the sterile containers were transported to laboratory in a thermal container for further processing. At the laboratory, each semen sample was incubated for 30 minutes in a humidified incubator (Panasonic, South Korea) at 37 °C for liquefaction of the semen. Then, the supernatant was replaced with 5 mL of DPBS, and the solution was carefully resuspended. Further, the cells were fixed with 4% PFA solution in DPBS for 10 minutes. After fixation, the solution was centrifuged at 1000 rpm and the supernatant was replaced with 5 mL of DPBS. The solution was then processed through the microfluidic chip using a syringe pump (Baoding Shenchen Precision Pump Co., Ltd, Baoding, China) at the flow rate of 1.7 mL/min. Finally, the cells were deposited with Cytospin™ 4 Cytocentrifuge onto four adhesive glass slides.

5.5.5. Immunofluorescence staining

The method of sequential labelling was applied to the cells deposited onto the glass slides for more specific and effective labelling of the isolated DTCs. Thus, three primary and three secondary antibodies were applied. The primary antibodies were: rabbit polyclonal recombinant anti-PSMA antibody (Abcam, Cambridge, UK), mouse monoclonal antibody anti-CK (Abcam, Cambridge, UK) and rabbit polyclonal recombinant anti-Glypican 1 antibody (Abcam, Cambridge, UK). The secondary antibodies were: goat anti-rabbit IgG H&L Alexa Fluor 568 (Abcam, Cambridge, UK), goat anti-mouse IgG H&L Alexa Fluor 488 (Abcam, Cambridge, UK), goat anti-rabbit IgG H&L Alexa Fluor 647 (Abcam, Cambridge, UK). All incubations were performed in a humidified chamber. The cells onto the glass slides were permeabilized with 0.2% PBS solution of Tween-20 (ThermoFisher Scientific, USA) detergent for 10 minutes at room temperature. After permeabilization, the glass slides were washed with DPBS 3 times for 5 minutes and air-dried. Further, glass slides were incubated with 3% solution of BSA in PBST (0, 05% solution of Tween-20 in PBS) for 1 hour at room temperature for minimization of unspecific binding of the antibodies. As the next step, the BSA+PBST solution was gently washed from the glass slide, and the solution of first primary antibody in PBST with 1% BSA was added onto the glass slides and incubated overnight at 4 °C. After, the glass slides were gently washed with DPBS 3 times for 5 minutes, air-dried and incubated with the 3% BSA PBST solution of secondary antibody for 1 hour. After, the glass slides were washed with DPBS 3 times for 5 minutes and air-dried. The incubations with other two primary and secondary antibodies were performed as described above. Finally, cells were mounted with a ProLong Gold Anti-Fade Mountant (Abcam, Cambridge, UK) containing DAPI, covered with a coverslip and observed under the confocal microscope.

5.5.6. Cell enumeration and data analysis

The glass slides were observed under scanning laser microscope Zeiss LSM 880, with the support of Zen Black software (ver 3.1). To identify the gold standard for the identification and enumeration of the isolated DTCs, approximately 1000 of PC3 (, or DU-145) cells were added to a healthy semen sample, processed through the chip and immunostained as described above. The immunostaned cells were then captured under the laser scanning microscope Zeiss LSM800 (ZeissAG, Germany) using Zen Black software, and the intensity of fluorescence signal from the cells was measured using the Fiji ImageJ software. Further, a mean (±SD) value of the signal intensity was measured and the value equal to mean-SD was indicated as a threshold. Therefore, the putative tumour cells were considered and counted as DTCs in case if the signal intensity was above the thresholds in all three channels (PSMA+, CK+, GPC-1+). In addition, to evaluate potential diagnostic and prognostic value of the technique, a correlation between the amounts of CTCs enriched from patients' blood, and PSA serum levels and Gleason score were estimated. The correlations were calculated using Microsoft Excel 2016 software.

5.6. Results

5.6.1. Spiral microfluidic device

The composed microfluidic device contained a microfluidic channel with the trapezoidal cross-section, one inlet, one bifurcation at its edge and therefore two outlets – one outlet for each of the two branches of the bifurcation (Figure1). Thus, cell suspension in DPBS, containing sperm cells and PCa tumour cells, was passed through the chip at the flow rate of 1.7 mL per minute by using the syringe pump. Accordingly, after processing through the chip, the cell suspension was divided into two liquid fractions: waste fraction containing most of the sperm cells, and targeted fraction containing most of the PCa tumour cells.

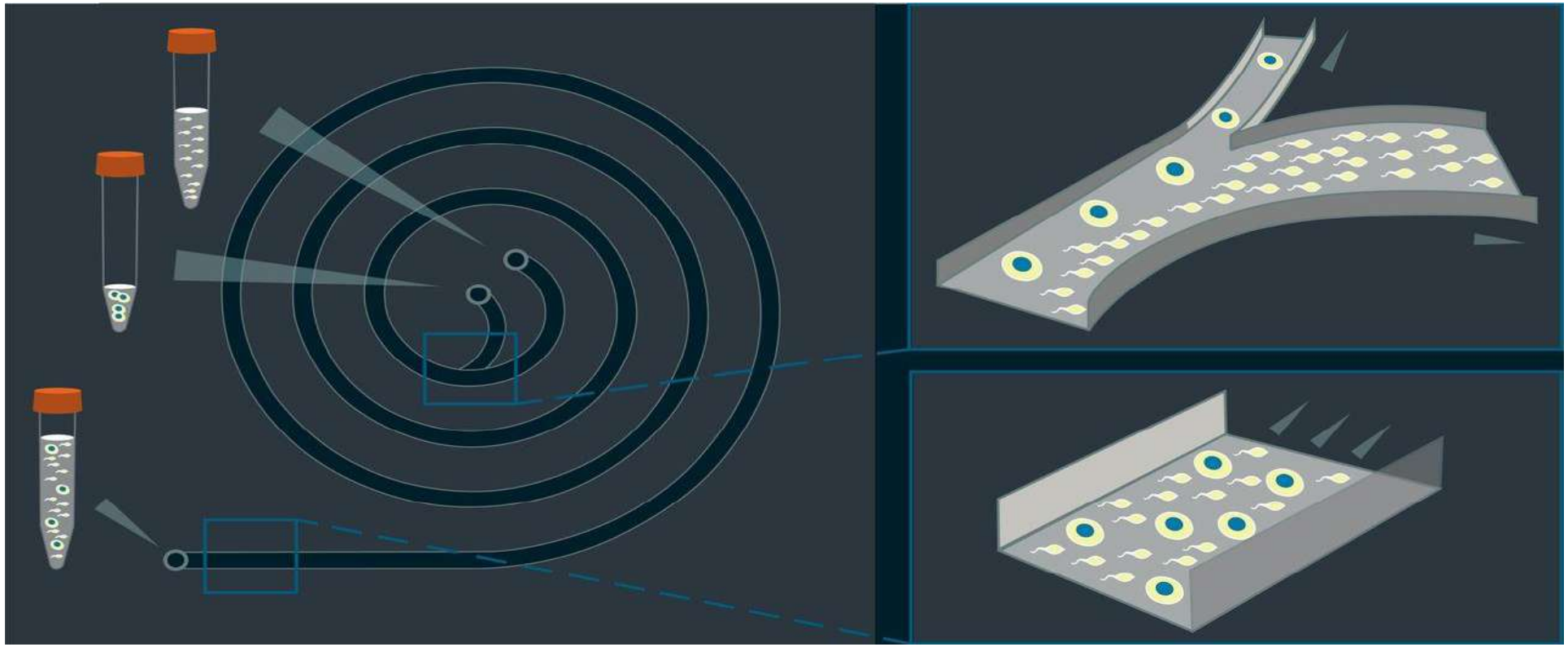


Figure1. Schematic representation of the spiral microfluidic channel for isolating DTCs from semen. First, a preliminary prepared semen sample is provided into the microchannel through the inlet under a continuous fluid flow at 1.7 mL/min. The preliminary prepared semen sample is represented by the suspension of the cells presenting in the semen, mainly sperm cells and DTCs, and DPBS as the liquid part. Thus, along the pass of the cell suspension through the spiral microchannel, being influenced by the inherent Dean vortex flows, larger DTCs tend to move to the inner wall of the microchannel while smaller sperm cells move to the outer wall of the microchannel. At the bifurcation site, sperm cells enter the outer

branch of the microchannel and therefore release through the outer outlet, while DTCs enter the inner branch of the microchannel and therefore release through the inner outlet.

5.6.2. Recovery rates of the PCa cell lines

According to the results of the experiment where the microfluidic chip was tested via processing the healthy semen sample spiked with cultural tumour cells (DU-145, or C3, or LNCaP), a potentially high efficiency of the chip in terms of isolating DTCs from the samples of PCa patients was identified. Thus, after the first processing, 85 (±8) %, 82 (±9) % and 83 (±6%) of DU-145, C3 and LNCaP cells, respectively, were collected from the initial amount of the cells spiked (Figure2). Therefore, all cell lines demonstrated a similar recovery rate at the percentage close to 80%. Also, more than 99% of sperm cells were isolated after the first processing. The additional processing of targeted fraction resulted in loss of 15 (±3) %, 12 (±6) % and 17 (±4) % of DU-145, C3 and LNCaP cells, respectively, presenting in the targeted fraction after the first processing. Further, second processing resulted in isolation of 13 (±5) % of the sperm cells presenting in the targeted fraction after the first processing. Thus, second processing resulted in loss of cancer cells and isolation of sperm cells at similar percentage around 15%. Therefore, additional purification of the targeted fraction from sperm cells via the second processing was considered unreasonable. Consequently, patients' samples were processed through the chip only once.

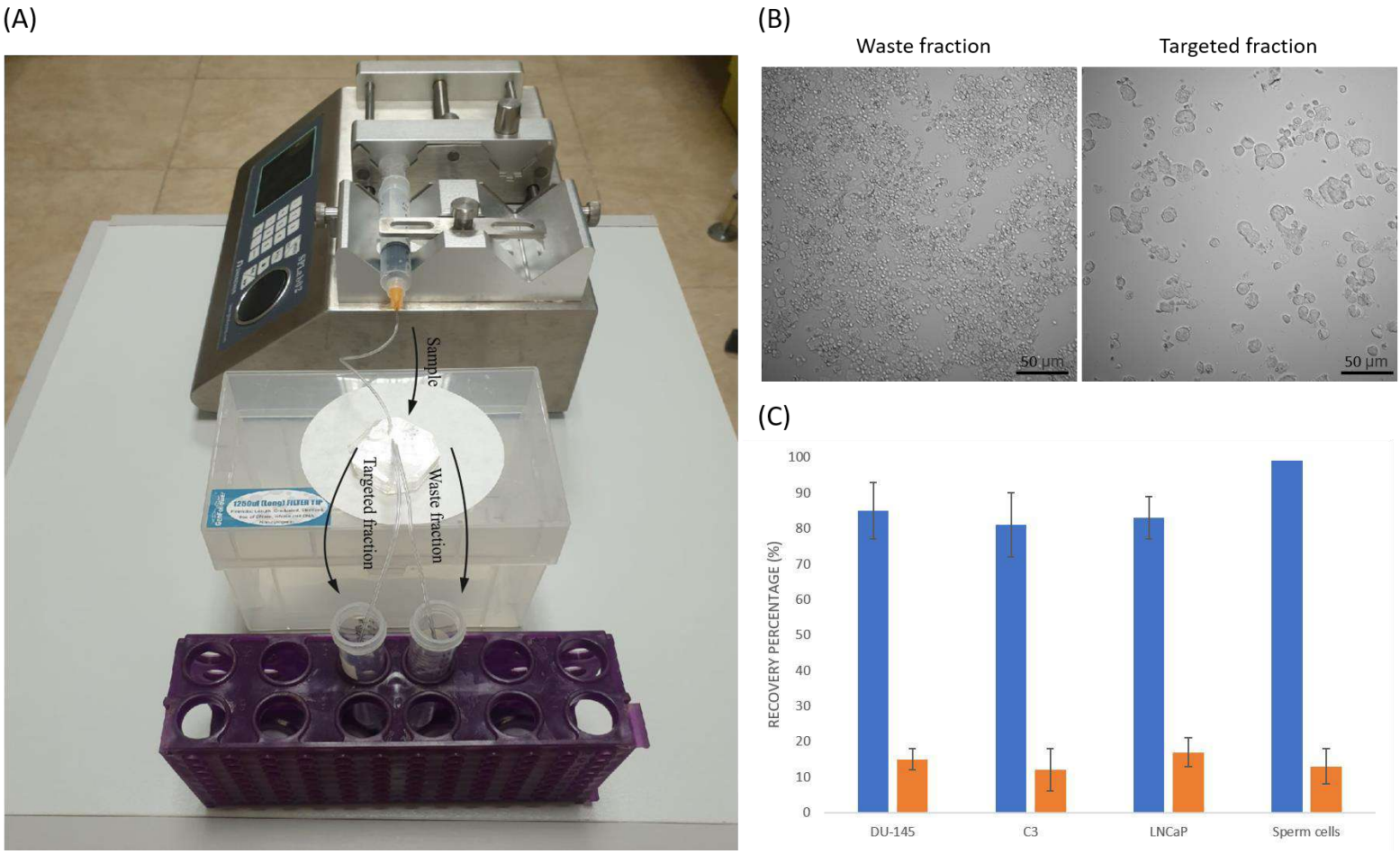


Figure2. Recovery rates of PCa cells from three different cell lines (DU-145, C3 and LNCaP) and sperm cells, as a result of spiking experiments where cell lines were added into a healthy sperm sample which was further processed through the chip. After processing, the sample was divided into two liquid fractions: targeted fraction containing most of the PCa cells and waste fraction containing most of the sperm cells (A). On the brightfield images obtained from each of the fractions it can be obviously seen that waste fraction is mainly represented by a very small cells – the sperm cells, while targeted fraction is represented by the large cells – PCa tumour cells. The blue bars demonstrate a percentage of the enriched PCa cells and separated sperm cells, achieved after processing of the sample, calculated from the total amount of the cells presenting in the spiked semen sample. After the first processing, targeted fraction was also processed through the chip. The orange bars demonstrate a percentage of the lost PCa cells and additionally separated sperm cells calculated from the total amount of the cells presenting in the targeted fraction obtained after the first processing (C).

5.6.3. Enrichment and counts of putative DTCs from semen

Semen samples from 15 patients with diagnosed PCa at its localized stage, and 15 healthy males at the age from 18 to 30 years, were collected. The isolated putative tumour cells were considered DTCs if they were >13 μm in size, had cell cytoplasm area at more than 232 μm^2 (mean at 284 μm^2 – SD at 52 μm^2, measured from DU-145 and PC3 cells) and area of the cell nucleus at more than 92 μm^2 (mean at 115 μm^2 – SD at 23 μm^2, measured from DU-145 and PC3 cells), and were positive for three antigens: CK (CK+), PSMA (PSMA+) and GPC-1 (GPC-1+). CK+ were considered the cells exhibiting the fluorescence signal at >1572 arbitrary units according to the mean (1771)-SD (199), PSMA+ were considered the cells exhibiting the fluorescence signal >1589 arbitrary units according to the mean (1867)-SD (278), and GPC-1+ were considered the cells exhibiting the fluorescence signal > 1150 arbitrary units according to the mean (1423)-SD (273), measured from DU-145 and PC-3 cells with Fiji ImageJ software. Thus, DTCs were found in semen samples collected from 15 out of 15 patients (100%), at the amount varied from 66.9 to 306.7 units per mL (Table1), with the median value at 103.5 and the mean value at 135.0 units. At the same time, in case of healthy donors, the cells expressing CK, PSMA and GPC-1 were identified in 2 of 15 samples (13,3%), with the number of identified cells at 1.2 and 3 units per mL. Also, a significant heterogeneity of DTCs, in terms of the intensity of expression of different antigens, was observed (Figure2).

