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Cancer-Devoted Liquid Biopsies and Applications to Prostate Cancer

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Abstract

Human Prostate cancer (PCa), the second most frequent in men, is facing two yet unsolved problems: first, at diagnosis, the prostate-specific antigen (PSA) blood test is not specific enough, which results in too much false positive detection; secondly, at the last tumor step, the metastatic castration-resistant prostate cancer (CRPC) is of very bad survival prognosis. Therefore, tools are urgently needed for helping the active surveillance before any treatment and for monitoring treatment of advanced prostate cancer for trying to slow down the fatal issue. This survey corresponds to a pertinent - but not complete - selection among many recent papers, searched in PubMed and Web of Science with the keywords "Liquid Biopsy" and "Prostate Cancer". It is aimed to give an overview of the existing methods searching for a satisfying reliable clinical test, i. e. obtained from easy accessible bodyfluids such as blood or urine, in order to replace the invasive needle tissue biopsies for frequent therapeutic control of PCa development. Taking into account the huge heterogeneity of the tumor process, elaborating a convenient liquid biopsy is also a necessary step towards a further individually-adapted precision medicine for curing prostate cancer. The most promising perspectives for clinical analysis of PCa through liquid biopsy will be discussed.

Keywords: Circulating tumor cells (CTCs); Circulating tumor DNA (ctDNAs); Tumor extracellular vesicles (tEVs); miRNAs; Castration-Resistant Prostate Cancer (CRPC)

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Introduction

Deciphering the human specific tumor processes through a mere analysis of the appropriate body fluids corresponds to a rather old dream. However, despite the incredible number of studies devoted to this purpose, this goal is not yet achieved into a few reliable specific cancer tests. This mini-review is intended as a critical overview of the different suggested liquid biopsies for human cancers, and more specifically for prostate cancer.

The standard for characterization of human solid tumors, when sufficiently big to escape immune surveillance, rests on needle tissue biopsies. However, these are both invasive and not always easy to perform. Moreover, depending on the cancer, it may take a "dormancy" period of many years before being detected. A sensitive liquid biopsy of tumor biomarkers expelled in the appropriate biofluids might be helpful both for controling any physiological modifications during the tumor dormancy, and mainly for helping cancer diagnosis and prognosis after treatment. All the characteristic tumor compounds, which might be expelled into the body fluids are potential biomarkers for liquid biopsy. Therefore a few liquid biopsy trials were concerned with macromolecular compounds measured by the "omics" technologies, such as proteomics, lipidomics and metabolomics. However, most of the searches for a specific cancer liquid biopsy focused on biomarkers harboring tumor genomic modifications, such as gene mutations or methylation, and on specific mRNas and miRNAs. Three main kinds of such complex biomarkers, shed from the tumor mostly into complete blood (or serum /plasma), were either viable circulating tumor cells (CTCs) [1], or circulating cell-free tumor DNAs (ctDNA) [2]. More recently, tumor cell-derived extracellular vesicles (tEVs) [3] brought a new interesting perspective for cancer liquid biopsy. Using recent literature, a short review will first be given of the previous main approaches for elaborating reliable human cancer liquid biopsies. The newly appeared EV-based liquid biopsy will be some more detailed. Lastly, a short review will be given of the current proposed liquid biopsies for helping prostate cancer diagnosis and for monitoring advanced prostate cancer during treatment.

Main Approaches of Liquid Biopsies for Human Cancers

Previous ongoing liquid biopsies for diagnosis and monitoring cancers

There are already many reviews dealing with this topic, so here is only a snapshot about the main current liquid biopsies for human cancers. As stated by Crowley et al. [2]* "a liquid biopsy, or blood sample, can provide the genetic landscape of all cancerous lesions (primary and metastases) as well as offering the opportunity to systematically track genomic evolution". Moreover, liquid biopsies from peripheral blood have prognostic and predictive impact across the most common type of solid tumours [4].

Liquid biopsy through viable circulating tumor cells (CTCs)

The term liquid biopsy of cancer was originaly introduced for the analysis of CTCs [1]*. This review focuses on the technologies used for the enrichment of CTCs. A new *in vivo* technology was mentioned, allowing the continuous enrichment of CTCs directly in the arm vein of the patient, which enables enrichment of CTCs from approximately 1.5 L of blood. Pantel and Alix-Panabières [5] discussed the main advantages and disadvantages of CTCs and ctDNA as biomarkers in clinical oncology, as well as their current challenges and future perspectives.

