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Exosomes: Key tools for cancer liquid biopsy

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Abstract: Precision medicine is based on the identification of biomarkers of tumor development and progression. Liquid biopsy is at the forefront of the ability to gather diagnostic and prognostic information on tumors, as it can be noninvasively performed prior or during treatment. Liquid biopsy mostly utilizes circulating tumor cells, or free DNA, but also exosomes. The latter are nanovesicles secreted by most cell types, found in any body fluid that deliver proteins, nucleic acids and lipids to nearby and distant cells with a unique homing ability. Exosomes function in signalling between the tumor microenvironment and the rest of the body, promoting metastasis, immune remodelling and drug resistance. Exosomes are emerging as a key tool in precision medicine for cancer liquid biopsy, as they efficiently preserve their biomarker cargo. Moreover, exosomes strongly resemble the parental cell, which can help in assessing the oxidative and metabolic state of the donor cell. In this respect, exosomes represent one of the most promising new tools to fight cancer. This review will discuss the clinical applications of profiling exosomal proteins and lipids by high-throughput proteomics and metabolomics, and nucleic acids by next generation sequencing, as well as how this may allow cancer diagnosis, therapy response monitoring and recurrence detection.

Introduction

Cancer is the second leading cause of death globally, but early diagnosis, prognosis and monitoring of therapy response in primary stages remain challenging (The global challenge of cancer, 2020). Late diagnosis and metastasis are a major cause of cancer deaths: the former is responsible for about 67% of solid tumor deaths (Dillekas *et al.*, 2019). Although cancer screening and detection technologies, as well as good treatment protocols are currently available, their limitations are many. There is the need for novel molecular biomarkers of the pre-invasive stage and for non-invasive bioptic procedures.

Traditional biopsies and surgical procedures are invasive and potentially harmful for complications. Their limitations include small sample size, unrepeatability and tumor inaccessibility such as for example, after surgery (Wang *et al.*, 2017; Palmirotta *et al.*, 2018). Also, samples may not reflect intrinsic tumor heterogeneity, which occurs at both genetic and phenotypic level throughout the different clinical stages (Siravegna *et al.*, 2017). The identification of metastatic biomarkers appears promising in shedding light into the mechanisms that control metastasis, to reduce cancer mortality. Notably, no metastasis-exclusive mutations have been found; instead, evidence suggests that metastasis is promoted by oncogenic driver mutations occurring via epigenetic mechanisms elicited in response to selective pressure of therapies, that interact with various physiological pathways (Patel *et al.*, 2021).

Liquid Biopsy

'Liquid biopsy' is a non-invasive test for genomic or proteomic assay of cancer-derived components in peripheral blood or body fluids (Poulet *et al.*, 2019). In the field of clinical oncology, the analysis of tumours using biomarker sources circulating in biofluids is drawing great interest as a safe alternative to solid biopsies (Quandt *et al.*, 2017) and an invaluable tool in precision medicine (Jiménez-Zenteno and Cerf, 2020). Conventional biopsies involve analysis of material obtained from biopsied samples. Advantages of tissue biopsies are the possibility to comprehensively reflect the tumor genetic profile than tissue biopsy, and analyse both the tumor and its microenvironment, facilitating the clinical decision-making. Disadvantages of tissue biopsies are invasiveness, difficulty in accessing the tumor cells, and lack of seriality when multiple analyses are necessary such as for example to monitor tumor progression. Liquid biopsies are fast, little costly minimally invasive, allow serial