Table1. Number of isolated DTCs and PCa characteristics in patients

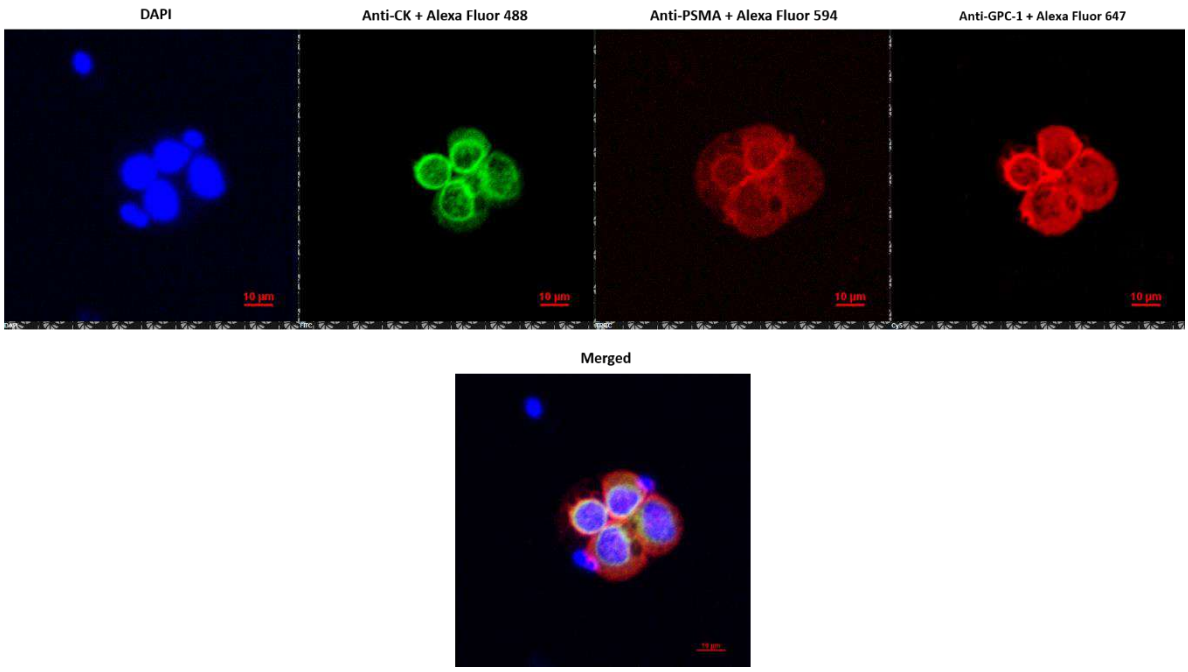
Patient number	n*	V**	n/V	TNM stage	Gleason score	PSA level
1	217	2.1	103.3	T1cN0M0	7	10.8
2	321	1.6	200.6	T2cN0M0	9	23
3	238	2.3	103.5	T2cN0M0	7	8.5
4	289	3.5	82.6	T2cN0M0	6	6.3
5	460	1.5	306.7	T1bN0M0	8	19
6	183	1.9	96.3	T1cN0M0	7	6.5
7	174	2.6	66.9	T2bN0M0	7	16
8	357	3	119.0	T2bN0M0	8	32.4

9	187	2.7	69.3	T1cN0M0	7	4.1
10	63	0.5	126	T1cN0M0	7	8.9
11	145	1.9	76.3	T1cN0M0	6	2.3
12	329	1.4	235	T1cN0M0	7	14.7
13	115	1.6	71.9	T1cN0M0	7	8.2
14	613	2.7	227	T2cN0M0	10	10.2
15	340	2.4	141.7	T2cN0M0	7	9.2

* - total amount of PSMA+,CK+,GPC-1+ cells in the patient's sperm sample

** - total volume of the patient's sperm sample

(A)



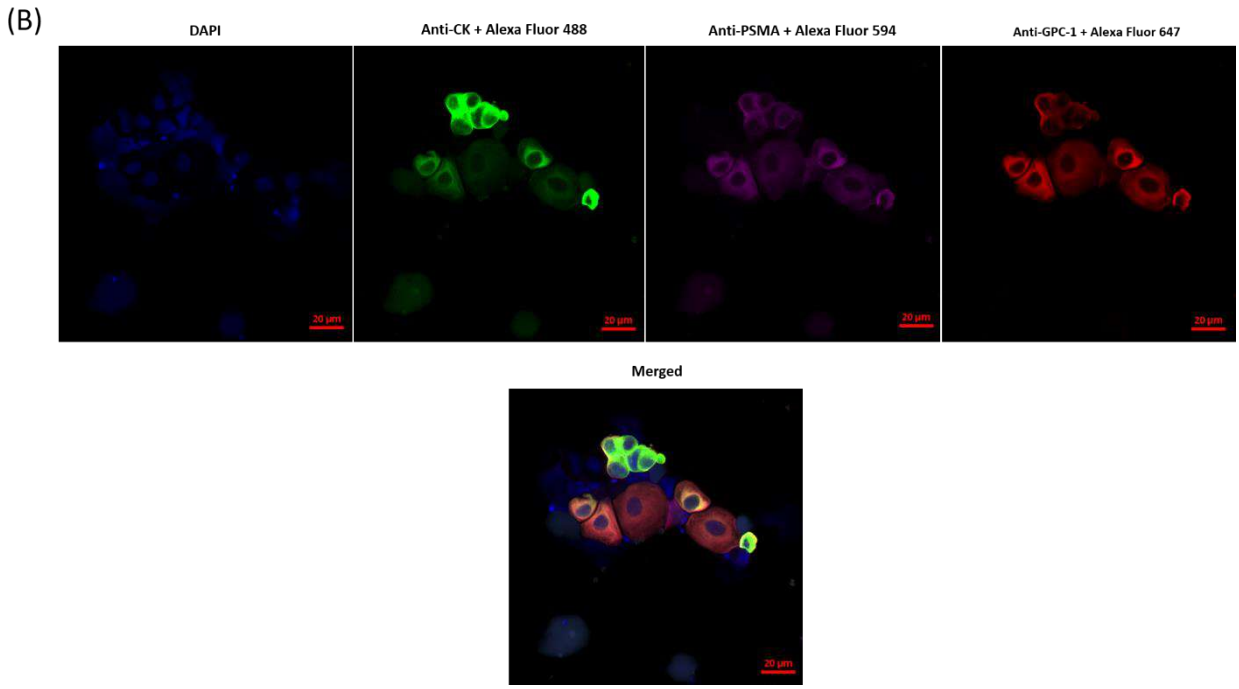


Figure2. A diversity of putative tumour cells isolated from the patients' semen samples. The putative tumour cells positive for all three antigens (PSMA, CK and GPC-1) were considered DTCs (A). At the same time, a high number of the putative tumour cells significantly positive for one or two of the three antigens was also observed in the patients' samples (B).

5.6.4. Analysis of potential clinical applicability of the method

To evaluate potential diagnostic and prognostic applicability of the developed method, correlations between the absolute amount of DTCs (n), or the amount of DTCs per mL of the semen (n/V), and conventional diagnostic/prognostic parameters such as PSA serum level (PSA) and Gleason score (GS), were identified. Thus, r values for n/V vs PSA and n/V vs GS were at 0.40 and 0.63, respectively. Further, r values for n vs PSA and n/V vs GS were at 0.42 and 0.73, respectively. Thus, weak positive correlation was determined between PSA serum level and total number of isolated DTCs, or the number of DTCs per mL of semen, with insignificant difference between the values. At the same time, moderate positive correlation was determined between GS and total number of isolated DTCs, or the number of DTCs per mL of semen, with no significant difference between the values (Figure3). Further, a weak positive correlation was identified between the amount of isolated DTCs and volume of a semen sample, with r value at 0.33.

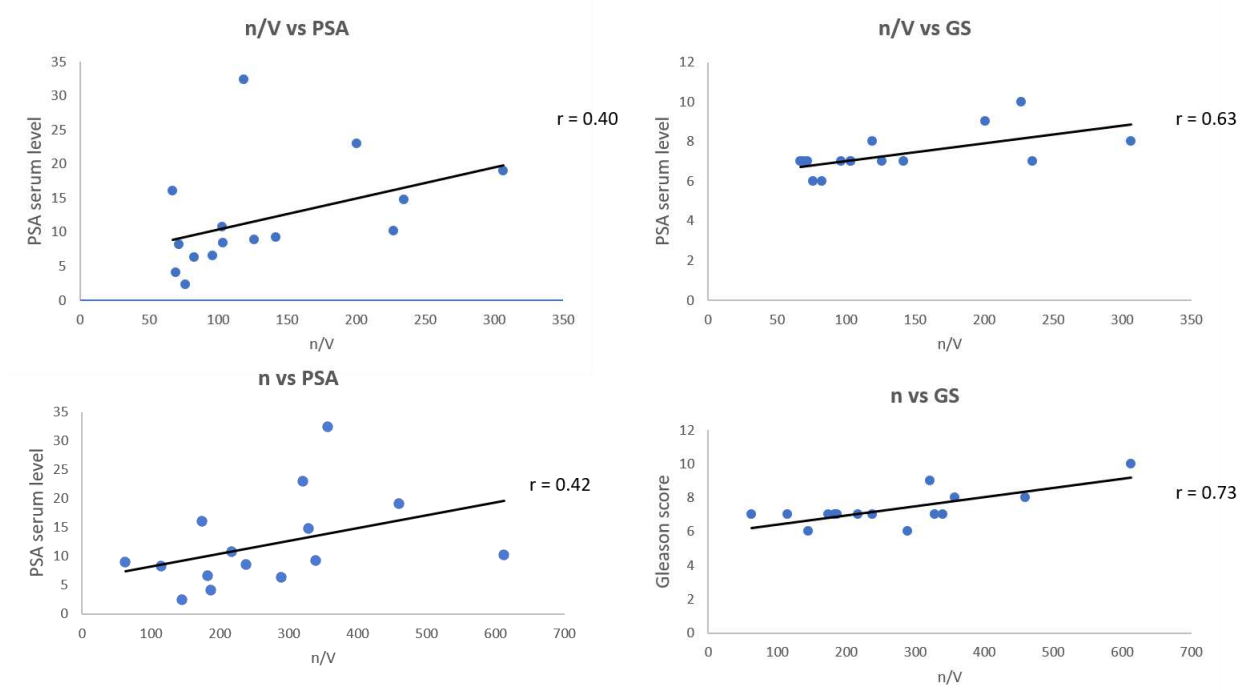


Figure3. Correlations between n/V vs PSA and n/V vs GS; n vs PSA and n vs GS. Thus, a relatively weak correlation was identified for n/V vs PSA with r value at r=0.40, and for n vs PSA with r value at r=0.42. At the same time, moderate correlation was identified for n/V vs GS with r value at r=0.63, and for n vs GS with r value at r=0.73.

5.7. Discussion

During the past decades, in clinical practice, PSA blood test has been the most trusted tool for early detection of PCa and its prognostics [17]. Another tool, conventionally used in diagnosing PCa, has been a tissue biopsy. However, both diagnostic tools have significant disadvantages. Thus, PSA blood test has a relatively low specificity, while tissue biopsy has only moderate sensitivity if performed without being accompanied by radiological techniques and may have serious complications [18]. Recently, in PCa management, liquid biopsy by means of microfluidics-based isolation and analysis of DTCs presenting in biological fluids was proposed as the perspective alternative to the existing conventional diagnostic and prognostic techniques [19]. However, according to the published data, liquid biopsy of neither blood [20, 21] nor urine [3] demonstrated an

outstanding performance in detecting PCa at its early stage. Therefore, investigation of other biological fluids as the potential sources of DTCs, particularly – semen, is of a great interest.

In this study, we tested the technique of microfluidic label-free isolation of PCa DTCs from semen of patients diagnosed with PCa at its early localized form. According to the obtained results, the technique demonstrated a high efficiency in separating sperm cells and isolating PCa DTCs. Thus, in the pilot experiments with healthy semen samples spiked with PCa cell lines, it was possible to separate approximately 99% of sperm cells and isolate more than 80% of PCa tumour cells. To ensure high specificity of the technique, immunocytochemistry panel containing multiple antigens, such as anti-CK, anti-PSMA and anti-GPC-1, was applied for detecting the isolated DTCs. All 15 of 15 patients with localized PCa (100%) had DTCs (CK+, PSMA+, GPC-1+ cells) in their semen, at the amounts varying from 66.9 to 306.7 units per mL and from 63 to 613 units per sample. At the same time, among the semen samples collected from healthy volunteers, 2 of 15 had putative PCa tumour cells (CK+, PSMA+, GPC-1+ cells) identified at the amounts of 1.2 and 3 units per mL. Thus, even if threshold for considering a PCa patient positive for DTCs is set at 3 units per mL, 100% (15/15) of patients would anyway be positive for DTCs. Therefore, the developed technique demonstrated an outstanding specificity and sensitivity in detecting PCa DTCs.

Another significant result of the study was the presence of correlations between the amounts of isolated DTCs and PSA serum level or GS. It is worth noting that, while the correlations between the absolute amount of DTCs or the amount of DTCs per mL of the semen and PSA serum level were weak, moderate positive correlations between the amounts of DTCs and GS could have been identified. This aspect may potentially have prognostic value in PCa risk stratification and treatment. Also, the proposed technique may potentially be applied for tracking the status of antigen expression by DTCs, which may be valuable for decision making during such approaches of localized PCa treatment as watchful waiting and active surveillance. At the same time, weak positive correlation was identified between the total amount of isolated DTCs and semen sample volume, which may be explained by either a small cohort of the PCa patients or the fact that most of the DTCs presenting in seminal ducts release with even a small amount of semen.

Overall, the conducted technique demonstrated great potential for being applied as the diagnostic and prognostic tool in PCa management. To better reveal such potential, a

study involving high number of the PCa patients and healthy volunteers is required, with the longitudinal investigation based on the analysis of multiple semen samples at different time points. Also, comparison of diagnostic and prognostic values between diverse antigen panels is of a high interest.

5.8. Conclusion

In this study, diagnostic and prognostic potential of the microfluidics-based label-free isolation of DTCs from semen of patients with localized prostate cancer was evaluated. In the pilot experiments, where healthy semen samples spiked with PCa cell lines were used, the proposed technique demonstrated high efficiency in isolating DTCs and separating sperm cells. Further, in the experiments with semen samples collected from patients with localized PCa, it was possible to isolate DTCs at the amounts varied from tens to hundreds of units per mL of the semen. Further, moderate positive correlations were identified between the amount of isolated cells and GS. These characterize the proposed technique as promising for being used in early diagnosis and prognosis of localized PCa.

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Chapter VI

Liquid biopsy via label-free microfluidic isolation of tumour cells from biological fluids in localized prostate cancer. What's better: blood, urine or semen?

Alexey S. Rzhevskiy [1,2,3,*], Alina Y. Kapitannikova [2], Denis V. Butnaru [3], Evgeniy V. Shpot [3], Majid Ebrahimi Warkiani [4], Andrei V. Zvyagin[1,2]

[1] ARC Centre of Excellence for Nanoscale BioPhotonics, MQ Photonics, Macquarie University, 2109 Sydney, Australia

[2] Institute of Molecular Medicine, Sechenov First Moscow State Medical University, 119991 Moscow, Russia

[3] Institute for Urology and Reproductive Health, Sechenov University, 119991 Moscow, Russia

[4] School of Biomedical Engineering, University of Technology Sydney, 2007 Sydney, Australia

[5] Department of Clinical Medicine, Faculty of Medicine and Health Sciences, Macquarie University, 2109 Sydney, Australia

* Correspondence: **alexey.rzhevskiy@hdr.mq.edu.au**

Keywords: prostate cancer, liquid biopsy, circulating tumour cells, disseminated tumour cells, microfluidics

6.1. Introduction to the chapter

According to the results presented in Chapter V, the developed technique of DTC isolation from semen demonstrated high efficiency in isolating PSMA+, CK+, GPC-1+ putative tumour cells from the semen of patients with localized PCa. Thus, all patients' samples contained PSMA+, CK+, GPC-1+ putative tumour cells at a very high amount of more than 60 of the cells per mL of the semen. At the same time, almost a total absence of such cell in the samples of healthy volunteers was observed. The indirect comparison of the techniques for DTC isolation from urine, blood and semen described in Chapters III, V and VI, respectively, identifies the technique of DTC isolation from semen as the most sensitive and specific, and allowing to enrich the highest number of DTCs for further analysis. However, to identify semen as the most preferable biological fluid for liquid biopsy of PCa with the aim of early diagnosis and prognostics of the disease, a direct comparison between all three biological fluids is required. Therefore, the current chapter represents the study on direct comparison of the liquid biopsy techniques on DTC isolation from semen, urine and blood, described in previous chapters.

6.2. Author's contribution

The PhD candidate (Alexey Rzhevskiy) conceptualized the study, designed and conducted the experiments, including isolation of DTCs from the biological fluids, ICC, and microscopy of the isolated DTCs. Also, he performed collection of the relevant data and interpretation of the results.

Candidate's principal supervisor Dr Andrei Zvyagin supervised the study and reviewed the manuscript. The adjunct supervisor Dr Majid Ebrahimi Warkiani participated in the conceptualization, design, and supervision of the study, provided advisory as the specialist in microfluidics, and reviewed the manuscript. Alina Kapitannikova participated in the development of ICC protocol and microscopy of the DTCs. Dr Denis Butnaru provided advisory as the specialist in urology and reviewed the manuscript. Dr Evgeniy Shpot organized collection of the clinical samples and reviewed the manuscript.