Jie et al. [6]* worked out the important problem of the still debated involvement of the epitelial-to-mesenchymental transition (EMT) in metastasis. EMT contributes to the generation of CTCs in epithelial cancers because it increases tumor cells invasiveness, promotes tumor cell intravasation and ensures tumor cell survival in the peripheral system. Some CTC-liquid biopsy studies have found that the expression of EMT and stemness markers in circulating tumor cells, in addition to CTC detection, can provide more information on tumor diagnosis, treatment, prognosis and research.

Liquid biopsy through circulating tumor DNA (ctDNAs)

Circulating DNA in human blood was already observed in 1948, whereas tumor-derived ctDNA was first claimed only in 1977, and confirmed in 1989, as noticed from the whole description of the origins, structures, and functions of circulating DNA in oncology [7]*. As an order of magnitude, a single human cell contains 6 pg of DNA and there is an average of 17 ng of DNA per ml of plasma in advanced stage cancer. If CTCs were the primary source of ctDNA, it would require over 2,000 cells per ml of plasma. In reality, there are on average less than 10 CTCs per 7.5 ml blood. The fraction of circulating DNA that is derived from the tumour can range between 0.01% and 93%, depending on the tumours and their apoptotic/necrotic states [2]. Han et al. [8]* recapitulate the current knowledge about circulating tumor DNA as biomarkers for cancer detection. They depict the landmarks in the detection of ctDNAs in patients with different cancers. They also compare different cancer detection methods, including CTCs, ctDNA, circulating RNA and exosomes, for their clinical utilities. Pantel and Alix-Panabières [9] focused again on the potential and challenges of liquid biopsy. Their conclusion was that the molecular and functional analysis of CTCs and ctNAs can be used as companion diagnostics to improve the stratification of therapies and to obtain insights into therapy induced selection of cancer cells.

Liquid biopsy through circulating RNAs (mainly miRNAs, but also mRNAs and non-coding long RNAs (lncRNAs)

Lombo et al. [10] envision the diagnostic potential of extracellular RNA (exRNAs) from biofluids. They state that the early discovery of small RNAs, including micro-RNAs (miRNAs) and noncoding RNAs (ncRNAs), secreted in biofluids has dramatically altered our understanding of the regulation of gene expression and its impact on human diseases. Different studies have demonstrated the existence of extracellular RNAs (exRNAs) in a variety of body fluids, where they are remarkably stable. exRNAs have tremendous potential for development as biomarkers for various cancers. The NIH supported the creation of an international consortium to coordinate large-scale studies to understand the role of exRNAs in health and disease. The exRNA communication program (ERCP) is partly involved with cancer. Investigators have identified ncRNAs in biofluids from patient's samples and they are now moving to validation for clinical applications in glioma, hepatocellular cancer and gastric cancer.

Liquid biopsy through tumor-educated platelets (TEPs)

Feller and Lewitzky [11] critically review the current circulating biomarkers. Then, they propose a diversion method, based on new biomarkers called TEPs, originating from a highly intriguing

Table 1 Figure or Table (s) suggested to be seen in a given reference[1,2].

Reference	1st author, year	Figure to see	Table (s) to see
[1]*	Alix-Panabières, C., 2013	Fig. 1	
[2]*	Crowley, E., 2013	Fig. 1	
[6]*	Jie, X. X, 2017	Fig. 1	
[7]*	Thierry, A. R., 2016	Fig. 20	
[8]*	Han, X., 2017	Fig. 1	Table 1
[12]*	Armstrong, D., 2018		Table 1
[13]*	Ciardiello, C., 2016	Fig. 2	Table 1
[14]*	Zhao, H., 2017		Table 1
[15]*	Li, A., 2017		Table 1
[16]*	Yang, Q., 2016		Table 11
[20]*	Zhang, W., 2017		Table 1
[23]*	Di, Meo, A., 2017		Tables 1, 2, 3
[26]*	Luo, J., 2016		Table 1
[30]*	Kretschmer, A., 2017		Table 3
[32]*	Hegemann, M., 2016		Table 1
[33]*	Miyamoto, D. T., 2016		Table 1

[1]All the references are open in the web

2 [ ]* : a reference with a star in the text, or in this table 1, means that it is suggested to see a given significant figure or Table (s) in the cited paper.

These suggestions are recapitulated in table 1 for clarity.

recent work of Best and colleagues see ref. 70 in [11]. This demonstrates that the ultimate cancer liquid biopsy still remains "a matter of dream" in the never-ending war against cancer.