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sampling, and monitoring of tumor heterogeneity. With respect to tissue biopsies, the relatively easy-to-obtain nature of bioliquids makes them an attractive alternative source for clinical application (Poulet *et al.*, 2019). Data from liquid biopsy are reproducible and highly specific, allowing to detect premalignant and early-stage cancers, to monitor response to treatment and therapy resistance (Quandt *et al.*, 2017). Cancer death rates have dropped by 26% between 1999 and 2018, according to the last update on cancer deaths (*An Update on Cancer Deaths in the United States*. Atlanta, GA: US Department of Health and Human Services, Centers for Disease Control and Prevention, Division of Cancer Prevention and Control; 2020), however, such improvement in cancer survival rates due the use of preventative measurements and precision medicine, mainly occurred in high-income countries (Girardi *et al.*, 2019). By contrast, access to tumor screening tests and cancer treatments in low-income countries are inadequate, with worse cancer outcomes and higher incidence rates (Kamaraju *et al.*, 2020). Early care and diagnosis need expensive bench top equipment and extensive training. Provided liquid biopsy option is implemented as portable user-friendly integrated microfluidic apparatuses this would render affordable and accessible the benefit of liquid biopsies even for low-income countries (Contreras-Naranjo *et al.*, 2017). Of course, before these devices can be used in clinical settings, validation and standardization of procedures and sample collection, storage and characterization must be achieved. Although liquid biopsy is not yet a routine test in clinical oncology practice, its application appears promising (Cescon *et al.*, 2020). Liquid biopsies are informative in early diagnostic screening and hopefully could predict recurrence or metastasis of malignancies. In fact, the organizations and foundations recognizing the impact of the global use of liquid biopsy have gathered in the International Liquid Biopsy Standardization Alliance (ILSA). ILSA has the scope to promote standardization-based programs and support resources and regulatory aspects in the field (Connors *et al.*, 2020).

The cancer-derived analytes can help in the early cancer diagnosis, assessment of tumor stage, identification of the micrometastases and of targets for tailored therapy, evaluation of recurrence risk, monitoring of treatment response. In patients with advanced stage disease, liquid biopsy can enable timely management of recurrences and choice of the appropriate treatment when resistance to targeted therapy is detected. In fact, liquid biopsy allows the dynamic follow-up of changes in tumour biology. Liquid biopsies may include detection of circulating tumor cells (CTCs), circulating cell-free DNA (cfDNA) and circulating tumour DNA (ctDNA), metabolites, tumor-educated platelets (TEPs), and exosomes (Exo) released by tumor cells.

Circulating tumor cells (CTCs) are cancer cells that shed from tumors and enter the circulatory system (Jimenez-Zenteno and Cerf, 2020). CTCs do not have defined morphological characteristics and are extremely rare, their frequency being lower than 10 cells/mL of blood, moreover their survival in the bloodstream is limited (Palmirotta *et al.*, 2018; Poulet *et al.*, 2019). CTC are found in the blood of patients with common tumors, but not in healthy subjects or patients with non-malignant diseases (Allard *et al.*, 2004). CTCs spread through circulation and may reside in specific permissive tissues, including bone marrow, in which case they are termed disseminated tumour cells (DTCs), always related to molecular residual disease (Sai and Xiang, 2018). CTCs are believed to be the metastatic precursors, having detached from the primary tumor site during the metastatic process that can establish metastatic loci in permissive niches (Masuda *et al.*, 2016). CTCs can be different from the primary tumor cells, having evolved under environmental and drug selection pressure (Ma and Jeffrey, 2020). CTCs exist in both single and clustered forms, which play different roles in metastasis: they form aggregates with other tumor cells or blood cells such as platelets, which protect them from the immune system (Heeke *et al.*, 2019). The CTC clustering in the circulation was shown to be also promoted by plakoglobin, a cell adhesion protein whose elevated expression is associated with higher breast cancer metastatic potential, and low survival rates (Lu *et al.*, 2015). On the other hand, diminished plakoglobin expression promoted epithelial to mesenchymal transition (EMT) (Lu *et al.*, 2015), in turn playing a pivotal role in all stages of cancer progression, and metastasis (Brabletz *et al.*, 2018). CTC can be detected in the peripheral blood of at least 10% of early-stage breast cancer and in up to 80% of metastatic breast cancer patients (Xu *et al.*, 2018). The presence of less than 5 cells/7.5 mL of blood before treatment was shown to be an independent predictor of progression-free survival in patients with metastatic breast cancer (Cristofanilli *et al.*, 2004). This result was confirmed by a meta-analysis on 50 studies with 6712 breast cancer patients, showing that therapy significantly reduced CTC-positive rate, which is in turn associated with longer progression-free survival (Yan *et al.*, 2017).