6.3. Abstract

In liquid biopsy, among the human body fluids, blood has been most widely investigated as the source of disseminated tumour cells (DTCs). However, in case of localized form of prostate cancer (PCa), neither of the existing liquid biopsy techniques demonstrated high efficiency in isolating DTCs from blood. In PCa, the biological fluids containing most of the DTCs are hypothetically blood, urine and semen. In the current manuscript, we report on the comparative study where it was attempted to isolate DTCs by means of microfluidic size-based approach from all three biological fluids – blood, urine and semen. According to the results of the study, in case of blood, it was possible to isolate only rare DTCs (CK+, PSMA+, CD45- cells) from low number (3/13) of patients. In case of urine, DTCs (CK+, PSMA+, GPC-1+ cells) could have been isolated from most of the patients (10/13). At the same time, in case of semen, DTCs (CK+, PSMA+, GPC-1+ cells) were isolated from all patients (13/13). Further, the amount of isolated DTCs per sample was significantly higher in comparison with both blood and urine. Also, a strong positive correlation was identified between the amount of DTCs isolated from semen and such conventional clinico-pathological parameter of PCa as PSA serum level. Thus, in accordance with the obtained results, it was concluded than the applied technique of microfluidic size-based isolation of DTCs from semen is perspective for early detection and prognostics of localized PCa.

6.4. Introduction

For the first time, disseminated tumour cells (DTCs) were described in the study by the Australian physician Thomas Ashworth in 1869, where cells morphologically similar to those of the primary tumour were identified in the blood of metastatic cancer patient [1]. However, liquid biopsy via isolation and analysis of DTCs has been most widely investigated only in the past decade. This has been first of all associated with a rapid development of microelectromechanical systems allowing to isolate DTCs. Until recent years, blood has been mostly investigated as the source of DTCs. However, the trend has started gradually shifting to the investigation of other biological fluids as the potential sources of DTCs. According to

the published data, In case of localized prostate cancer (PCa) [2], liquid biopsy of blood was considered only moderately efficient due to in average low amount of DTCs in 1 mL of the biological fluid [3]. Therefore, urine has been considered the most relevant alternative to blood as the source of DTCs, and promising data was demonstrated in several studies [4-6]. At the same time, a limited data has been published so far on using semen as a source of DTCs in liquid biopsy [7], while it is thought that PCa tumour cells may release to all three biological fluids: blood, urine and semen (Figure1).

According to the results of our previous study, where the microfluidic chip and the corresponding technique for isolating PCa DTCs from urine were developed, DTCs (GPC-1+ cells) were isolated from the urine of 12 out of 14 patients (86%) [6]. The number of such cells varied from 4 to 194 units between patients, with the median count of the DTCs at 22 units. In case of the control group, urine samples of 11 of 14 (79%) healthy volunteers were negative in terms of GPC-1+ cells, with the number of cells in positive cases al less than 8 units. Thus, a preliminary sensitivity and specificity of the developed method was identified at around 80%. At the same time, as it has been recently demonstrated by our research group, a preliminary sensitivity and specificity of the microfluidics-based isolation of PCa DTCs from the semen was identified at as much as 100%. However, to identify the most efficient technique for microfluidic isolation of DTCs, the study on direct comparison of the techniques is required.

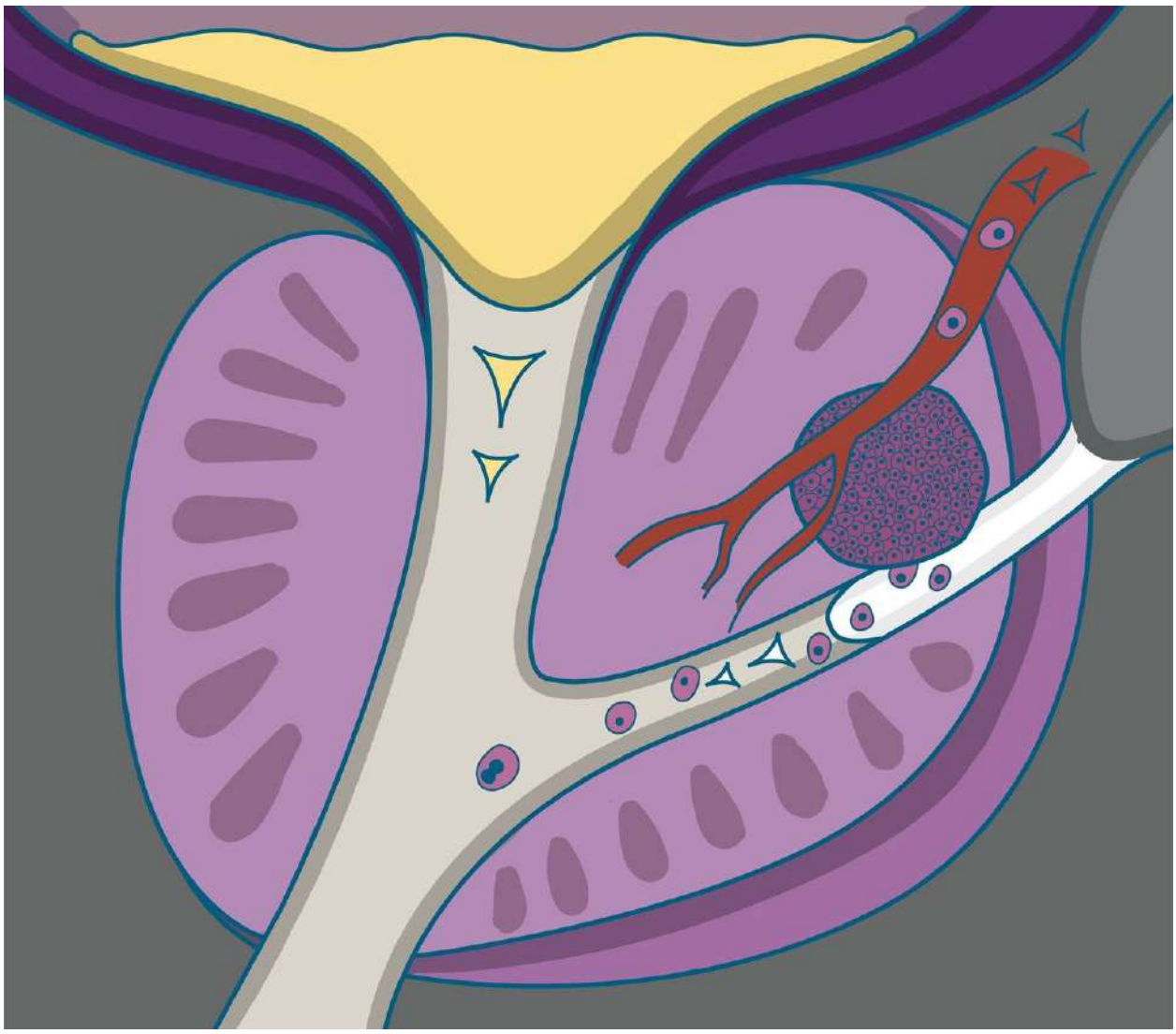


Figure1. Possible directions of releasing for PCa DTCs from the primary tumour. PCa tumour is most commonly formed on a peripheral side of the prostate gland and therefore does not usually interact with urethra at its early stages of the development [8]. Thus, the most frequent directions for releasing of PCa DTCs are blood vessels and prostatic ducts or seminal ducts. From seminal ducts, DTCs may further proceed to urethra via the anatomical junction and therefore present in urine samples of PCa patients.

Accordingly, the aim of the current study was to perform a direct comparison between urine, blood, and semen, as the sources of DTCs. Thus, in the described study, all three biological fluids were collected from all patients and subsequently analyzed. For isolating DTCs from the biological fluids, we used the same microfluidic technology, previously successfully adapted for isolating DTCs from blood [9-13] and urine [6] Also, a

similar immunohistochemical method was used for the identification if DTCs isolated from all the biological fluids.

6.5. Materials and Methods

6.5.1. Microfluidic device

For isolating tumour cells from urine, the microfluidic chip described by Rzhevskiy et al. was used [6]. In case of blood and semen, the microfluidic chip which was previously developed by Warkiani et al. and successfully applied for isolating DTCs from blood of patients with diverse types of cancers including breast [14], head and neck [10], and lung cancer [11], was applied. In general, the microfluidic chip was composed of polydimethylsiloxane and contained the spiral microfluidic channel with a bifurcation at its edge, one inlet and two outlets. Thus, if describing the process of DTC isolation, a biological fluid, preliminary prepared as described in the “Clinical samples” section, was first provided into the spiral microchannel under a continuous fluid flow by using a syringe pump. Then, the fluid passed through the microchannel and divided into two fractions at the bifurcation site, with one fraction (targeted fraction) containing most of the tumour cells and other fraction (waste fraction) containing most of the healthy cells. Finally, each of the fractions released through one of two outlets, and targeted fraction was further analyzed.

6.5.2. Cell culture

Three PCa cell lines were used in the current study: DU-145, PC3 and LNCaP. The cell lines were cultured in a CO2 incubator (Panasonic, South Korea) at 37 °C and 5% CO2. For culturing of DU-145 and PC3 cell lines, the RPMI-1640 medium (Thermofisher Scientific, USA) was used. The medium was combined with Glutamax supplement (Gibco, USA), 10% heat-inactivated and 0.22 μm filtered fetal bovine serum (Life Technologies, Inc), and 1% Penicillin-Streptomycin (Life Technologies, Inc). The LNCaP cell line was cultured with the

RPMI-1640 medium (Thermofisher Scientific, USA) with an addition of 10% heat-inactivated, and 0.22 μm filtered fetal bovine serum (Life Technologies, Inc), 1% insulin-transferrin (Paneco, Russia), 1% HEPES pH 7.2-7.5 (Paneco, Russia), 1% gentamycin (Paneco, Russia) and 1% nonessential amino acid solution (Gibco, USA). A subculturing of the cell lines was performed by using T-25 and T-75 filtered flasks (Corning, USA). For cell dissociation, 0.25% (w/v) Trypsin- 0.53 mM EDTA solution (Thermofisher Scientific, USA) was used. The replacement of the medium was performed every 2-3 days. To prevent mycoplasma infection, before usage, the cell lines were evaluated by means of STR DNA profiling.

6.5.3. Spiking experiments

In case of urine, a predetermined number of cells from each of the cell lines (DU-145, or LNCaP, or C3) at approximately 1000 units was separately added into 20 mL of healthy urine sample. The sample was then preliminary prepared as described in the "Clinical samples" section, and processed through the chip with the syringe pump (Baoding Shenchen Precision Pump Co., Ltd, China) at the flow rate of 1.7 mL/min. The cells presenting in the targeted fraction, obtained after processing of the biological fluids, were fixed with 4% paraformaldehyde and sedimented onto adhesive glass slides (ThermoFisher Scientific, USA) using a Thermo Scientific™ Cytospin™ 4 Cytocentrifuge (ThermoFisher Scientific, USA). On the glass slides, after being air-dried, the deposited cells were labelled with the antibody panel containing the following antibodies: Anti-prostate specific membrane antigen (anti-PSMA) (Abcam, Cambridge, UK) + AlexaFluor568 (Abcam, Cambridge, UK), anti-Cytokeratin (anti-CK) (Abcam, Cambridge, UK) + AlexaFluor488 (Abcam, Cambridge, UK), anti-glypican-1 (anti-GPC-1) (Abcam, Cambridge, UK) + AlexaFluor647 (Abcam, Cambridge, UK). In case of semen, the predetermined number of tumour cells at approximately 1000 units from each of the cell lines was separately added into a 2-3 mL whole semen sample provided by a healthy male. The sample was then preliminary prepared as described in the "Clinical samples" section and processed through the microfluidic chip. Then, the obtained targeted fraction was further fixed, deposited onto the glass slides, and labelled with the antibody panel as described above for the case with urine. In case of blood, the predetermined number of tumour cells at approximately 1000

units from each of the cell lines was separately added into a 7.5 mL of healthy blood. The sample was then preliminary prepared as described in the “Clinical samples” section and processed through the microfluidic chip. Then, the cells were fixed and deposited onto the glass slides as described above. Then, the deposited cells were immunostained with the antibody panel containing the following antibodies: anti-PSMA + AlexaFluor568, anti-CK + AlexaFluor488 and anti-CD45 (Abcam, Cambridge, UK) + AlexaFluor647. After immunostaining, the glass slides were mounted with the ProLong Gold Anti-Fade Mountant (Abcam, Cambridge, UK), covered with a cover slip and observed under a confocal microscope. The tumour cells were counted, and the percentage of the isolated tumour cells from their initial amount was calculated for the cases with each of the cell lines and each of the biological fluids.

6.5.4. Clinical samples

A part of the study that includes collection and processing of the semen, blood, or urine samples collected from PCa patients, and also collection and analysis of relevant clinical data, was performed under the ethical approval provided by Sechenov University Local Ethics Committee (extraction from protocol № 17-19). The samples were collected at Sechenov University Clinical Hospital № 2, Moscow, Russia. All patients provided written informed consent for collection of the samples and provision of their clinical data. The samples were collected and analyzed in a non-blinded manner; all three biological fluids were collected from 13 patients with localized PCa. All samples were processed within 2 hours post-collection. In case of blood, 7.5 mL was collected in the VACUETTE K2 EDTA tube (Greiner Bio-One, Austria). Then, the blood was incubated for 10 minutes with RBC buffer (BioLegend, USA) at the volume ratio of blood to buffer at 1/3. After, the solution was centrifuged, and the supernatant was replaced with 10 mL of DPBS; further, for better purification, the cell suspension in DPBS was centrifuged again, and the supernatant was replaced with 5 mL of DPBS. Then, the cell suspension was processed through the microfluidic chip, deposited onto the glass slides, and labelled as described for blood in the “Spiking experiments” section.

In case of urine, approximately 20 mL were collected in a sterile contained prior to

the collection of semen. After, the sample was centrifuged and the supernatant was replaced with 10 mL of DPBS; after, the cell suspension was centrifuged again and replaced with 5 mL of DPBS. Further, the cell suspension was processed through the microfluidic chip, deposited onto the glass slides, and labelled as described for urine in the "Spiking experiments" section.

In case of semen, the whole sample was collected right after the collection of urine sample. Each sample was collected into a sterile container, then incubated in the CO2 incubator (Panasonic, South Korea) at 37 °C for 30 minutes. After, the sample was centrifuged, the supernatant was collected and replaced with 10 mL of DPBS; the obtained cell suspension was also centrifuged, and the supernatant was replaced with 5 mL of DPBS. Then, the cell suspension was processed through the microfluidic chip, deposited onto the glass slides, and labelled as described for semen in the "Spiking experiments" section.

All glass slides were then observed under the confocal microscope, and the abnormal cells were captured using Zen Black software (ver. 3.1).

6.5.5. Immunofluorescence staining

To provide a high-quality ICC labelling of the isolated DTCs, a method of sequential labelling was applied. Two antibody panels were applied: one panel – for urine and semen, and another panel – for blood. The antibody panels contained the types of antibodies as described previously in the "Spiking experiments" section. A humidified chamber was used for performing all incubations. Thus, after the isolated cells were deposited onto the glass slides, cell permeabilization was performed by using 0.2% PBS solution of Tween-20 (ThermoFisher Scientific, USA) for 10 minutes. After the permeabilization, the glass slides were washed three times for five minutes and then air-dried. Further, the cells were incubated with a 3% bovine serum albumin solution (BSA) in PBST, for the duration of 1 hour. Then, the glass slides were washed as described above, and the incubation with the first primary antibody in PBST with 1% BSA was performed overnight at 4 °C. After, the glass slides were washed as described above and incubated with an appropriate secondary antibody for 1 hour. Further, the glass slides were washed as described above. Incubations with other two primary and two secondary antibodies were performed as described above.

After all incubations, the cells were mounted with the ProLong Gold Anti-Fade Mountant, covered with the cover slip, and observed under the confocal microscope.

6.5.6. Cell enumeration and data analysis

The glass slides were observed under the laser scanning microscope Zeiss LSM 880 (ZeissAG, Germany) and the images of the DTCs were captured using Zen Black software (ver 3.1). To identify a gold standard for identification of DTCs isolated from the samples of biological fluids collected from the patients, the cultural cells (DU-145, PC3) immunostained in the spiking experiment were captured under the laser scanning microscope and analyzed with the FiJi ImageJ software. Thus, a mean (±SD) value of the signal intensity from diverse antibodies was measured (Anti-PSMA+AlexaFluor568, or anti-GPC-1+AlexaFluor647, or anti-CK+AlexaFluor488) and the value equal to mean-SD was indicated as the threshold for identifying the cells isolated from the patients' samples as DTCs. To identify a gold standard for signal intensity of CD45+AlexaFluor647, a healthy blood sample was processed through the chip and the targeted fraction was immunostained with CD45+AlexaFluor647. The value of the signal, measured from white blood cells (WBCs), equal to mean-SD was set as the threshold for differentiating DTCs from WBCs in blood. The presence of the cell nucleus (DAPI+) was also taken as an essential criterion. In addition, the size of the cell (>13 μm), the area of the nucleus and the area of the cytoplasm was also taken into account.

