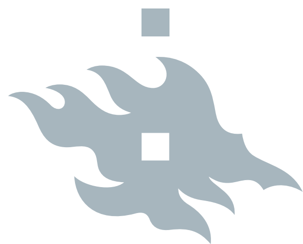

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Extracellular Vesicles: Prospects in Prostate Cancer Biomarker Discovery and Drug Delivery

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Extracellular Vesicles: Prospects in Prostate Cancer Biomarker Discovery and Drug Delivery

by

Elisa Lázaro Ibáñez

ACADEMIC DISSERTATION

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Helsinki 2017

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Abstract

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Extracellular vesicles (EVs), including exosomes, microvesicles, and apoptotic bodies are a heterogeneous population of membrane particles released by cells to the extracellular space and into biofluids during normal physiological and pathological processes. EVs have been recognized as powerful vehicles for intercellular communication due to their capacity to transfer lipids, proteins, and nucleic acids, thereby influencing the properties and functions of recipient cells. Cells generate EVs with a unique composition based on their characteristics, which has a special relevance in the study of diseases such as cancer. Since specific molecular signatures can be passed on to tumor EVs, they are prime candidates for implementation as cancer biomarkers and in the delivery of therapeutics. Thus, exhaustive research is currently targeted towards elucidating the role of EVs in cell-to-cell communication and their therapeutic and diagnostic use.

This thesis aims at broadening our understanding of the applicability and functional relevance of the use of EVs as prostate cancer biomarkers and therapeutic delivery vehicles. First, the practical use of EVs as a source of nucleic acid biomarkers in prostate cancer was assessed by exploring the DNA and RNA content of vesicles. Genomic DNA analysis of apoptotic bodies, microvesicles, and exosomes were performed to detect mutations within the EV cargo. The results were validated in plasma EVs of prostate cancer patients, from which the presence of prostate cancer-relevant genes was identified. Next, the prostate cancer-specific messenger RNA signatures of microvesicles and exosomes were analyzed. Unique nucleic acid signatures distinctive for the cell origin were found in the form of differential levels of mRNA transcripts from EV subpopulations. Overall, the nucleic acid content of EVs provided a new source of diagnostic information that could contribute to early prediction and diagnosis of prostate cancer, especially if combined. The role of EV-mediated intercellular communication was shown by comparing the uptake efficiencies and functional effects of EVs from prostate cancer cells of different metastatic status with non-cancer EVs. Additionally, the ability of EVs to carry and deliver a chemotherapeutic drug, together with their cytotoxic effects on prostate cancer cells were also analyzed. While EV uptake, in general, was an active and continuous process, the internalization rate and the subsequent functional effects of EVs on recipient cells differed based on the vesicle origin. EVs derived from cells of a metastatic source were more efficiently internalized than primary prostate cancer or benign prostate epithelial cell-derived EVs. Similarly, those EVs also induced a more proliferative and migratory phenotype in the recipient cells. Applying prostate cancer EVs in the *in vitro* delivery of paclitaxel to prostate cancer cells, resulted in an enhanced cytotoxic effect of paclitaxel mediated by EV delivery compared to the free drug.

In summary, the results presented in this thesis support the concept that EVs can be utilized in both biomarker discovery and drug delivery fields as multifunctional tools for diagnosis and treatment of diseases such as prostate cancer. The studies presented here will also contribute to set the bases for further functional analysis of the roles of EVs in cell-to-cell communication. This new era of research could lead to faster, non-invasive, and more individualized diagnosis and improved treatments tailored to the specific needs of the patients.

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One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away. — Stephen Hawking

To my parents

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List of original publications

This thesis is based on the following publications, which are referred to in the text by roman numerals (**I-IV**).

I **Lázaro-Ibáñez E.**, Sanz-Garcia A., Visakorpi T., Escobedo-Lucea C., Siljander P., Ayuso-Sacido Á*., Yliperttula M*. Different gDNA content in the subpopulations of extracellular vesicles: Apoptotic bodies, microvesicles, and exosomes. Prostate. 74(14):1379-90, 2014.

II **Lázaro-Ibáñez E.**, Lunavat T.R., Jang SC., Escobedo-Lucea C., Oliver-De la Cruz J., Siljander P., Lötvall J*., Yliperttula M*. Distinct prostate cancer-related mRNA cargo in extracellular vesicle subsets from prostate cell lines. BMC cancer. 17 (1):92, 2017.

III **Lázaro-Ibáñez E.**, Neuvonen M., Takatalo M., Thanigai Arasu U., Capasso C., Rhim JS., Rilla K., Yliperttula M., Siljander P. Metastatic state of parent cells influences the uptake and functionality of prostate cancer cell-derived extracellular vesicles. *Submitted*.