CTCs isolation is technically challenging. Current CTC capture methods are either “label-free”, i.e., based on their physicalproperties (e.g., size, density, mechanical and electrical properties) or affinity-based (e.g., utilizing antibodies against protein markers) (Ferreira *et al.*, 2016). Among the latter, an efficient biomarker for CTC capture in breast cancer patients’ blood is the epithelial membrane protein 2 (EMP2) cancer-promoting protein, highly expressed in the epithelial malignancies (Chen *et al.*, 2019). An automated enrichment and immunocytochemical detection system for CTCs CellSearch Circulating Tumor Cell Kits, as EpCAM+/CK+/CD45-cells, in 7.5 mL of blood. The most used marker is in fact theepithelial cell adhesion molecule (EpCAM) (Ferreira *et al.*, 2016; Andree *et al.*, 2016). To date, the CellSearch Circulating Tumor Cell Kit (Menarini Silicon Biosystems, Firenze, Italy) assay, using immunomagnetic technique and cytometry imaging is the only US Food and Drug Administration (FDA) approved CTC diagnostic technology (Palmirotta *et al.*, 2018). A blood test, called CancerSEEK, can detect cancer types using combined assays for genetic alterations and protein biomarkers. CancerSEEK tests were positive in a median of 70% of eight common cancer types, among which ovary, liver, pancreas (Cohen *et al.*, 2018).

Platelets were found to be ‘educated’ (Tumor-Educated Platelets, TEPs) by local and systemic conditions in a number of diseases altering their transcriptome, including cancer. In fact, platelets can sequester RNAs released by cancer cells. The RNA profiles of TEPs from cancer patients

are altered, as compared with healthy donors (In't Veld and Wurdinger, 2019). Hence, TEPRNA repertoire appears a promising biomarker source (Best *et al.*, 2017).

Cell-free nucleic acids include DNA (cfDNA), and RNAs. cfDNA is short, fragmented DNA released from apoptotic or necrotic cancer cells able to provide information on cancer cells genetic and epigenetic mutations (Chen *et al.*, 2020). However, due to its short half-life in the circulation, cfDNA applicability is limited, moreover, its sequencing needs highly sensitive assays such as next-generation sequencing (NGS) (Jimenez-Zenteno and Cerf, 2020). The development of novel techniques for the analysis of low-abundance DNA has allowed to exploit the potential of circulating tumor DNA (ctDNA) as a marker of cancer disease burden, predictor of therapy response or resistance (Cescon *et al.*, 2020). TruSight™ Oncology 500 circulating tumor DNA (Illumina, San Diego, CA) assay enables comprehensive genomic profiling of ctDNA for liquid biopsy profiling assay designed to identify known and emerging tumor biomarkers, including small variants, splice variants, and fusions. Importantly, the TruSight™ Oncology 500 measures TMB and microsatellite instability (MSI), by sequencing cancer-related genes (Wang *et al.*, 2017). However, as its abundance is low and quality often poor, ctDNA extraction needs improvement and ctDNA-based diagnostic tests still need validation.