Hence, all three biological fluids were directly compared in terms of two antigens: CK and PSMA. In this comparative branch, DTCs were considered PSMA+ and CK+ for urine and semen, and PSMA+, CK+ and CD45- for blood. The other comparative branch included two biological fluids: urine and semen. In this comparative branch, DTCs were considered PSMA+, CK+, GPC-1+ for both urine and semen. Also, a potential prognostic applicability of three different liquid biopsy approaches utilizing different biological fluids – blood, urine, and semen, were evaluated by estimating correlations between the amounts of isolated DTCs and PSA serum level, or Gleason score.

For better presentation of the information described in subsections of the "Materials and Methods" section, a whole process from sample collection to data analysis obtained after processing of the collected samples through the microfluidic chip is depicted on Figure2.

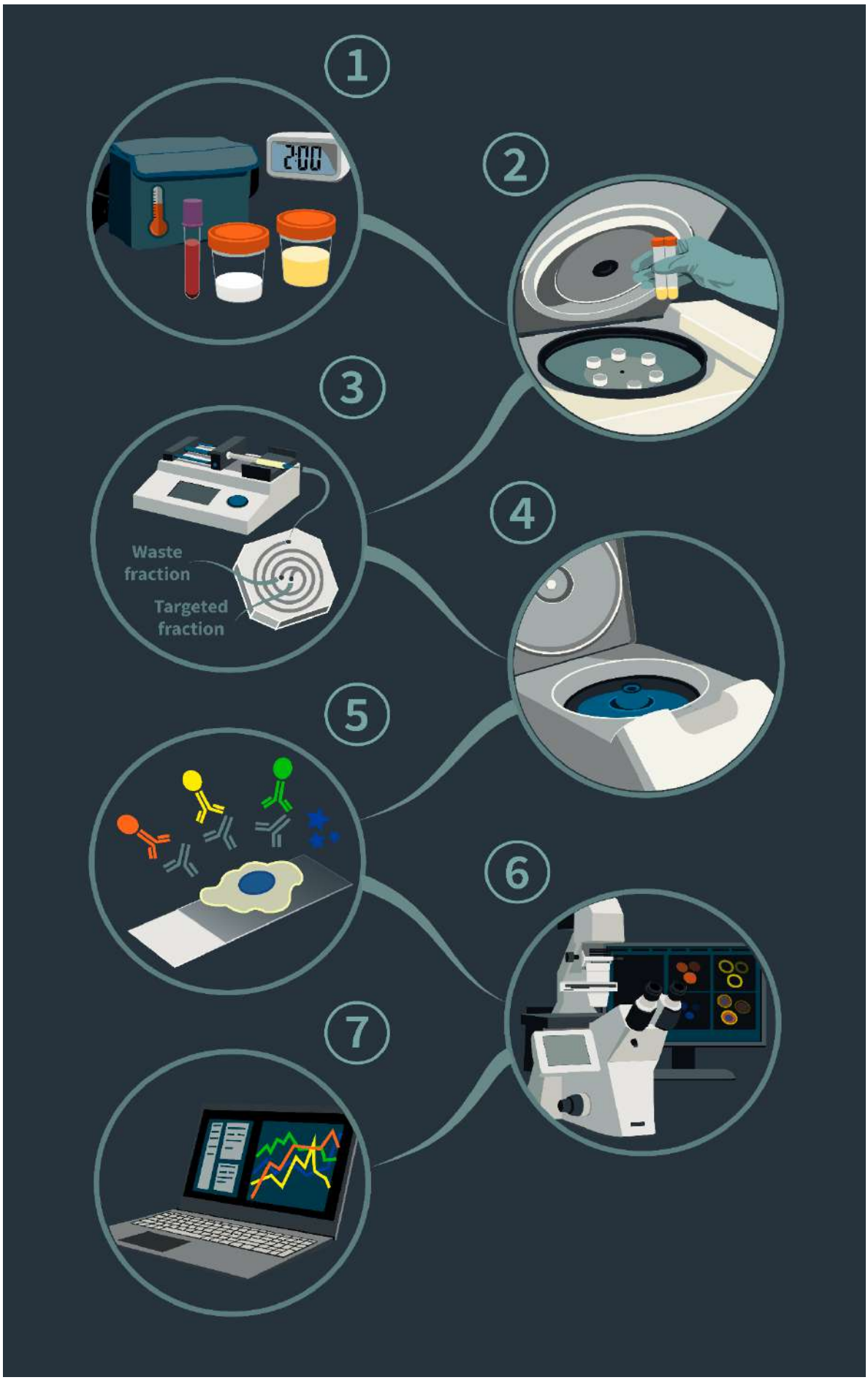

1
2
3
Waste fraction
Targeted fraction
4
5
6
7

Figure2. A general step-by-step schematic representation of the microfluidic label-free liquid biopsy technique by means of isolating DTCs with the spiral microfluidic chip, followed by ICC labelling of the isolated DTCs and their identification under a confocal microscope, and data analysis: 1) The blood/urine/semen sample is collected and transported to the laboratory within 2 hours to prevent possible cytolysis of DTCs presenting in the sample; 2) Special preparation of the sample. In case of blood – lysis of RBCs, centrifugation and replacement of supernatant with DPBS. In case of semen – incubation for 30 minutes at 37 ^{0}C, centrifugation and replacement of supernatant with DPBS. In case of urine – centrifugation and replacement of supernatant with DPBS; 3) Processing of the sample through the spiral microfluidic chip, with further division of the sample into the targeted fraction containing most of DTCs presenting in the sample and waste fraction containing most of the normal cells presenting in the sample; 4) Sedimentation of the cells presenting into the targeted fraction onto the glass slides via centrifugation; 5) ICC labelling of the cells deposited onto the glass slides with the antibodies specific to PCa antigens; 6) Visualization and capturing of ICC labelled cells under a confocal microscope; 7) Quantitative analysis of the collected images.

6.6. Results

6.6.1. Spiral microfluidic device

The pilot experiments, where healthy samples spiked with PCa cell lines were processed through the chip, demonstrated the following results in terms of the isolation efficiency: 1) In case of blood, it was possible to collect 82 (±7) % of DU-145 cells, 78 (±4) % of PC3 cells and 83 (±3) % of LNCaP cells ; 2) In case of urine, it was possible to collect 80 (±3) % of DU-145 cells, 85 (±6) % of PC3 cells and 85 (±5) % of LNCaP cells; 3) 2) In case of semen, it was possible to collect 84 (±4) % of DU-145 cells, 82 (±7) % of PC3 cells and 81 (±6) % of LNCaP cells.

6.6.2. Enrichment and counts of isolated putative DTCs

Urine, semen and blood were collected from 13 patients with localized PCa. In case of the comparison between all biological fluids – urine, blood and semen, DTCs were considered the cells >13 μm in size, with a high nucleus to cytoplasm size ratio or multiple nuclei, and positive for PSMA and CK. The additional requirement for putative DTCs isolated from blood was a negative status in terms of CD45 antigen. In case of the comparison between urine and semen, DTCs were considered the large cells (>13 μm), with a high nucleus to cytoplasm size ratio or multiple nuclei, and positive for three antigens: PSMA, CK and GPC-1.

In case of blood, CK+ cells exhibiting the fluorescence signal at >1495 arbitrary units according to the mean (1341)-SD (146), PSMA+ cells exhibiting the fluorescence signal >1618 arbitrary units according to the mean (1872)-SD (254), and CD-45- cells exhibiting the fluorescence signal at <679 arbitrary units according to the mean (735)-SD (56), measured from DU-145 and PC-3 cells with Fiji ImageJ software, were registered as DTCs. The inclusion criterion was also cell size at more than 13 μm, cell cytoplasm area at more than 239 μm^2 (mean 273 μm^2 - SD 32 μm^2) and the area of the cell nucleus at more than 88 μm^2 (mean 92 μm^2 – SD 4 μm^2).

In case of urine, CK+ cells exhibiting the fluorescence signal >1499 arbitrary units according to the mean (1810)-SD (311) arbitrary units, PSMA+ cells exhibiting the fluorescence signal >1552 arbitrary units according to the mean (1738)-SD (186) arbitrary units, and GPC-1+ cells exhibiting the fluorescence signal at > 1017 arbitrary units according to the mean (1374)-SD (257) arbitrary units, measured from DU-145 and PC-3 cells with ImageJ, were registered as DTCs. The inclusion criterion was also cell size at more than 13 μm, cell cytoplasm area at more than 229 μm^2 (mean 271 μm^2 - SD 42) and the area of the cell nucleus at more than 101 μm2 (mean 125 μm2 – SD 24).

In case of semen, CK+ cells exhibiting the fluorescence signal >1572 arbitrary units according to the mean (1771)-SD (199) arbitrary units, PSMA+ cells exhibiting the fluorescence signal >1589 arbitrary units according to the mean (1867)-SD (278) arbitrary units, and GPC-1+ cells exhibiting the fluorescence signal > 1150 arbitrary units according to the mean (1423)-SD (273) arbitrary units, measured from DU-145 and PC-3 cells with

ImageJ, were registered as DTCs. The inclusion criterion was also cell size at more than 12 μm, cell cytoplasm area at more than 232 μm^2 (mean 284 μm2 - SD 52 μm^2) and the area of the cell nucleus at more than 92 μm^2 (mean 115 μm^2 – SD 23 μm^2).

Thus, in the first comparative case (Table1), DTCs (PSMA+, CK+, GPC-1+) (Figure3) were found in the semen samples from 13 of 13 (100%) patients, and in the urine samples from 10 of 13 (76.9%) patients. For semen, the total amount of isolated DTCs varied from 17 to 560 units, with the median value at 168 units; for urine - from 0 to 85 units with the median value at 18 units. The amount of DTCs per mL varied from 47 to 280.8 units, with the median value at 109.6, for semen; for urine - from 0 to 4.3, units with a median at 0.9 units. In the second comparative case, DTCs (PSMA+, CK+; CD45-) were found in the semen samples from 13 of 13 (100%) patients, in the urine samples from 12 of 13 (92.3%) patients, and in the blood samples from 3 of 13 (23.1%) patients. For semen, the total amount of isolated DTCs varied from 49 to 810 units, with the median value at 304; for urine – from 0 to 107 units, with the median value at 25; for blood – from 0 to 6 units, with the median value at 0 units.

Table1. Amounts of DTCs isolated from blood, urine and semen of patients with non-metastatic PCa, and conventional clinico-pathological data for the patients.

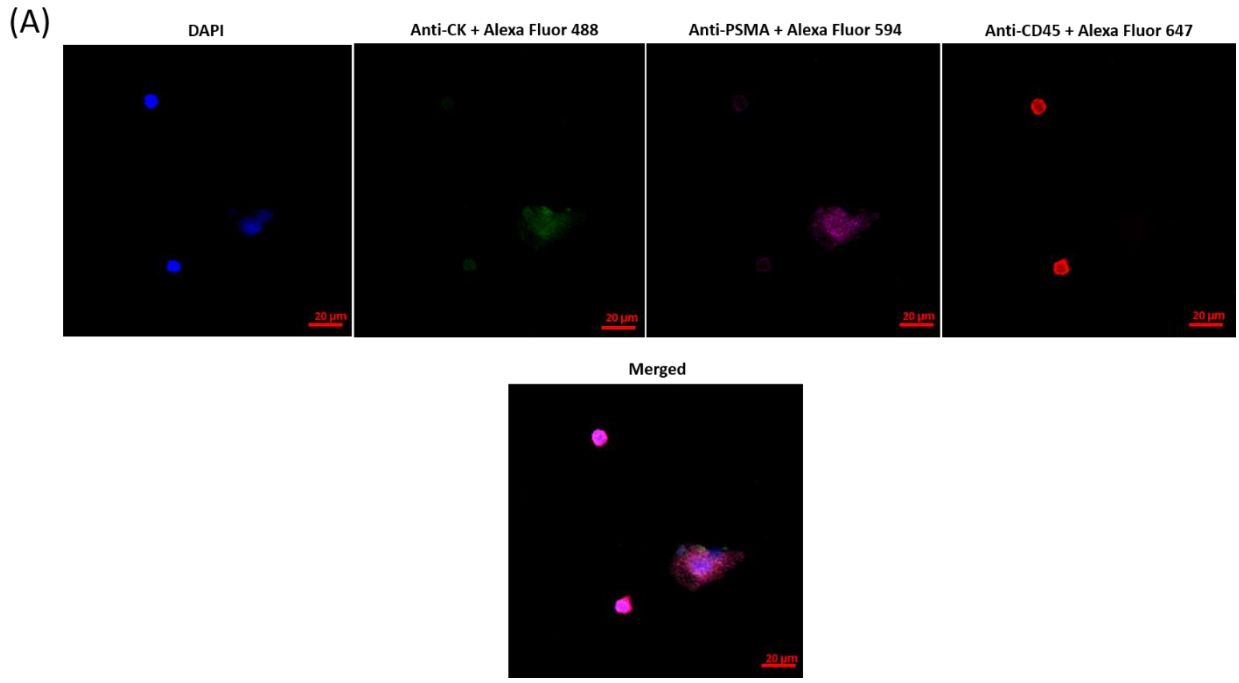
Patient number	n1* (CK+, PSMA+, GPC-1+ cells)				n2**** (CK+, PSMA+; CD45- cells)						TNM stage	GS	PSA
	urine		semen		blood		urine		semen				
	n**	n/V***	n	n/V	n	n/V	n	n/V	n	n/V			
1	8	0.4	199	150.9	0	0	15	0.75	351	266.2	T2aN0M0	7	10.5
2	9	0.5	401	133.3	0	0	19	0.95	564	187.5	T2cN0Mx	6	7.4
3	31	1.6	337	109.6	1	0.1	44	2.2	480	156.1	T1cN0M0	7	8.2
4	48	2.4	84	159	0	0	53	2.65	186	352.1	T1cN0M0	7	11
5	0	0.0	45	161.2	0	0	7	0.35	67	240.0	T1cN0M0	7	8.3
6	0	0.0	27	47	0	0	0	0	89	154.9	T1cN0M0	7	1.6
7	56	2.8	560	280.8	6	0.8	78	3.9	810	406.2	T2cN0Mx	8	159
8	23	1.2	128	50.1	0	0	31	1.55	315	123.3	T2bN0M0	8	5.1
9	0	0.0	17	47.4	0	0	1	0.05	49	136.6	T1cN0M0	6	2.4
10	85	4.3	399	181.7	2	0.3	107	5.35	572	260.5	T1cN0M0	7	40
11	14	0.7	176	67.8	0	0	17	0.85	258	99.4	T2aN0Mx	7	5.4
12	18	0.9	145	105.3	0	0	25	1.25	266	193.2	T3bN0M0	7	6.8
13	33	1.7	168	63	0	0	38	1.9	304	114.0	T3bN0M0	6	6.7

* - the amounts of DTCs as CK+, PSMA+, GPC-1+ cells

** - total amount of DTCs per liquid biopsy sample

*** - total amount of DTCs per mL of the liquid biopsy sample

**** - the amounts of DTCs as CK+ and PSMA+ cells (for urine and semen); and CD45- cells (for blood)



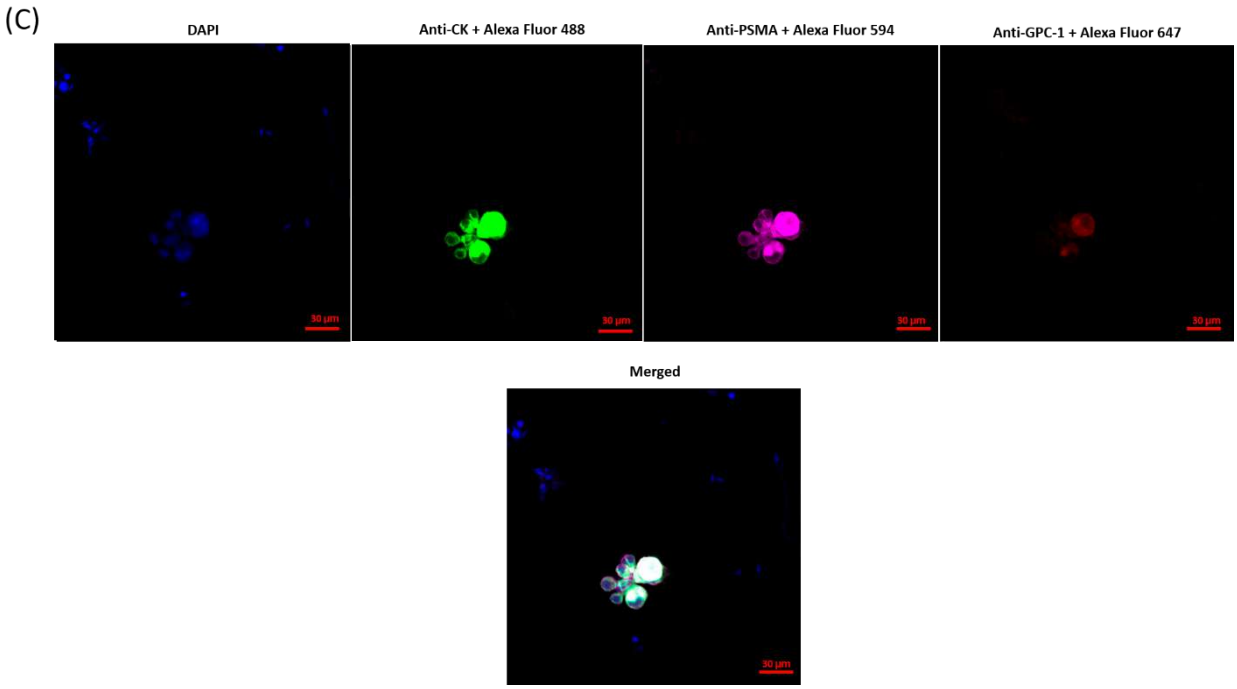


Figure3. DTCs isolated from diverse biological fluids – blood, urine and semen. In case of blood, the DTCs were the CK+, PSMA+ cells, with the lack of CD45 expression, differently from lymphocytes (CK-, PSMA-, CD45-) which also presented in the blood samples (A). In case of urine, the DTCs were the CK+, PSMA+, GPC-1+ cells, differently from diverse normal epithelial (CK-, PSMA-, GPC-1-) cells which also presented in the urine samples (B). In case of semen, the DTCs were the CK+, PSMA+, GPC-1+ cells, differently from sperm cells (CK-, PSMA-, GPC-1-) and normal epithelial which also presented in the semen samples (C).