New liquid biopsy through tumor cell-derived extracellular vesicles (tEVs) including exosomes, and miRNas

Armstrong and Wildman [12]* envision the promise of EV-mediated continuous liquid biopsies. EVs have been found in all domains of life, including Archae, Bacteria, and Eukaryotes. EVs are also found in almost every human body fluid, including urine, blood, saliva, cerebrospinal fluid (CSF), synovial fluid, semen, breast milk, amniotic fluid, lymph, and broncho alveolar lavage fluid. They summarized tissues, cells, and fluids in which EVs have been identified as listed in Vesiclepedia. Using EVs for diagnosis is already the matter of many patents, but their most promising use is a remote sensor of organs which are innaccessible to routine monitoring.

Ciardiello et al. [13]* describe the main different EV classes (exosomes, ectosomes, large oncosomes and apoptotic bodies), analysing their different physical and biological properties, and specifically focusing on their main EV function of cell-to-cell communication between cancer cells and different components of the tumor microenvironment. Exosome and MV subtypes can mediate single and double-stranded DNA transfer and one fascinating hypothesis is that EVs might partly function by horizontal transfer of genes with pre-existent mutations. Moreover, numerous studies have reported enrichment of miRNAs in EVs released by different types of cancer cells and present in biological fluids from cancer patients.

Zhao et al. [14]* review the key role of EVs in the metastatic process, showing a summary of papers demonstrating exosomes / EVs role in tumor metastasis.

Wu et al. [3] are concerned with the three main EV classes (exosomes, MVs and apoptotic bodies) as critical mediators of extracellular communication. While tumor cell-derived EVs are capable of reprogramming stromal cells to generate a proper tumor cell niche, stromal-derived EVs profoundly affect the growth, resistance, and stem cell properties of tumor cells. This review discusses these EVs-mediated reciprocal communications in different types of cancers, and the therapeutic opportunities of utilizing EVs as emerging targets for cancer diagnosis and therapy.

Li et al. [15]* highlight the unique features of exosomal proteins and summarize their recent use in cancer diagnosis and prognosis, with emphasis on the clinical use of exosome proteomics. There are over 10^9/ml exosomes in human blood and individual exosomes typically expose 10-100 surface antigens. They show some exosomal proteins as potential diagnostic markers in various tumors. These exo-based markers can be identified for early stage cancer detection, as well as to predict clinical outcome. However, most of the corresponding clinical research is still based on the case data from a single group/hospital.

Yang et al. [16]* face the emerging role of extracellular vesicle-derived miRNAs. Accumulating evidence demonstrates that EV-derived miRNAs have key roles in regulating various aspects of cellular homeostasis, including proliferation, survival, migration, metastasis and the immune system etc. The review aims to summarize the recent advances in EV-derived miRNAs in a variety of tumor types and stem cells.

Torrano et al. [17] provide an updated view of the potential of exosomes and microvesicles as cancer biomarkers and the available technologies for their isolation. Despite predicting an exciting bright future for EVs and their applicability in liquid biopsy, they recognize that there is still a long way and many challenges to solve for their clinical use as biomarkers of disease.

Shao et al. [18] compare the technological challenges posed by CTCs and exosomes for clinical translation: CTCs are rare and short-lived, whereas exosomes are small. They overview their respective characteristics and describe the emerging technologies developed to address these technical obstacles towards using the unique clinical opportunities of these biomarkers.

Fisher et al. [19] showed that EVs released from human bone marrow derived mesenchymal stromal cells (BM-hMSC) carry high molecular DNA in addition to proteins and RNAs. The DNA isolated from EVs is not only inside the EVs but significant amounts were also associated to the outer membrane. Their results suggested that EV-DNA is not organized in nucleosomes and is not derived from apoptotic or necrotic cells. Next generation sequencing showed that EV-associated DNA derived virtually from the entire genome. Considering the size of EV within 30-1000 nm, sufficient space is being available to pack RNA, proteins and high-molecular DNA. Thus, within the calculated internal volume of exosomes ($4.2\text{-}380 \times 10^{-24}$ l), a total cargo of ≤ 100 proteins and ≤ 10,000 net nucleotides of nucleic acid is likely. Moreover, they presented a pilot study as a first proof-of-principle of EV-mediated horizontal DNA transfer into recipient cells.