Exosomes

A major component of the cell secretome is that of extracellular vesicles (EVs). According to the updated guidelines of the International Society for Extracellular Vesicles of 2018 (MISEV2018), EV is a collective term for the lipid-bound nanoparticle, not able to replicate, released by cells and present in all bodily fluids (Thery *et al.*, 2018). The EVs molecular cargo (i.e., proteins, lipids, nucleic acids, and metabolites) plays a signalling role between the cell of origin and the recipient cells (Raposo and Stoorvogel, 2013; van Niel *et al.*, 2018) in a diverse array of functions, thereby comprising an inter-kingdom communication (Chen *et al.*, 2021). EV secretion is an evolutionarily conserved constitutive and regulated process. After release into the extracellular space, EVs reach their target cells where their information is conveyed. Uptake of EVs seems dependent on the recipient cell and can require specific receptors, or involve direct fusion to the plasma membrane, phagocytosis, or endocytosis either clathrin- or caveolin-dependent (Stahl *et al.*, 2019). Therefore, differently from the previous MISEV guidelines, that distinguished exosomes (20–150 nm, deriving from multivesicular bodies), microvesicles (100–1,000 nm, directly budding form the plasmamembrane), and apoptotic bodies (1,000–5,000 nm) (Raposo and Stoorvogel, 2013; Lotvall *et al.*, 2014) the collective term "EVs" is now recommended. EVs can be classified based on their size and biogenesis, as apoptotic bodies (800–5000 nm), microvesicles (MV, 100–1000 nm), and exo (Exo, 30–120 nm) (Maas *et al.*, 2017). Although some markers, such as principally tetraspannins (CD9, CD81, and CD63), and annexins are enriched on Exo (Vlassov *et al.*, 2012; Dear *et al.*, 2013; Abramowicz *et al.*, 2016), there can be some overlap in EV size and markers, therefore classification should primarily based on the size and biogenesis (Thery *et al.*, 2018). It has been suggested to name EVs that do not precipitate at 10,000×*g* small EVs (sEVs, i.e., exosomes), and large EVs (lEVs, i.e., MV, apoptosomes and oncosomes) those that do (Kowal *et al.*, 2016). MV, also referred to as *ectosomes*, derive from direct budding from the plasma membrane. Apoptotic bodies are released from apoptotic or tumor cells. Exo, in particular, originate by inward budding of late endosomes/multivesicular bodies (MVB) that release their contents in the extracellular environment fusing with the plasma membrane (Kowal *et al.*, 2014). Exo are found in a vast range of biological fluids as well as in cell culture media (Rashed *et al.*, 2017; van Niel *et al.*, 2018). Exo are released by a variety of mammalian cells, thereby including cancer cells, that were shown to release more Exo than healthy cells (Sun *et al.*, 2018). Recently, Exo have gained interest as biomarkers source for cancer diagnosis and treatment, due to their valuable cargo of proteins, RNAs and microRNAs (miRNAs), DNA and lipids, along with their role as mediators of intercellular communication, immune response and disease development (Chaput and Thery, 2011; Keerthikumar *et al.*, 2016; Rashed *et al.*, 2017). EVs have become a widely studied novel source of disease biomarkers and possible therapeutic agents (Cocucci and Meldolesi, 2015; Maas *et al.*, 2017; Panfoli and Bruschi, 2020). Numerous studies have explored the immense therapeutic potential of Exo that can be collected from bodily fluids by minimally invasive procedures and can be precisely controlled by nanoscale particle assays (Kalluri and LeBleu, 2020). By proteomic and biochemical analyses, we have reported a novel characteristic of human mesenchymal stem cell (MSC) and urinary Exo, i.e., they can consume oxygen to aerobically synthesize ATP (Bruschi *et al.*, 2015; Panfoli *et al.*, 2016; Bruschi *et al.*, 2016). Such metabolic capacity appears consistent with the Exo prolonged permanence in the circulation (Bruschi *et al.*, 2018).

Exosome isolation techniques

The choice of the proper method for isolation and analysis of clinical grade Exo is challenging (Li *et al.*, 2017; Zhang *et al.*, 2020). There is still lack of standard procedures for single biological fluids for specific downstream clinical diagnostics applications. The current isolation techniques rely either on size differences between EVs (ultracentrifugation, precipitation, filtration, chromatography), or on specific surface markers (immunoaffinity-based methods) (Li *et al.*, 2020). The gold-standard is ultracentrifugation, whoseonly drawback is low Exo recovery. Precipitation is simple and fast, allowing one-step EV isolation, but lacks selectivity setting purity low. Filtration through membranes with appropriate pore size has been used, often combined with ultracentrifugation. However, clogging effects can lower Exo yields. Density gradient centrifugation (on either sucrose or iodixanol) isolates EVs based on in size, mass, and density: it is efficient in separating single EV subpopulations and EVs from contaminant aggregates. Size-exclusion chromatography (SEC) has also been used to separate Exo from protein aggregates, generally after a centrifugation or filtration step (Nordin *et al.*, 2015). Immunoaffinity-based approaches utilize antibody coated magnetic beads to