6.6.3. Comparison of potential clinical applicability of the techniques

A potential clinical applicability of liquid biopsy techniques based on DTCs isolation from urine and semen was compared. For this purpose, correlations between the amounts of DTCs isolated from the biological fluids and PSA serum level, or GS, were identified (Figure4). The technique of blood liquid biopsy was excluded from the analysis due to a negligible amount of DTCs, which could have been isolated from the patients' blood samples.

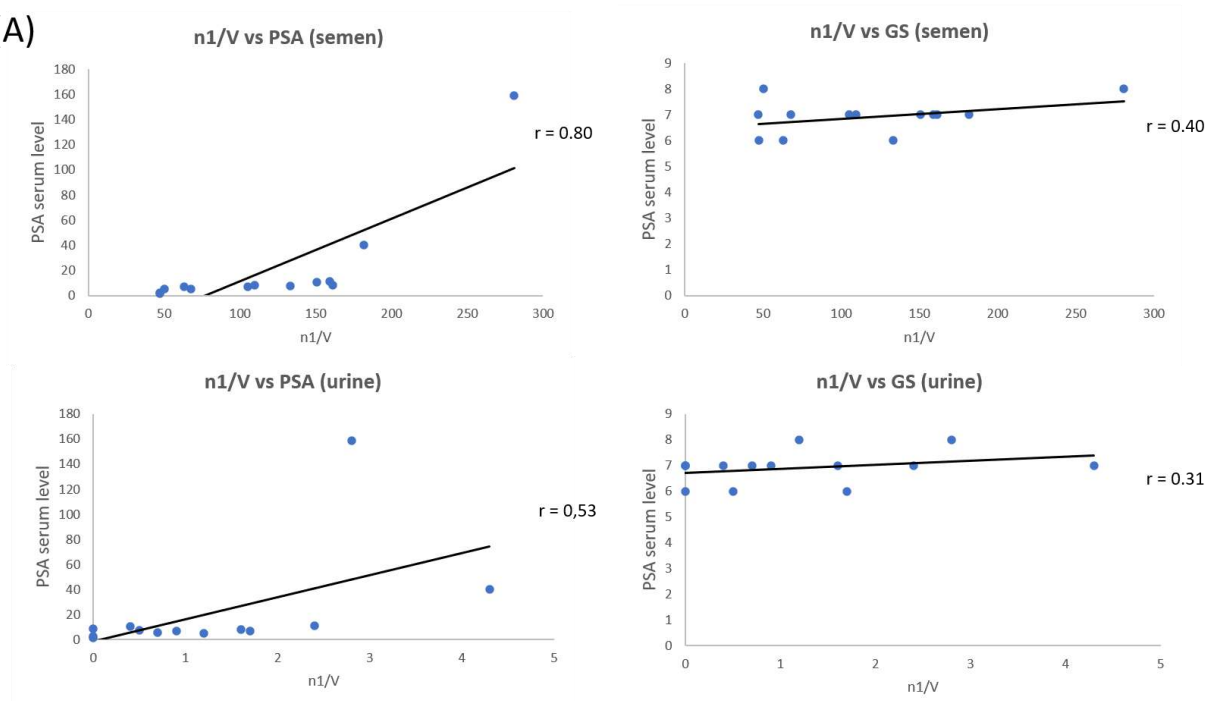
According to the obtained result, in the first comparative group where DTCs were considered CK+, PSMA+, GPC-1+ cells, strong positive correlation (r=0.80) was identified between PSA serum level and the amount of DTCs per mL of semen. Further, moderate

positive correlations were found between GS and the amount of DTCS per mL of semen (r=0.40), and between PSA serum level and the amount of DTCS per mL of urine (r=0.53). Weak positive correlation was identified between GS and the amount of DTCS per mL of urine (r=0.31).

In the second comparative group, where DTCs were considered CK+, PSMA+ cells, as well as in case the first comparative group, strong positive correlation (r=0.70) was identified between PSA serum level and the amount of DTCs per mL of semen. moderate positive correlations were found between GS and the amount of DTCS per mL of semen (r=0.41), and between PSA serum level and the amount of DTCS per mL of urine (r=0.60). Weak positive correlation was identified between GS and the amount of DTCS per mL of urine (r=0.35).

Nevertheless, it should be noted that such parameter as PSA was not distributed normally among the patients according to the Kolmagorov-Smirnov test of normality (*p = 0.0078).* It can be seen from Table1 that the most significant outlier was the PSA value at 159 which belongs to the patient №7. Thus, if the patient №7 is withdrawn from the statistical analysis, PSA would be distributed normally and the correlations would be as follows: r = 0.67 for n1/V vs PSA (semen), r = 0.85 for n1/V vs PSA (urine), r = 0.48 for n1/V vs PSA (semen), and r = 0,89 for n2/V vs PSA (urine). Thus, it can be seen that recalculated r values also represent either moderate or high correlations in all of the cases.

(A)



(B)

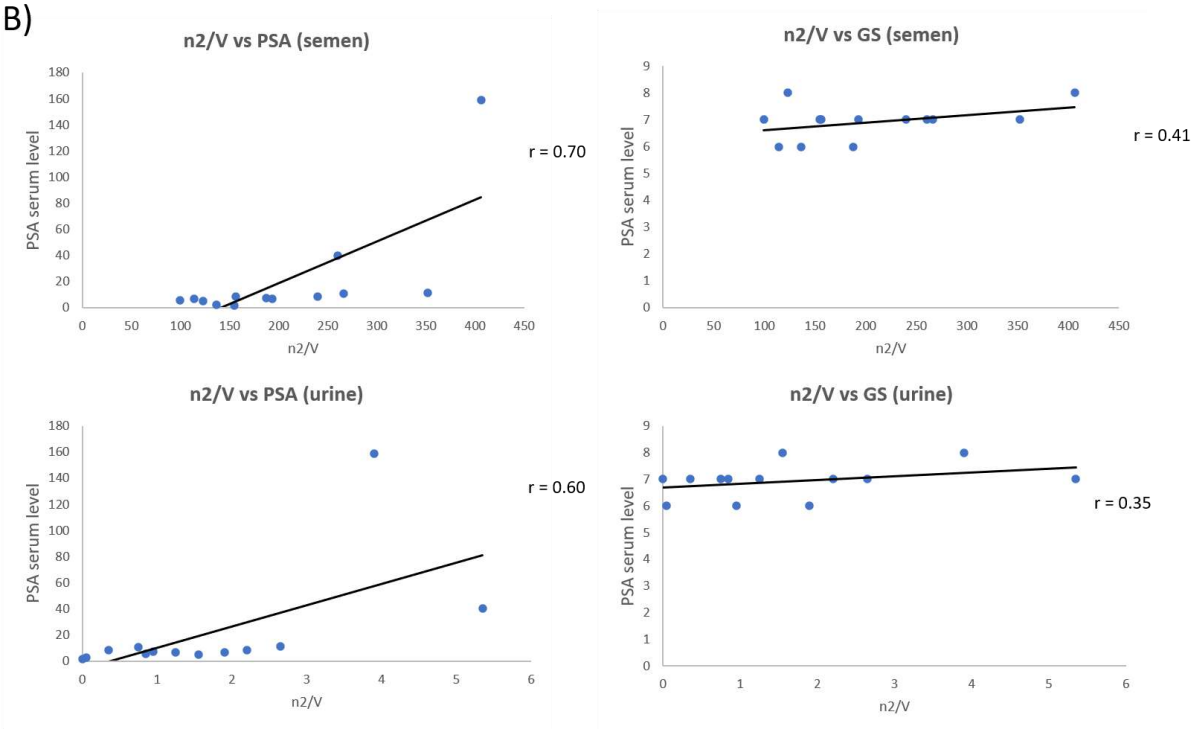


Figure4. Correlations between n1/V vs PSA (semen), and n1/V vs PSA (urine); n1/V vs GS (semen), and n1/V vs GS (urine); n2/V vs PSA (semen), and n2/V vs PSA (urine); n2/V vs GS (semen), and n2/V vs GS (urine). The strongest positive correlations were identified between n1/V vs PSA (semen) with r = 0.8, and n2/V vs PSA (semen) with r = 0.7. Moderate positive correlations were identified between n1/V vs PSA (urine) with r = 0.53, n1/V vs GS (semen) with r = 0.40, n2/V vs PSA (urine) with r = 0.60, and n2/V vs GS (semen) with r = 0.41. Weak positive correlations were identified between n1/V vs GS (semen) r = 0.31, and n2/V vs GS (semen) r = 0.35.

6.7. Discussion

Previously, label-free isolation of DTCs from blood of non-metastatic PCa patients was described in the studies where different liquid biopsy techniques were applied. According to the data, published by different research group, sensitivity of the techniques for isolating DTCs from blood of non-metastatic PCa patients was commonly below 50% and the amount of isolated DTCs was usually marginal [15-18]. In the current study, for isolating PCa DTCs from blood, it was attempted to apply the microfluidic label-free technique which was previously developed [13] and successfully tested for isolating DTCs from blood of patients with diverse types of cancer. However, in this study, the technique did not

demonstrate high output in terms of the amount of isolated DTCs (CK+, PSMA+, CD45- cells) from 7.5 mL blood samples collected from non-metastatic PCa patients.

The second biological fluid, investigated for presence of PCa DTCs, was urine. In the current study, the microfluidic technique for isolating PCa DTCs from urine, previously described by our research group in the study by Rzhevskiy et al. [6], was applied. According to the results of that study, PCa DTCs, which were considered the GPC-1+ cells, could have been isolated from high volume urine samples (30-100 mL) of 12 (86%) of 14 localized PCa patients at the total amount varying from 11 to 194 units per sample. However, despite the positive results, the study had several limitations. First, considering the throughput of the microfluidic technology at 1.7 ml/min, processing of the high-volume samples significantly decreased the attractiveness of practical implementation of the technique as the liquid biopsy technique due to the prolonged processing time. Second, the usage of only a single antibody for ICC identification of the isolated putative DTCs could has compromised specificity of the technique. Thus, in the current comparative study, a reasonable amount of the patients' urine at 20 mL was used for the analysis and the isolated putative DTCs were labelled with the antibody panel containing three primary antibodies specific to PCa – anti-CK, anti-PSMA and anti-GPC-1. According to the results, DTCs (CK+, PSMA+, GPC-1+ cells) could have been isolated from 10 (76.9%) of 13 patients at the total amount varying from 0 to 85 units per sample, or from 0 to 4.3 units per mL. Also, a moderate correlation was identified between PSA serum level and the amount of DTCs per mL. Also, for the case when DTCs were considered the CK+, PSMA+ cells, no significant difference in the results was indicated. Thus, the technique for isolating DTCs from urine demonstrated much higher capacity in isolating DTCs compared to blood. Also, the results obtained in the current study have in general corresponded to the results, which were previously presented in the study by Rzhevskiy et al. [6].

The third biological fluid, investigated as a potential source of PCa DTCs, was semen. According to the obtained results, efficiency of isolating DTCs from the semen had significantly overcome the efficiency identified in the cases with urine and blood. Thus, in case of semen, DTCs could have been isolated from the samples of 13 (100%) of 13 patients. The total amount of isolated DTCs (CK+, PSMA+, GPC-1+ cells) varied from 17 to 560 units, with the amount of DTCs per mL varying from 47 to 280.8 units. Further, strong positive

correlation was identified between PSA serum level and the amount of isolated DTCs per mL.

Overall, to our knowledge, this is the first in kind comparative study where three different biological fluids – blood, urine and semen were compared in terms of presence of PCa DTCs. As it was demonstrated, microfluidic size-based liquid biopsy of semen demonstrated an outstanding potential for being used as the diagnostic and prognostic technique in managing localized PCa. Thus, for better evaluation of the potential practical applicability of the technique, further study with the involvement of statistically significant number of patients and healthy controls is required.

6.8. Conclusions

In this study, a comparison between three biological fluids – blood, urine and semen collected from patients with non-metastatic PCa, was performed in terms of the efficiency of DTC isolation by means of microfluidic label-free liquid biopsy. According to the results of the study, marginal number of DTCs (CK+, PSMA+, CD45- cells) could has been isolated from the blood samples of only few of the involved patients, which in general corresponds to the results published by other authors. In case of urine, the results were more promising compared to blood, with total amount of isolated DTCs (CK+, PSMA+, GPC-1+ cells) varying from 0 to 85 units per sample and the amount of DTCs per mL varying from 0 to 4.3 units. At the same time, only 10 of 13 patients had DTCs in their urine samples. Finally, the most promising results were obtained for semen. Thus, the total amount of DTCs (CK+, PSMA+, GPC-1+ cells) isolated from semen varied from 17 to 560 units, with the amount of DTCs per mL varying from 47 to 280.8 units. Notably, DTCs could have been isolated from semen samples of all 13 of 13 patients.

6.9. References

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Chapter VII

General discussion and conclusions

During the last decade, microfluidic size-based label-free liquid biopsy by means of DTC isolation from biological fluids has attracted significant attention as the technique for diagnosis and prognostics of malignant tumours. In its turn, early diagnosis of PCa requires a multistage complex approach and therefore novel diagnostic tools, which would facilitate to more specific and sensitive detection of localized PCa. In this thesis, the development of new approaches in the microfluidic size-based isolation of DTCs from urine and semen of patients with localized PCa, was described. Also, an efficiency of the technique previously developed for isolating DTCs from blood, was investigated.

In Chapter III, the development of a chip for label-free isolation of DTCs from large volumes of urine relying on principles of a so-called inertial microfluidics, and a corresponding method including immunocytochemical identification of the isolated DTCs by using the anti-GPC-1 primary antibody MIL-38 (Minomic International Ltd, Australia), were described. As a result, the technique demonstrated a potential capacity for isolating 85 (±6) % DTCs presenting in urine sample. Also, putative DTCs (GPC-1+ cells) were found at a significant amount of >8 units in 11 out of 14 patients (79 %), while among healthy volunteers only 3 of 11 urine samples contained GPC-1+ cells at the amount of 8 $\leqslant$. Thus, potential specificity and sensitivity of the developed technique was close to 80%. Also, moderate positive correlation ($r = 0.48$) between the amount of DTCs isolated from patients' urine samples per mL and GS score was identified. Hence, the developed technique was considered promising for being a supplement to the existing standard diagnostic tests used in diagnosis of localized PCa such as GS score and PSA serum level. Also, the conducted study has proven a high specificity of anti-GPC-1 antibody, particularly the MIL-38 antibody provided by Minomic International Ltd, for immunocytochemical identification of PCa tumour cells. Therefore, relying on the presented data and data recently published by different research groups [1, 2], anti-GPC-1 antibodies may be recommended for being included in ICC antibody panels specific for PCa. Future development of the described

technique requires a prospective study involving a preliminary estimated statistically significant number of patients and healthy volunteers, with longitudinal follow up for the patients. Also, addition of more antibodies specific to PCa for ICC identification of putative DTCs, including anti-PSMA and anti-CK, would potentially increase specificity of the technique. Such study would provide an opportunity to characterize prognostic potential of the technique more precisely, and to better evaluate and improve performance of the technique. However, limitations of the technique should be also considered. Thus, even though the processing speed at 1.7 mL/min is relatively fast, processing of large urine volumes through the developed chip with a peristaltic pump as depicted on Figure2 (Chapter III) may take more than one hour. Hence, urine volume should be adjusted to the optimal value which would allow to collect a number of DTCs sufficient for reliable analysis, for a short time. Nevertheless, due to a complex and multistage character of PCa management, it should be noticed that the field of diagnostic and prognostic techniques for PCa management first of all demands alternatives rather than supplementary techniques. Therefore, the techniques based on usage of other biological fluids should also be considered.