Liquid biopsy and cancer-targeted precision medicine

Liquid biopsy plays an important role in the precision medicine field. Zhang et al. [20]* performed a critical comparison between CTCs, cfDNA and exosomes as biomarkers for cancer liquid biopsy. They compared the source, characteristics, technology of detection and current situation of the three methods. Liquid biopsy is more sensitive and more accurate and provides substantially more information regarding prognosis and treatment direction than conventional imaging and tumor markers, however many hurdles need to be overcome before any liquid biopsy being used in a clinical setting.

Sumanasuriya et al. [21] also questionned the application of liquid biopsies in cancer targeted therapy. They considered circulating nucleic acids (cfDNAs, RNA and microRNAs, tumor educated platelet mRNA), circulating tumor cells (CTCs) and immune cell studies. They recognize the interest of circulating biomarkers and state that CTCs and cfDNAs are complementary non-invasive approaches for such biomarkers studies. However, only rigorous validation will allow these biomarkers to transform cancer care.

Wang S and Wang J [22] asked whether liquid biopsies are ready for prime time of clinical applications. They discussed the molecular targeted therapy represented by EGFR tyrosine kinase inhibitors (TKIs), which has become a paradigm for precision management of NSLC. They wondered whether there is current evidence sufficient to support using EGFR mutation detection assays based on liquid biopsy in routine clinical practice. As a result of a large multicenter (in Europe and Japan) diagnostic study intended to investigate the utility of plasma ctDNA EGFR mutation testing, it was concluded that EGFR mutation status in ctDNA could not be correlated in this global assess study with the clinical outcome of EGFR TKIs. This helped to demonstrate the remaining challenges of plasma ctDNA detection in the real world.

Di Meo et al. [23]* give a very interesting perspective for applying liquid biopsy towards precision medicine in urologic malignancies, especially for prostate cancer. They summarize the results obtained with different liquid biopsies. They analyse the advantages and limitations of cell-free DNA. They enumerate the plateforms used to analyse cfDNA in circulation and the diagnostic, prognostic and predictive applications of cfDNA for different urologic cancer types. They consider the same properties of other circulating molecules, such as CTCs, miRNAs, lncRNAs, mRNAs, proteins, peptides, and exosomes. They conclude that so far, the most exciting applications of liquid biopsies seem to be prognosis and early assessment of treatment failure.

Liquid biopsies for human prostate cancer

As for all cancers, an early diagnosis of PCa is quite important for the succes of the following therapy. Before the advent of current liquid biopsy, PCa diagnosis rested mainly on prostate-specific antigen (PSA) blood test and digital rectal palpation (DRE). The diagnosis should be conforted by needle biopsies of the potential tumoral prostate. However, the too low specificity of the PSA test resulted in too much false positive PCa detection followed with detrimental therapy. Therefore, the current trend is to perform an "active surveillance" of the patient before any harmful treatment. Both for helping this active monitoring, and even more for elaborating a reliable PCa diagnosis, all the efforts concerning liquid biopsies might be fruitful in the future. However, contrary to other cancers, PCa is not endowed with very specific mutations at the initiation of the tumoral process, which lowers the hope of finding significant alterations in the body fluids for early PCa diagnosis by both CTCs and ctDNAs liquid biopsies. This explains why researchers are, for the moment, more focused on using liquid biopsies for monitoring advanced PCa.

After surgical removal of a PCa tumor, different treatments might be tried, and the PSA test is, then, useful for monitoring the treatment efficiency. During the last decade, many pharmaceuticals have been elaborated against advanced PCa, especially the anti-androgen therapy, with abiratirone and enzetalumide. But even with a noticable initial benefit in most patients, acquired resistance to these drugs inevitably leads up to the last lethal step of metastatic PCa, called castration-resistant prostate cancer (CRPC), mainly with bone metastasis, and others, like visceral metastasis. It is utmostly important to efficiently monitor the treatment of advanced PCa, in order to prevent, or at least delay, the lethal issue of PCa. Inasmuch as repeated invasive needle biopsies cannot be envisioned for this cancer, liquid biopsies are, indeed, good candidates for monitoring advanced PCa, in order to efficiently try to modify the therapy.

Here is a short survey of ten recent studies shedding light on the state-of-art of liquid biopsies for PCa.

Unique among the selected papers, Lima et al. [24] performed an update in metabolomics studies of biomarkers in prostatic fluid, blood, plasma/serum, urine, tissues for human PCa. Sarcosine, one of the most promising biomarkers identified to date remains a controversial issue in the clinic.