capture Exo expressing specific proteins (Kowal *et al.*, 2016). Exo isolation protocols often combine different methods. A study examined the comparative efficiencies the efficacy of six protocols among which some combinations, for the isolation of urinary Exo: the purest Exo proteins for subsequent proteomic analysis came from ultracentrifugation, or ultracentrifugation on a 30% sucrose cushion, while nano-membrane ultrafiltration centrifugal concentrator (Vivaspin 20) performed less efficiently (Alvarez *et al.*, 2012). The commercial Exo isolation kits (ExoQuick^{TM} Exosome precipitation), that use polymeric additives to precipitate Exo with a standard centrifuge, according to the Authors, need some modifications in order toyield high quality Exo RNAs (Alvarez, 2014). Consistently, a study comparing the efficiency of six commercial kits (exoEasy, ExoQuick, Exo-spin, ME kit, ExoQuick Plus and Exo-Flow) in obtaining pure Exo from healthy sera, found that the highest purity was achieved with ExoQuick Plus and exoEasy, while the lowest with ME kit and ExoQuick (Macias *et al.*, 2019). The isolation of Exo from serum by differential ultracentrifugation and by commercial Exo-spin^{TM} columns was compared recently, and it was found that Exo isolated from blood with either Exo-spin^{TM} or ultracentrifugation were similar, but yields were lower with Exo-spin^{TM} (Małys *et al.*, 2021). Also, residual matrices may influence downstream analyses (Paolini *et al.*, 2016). Optimization is needed to maximize yield and minimize impurities, in function of the biological sample. Microfluidics offers incredible potential, enabling rapid isolation and analysis of clinical grade Exo for diagnostic applications (He and Zeng, 2016). A significant step toward diagnostic accuracy for optimal clinical applications are the microfluidic-based systems, able to perform integrated on-chip exosome isolation, detection, and quantification within a single device. These on-chip multiplexed assays platforms offer several advantages in terms of timing, and sensitivity when processing of small sample volumes, as in the clinical settings (Contreras-Naranjo *et al.*, 2017). These versatile platforms for Exo separation in lab-on-a-chip format can be integrated with multiple processes in a single instrument, reducing the risk of cross-contamination (Liu *et al.*, 2017). The most used of these approaches is immunoaffinity captureof Exo by antibodies immobilized on solid surfaces. A microfluidic platform for multi-scale filtration has been developed (Chen *et al.*, 2020). Acoustic trapping of microparticles on seed microbeads against the flow direction inside a capillary been performed (Wu *et al.*, 2017). Downstream analysis to assess Exo quantity and purity typically involves size characterization by dynamic light scattering (DLS), TEM microscopy to observe morphology, surface marker analysis by immunoassays, and characterization of nucleic acid content (Oosthuyzen *et al.*, 2013; Veziroglu and Mias, 2020).

Exosomes for Liquid Biopsy

Exo are drawing attention in the field of liquid biopsy, as they bear an enormous potential to enable personalized medicine (He and Zeng, 2016; Zhou *et al.*, 2020; Li *et al.*, 2021). Already in 2010, the American Society of Clinical Oncology suggested that blood Exo would be a better choice than CTCs for monitoring cancer progression (Pawlowski *et al.*, 2010). In fact, a higher level of blood Exo is found in tumor patients, respect to healthy individuals, independently of tumor histology (Zhong *et al.*, 2021). Exo can be efficiently isolated from a small amount of human bodily fluids and easily classified by homogeneous size, cup-shaped appearance and specific protein markers which can be exploited to separate them from other subcellular vesicles (Li *et al.*, 2020). Moreover, Exo are remarkably stable in the circulation and their cargo is protected from enzymatic degradation by the lipid membrane. Moreover, the release of EVs, among which Exo, is enhanced in cancer (Inal *et al.*, 2013).

The Exo cargo can be investigated by high throughput technologies, such as proteomics and NGS (Abramowicz *et al.*, 2016; Veziroglu and Mias, 2020) and provide a lot of potential information on the parent cancer cell in a simple manner. Moreover, Exo conceivably come from heterogeneous cancer cells, therefore their cargo would not only represent the primary, but also the metastatic tumor cells, as well as the crosstalk among the cancer cells and their environment. Therefore, Exo could unveil the molecular tools enabling of the original cancer to disseminate and metastasize (Fig. 1). In fact, tumor-derived Exo are believed to promote cancer progression and metastasis, by setting the premetastatic niche priming organs for cancer metastasis (Adem *et al.*, 2020). It has been long known that melanoma-derived Exopromote the process of metastasis by modulating