IV Saari H*., **Lázaro-Ibáñez E***., Viitala T., Vuorimaa-Laukkanen E., Siljander P., Yliperttula M. Microvesicle- and exosome- mediated drug delivery enhances the cytotoxicity of Paclitaxel in autologous prostate cancer cells. Journal of Controlled Release. 28;220, (Pt B):727-37, 2015. *Equal contribution.

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Personal contribution

Publication I

The author contributed to the experimental design of the study, performed all the experiments, contributed to the analysis and interpretation of the data, and wrote the manuscript with co-authors.

Publication II

The author conceived and designed the study, conducted all the experiments, and collected and analyzed the data. The author wrote the manuscript with co-authors.

Publication III

The author conceived and designed the study with contributions from co-authors. The author performed the extracellular vesicle isolation and characterization experiments, cell uptake, cell cycle, proliferation, and migration studies. The author co-analyzed the data, and wrote the manuscript with co-authors.

Publication IV

The author contributed to the experimental design of the study, performed the extracellular vesicle isolation and characterization experiments, and cell studies including uptake. The author interpreted and co-analyzed the data, and co-wrote the manuscript with co-authors.

Additional publications

List of additional publications not included in this thesis.

García-Romero N*., Carrión-Navarro J*., Esteban-Rubio S*., **Lázaro-Ibáñez E.**, Peris-Celda M., Alonso M.M., Guzmán-DeVilloria J., Fernández-Carballal C., Ortiz de Mendivil A., García-Duque S., Escobedo-Lucea C., Prat-Acín R., Belda-Iniesta C., Ayuso-Sacido A. DNA sequences within glioma-derived extracellular vesicles can cross the intact Blood-Brain Barrier and be detected in peripheral blood of patients. Oncotarget. 8(1): 1416-28. 2016

Mustonen A., Nieminen P., Joukainen A., Jaroma A., Kääriäinen T., Kröger H., **Lázaro-Ibáñez E.**, Siljander P.R-M., Kärjä V., Härkönen, K., Koistinen, A., and Rilla, K. First *in vivo* detection and characterization of hyaluronan-coated extracellular vesicles in human synovial fluid. J Orthop Res. 2016.

Smith Z., Lee C*., Rojalin T*., Carney R*., Hazari S., Knudson A., Lam K., Saari H., **Lázaro-Ibáñez E.**, Viitala T., Laaksonen T., Yliperttula M., Wachsmann-Hogiu S. Single exosome study reveals subpopulations distributed among cell lines with variability related to membrane content. J Extracell Vesicles. 7;4:28533, 2015.

Molina I., **Lázaro-Ibáñez E.**, Pertusa J., Debón A., Martínez-Sanchís J.V., Pellicer A. A minimally invasive methodology based on morphometric parameters for day 2 embryo quality assessment. Reprod BioMed Online. 29(4):470-80, 2014.

Oliver-De La Cruz J., Carrión-Navarro J., García-Romero N., Gutiérrez-Martín A., **Lázaro-Ibáñez E.**, Escobedo-Lucea C., Perona R., Belda-Iniesta C., Ayuso-Sacido A. SOX2+ cell population from normal human brain white matter is able to generate mature oligodendrocytes. PLoS ONE. 5;9(6): e99253. 2014.

Perez-Garcia A., Carrion-Navarro J., Bosch-Fortea M., **Lázaro-Ibáñez E.**, Prat-Acin R., Ayuso-Sacido A. Genomic instability of surgical sample and cancer-initiating cell lines from human glioblastoma. Front Biosci. 1;17:1469-79, 2012.

*Equal contribution

Abbreviations

ABs	Apoptotic bodies
Alix	ALG–2-interacting protein X
CAFs	Cancer-associated fibroblast
CFSE	Carboxyfluorescein succinimidyl ester
Cryo-EM	Cryo-electron microscopy
DiI	$DiIC_{18}(5)$-DS
DiO	SP-$DiOC_{18}(3)$
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal sorting complex required for transport
EVs	Extracellular vesicles
EXOs	Exosomes
FASN	Fatty acid synthase
FBS	Fetal bovine serum
gDNA	Genomic deoxyribonucleic acid
HIFs	Hypoxia inducible factors
LOs	Large oncosomes
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem/stromal cells
mtDNA	Mitochondrial DNA
MVB	Multivesicular bodies
MVs	Microvesicles
NTA	Nanoparticle tracking analysis
PCA-3	Prostate cancer antigen 3
PCR	Polymerase chain reaction
PI	Propidium iodide
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
PTEN	Phosphatase and tensin homolog
PtX	Paclitaxel
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
SFM	Serum free media
TEM	Transmission electron microscopy
TP53	Tumor protein p53
TSG101	Tumor susceptibility 101
UPLC	Ultra performance liquid chromatography