Assinder and Bhoopalan [25] state a promising future for PCa diagnostics, especially by using the most frequently deregulated circulating miRNAs (miR21, miR141 and miR24), with miR-141 consistently increased in men with high risk PCa, metastatic PCa and CRPC. These miRNAs have also been shown to be altered in tissue biopsies and at least for miR-21, in urine. When combined with two other miRNAs enriched in urine (miR19a and miR19b), miR-21 has greater diagnostic power than PSA.

Luo [26]* reviewed non-invasive actionable biomarkers for metastatic PCa. Prior to 2004, there was no approved therapies for mCRPC. Today there are six FDA-approved drugs for mCRPC, including AR-directed therapies (abiraterone acetate and enzalutamide), taxane chemotherapies (docetaxel and cabazitaxel), immunotherapy (sipuleucel-T) and the bone-targeting radiopharmaceutical radium-223. The survival benefit of each individual therapies has been established in definitive clinical trials. However not all men benefit from the therapies and almost invariably, treated men will stop responding and develop resistance shortly after initiation of the therapies. Liquid biopsies that can be sampled non-invasively during the course of the treatment might be of critical importance in clinical development of biomarkers to facilitate treatment selection. He highlights the most promising biomarkers for mCRPC that have been evaluated in clinical studies so far, focusing mainly on CTC- and cTDNA-based detection of AR aberrations.

In Editorials, Chalfin et al. [27] questioned the too rigid selection of CTCs in human blood, mostly based on targeting expression of the cell adhesion epithelial marker EpCAM. They defined smaller "non-traditional" CTCs with weak/absent cytokeratin (CK) expression and point the interest of a selection-free assay from Epic Science (San Diego, CA, USA) for identification of phenotypically diverse CTCs, that may provide prognostic information in patients with metastatic CRPC.

Cheng et al. [28] presented the successive different steps in using CTCs for PCa. Until recently, clinical applications of CTCs have been limited to using enumeration as a prognostic tool in Oncology. Prior to starting cytotoxic chemotherapy, mCRPC, patients with unfavorable CTC counts (> 5 CTCs / 7.5 ml blood) had shorter overall survival (OS). Post-treatment, unfavourable CTC counts also predicted shorter OS. The OS profile followed

the variation of CTC counts from unfavorable to favorable or the reverse. This showed that CTC enumeration can provide insight to disease progression in patients and is a better predictor of OS than serum PSA changes. New applications of emerging CTC technologies concern the morphological phenotyping of CTCs and the biochemical analysis of the CTC-based biomarkers. Not only CTCs range in size [27], with nuclei smaller than 9 µm, as observed in one case of CRPC with visceral metastasis, but CTCs also form clusters, ranging from 2 to 50 cells, which might be precursors of metastases. An example of success in studying biology specific to CRPC lies in the work done looking at the androgen receptor (AR) splice variants, such as AR-V7. In CTCs from mCRPC patients, treated with new AR-targeted therapies such as abiraterone and enzulatemide, expression levels or AR-V7 were associated with a greater likelihood of developing AR-targeted therapy resistance. This could aid physicians in selecting between taxanes and AR-targeted therapy. On the other hand, many modern genomic tools has allowed genome amplification and single cell DNA sequencing. Two groups independently reported many similarities between PCa tissue samples and single CTCs. CTC-based approaches will certainly have a significant impact on the care of men with PCa.

Crona and Wang [29] commented the use of liquid biopsies to identify genomic factors associated with therapy resistance in CRPC, and detailed the use of ctDNAs for that purpose. They discussed the known complex mechanisms mediating resistance to abiraterone and enzelutamide. They mentioned the variations of AR copy number and point mutations, together with the detection of AR-V7 in different patients with parallel studies about their PSA response and clinical outcomes. They highlighted a fundamental difference between ctDNAs, composed of nucleic acid fragments not associated with cells, and CTCs which are intact cells. The detection of AR-V7 mRNA expression in CTCs from CRPC patients predicts for resistance to enzelutamide and abiraterone.

Kretschmer and Tilki [30]* reviewed the biomarkers in PCa and their current and future utility. A summary of studies investigating CTC and cfDNA predictive biomarkers in patients with mCPRC is provided.