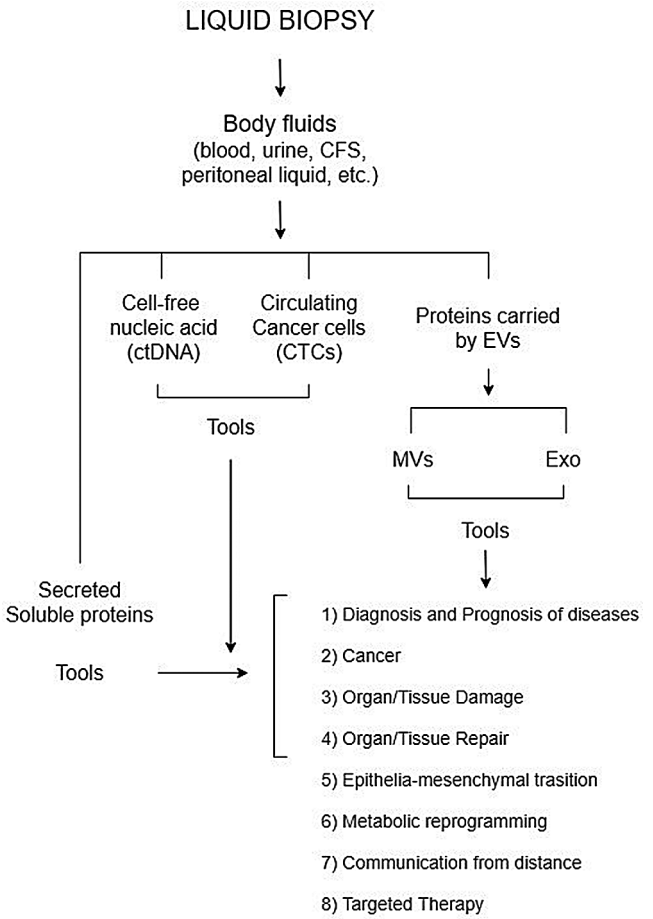

FIGURE 1. Figure illustrates the use of extracellular vesicle proteins for liquid biopsy.

the invasive capacity of malignant cells (Wu *et al.*, 2019; Gowda *et al.*, 2020). Exosomal miR-23a plays an important role in promoting tumor progression (Cui *et al.*, 2018).

State of the art

There has been a tremendous increase in publications on the use of Exo for cancer management (Zhou *et al.*, 2020). Exo are emerging as a novel tool in the field of precision medicine (Kim *et al.*, 2015) and for cancer liquid biopsy (Li *et al.*, 2021). In fact, Exo contain a wealth of information both on their membranes and as a cargo for diagnosis, response to therapy, metastasis, recurrence assessment of tumors.

The use of Exo for liquid biopsy has been demonstrated to hold a great potential for a variety of cancers. Exo are emerging as a valuable source of biomarkers in the effort to improve the prognosis of breast cancer, presently the second cause of cancer mortality in women worldwide (Meng *et al.*, 2019; Wang *et al.*, 2021). One of the recently identified Exo protein biomarkers for the diagnosis of breast cancer was CD82, a tetraspanin that is specifically expressed in Exo microdomains (TEMs) (Wang *et al.*, 2019). The first knowledge base for exosome-based biomarker discovery for breast cancer ExoBCD has recently been developed, that identified 36 promising biomarkers, among which the most promising were *IGF1R* and *FRS2* (Wang *et al.*, 2021). Exo were also shown to participate in a non-targeting effect of breast radiotherapy, i.e., promotion of secondary tumorigenesis through their action on the endothelium: Exo isolated form conditioned media from X-ray irradiated human breast cancer cells (MCF-7) promoted angiogenesis in human umbilical vein endothelial cells (HUVECs), *in vitro* (Jabbari *et al.*, 2019).

Exo appear also promising in liquid biopsy for ovarian cancer, the most lethal gynaecologic malignancy, for early diagnosis, and even in general population screening (Giannopoulou *et al.*, 2018). In fact, Exo released by the primary ovarian tumor play a pivotal role in metastatic invasion, preparing the pre-metastatic niche (Feng *et al.*, 2019). In particular, the lipidomic profile of exosomes from human ovarian cancer cells (SKOV-3) and ovarian epithelial cancer cell line (HOSEPiC), had a different and individual lipid profile (Cheng *et al.*, 2020) Moreover, in ovarian cancer Exo can be isolated from both ascites and blood.

Exo are valuable diagnostic/prognostic biomarkers for lung cancer, the leading cause of cancer-related death worldwide, due to failure to timely detect the disease the leading cause of cancer-related death worldwide, due to failure to timely detect the disease (Cui *et al.*, 2018). The abundance of miR-3182 in blood Exo was shown to be able of distinguishing non-small cell lung cancer (NSCLC) from benign lung tumours with high sensitivity and specificity, suggesting the potential use of miR-3182 as a biomarker for early NSCLC diagnosis (Visan *et al.*, 2022). A panel of six up-regulated miRNAs (miR-19b-3p, miR-21-5p, miR-221-3p, miR-409-3p, miR-425-5p and miR-584-5p) was developed for the diagnosis of lung adenocarcinoma in peripheral plasma. In particular, the first three cited miRNAs were significantly upregulated in Exo from samples from lung adenocarcinoma (Zhou *et al.*, 2017).