In Chapter IV, potential of the previously developed liquid biopsy technique for early diagnosis of PCa, based on microfluidic size-based label-free isolation of DTCs from blood, was evaluated. In previous studies, performed by the candidate's Adjunct Supervisor Dr Majid Warkiani in collaboration with different research groups, the technique demonstrated high capacity for isolating DTCs from blood of patients diagnosed with head and neck, breast, or lung cancer. However, in the current study, only a limited amount of putative DTCs could have been isolated from a standard blood portion at 7.5 mL from only a small number of patients diagnosed with localized PCa. Thus, blood samples from 3 of 8 (37.5 %) patients were positive for putative DTCs, with the amount of isolated DTCs varying from 0 to 3 units and the median value at 0 units. In case of healthy volunteers, putative DTCs were found in 0 of 0 (0%) blood. Notably, as well as in case with urine, the speed of blood processing through the microfluidic chip was at 1.7 mL/min which allowed to process 7.5 mL of the blood in several minutes. However, even though the described blood liquid biopsy technique is advantageous in terms of the processing speed in comparison with the urine liquid biopsy technique described in Chapter III, it demonstrated a significantly lower sensitivity. At the same time, differently from the case with urine, the immunocytochemical

identification of the isolated DTCs was performed by using three primary antibodies (anti-PSMA, anti-CK, anti-CD45) instead of only one anti-GPC-1 antibody. Thus, in case of blood, DTCs were considered PSMA+, CK+ and CD45-, and therefore a direct comparison between two liquid biopsy techniques could not have been performed. To further improve the blood liquid biopsy technique, a combination of different primary antibodies specific to PCa DTCs, as well as involvement of larger cohort of patients and healthy volunteers to the study, may be performed. However, according to the published investigations of liquid biopsy techniques via DTC isolation of blood from patients diagnosed with PCa at its localized form, additional measures may not significantly increase specificity and sensitivity of the technique due to a negligible number of DTCs per mL of blood.

In Chapter V, the investigation of the applicability of semen as the fluid for liquid biopsy by means of the microfluidic label-free isolation of PCa DTCs, was described. It was hypothesized that the DTCs presenting in urine originate from the seminal ducts. For isolating DTCs from semen, the same chip as in case with blood was used. The choice was justified by cellular homogeneity of the semen, in which >99,9% of the cells are sperm cells which are normally significantly smaller than 13 μm. Thus, the microfluidic chip described in Chapter IV, with the cell isolation cutoff at 13 μm, was considered potentially effective for the case with semen. In this study, the antibody panel containing anti-PSMA, anti-CK and anti-GPC-1 antibodies was used for labelling the isolated DTCs, and therefore DTCs were considered PSMA+, CK+, CGP1+ cells. According to the results, all 100% of the involved patients (15 of 15) had DTCs in their semen samples at the amounts varying from 66.9 to 306.7 units, with the median value at 103.5 and the mean value at 135.0 units. In case of healthy volunteers, 2 of 15 (13.3%) patients had DTCs found in their semen samples at the amounts varying from 1.2 to 3 units per mL. Thus, even if the number of DTCs at 3 units per mL is determined as a cutoff value, 15 of 15 (100%) patients would anyway be significantly positive for DTCs. Additionally, a moderate positive correlation with the r value at 0.73 was identified between the total amount of isolated DTCs per semen sample and GS score. Therefore, the described liquid biopsy technique based on DTC isolation from semen demonstrated a significantly higher sensitivity and specificity, and also a better correlation between the amount of isolated DTCs and GS score, in comparison with the techniques of either blood or urine liquid biopsy. As well as in case of urine, the anti-GPC-1 antibody demonstrated its high specificity to PCa DTCs. However, in the current study, a high

specificity of the anti-GPC-1 antibody to PCa DTCs was more obvious due to the usage of the antibody panel which additionally included the primary antibodies well known for their high specificity to PCa DTCs, particularly: anti-PSMA and anti-CK [3, 4]. Even though direct comparison between two different liquid biopsy techniques, based on either urine or semen processing, could not have been performed due to the fact that in case of urine only anti-GPC-1 antibody was applied for the ICC labelling of the isolated DTCs, the obtained results may partly justify the hypothesis regarding seminal fluid as a primary origin of DTCs in urine. Further improvement of the technique requires the analysis of semen samples from a predetermined statistically significant number of patients and healthy volunteers, which would allow to obtain data required for reliable estimation of specificity/sensitivity of the technique, and also to determine correlations between the amount of PCa DTCs and GS score or PSA serum level more accurately. In a context of possible future investigations, one of the most important issues is to determine if there is an association between the localization of PCa tumour within the prostate gland and either quantity or quality of the isolated DTCs. Additionally, a part of the technique involving ICC identification of the isolated DTCs requires a thorough investigation to indicate the most efficient types of primary antibodies, as well as their number, to create the most reasonable antibody panel owning such features as high specificity/sensitivity and cost-effectiveness. Such possible primary antibodies, additional to the existing antibody panel, may be based on anti-epithelial cell adhesion molecule [5] and anti-prostate specific antigen [6]. At the same time, despite very promising results obtained in the current study, it should be noted that the technique has significant limitations. Thus, PCa is the disease which is usually developed in men after 50 years of age, with 60% of new cases occurring over the age of 65 years [7], who are at a high risk of erectile dysfunction. According to the published data, more than 50% of men have moderate to severe erectile dysfunction at the age over 60 years [8]. Thus, erectile dysfunction is expected to be the most frequent cause for difficulty of semen donation. However, erectile dysfunction, excluding its severe forms, can be overcome by the prescription of sildenafil-based medications [9] prior to the semen donation, and therefore this limitation may be overcome to a particular extent. Another possible serious limitation is retrograde ejaculation – a condition at which semen enters bladder instead of being ejected out of the body due to the overrelaxation of the bladder neck muscle [10]. Such condition may be caused by prior endoscopic surgeries on bladder or prostate [11],

side effects of certain medications [12], or damaged nerves responsible for bladder innervation [13]. It should be also considered that the results obtained in the current study could not have been directly compared with the results of the studies described in Chapters III and IV. To perform such comparison, simultaneous collection of all three biological fluids (blood, urine, semen) from the involved patients and usage of the same/similar antibody panel for identifying DTCs isolated from each biological fluid was required.

In Chapter VI, the study on direct comparison of three liquid biopsy techniques based on microfluidic size-based label-free isolation of DTCs from urine/blood/semen, previously described in Chapter III, IV and V, was presented. In this study, DTCs isolated from urine and semen were labelled with the antibody panel containing anti-PSMA, anti-CK and anti-GPC-1 primary antibodies, while the antibody panel applied for detecting DTCs from blood contained anti-PSMA, anti-CK and anti-CD45 primary antibodies. Hence, in case of the comparison between urine and semen, DTCs were considered PSMA+, CK+, GPC-1+ cells; while in case of the comparison between urine, semen and blood, DTCs were considered PSMA+, CK+ cells for urine and semen, and PSMA+, CK+, CD45- for blood. Thus, in the first comparative case of 13 patients, DTCs were isolated from urine samples of 10/13 (76.9%) and semen samples of 13/13 (100%) patients. The amount of isolated DTCs per mL varied from 47 to 280.8 units and from 0 to 4.3 units in case of urine and semen, respectively. In case of urine, correlations between the amount of isolated DTCs and GS or PSA serum level had r values at 0.31 and 0.53, respectively. In case of semen, correlations between the amount of isolated DTCs and GS or PSA serum level had r values at 0.40 and 0.80, respectively. Therefore, in the first comparative case, semen contained significantly higher amount of DTCs than urine. Also, differently from the urine samples, DTCs could have been isolated from semen samples of all patients. Further, correlation coefficient between the amount of isolated DTCs and PSA serum level was also significantly higher in case of semen. Thus, considering the results from the first comparative case, liquid biopsy of semen demonstrated much higher value in terms of potential applicability for early PCa diagnosis and prognosis of PCa. In the second comparative case, the amounts of isolated DTCs per mL varied from 0 to 0.08, from 0 to 5.35 and from 96.4 to 406.2 in case of blood, urine and semen, respectively. Out of 13 patients, DTCs were isolated from urine samples of 12 (92.3%), semen samples of 13 (100%) and blood of 3 (23.1%) patients. The r values for correlations between the amount of isolated DTCs per mL and PSA serum level were 0.60

and 0.70 in case of urine and semen, respectively. Hence, as well as in the first comparative case, in the second comparative case semen was also identified as the biological fluid providing the most valuable diagnostic and prognostic data in liquid biopsy of non-metastatic PCa. To better determine difference between the biological fluids in terms of their specificity and sensitivity, the study including a control group of healthy volunteers is required. Also, due to the issue of frequent differential diagnosis between PCa and benign prostate hyperplasia (BPH) [14], inclusion of the control group of patients with BPH is also relevant. The future study will also require a more rigid stratification of patients, taking into account their comorbidities and functional state of the prostate gland as well as urinary tract.

Overall, according to the results of the investigations described in the current thesis, microfluidic size-based label-free approach for isolating PCa DTCs from biological fluids was proven efficient as the means for liquid biopsy of the disease. The biological fluids may be ranked in the following order in terms of the diagnostic and prognostic value in localized PCa: 3-blood, 2-urine, 1-semen. The third place given to blood is justified by a relatively low number of isolated DTCs, and also low sensitivity of blood liquid biopsy for detecting PCa at its early stage, even though blood is the biological fluid most commonly used for liquid biopsy. To our belief, isolation of DTCs from blood does not have future as the technique for early diagnosis and prognosis of PCa and is not required to be further investigated. The second place given to the urine is justified by a relatively high specificity and sensitivity of the urine liquid biopsy technique and a relatively high amount of isolated DTCs. Another significant advantage of urine as the source of DTCs is its high accessibility and possibility to collect high amounts of the fluid. However, with the developed technique, processing of large urine volumes of more than 30 mL is unreasonable due to its extensive duration. At the same time, it was demonstrated that isolation of DTCs from the amount of urine at 20 mL may not provide sufficient number of DTCs for the analysis. Also, it is hypothesized that the number of DTCs may alter in urine due to numerous factors, and therefore protocol of urine collection has to be optimized to improve the developed technique. As one of the approaches increasing the amount of DTCs in urine sample, digital rectal examination may be proposed prior to urination [15]. Therefore, as a resume, the developed technique of DTC isolation from urine has a prospect for being further investigated. Finally, the first place given to semen is justified by a huge amount of DTCs presenting in a standard semen portion

at 2-3 mL, and sensitivity/specificity at 100% according to the initial results obtained in the study described in Chapters V and VI. According to the data presented in Chapter V, the total amount of DTCs (PSMA+, CK+, GPC-1+ cells) isolated from semen varied from 63 to 613 units, with the median number at 103.5 units. Thus, semen is the biological fluid containing the highest number of PCa DTCs per sample, as well as per mL, among all three biological fluids investigated in this thesis. Hence, the developed liquid biopsy technique of semen is very promising and is worth of being further investigated and has a potential for being introduced into a clinical practice. For this purpose, reliable specificity and sensitivity of the technique must be identified. Hence, after final optimization of the protocol for isolating PCa DTCs and development of the reasonable immunofluorescence assay, a blinded study including a predetermined statistically significant cohort of patients suspected but not yet diagnosed with PCa is required.

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Appendix A – Rapid and Label-Free Isolation of Tumour Cells from the Urine of Patients with Localized Prostate Cancer Using Inertial Microfluidics. Original publication.

cancers

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Article

Rapid and Label-Free Isolation of Tumour Cells from the Urine of Patients with Localised Prostate Cancer Using Inertial Microfluidics

Alexey S. Rzhevskiy [1,2,3,*], Sajad Razavi Bazaz [4], Lin Ding [4], Alina Kapitannikova [1,2], Nima Sayyadi [1,5], Douglas Campbell [6], Bradley Walsh [6], David Gillatt [3], Majid Ebrahimi Warkiani [2,4,*] and Andrei V. Zvyagin [1,2,*]

1 ARC Centre of Excellence for Nanoscale BioPhotonics, MQ Photonics, Macquarie University, 2109 Sydney, Australia; a.kapitannikova@gmail.com (A.K.); nima.sayyadi@mq.edu.au (N.S.)
2 Institute of Molecular Medicine, Sechenov First Moscow State Medical University, 119991 Moscow, Russia
3 Department of Clinical Medicine, Faculty of Medicine and Health Sciences, Macquarie University, 2109 Sydney, Australia; david.gillatt@mq.edu.au
4 School of Biomedical Engineering, University of Technology Sydney, 2007 Sydney, Australia; Sajad.RazaviBazaz@student.uts.edu.au (S.R.B.); Lin.Ding@student.uts.edu.au (L.D.)
5 Department of Molecular Sciences, Macquarie University, 2109 Sydney, Australia
6 Minomic International Ltd., 2113 Sydney, Australia; douglas.campbell@minomic.com (D.C.); Brad.Walsh@minomic.com (B.W.)
* Correspondence: alexey.rzhevskiy@hdr.mq.edu.au (A.S.R.); majid.warkiani@uts.edu.au (M.E.W.); andrei.zvyagin@mq.edu.au (A.V.Z.); Tel.: +61-457-248-832 (A.S.R.); Tel.: +61-424-100-396 (M.E.W.); Tel.: +61-298-507-760 (A.V.Z.)

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Abstract: During the last decade, isolation of circulating tumour cells via blood liquid biopsy of prostate cancer (PCa) has attracted significant attention as an alternative, or substitute, to conventional diagnostic tests. However, it was previously determined that localised forms of PCa shed a small number of cancer cells into the bloodstream, and a large volume of blood is required just for a single test, which is impractical. To address this issue, urine has been used as an alternative to blood for liquid biopsy as a truly non-invasive, patient-friendly test. To this end, we developed a spiral microfluidic chip capable of isolating PCa cells from the urine of PCa patients. Potential clinical utility of the chip was demonstrated using anti-Glypican-1 (GPC-1) antibody as a model of the primary antibody in immunofluorescent assay for identification and detection of the collected tumour cells. The microchannel device was first evaluated using DU-145 cells in a diluted Dulbecco's phosphate-buffered saline sample, where it demonstrated >85 (±6) % efficiency. The microchannel proved to be functional in at least 79% of cases for capturing GPC1+ putative tumour cells from the urine of patients with localised PCa. More importantly, a correlation was found between the amount of the captured GPC1+ cells and crucial diagnostic and prognostic parameter of localised PCa—Gleason score. Thus, the technique demonstrated promise for further assessment of its diagnostic value in PCa detection, diagnosis, and prognosis.

Keywords: prostate cancer; inertial microfluidics; cell separation; tumour cells; glycoprotein

1. Introduction

According to GLOBOCAN 2018, prostate cancer (PCa) is the second most frequent cancer as well as the second leading cause of the cancer deaths in males worldwide [1]. Statistical reports show that only a minor decrease in cancer-related mortality has been achieved during the past decade. This was

primarily due to a lack of efficient techniques for detection and prognosis of PCa, as well as monitoring the treatment outcomes [2].

Currently, the gold standard for PCa detection is a costly multistage process, which conventionally includes the prostate-specific antigen (PSA) blood test and subsequent tissue biopsy. However, these diagnostic techniques have significant limitations. For instance, the PSA test is sensitive to diverse disorders, including benign prostatic hyperplasia and prostatitis; therefore, its specificity is relatively low [3]. On the other hand, the tissue biopsy is a highly specific yet invasive examination with possible adverse side effects as local bleeding and infectious complications including drug resistant bacterial strains. These shortcomings call for development of cost-effective and reliable alternatives to the existing diagnostic tests for PCa, which would first of all reduce the number of unnecessary tissue biopsies. Liquid biopsy via isolation of circulating tumour cells (CTCs) is a promising diagnostic tool capable of supplementing state-of-the-art PCa diagnostics [4]. However, standard liquid biopsy of the blood samples from PCa patients suffers from low CTC yield, especially in the case of the localised forms of cancer, implying a requirement for providing a large volume of blood for PCa detection. This requirement makes the feasibility of the standard liquid biopsy questionable. Urine represents an obvious and natural choice of the biological fluid to yield diagnostically relevant amounts of cancer cells from patients with diverse urologic cancers including PCa [5,6]. Since a prostate gland is anatomically connected to the lower part of the urinary tract in males, diverse cells can be shed from the gland into the urinary tract, including tumour cells, which can be concomitantly washed out in the process of urination. The major advantages of the tumour cell isolation via liquid biopsy of the urine in comparison with the isolation of CTCs from the blood, as a truly non-invasive method, is a lack of limitation in the sample volume. Compared with blood, urine can be readily collected, stored, and transported. The urine collection process has enviable patient compliance and does not require a skilled medical professional.