1 Introduction

The current diagnosis and development of cancer treatments is increasingly dependent on the understanding of the patient's unique molecular and genetic characteristics. Predictive cancer biomarkers are crucial tools in personalized medicine, as they enable the selection of patients that are most likely to benefit from targeted therapies (Schork, 2015). A major challenge in personalized medicine is the identification of biomarkers with diagnostic and prognostic value. Despite the increased in research devoted to identifying new cancer biomarkers only a limited number have been approved for clinical use by the Food and Drug Administration (FDA) (Goossens et al., 2015). New cancer biomarkers are needed in order to predict patient survival, monitor the disease progression, and predict the response to therapies. Additionally, the development of novel cancer treatments and the customization of current therapeutic strategies are also important in order to increase the health outcomes of cancer patients. Drug delivery systems have been extensively used in the treatment of cancer as they improve the pharmacological properties of the drugs (Allen and Cullis, 2004). However, important limitations including reduced specificity, targeting, biocompatibility, and limited ability to penetrate tissues, are major drawbacks of some of the current drug delivery systems (Fais et al., 2016).

The analysis of cancer-derived extracellular vesicles (EVs) has given new insights into the biomarker discovery and drug delivery fields. EVs are secreted as nature´s intercellular transport vesicles into blood, urine, and other biofluids, and thus, they interact with many diverse cell types, mediating physiological and pathological functions (Raposo and Stoorvogel, 2013; Yáñez-Mó, Siljander et al., 2015). The discovery that EVs carry an array of bioactive molecules such as lipids, proteins, and nucleic acids that can be shuttled between cells demonstrates the relevant participation of EVs in the complex framework of cell signaling and communication (Barry et al., 1997; Deregibus et al., 2007; Ratajczak et al., 2006; Valadi et al., 2007). EVs released by cancer cells are known to participate in tumor development and in the acquisition of the cancer hallmark capabilities, having a clear impact on cancer-sustaining processes such as angiogenesis, tumor proliferation, invasion, and metastasis (Kanada et al., 2016; Rak, 2013). On that basis, EVs can be harnessed for cancer diagnostics, prognostics, and treatment monitoring. EV levels and their cargo, particularly proteins, RNA, and DNA, vary in different conditions and disease stages, reflecting the status of the cancer cells, thereby providing a snapshot of the tumor. EVs have also attracted considerable interest for their potential use as effective, targeted, and non-immunogenic therapeutic agents and drug delivery carriers (Ha et al., 2016; Stremersch et al., 2016a). Thus, elucidation of the molecular mechanisms that underlie the release and uptake of different EV subtypes, together with new strategies to specifically target cancer cells by EVs, will be essential to increase our understanding of the role of EVs in intercellular communication. This knowledge will also be an advantage for the design of engineered drug delivery vehicles by using EVs as a blueprint.

This thesis aimed to assess the use of EVs as a potential source of biomarkers and as therapeutic drug carriers using prostate cancer as a disease model. First, the nucleic acid content of EV subpopulations, including genomic DNA (gDNA) and messenger RNA (mRNA), was explored by examining specific prostate cancer mutations and transcript signatures in EV subsets, and further evaluated for their possible utility in prostate cancer diagnosis. Next, the differences of prostate cancer and non-cancer derived EVs in cell-to-cell communication were examined, and the feasibility of autologous prostate cancer cell-derived EVs in the *in vitro* delivery of paclitaxel was tested.

2 Literature review

2.1 Extracellular vesicles

2.1.1 The emergence of EVs in life sciences

The first work on EVs dates from the mid-1940s (Chargaff and West, 1946), with the discovery that platelet-free plasma contained a clotting factor that could be isolated by ultracentrifugation, which more than twenty years later was described as “platelet dust” (Wolf, 1967). Initial observations also included matrix "vesicles" identified during bone calcification (Anderson, 1969). Until the early 1980s, EV studies were mainly limited to the understanding of the cell function without considering soluble factors or molecules released to the extracellular medium. During the 1980s, another vesicular secretion pathway was suggested in which vesicles formed within multivesicular bodies (MVBs) were secreted to the extracellular space as a form of cellular waste release (Harding and Stahl, 1983; Pan and Johnstone, 1983). Indeed, the term “exosome” was first introduced when referring to membrane fragments isolated from biofluids (Trams et al., 1981), and proposed for vesicles of endosomal origin in the late-1980s (Johnstone et al., 1987).

This early work inspired a new era of vesicle research prospering in the 2000s with EVs as a main focus. Our understanding of the relevance of EVs as multifunctional mediators of cell-to-cell communication has exponentially increased over the last decade and the EV field has rapidly expanded to explore the different areas where EVs may participate. Of special interest to the EV field is the marked relation of EVs to the different aspects of cancer development and their novel application as drug delivery vehicles. **Figure 1** shows the evolution and the fast-growing interest in research focusing on the use of EVs in cancer biomarker discovery and drug delivery.