Minciacchi et al. [31] review the current literature on the molecular profile of prostate cancer-derived EVs in model systems and patient biological fluids, in an attempt to draw some practical and universal conclusions on the use of EVs as a tool for liquid biopsy in clinical specimens. They conclude that EV analysis holds strong promises for the development of non-invasive biomarkers in patients with prostate cancer. Implementation of modern methods for EV isolation and characterization will enable to interrogate circulating EVs *in vivo.*

Hegemann et al. [32]* asked whether liquid biopsy is ready to guide therapy in advanced PCa. They compared CTCs, ctDNAs and circulating RNAs for liquid biopsies of advanced PCa. They suggested that selection methods using more specific target proteins like PSA or prostate–specific membrane antigen (PMSA) may be able to more specifically detect PCa CTCs. They summarised the clinical studies about markers of drug resistance, using molecular characterisation of CTCs and ctDNAs. They concluded that the wide variety in platforms used for both CTC and ctDNA analyses greatly impairs comparisons between studies. However, the three liquid biopsies will likely be an important component of personalised treatment strategies in the future.

Miyamoto et al. [33]* asked whether cell-free and tumor cell-based biomarkers in men with metastatic PCa might become tools for real-time precision medicine. They show prognostic and predictive value of individual circulating biomarkers in mCRPC from selected publications. After a thorough critical evaluation of the circulating biomarkers, they conclude that "we are moving closer to using liquid biopsies to tailor treatment for individual patients in real time".

Discussion and Perspectives

One of the most striking characteristics of all the cancer-devoted liquid biopsies is the long-time lapse between the first observation of any biomarker of interest and its clinical translation. For example, T. Ashworth discovered CTCs in the 1860s, while using a microscope to examine peripheral blood [20], but only in 2008, the FDA from USA cleared its clinical utilization through the unique CellSearch (Veridex) System with a precise definition of CTCs. With ctDNAs, circulating DNAs were first observed in 1948, but tumor ctDNAs were only mentioned in 1977 and confirmed in 1989. However, despite the tremendous number of researches, there is, yet, no consensus for clinical applications with standardized methodologies. Any tumor progression corresponds to a quite heterogeneous process, much more difficult to control than any well-defined physiological process. This is well illustrated by Heitzer et al. [34], when comparing the fetal cDNA circulating into mother's blood and the circulating tDNA in any cancer disease. Observed for the first time in 1997, the fetal cDNA is rather homogeneous, and 9 weeks after conception, it corresponds to about 10% of the total cDNA in the mother's blood, which is enough for a reliable characterization. Although much more favorable than the heterogeneous ctDNA, with minor but potentially important sub-populations originating from heterogeneous tumor parts, a mother's blood test for detecting fetus trisomies had to wait about two decades for achievement. Both above discussed liquid biopsies are faced with the same challenge of focusing on rare significant circulating biomarkers among a huge number of physiological blood cells or normal cDNAs, respectively. They are both highly dependent on the technological progresses for achieving a significant separation and characterization of the biomarkers. Moreover, they urgently need large scale validations with standardized methods in order to reach the clinic. Such a multicenter organization, initiated in USA is already ongoing for the more recent RNA- liquid biopsies [10].

Likewise, tEVs, ranging in size between 0.4 and 1.2 µm, were first described in 1978, in cell lines derived from patients with Hodgkin's disease. Only more than two decades later, was it formally proven that EVs are not artifacts and can affect tumor progression by promoting angiogenesis, tumor invasion, and

immune escape [13]. With regard to EVs, the general knowledge has been greatly increasing, since the Fundation of ISEV in 2012, together with numerous technological advancies for EVs isolation and characterisation. However, EVs-based liquid biopsies are still in infancy and faced with challenges concerning EVs precise identification and characterisation. It is to be noticed that rather few studies are concerned with the DNA cargo of EVs, which are not yet much involved with precise DNA modifications, such as mutations or methylation changes, already important for the other cancer liquid biopsies. It is also important to stress that the whole human body inholds a myriad of differentiated cells (10^{14}, as an order of magnitude) with about 10 times more bacteria, 100 times more viruses and 1000 times more EVs, even increasing in presence of cancer. Therefore, EVs do not suffer of the recognized scarcity of CTCs and ctDNAs present in blood. Nevertheless, deciphering EVs heterogeneity in order to focus only on the most significant EV subpopulations for cancers liquid biopsies is, indeed, the biggest challenge to solve presently.

Conclusion

Owing to my own experience with *Dictyostelium discoideum* cells-derived EVs as a model for eukaryotic EVs [35] and to the impressive increasing evidence of EVs-mediated intercellular communication, specifically during cancer progression towards metastasis, I guess that tEVs will become in the future very important players for liquid biopsies of human cancers and a help for individual precision medicine, especially for advanced prostate cancer.

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