Recently, isolation of tumour cells from biological samples via microfluidic technology has gained significant attention. Among the existing microfluidic modalities, inertial microfluidics based on a spiral microchannel device configuration has experienced a massive growth as exemplified by the separation of CTCs and circulating fetal trophoblasts [7–10], identification of bacterial spoilage from beers [11], blood fractionation [12], enrichment of circulating head and neck tumour cells [13], and separation of microalgae [14]. The technique is attractive for its cost-effectiveness and high throughput. The technology exploits inertial lift and Dean drag forces exerted on cells and particles flowing through the spiral microchannel. The interplay of these two forces causes lateral migration of cells and their focussing at equilibrium positions. As the amount of these two forces is directly related to the cell size, the cell eventually equilibrates at a lateral position, which precisely corresponds to its diameter. As a result, the larger cells (or particles, or both) tend to focus at regions near the inner wall, whereas the smaller cells (or particles, or both) tend to move away from it.

In this communication, we report on the development of a microfluidic chip capable of collecting PCa tumour cells from the urine using the principles of cell sorting employing inertial microfluidics. Isolated tumour cells were assayed via immunocytochemistry with monoclonal mouse anti-glypican-1 (anti-GPC-1) [15] antibody MIL-38 (Minomic International Ltd., Sydney, NSW, Australia) previously reported to be highly specific and sensitive in the detection of PCa [16]. MIL-38 was used as a model primary antibody. The GPC-1 is a heparan sulphate proteoglycan which is found attached to the cancer cell surface and which has recently received significant attention as a biomarker for PCa, especially in terms of evaluating its aggressiveness and growth [17]. Furthermore, to showcase the versatility of the developed microchannel in terms of diagnosis and prognosis of localised PCa, correlations between the number of isolated tumour cells and conventional diagnostic parameters of PCa such as the level of prostate-specific antigen (PSA) in blood and Gleason score (GS) were analysed.

2. Methods

2.1. Device Design and Fabrication

A spiral microchannel device for the isolation of PCa cells from urine samples was designed, fabricated, and tested. This device represented an adaptation of the microfluidic technology previously developed for the isolation of CTCs from peripheral blood [9,18]. Briefly, polydimethylsiloxane (PDMS) pre-polymer with the curing agent (Sylgard 184, DowsilCorp., Midland, MI, USA) were first mixed at the ratio of 10:1 and then was degassed in a vacuum chamber. This mixture was poured onto an aluminium mold with subsequent baking in a laboratory oven for 2 hours at 70 °C. The mold can be fabricated via a milling process [19] or 3D printing technology [20]; in this study, we used a milling process. Furthermore, the cured PDMS was peeled from the mold, and holes with 1.5 mm diameter were pierced with a Uni-Core™ Puncher (Sigma-Aldrich Co. LLC., Singapore) at the sites designated for the inlet and outlets of the chip. Eventually, the PDMS microchannel was irreversibly bonded to another layer of PDMS using an oxygen plasma machine (Harrick Plasma, Ithaca, NY, USA).

2.2. Experimental Setup and Procedure

The efficiency of the spiral microfluidic chip for capturing PCa tumour cells was evaluated by spiking a predetermined number of DU-145 cells (ATCC HTB-81) (approximately 1000) into 50 mL of Dulbecco's phosphate-buffered saline (DPBS). The solution was injected into the chip using a peristaltic pump (Baoding Shenchen Precision Pump Co., Ltd, Baoding, China) at a 1.7-mL/min flow rate. When the volume of DPBS in the sample tube decreased to 1 mL, enriched cells were deposited onto an adhesive glass slide (ThermoFisher Scientific, Scoresby, VIC, Australia) using a Thermo Scientific™ Cytospin™ 4 Cytocentrifuge (ThermoFisher Scientific, Scoresby, VIC, Australia) and then air-dried. To determine if there were cells in the waste outlet, DPBS in the waste tube was centrifuged, and cell pellets were deposited onto 4 adhesive glass slides with Cytospin™ 4 Cytocentrifuge for further quantitation. Deposited cells on slides were fixed with ice-cold acetone for 3 min at –20 °C and mounted with a Fluoroshield medium containing 4′,6-diamidino-2-phenylindole (DAPI) Prolong Gold Antifade Reagent(ThermoFisher Scientific, sScoresby, VIC, Australia) covered with a coverslip and counted using a fluorescence microscope (Zeiss Axio Imager Z2 Upright Microscope, North Ryde, NSW, Australia).

2.3. Ethics Statement and Clinical Sample Preparation

A part of this study that includes collection and analysis of clinical samples from patients with localised PCa was conducted under an ethical approval provided by Macquarie University Human Research Ethics Committee (HREC No: 5201500707), Australia. All patients and healthy volunteers provided written informed consent for collection of the samples and provision of their clinical data. The samples were collected and analysed in a non-blinded manner, 14 midstream urine samples from healthy volunteers and fourteen midstream urine samples from patients with localised PCa were acquired. The midstream specimen of urine was chosen for analysis to avoid possible channel occlusion with debris which may present in the first pass specimen. The volume of urine collected from patients and healthy volunteers varied from 30 to 100 mL. Prior to sample processing, the amount of cells per mL of urine was identified with an automated cell counter (TC20™ Automated Cell Counter, Bio-Rad, Gladesville, NSW, Australia). After the sample collection, samples were processed through the spiral microchannel on the same day with the peristaltic pump at the optimum flow rate of 1.7 mL/min. During the sample processing, once the volume reduced to approximately 5 mL, 5 mL of DPBS was added, and the processing was continued until the volume of solution decreased to approximately 1 mL. Afterwards, cells from the solution were sedimented with Cytospin™ 4 Cytocentrifuge onto four adhesive glass slides. The glass slides were then air-dried and fixed with ice-cold acetone for 3 min at –20 °C and processed either immediately or stored at 4 °C in a dry chamber and analysed within a week. The schematic representation of the sample processing is illustrated in Figure 1.

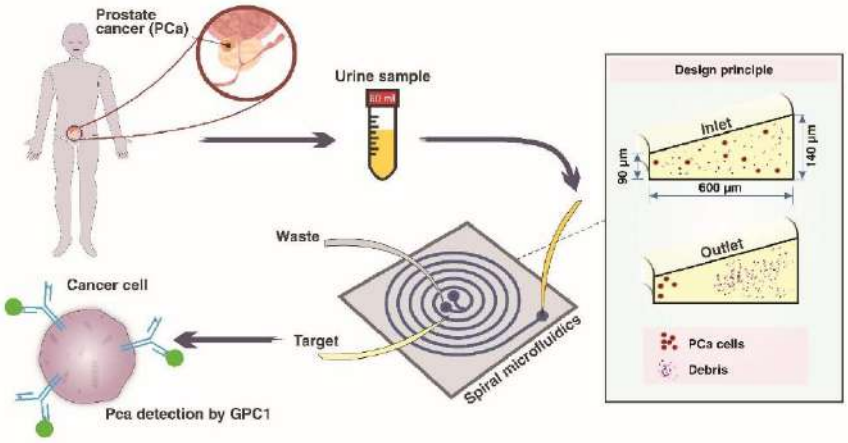

Figure 1. A schematic representation of the workflow for PCa cell detection from the urine sample employing a spiral microfluidic chip. First, PCa cells shed from the prostate gland into the urethra are collected into a container in the process of urination. Then, the collected PCa cells present in urine are isolated via processing through the spiral microchannel. Finally, the collected cells are labelled with fluorescent antibodies, i.e., anti-GPC-1 and immunoassayed under a microscope.

2.4. Immunofluorescence Staining

All incubations were performed in a humidified chamber. Cells were fixed on the surface of dry glass slides, rehydrated in DPBS for 3 min, followed by blocking with 1% Bovine Serum Albumin (BSA) solution in DPBS for 1 h at room temperature. Cells were then incubated for 2 h at room temperature with an MIL-38 anti-GPC-1 mouse primary antibody diluted in blocking solution (1% BSA) at 10 µg/mL. At this step, one of the 4 slides was incubated with the blocking agent instead of the secondary antibody and was used as a secondary antibody alone control to monitor the non-specific binding of second antibody. Then, the glass slides were washed with DPBS 3 times for 5 min and air-dried. Then, cells were incubated for 1 h with goat-anti-mouse Alexa Fluor 488 (Abcam, Australia) diluted in the blocking solution at 4 µg/mL and washed with DPBS 3 times for 5 min after the incubation. Finally, cells were mounted with a Fluoroshield medium containing DAPI, covered with a coverslip, and observed under a fluorescence microscope.

2.5. Cell Enumeration and Data Analysis

To identify a quantitative gold standard for enumeration of GPC-1$^+$ putative tumour cells collected from the urine samples, approximately DU-145 cells were added to a healthy urine sample, processed with the chip, immunostained as described above, captured under the fluorescence microscope, and the intensity of fluorescence signal from the cells was measured with ImageJ software. Then, a mean (±SD) for the signal intensity was calculated, and the intensity at the value equal to mean–SD was chosen as the threshold. Above this threshold, cells from the urine samples of PCa patients or healthy volunteers were registered as putative tumour cells and counted. Thus, in the case of cells isolated from the urine sample of PCa patients, during observation under the microscope, images of suspicious cells were captured, and the cells were analysed with ImageJ software for signal intensity. GPC-1$^+$ cells whose signal intensity was higher than the mean–SD (determined using DU-145 cells) were registered as putative tumour cells and counted. Finally, to evaluate potential diagnostic and prognostic value of the technique, correlations between the amount of GPC-1$^+$ cells collected from the patients and the levels of conventional PCa markers such as the blood PSA level and GS were measured. To further evaluate the applicability of the developed microfluidic device, correlations between the amount of collected

GPC-1$^+$ cells, the volume of the urine sample and total amount of cells in urine sample (mL^{-1}) were measured. The correlations were calculated using Microsoft Excel 2016 software (Microsoft, Redmond, WA, USA).

3. Results

3.1. Spiral Microfluidic Device

In this study, we report a novel, efficient, and non-invasive method for isolation of tumour cells from urine samples. This is the first time, to the best of our knowledge, that detection of PCa cells using the urine samples based on inertial microfluidics in a spiral channel has been reported.

Prostate cancer cells experience two major inertial lift and Dean drag forces inside the spiral channel. These forces can be calculated by Equations (1) and (2):

$$F_L = \rho\left(\frac{U_{max}}{D_h}\right)^2 C_L a^4, \quad (1)$$

$$F_d = 5.4 \times 10^{-4} \pi \mu De^{1.63} a\,, \quad (2)$$

where U_{max} indicates the maximum fluid velocity, ρ is the fluid density, μ denotes the dynamic viscosity of the fluid, C_L is the lift force coefficient, D_h represents the channel hydraulic diameter, a is the particle diameter, and De is Dean number. Considering these equations, a particle (or cell) of the diameter a experiences F_L and F_d different uniquely determined by the a [11]. As schematically shown in Figure 1, the larger cells (prostate cancer cells) are focussed at the inner wall (where its height is purpose-designed to be smaller than that of the outer wall), whereas the smaller cells and debris drift to the outer wall.

Generally, the ratio of particle diameter to the height of channel, as well as the hydraulic diameter of microchannel play a critical role in the particle focussing. The channel cross-section designed in this study was carefully analysed to focus PCa cells (~15–20 μm) featuring a trapezoidal cross-section with the base of 600 μm, inner wall of 90 μm, and outer wall of 140 μm. The bifurcation at the outlet of the channel was placed at a location 350 μm to the inner wall. Upon the optimisation of the channel flow rate, DU-145 cells in DPBS solution were introduced from the inlet via a peristaltic pump. The results demonstrated that the flow rate of 1.7 mL/min was optimal where 85 (±6) % of cells were collected through the target outlet (Figure 2).

3.2. Collection of GPC-1$^+$ cells from Urine

Midstream urine samples in volumes ranging from 30 to 100 mL were collected from 14 patients with localised PCa and processed using our spiral microfluidic chip. GPC-1$^+$ cells exhibiting the fluorescence signal >1781 arbitrary units, according to the mean (2069)-SD (288) arbitrary units measured from DU-145 cells with ImageJ were registered as putative tumour cells. Mostly, these cells exhibited round nuclei and a high ratio of nuclear to cytoplasmic size. We note that these cells were located in groups or clusters (Figure 3A,B). Putative tumour cells were detected in 12 out of 14 patients (86%). The total amount of detected cells (n) varied from 4 to 194 among the samples and patients, with the median value of 22. In the case of healthy volunteers, 11 samples out of 14 (79%) were negative in terms of GPC-1$^+$ cells. It is worthy to note that the urine samples of the 3 healthy volunteers registered as GPC-1$^+$ positive, contained only <8 cells. Thus, in case of PCa patients, only those patients whose urine samples contained $n > 8$ were considered positive with confidence. Accordingly, the number of such positive patients was 11 out of 14 (79%) (Table 1).

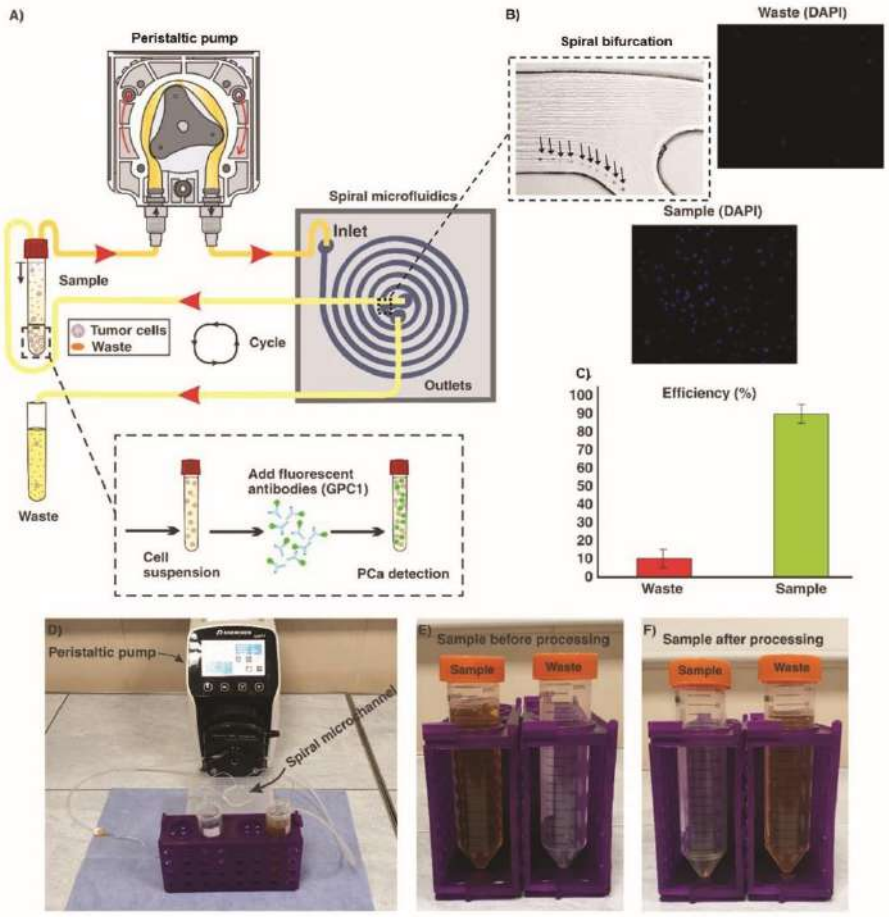

Figure 2. (**A**) schematic representation of the processing of a urine sample containing PCa cells through the spiral microfluidic chip. Samples are introduced via a peristaltic pump and then recycled until 1 mL of the urine sample remains in the sample tube. Then, PCa assaying is implemented by the use of fluorescent antibodies; (**B**) illustration of the bifurcation of the spiral, target (sample), and waste outlets. The results show that most of cells were collected through the target outlet. The flow rate of 1.7 mL/min was selected as the optimum flow rate for the separation efficiency of PCa cells; (**C**) 85 (±6) % of cells were collected through the sample outlet of the chip; (**D**) experimental setup used in this study; (**E**) state of urine sample before processing, and (**F**) after processing, when about 1 mL of sample remains in the tube. The remaining 1-mL contains most of PCa cells and is subsequently analysed as described.

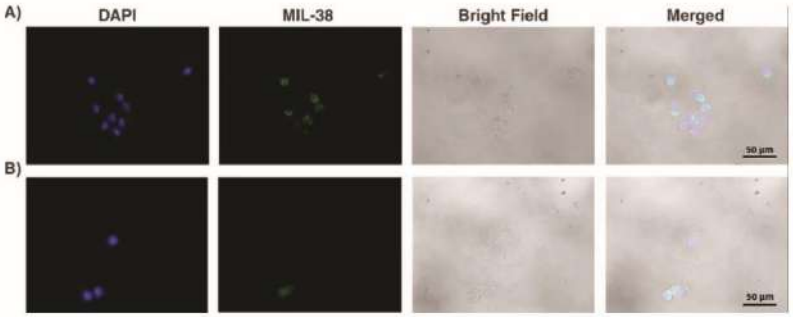

Figure 3. Numerous glypican-1+ (GPC-1$^+$) cells (**A**), two GPC-1$^+$ cells and one GPC-1$^-$ squamous epithelial cell (**B**) isolated from the samples of PCa patients. The GPC-1$^+$ putative tumour cells typically featured a high ratio of the nucleus to cytoplasm size and were prone to grouping or clustering.