The potential of urinary Exo for urologic tumour lipid biopsy has also been exploited. Prostate Cancer (PCa) has a great prevalence, and there is the lack of specific prognostic tests to differentiate aggressive from indolent forms. Exo mRNA profiling has been proposed as diagnostic procedure for PCa as an alternative to PSA-based screening, which appears inadequate and responsible of over diagnosis. A quantitative lipidomic analysis of urinary Exo was performed in 15 prostate cancer patients and 13 healthy controls: nine lipid species were found to be significantly different among the two groups, besides, an alteration in sphingolipids was observed (Skotland *et al.*, 2017). A high-throughput, profiling platform for spherical nucleic acid-based miRNA (Scano-miR bioassay) was developed, able to identify the molecular signature specific for very high-risk aggressive PCa (Alhasan *et al.*, 2016).

Pancreatic cancer (PC) is a latent lethal malignancy, whose late diagnosis contributes to its poor prognosis. Several studies recently reported the promising diagnostic value of serum Exo in PC early detection and diagnosis (Lu and Risch, 2016). The potential of blood Exo RNAs in PC early detection was confirmed by a recent study showing that PC patients have a distinct blood Exo RNA signature, respect to individuals without PC. In particular, the Exo levels of *HIST2H2AA3*, *LUZP6* and *HLA-DRA* were considered a signature able to distinguish with high sensitivity and specificity PC patients from healthy controls (Wu *et al.*, 2021). Serum Exo from most PC patients were shown to express selected protein markers (CD44v6, Tspan8, EpCAM, MET and CD104, expressed in Exo of PC cell lines), and to contain miRNAs (miR-1246, miR-4644, miR-3976 and miR-4306), which significantly improve PC diagnosis (Madhavan *et al.*, 2015). A case-control study profiled eight long RNAs (FGA, KRT19, HIST1H2BK, ITIH2, MARCH2, CLDN1, MAL2 and TIMP1) from blood extracellular vesicles from subjects with pancreatic ductal adenocarcinoma (PDAC), respect to controls without PDAC: by a support vector machine algorithm, the study identified a d-signature able to identify PDAC at a resectable stage, which could sensibly improve the PDAC prognosis (Yu *et al.*, 2020). Interestingly, urine Exo also were shown to contain several markers associated with PC (Madhavan *et al.*, 2015). The possibility to find diagnostic biomarkers that are not exclusive of the urinary tract and the kidney in the urinary Exo, holds an immense potential for liquid biopsy (Franzen *et al.*, 2016; Panfoli, 2017). Long non-coding RNA (lncRNA) CCHE1 plays a role in ovarian cancer metastasis, as demonstrated by its silencing, which suggests its potential role as a biomarker for its diagnosis (Chen *et al.*, 2020).

The potential of Exo as biomarker source for therapeutic applications has been highlighted also for liver diseases. Exo are released by hepatocytes as part of a crosstalk among them and non parenchymal cells (hepatic stellate cells, liver sinusoidal endothelial cells, and cholangiocytes). In liver, Exo function in regulating regeneration upon liver injury but can also play a role in priming the tumour microenvironment promoting cancer progression (Sung *et al.*, 2018).

CRC-derived Exo were shown to promote metabolic reprogramming and immuno-suppressive signals facilitating the formation of the pre-metastatic niche (Mannavola *et al.*, 2019). Therefore, as CRC cells secrete Exo that convey

tumorigenic signals to stromal cells, Exo represent a promising target of liquid biopsy also for colorectal cancer (CRC) since its early phases.

Melanoma is one of the most aggressive cancers, with growing incidence rates. Lack of markers for the early detection of the disease still prevents proper treatment. Melanoma-derived Exo are known to promote the process of metastasis by setting the pre-metastatic niche (Gowda *et al.*, 2020). A study isolated simultaneously CTCs and Exo from blood samples using the dual-utilization OncoBean (DUO) device and found that both express melanoma-associated genes (Kang *et al.*, 2020). Moreover, blood of melanoma patients possessed three times more exosomal protein mL^{-1} than healthy donors (Kang *et al.*, 2020).

Exo-based diagnosis appears promising also for brain cancer, as it could allow bypassing tissue biopsy, especially in patients with high surgical risk. Exo isolated from serum of 96 high-grade glioma patients could reliably detect the tumorigenic epidermal growth factor receptor variant III (*EGFRvIII*), typical of high-grade gliomas (Manda *et al.*, 2018).

Exo from dendritic cells (DCs), named dexosomes were shown to transfer the antigen-MHC complexes to naïve DCs, as they express the major histocompatibility complex class I/II (MHC I/II). This ability has been exploited to *ex vivo* induce DCs to present tumorantigens, and use them as cancer vaccines. These were shown to be safe for administration into patients to induce a tumor-specific immune response, in two phase I and one phase II clinical trials. In particular, in malignant melanoma, dexosomes elicited NK-related immune responses (Bol *et al.*, 2019).

Advantages of Exosomes for Liquid Biopsy

The use of Exo for cancer liquid biopsy has several advantages, respect to TEPs, CTCs and ctDNA (Li *et al.*, 2021). In fact, TEP RNAs can be affected by drugs or immunological status. CTCs are phenotypically heterogeneous and require rapid processing after isolation (Abramowicz *et al.*, 2016). By contrast, is the availability of integrated microfluidic platforms for cheap and rapid separation of Exo, which require small sample volumes and allowing automation (Li *et al.*, 2020). Limitations of ctDNA regard its chemical characteristics and its variable concentration in blood, in fact it accounts for a fraction of cfDNA, which mostly derives from non-malignant cells. ctDNA can also be influenced by multiple tumor, and anatomical factors, as well as unrelated somatic mutations (Li *et al.*, 2021). Notably, it is possible to detect cancer cell DNA aberrations also using Exo. A disadvantage in the use of Exo is the technical difficulty isolation and detection techniques, and the impossibility to image them acquiring morphological information as can be done on CTCs (Masuda *et al.*, 2016).

The most important advantage of using Exo for liquid biopsies is the possibility to identify all at the same time proteins, nucleic acids and lipids (Cheng *et al.*, 2020) specifically packed so to convey an integrated information for the homing at distance from the primary tumor site, meantto build the premetastatic niche (Masuda *et al.*, 2016), also reflecting the stromal along with the malignant cells. Furthermore, in this respect Exo offer the possibility to identify surface *vs.* cargo proteins. Notably, the proteins the Exo carry mediate a non-conventional form of protein secretion, as they lack a signal peptide (Inal *et al.*, 2013) and interestingly among them the five complexes of the redox chain were found functionally expressed (Bruschi *et al.*, 2015; Panfoli *et al.*, 2016; Bruschi *et al.*, 2016). Consistently, ExoCarta (http://www.exocarta.org), reports the expression of several of the subunits of the F_1F_o-ATP synthase and of the respiratory chain complexes. Human urinary and umbilical cord mesenchymal stem cell Exo produce ATP and display a respiratory ability independent of whole mitochondria (Bruschi *et al.*, 2015; Panfoli *et al.*, 2016; Bruschi *et al.*, 2018). Such ecto-ATP can be metabolizedto adenosine by ectonucleotidases such as CD73, expressed on extracellular vesicles, presumably as a part of inflammatory signalling mechanism (Schneider *et al.*, 2021). Therefore, besides bearing a potential for biomarker for cancer liquid biopsy, Exo can convey information on the bioenergetic and oxidative state of the individual: this was shown to be the case for Mesenchymal Stem Cell exosomes from preterm and term newborns (Panfoli *et al.*, 2016). In fact, stem cells produce Exo that promote angiogenesis and appear to be a promising tool for the treatment of ischemic diseases (Babaei and Rezaie, 2021).

Conclusion

The identification and selection of valuable biomarkers from biofluids remains a challenge. Liquid biopsy is a novel, minimally invasive emerging technique alternative to tissue biopsy for malignancies (Martins *et al.*, 2021), for the advantage of allowing serial sampling. In particular, the potential of Exo for liquid biopsy is undebatable, due to their unique potential in revealing the role of a specific combination of lipids, nucleic acids and surface/cargo proteins in cancer dissemination and metastasis, the most important cause of cancer death (Dillekas *et al.*, 2019). There is the need for standardization of Exo isolation and storage protocols from the different body fluids, to reach analytical consistency, and for validation from clinical trials to support applicability in monitoring cancer. Nonetheless, it can be foreseen that new/standardized methods for Exo capture and analysis of Exo from body fluids will be implemented, and, after demonstration of feasibility, their use for liquid biopsy may hopefully be inserted into guidelines.

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