Table 1. The number of glypican-1$^+$ (GPC-1)$^+$ cells detected in the urine samples and relevant clinical diagnostic test results. The number of cells with high GPC-1$^+$ expression (n) isolated from the patients' and urine samples varied from 4 to 194 units with the median value of 22. n was <8 in the urine samples of the healthy volunteers, i.e., 11 (79%) out of 14 healthy volunteers were registered PCa-negative in terms of the n -number. The number of patients positive with confidence for GPC-1$^+$ cells ($n > 8$) was 11 out of 14 (79%).

Patient Number	n	Urine Sample Volume (mL)	N	Blood PSA level (ng/mL)	Total GS
1	23	90	1.6×10^4	5.4	6
2	21	30	8.1×10^3	8.7	7
3	37	100	1.3×10^4	7	8
4	16	60	6.3×10^4	4.4	6
5	4	60	4.4×10^3	11	8
6	10	40	5.2×10^4	4.9	7
7	37	30	1.5×10^4	7.2	7
8	12	90	8.3×10^4	3.9	7
9	194	40	3.6×10^4	7.9	8
10	42	50	7.4×10^4	11.6	6
11	0	40	1.9×10^4	1.3	6
12	37	30	9.7×10^3	11	7
13	11	50	1.5×10^4	5.5	6
14	0	70	2.7×10^3	5.6	6

n—number of GPC1+ cells; N—total number of cells in the analysed sample.

Low negative correlation between the amount of GPC-1$^+$ cells and the volume of urine sample ($r = -0.18$, where r is correlation coefficient) and negligible positive correlation between the amount of GPC-1$^+$ cells and the total number of cells per mL of the initial urine sample ($r = 0.02$) were identified. At the same time, moderate positive correlation between the volume of the urine sample and the total number of cells (mL$^-$1) ($r = 0.32$) was identified. Among all 28 samples analysed, 1 (4%) sample was discarded due to the high concentration of non-cellular elements (i.e., urine crystals).

3.3. Analysis of Potential Diagnostic Applicability of the Method

To evaluate the flexibility of the method proposed in this study for early detection and diagnosis of PCa, correlations between the amount of isolated putative tumour cells and conventional diagnostic parameters of PCa were analysed. Thus, correlations between the amount of GPC-1$^+$ cells (n) and PSA level, or total GS were recognised (Figure 4). Furthermore, correlations between the ratio of n/V (where V is volume of urine sample), or the ratio n/N (where N is a total number of cells in

urine sample), and PSA level, or GS, were also identified. As a result, low positive correlations were identified between n and PSA level ($r = 0.27$) as well as n/V and PSA level ($r = 0.30$), moderately positive correlations between n and GS ($r = 0.48$), and between n/V and GS ($r = 0.46$), and moderately positive correlations between n/N and PSA level ($r = 0.47$) and GS ($r = 0.61$).

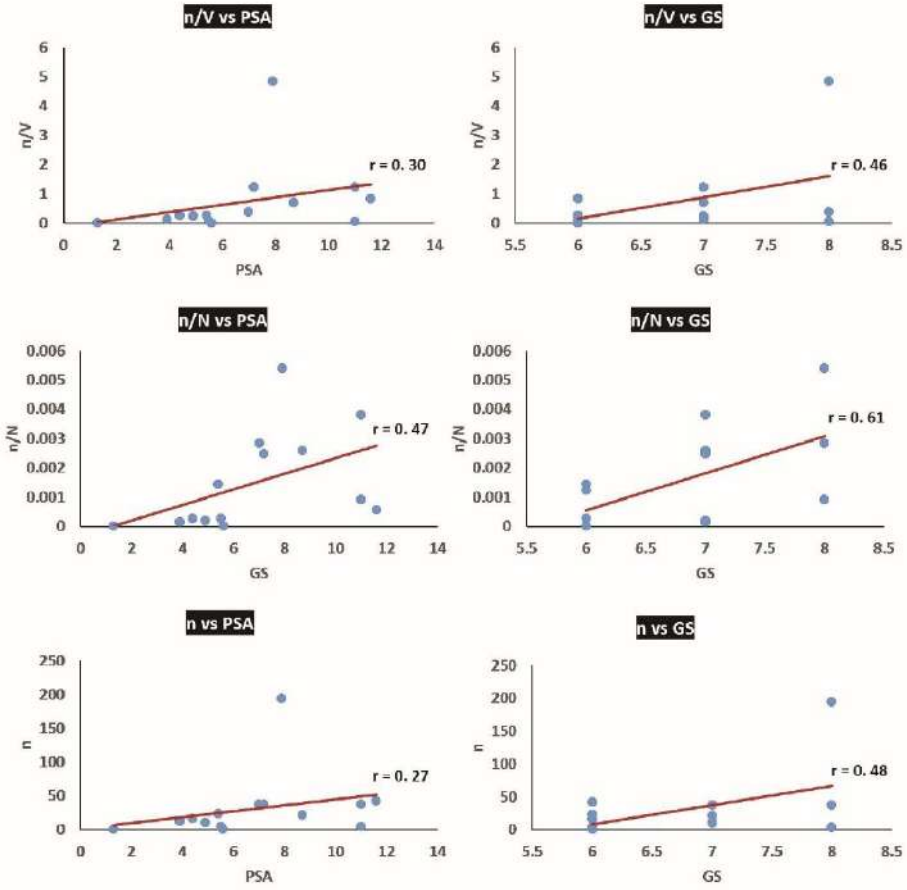

Figure 4. Correlations between the amount of GPC1+ cells (n), n/V (V, volume of urine sample), n/N (N, total number of cells in urine sample) and conventional clinicopathological parameters of the PCa–prostate specific antigen (PSA) level and Gleason score (GS). The lowest correlation was identified between n and PSA level, and the highest correlation was identified between n/N and GS.

4. Discussion

PSA screening is the most common method of early detection of PCa. A prostate biopsy remains the only definitive diagnostic test for the presence of PCa. However, the high prevalence of false positive PSA tests gives rise to a large number of un-necessary prostate biopsies. There remains a high clinical need for tests better able to guide a decision to proceed to prostate biopsy. This provides an opportunity to choose the right time for surgical intervention in the case of the parameter increments [21]. However, the specificity of PSA screening is not high and a GS is obtained via an invasive tissue biopsy causing significant discomfort to a patient. In recent years, early diagnosis and prognosis of PCa by means of

CTC isolation via liquid biopsy of blood has been broadly investigated. However, progress has been minimal and the technique is not efficient due to the low number of CTCs in a standard sample of 7.5 mL of peripheral blood [22]. As an alternative, in this study, we developed and investigated a high-throughput microfluidic chip for enrichment of PCa tumour cells derived from the urine.

In the pilot enrichment of DU-145 cells from DPBS, the chip demonstrated 85 (±6)% efficiency in capturing cells at the optimum flow rate i.e., 1.7 mL/min. For urine sample analysis, 12 out of 14 PCa patient samples (86%) were positive, and 11 out of 14 patients (79%) were positive with confidence in terms of cells with the high level of GPC-1$^+$ expression—the cells which were registered as putative tumour cells in the current study. At the same time, 11 out of 14 healthy volunteers (78%) were identified as PCa-negative in terms of GPC-1$^+$ cells. Such results corresponded to the specificity and sensitivity of anti-GPC1 primary antibody MIL-38 used as a model primary antibody for the detection of putative tumour cells [16]. A median amount of GPC-1$^+$ cells of 22 units, captured from patient samples, identifies urine as a preferable medium for liquid biopsy of localised PCa. Furthermore, low negative correlation ($r = -0.18$) identified between the amount of captured GPC-1$^+$ cells (n) and the volume of the urine (V) suggested that the major amount of n were released in the first stream of the urine—a reasonable assumption considering the anatomical connection of the prostate gland to urethra [23]. This assumption is corroborated by moderately positive correlation between the total number of cells in urine samples (N) and V ($r = 0.32$) alongside a positive correlation between N and n ($r = 0.02$). This seems to indicate that other cells normally present in urine are gradually released during urination.

The low positive correlation between n/V and PSA level ($r = 0.30$) suggests that larger volumes of urine do not contain proportionally greater n, and the first stream of urine is sufficient to collect the majority of PCa cells. Therefore, it can be concluded that ≤30 mL of the voided urine is likely to be optimal for rapid isolation of tumour cells using our spiral microfluidic chip. The low positive correlation between n and PSA level ($r = 0.27$) can be explained by the relatively low specificity of the PSA level increment as a marker for the PCa progression and aggressiveness [24]. However, the moderately high correlation between n/N and PSA level ($r = 0.47$) is noteworthy. The highest positive correlation in this study was achieved between n/N and GS ($r = 0.61$) and can be explained by the high specificity of GS as a marker of the progression and aggressiveness of localised PCa [25]. This assumption is corroborated by the moderate correlation between n and GS ($r = 0.48$) and between n/V and GS ($r = 0.46$).

The reported technique lends itself to some straightforward improvements. Fine-tuning of the chip by including an additional outlet to remove large waste elements will enable handling of a greater variety of urine samples, including samples containing a large amount of urine crystals, which we had to discard. The use of more specific and sensitive PCa antibodies or their combination will improve the immunocytochemistry assaying and increase the sensitivity and specificity of our technique.

5. Conclusions

For the first time, to the best of our knowledge, we introduce here a spiral microfluidic chip capable of rapid and label-free isolation of tumour cells from urine. The spiral microchannel used in this study had a trapezoidal cross-section with a width of 600 µm, an inner wall of 90 µm, and outer wall of 140 µm. The microchannel was first tested using a spiked cancer cell line, proving its high efficiency: separation of ca 86% of cancer cells at the optimum flow rate of 1.7 mL/min. Secondly, ≥79% of the analysed clinical samples from urine of patients with localised PCa were positive for GPC-1$^+$ putative tumour cells. Thirdly, moderate correlations were observed between the ratio of GPC-1$^+$ cells (n) to the total number of isolated cells (N) and PSA; n and GS; and n/V (V, urine volume) and GS. These results demonstrate promise of the spiral inertial microfluidic technique in terms of diagnosis and prognosis of localised PCa by liquid biopsy of urine, paving the way for inexpensive rapid, non-invasive diagnosis, as well as screening and monitoring therapeutic outcomes of PCa and other urology cancers.

Author Contributions: Conceptualization, A.S.R. and D.G.; Data curation, A.S.R.; Formal analysis, L.D.; Investigation, A.S.R. and L.D.; Methodology, A.S.R., S.R.B., N.S., D.C., D.G., and M.E.W.; Project administration, A.V.Z.; Resources, B.W., M.E.W. and A.V.Z.; Software, S.R.B.; Visualization, A.K.; Writing—original draft, A.S.R., S.R.B., L.D. and A.K.; Writing—review and editing, N.S., D.C., B.W., D.G., M.E.W., and A.V.Z. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Appendix B – Ethics approvals

Office of the Deputy Vice-Chancellor
(Research)

Research Office
Research Hub, Building C5C East
Macquarie University
NSW 2109 Australia
T: +61 (2) 9850 4459
http://www.research.mq.edu.au/
ABN 90 952 801 237

MACQUARIE University
SYDNEY·AUSTRALIA

22 October 2015

Dear Prof Walsh

Reference No: 5201500707

Title: Biomarkers for early diagnosis and prognosis of cancer

Thank you for submitting the above application for ethical and scientific review. Your application was considered by the Macquarie University Human Research Ethics Committee (HREC (Medical Sciences)) at its meeting on 24 September 2015 at which further information was requested to be reviewed by the HREC (Medical Sciences) Executive.

The requested information was received with correspondence on 11 September 2015.

I am pleased to advise that ethical and scientific approval has been granted for this project to be conducted at:

- Macquarie University

This research meets the requirements set out in the *National Statement on Ethical Conduct in Human Research* (2007 – Updated March 2014) (the *National Statement*).

This letter constitutes ethical and scientific approval only.

Standard Conditions of Approval:

1. Continuing compliance with the requirements of the *National Statement*, which is available at the following website:

http://www.nhmrc.gov.au/book/national-statement-ethical-conduct-human-research

2. This approval is valid for five (5) years, subject to the submission of annual reports. Please submit your reports on the anniversary of the approval for this protocol.

3. All adverse events, including events which might affect the continued ethical and scientific acceptability of the project, must be reported to the HREC within 72 hours.

4. Proposed changes to the protocol must be submitted to the Committee for approval before implementation.

It is the responsibility of the Chief investigator to retain a copy of all documentation related to this project and to forward a copy of this approval letter to all personnel listed on the project.

Should you have any queries regarding your project, please contact the Ethics Secretariat on 9850 4194 or by email ethics.secretariat@mq.edu.au

The HREC (Medical Sciences) Terms of Reference and Standard Operating Procedures are available from the Research Office website at:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/human_research_ethics

The HREC (Medical Sciences) wishes you every success in your research.

Yours sincerely

Professor Tony Eyers
Chair, Macquarie University Human Research Ethics Committee (Medical Sciences)

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research* (2007) and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.

Details of this approval are as follows:

Approval Date: 22 October 2015

The following documentation has been reviewed and approved by the HREC (Medical Sciences):

Documents reviewed	Version no.	Date
NEAF	Revision 2	Received 11/9/15
Correspondence from Prof Walsh responding to the issues raised by the HREC (Medical Sciences)		Received 11/9/15
MQ Participant Information and Consent Form (PICF)	2.0	11/9/15
Data collection tool	1.0	11/9/15

Federal State Autonomous Educational Institution of Higher Education
I.M. SECHENOV FIRST MOSCOW STATE MEDICAL UNIVERSITY
(Sechenov University)

LOCAL ETHICS COMMITTEE

8 Trubetskaya Street, Moscow, 119991 T.: 8(495) 622-97-06, fax: 8 (495) 622-97-56
iec@lmsmu.ru; iec@sechenov.ru

Extract from Minutes No. 17-19
of the Local Ethics Committee meeting
of 11.12.2019

Present:
Chairperson of the Committee - V.N. Nikolenko
Deputy Chairperson of the Committee - E.L. Rebrova
Committee members: I.I. Ermolaeva, N.G. Berdnikova, N.I. Borisova, E.V. Dubograi, E.A. Smolyarchuk

A quorum is present, the meeting is considered to be duly constituted.

The committee considered the following: approval of **research work** on topic: "Biomarkers for early diagnosis and prediction of development of cancer of the prostate gland by means of liquid biopsy" on the basis of the Federal State Autonomous Educational Institution of Higher Education I.M. Sechenov First Moscow State Medical University (Sechenov University): 119991, Moscow, Trubetskaya st. 8, bldg. 2, University Clinical Hospital No. 2: 119435, Moscow, Bolshaya Pirogovskaya st. 2, bldg. 1.

The main researcher is Shpot, Evgenii Valerievich.

Provided documents:

1. Minutes of the meeting of the department on the approval of the topic.
2. Annotation of research work.
3. Patient information sheet with informed consent form - 2 versions.
4. List of clinical centers where the study is planned.
5. Cutticulum vitae (CV) of all study personnel, dated and signed.
6. Commitment to confidentiality of all research personnel.
7. Information on the criteria for inclusion of patients in the study.

The Committee has resolved: to approve the **research work** on topic: "Biomarkers for early diagnosis and prediction of development of cancer of the prostate gland by means of liquid biopsy" on the basis of the Federal State Autonomous Educational Institution of Higher Education I.M. Sechenov First Moscow State Medical University (Sechenov University): 119991, Moscow, Trubetskaya st. 8, bldg. 2, University Clinical Hospital No. 2: 119435, Moscow, Bolshaya Pirogovskaya st. 2, bldg. 1.

Executive Secretary [*original signed*] **I.I. Ermolaeva**

23.12.2019

[Stamp]: **LOCAL ETHICS COMMITTEE**
Federal State Autonomous Educational Institution of Higher Education I.M. Sechenov First Moscow State Medical University (the Sechenov University) (Sechenov University)
T. 8 (495) 622-97-0

* * *

Translator's disclaimer: Signatures and round seal affixed. Emblem of the Russian Federation appears throughout the document. Spelling of names may vary due to possible variations in transliteration. Translator does not accept responsibility for slight discrepancies in academic and professional terminologies. Translated from the original document on 16/06/2021 by Alexei Vikulov, NAATI certified professional translator.

ALEXEI VIKULOV
CPN7TH80E
RUSSIAN <-> ENGLISH
VALID TO 08/04/2024
SIGNATURE:
TRANSLATION DATE: See disclaimer
CERTIFIED TRANSLATOR