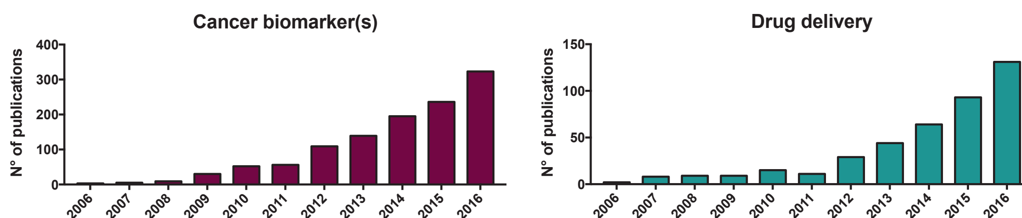

Figure 1. Bar graphs showing the number of publications referring to the use of EVs in cancer biomarker and drug delivery. An advanced search was performed in the Web of Science (accessed 16.01.17) to find, for each year, from 2000 to 2016, all articles in English with the terms “extracellular vesicles, ectosome(s), exosome(s), microvesicle(s), microparticle(s), apoptotic bod(ies), prostasome(s), oncosome(s)” and “cancer biomarker(s)” or “drug delivery”. The term microparticle(s) was excluded from the drug delivery search since it has a different meaning in pharmacological settings. The figure is intended to show the expansion of the use of EVs as cancer biomarkers and drug delivery vehicles. No comparison to other fields was made, since the number of publications was not normalized to the total number of scientific life-sciences publications per year.

The terminology used to refer to EV subpopulations is diverse and has previously been based on the EV size and cellular origin, or EV presence outside or inside the cells. The term EVs was proposed to encompass all types of membrane vesicles released into the

extracellular space, regardless of their differences in biogenesis and composition (György et al., 2011). However, there is no consensus in either terminology (Gould and Raposo, 2013) or EVs classification (Witwer et al., 2013), mainly because the present purification methods often result in mixtures of heterogeneous vesicle subsets. Despite the difficulty in isolating and characterizing EVs in a standardized manner, the general criteria classify EV subpopulations based on their biogenesis. Additional factors such as density, cellular origin, size, and cargo, have also been used to classify EVs (Colombo et al., 2014). In the following review of literature, different characteristics related to EV formation, composition, and functions are described.

2.1.2 Biogenesis and secretion of EVs

The diversity, abundance, and molecular composition of EVs reflect not only the state and identity of their parent cells, but also the diversity of the biogenetic pathways (Théry et al., 2009). Currently, EVs are most often classified into three main categories based on their biogenesis: exosomes (EXOs), microvesicles (MVs), and apoptotic bodies (ABs).

The biogenesis of EXOs is initiated by inward invagination of the cellular plasma membrane forming the early endosomes (**Figure 2**). While the early endosomes mature into late endosomes, the membrane undergoes a series of inward invaginations leading to the formation of ~40–100 nm intraluminal vesicles (ILVs) engulfing cytosolic components and incorporating peripheral and transmembrane proteins. The endosomal sorting complexes required for transport (ESCRT), including ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, ALG-2-interacting protein X (Alix), and tumor susceptibility 101 (TSG101), trigger the formation of ILVs in late endosomal multivesicular bodies (MVBs) (Huotari and Helenius, 2011; Hurley and Hanson, 2010; Raiborg and Stenmark, 2009; Thery et al., 2001). The MVBs can either be targeted for degradation in the lysosome by ubiquitin-dependent interactions with ESCRT-0, ESCRT-I, and ESCRT-II (Johnstone et al., 1987), or may fuse with the plasma membrane in an ubiquitin-independent manner, secreting the EXOs (Théry et al., 2002). Several proteins participate in this process, including Alix, a protein that interacts with ESCRT-III binding syntenin, providing a distinctive signature to avoid lysosomal degradation (Baietti et al., 2012; Hurley and Odorizzi, 2012). Also, GTPases such as Rab5, Rab7 (Baietti et al., 2012; Vanlandingham and Ceresa, 2009), and Rab11 (Savina et al., 2002) regulate endocytic trafficking and cargo segregation, while Rab27 and Rab35 regulate the secretion of EXOs (Hsu et al., 2010). Other proteins involved in the EV biogenesis and trafficking of biological membranes include soluble NSF attachment protein receptors (SNAREs) (Chen and Scheller, 2001), ADP-ribosylation factor 6 (ARF6) and its effector phospholipase D2 (Ghossoub et al., 2014), and TSG101 and vacuolar protein sorting 4 (Bishop and Woodman, 2000; Buschow et al., 2005). The mechanisms and molecules that regulate the fusion of MVBs with the plasma membrane are not yet fully understood. The ESCRT machinery is considered to be mainly involved in sorting proteins destined for lysosomal degradation, whereas sorting cargo into the MVBs and EXOs is ubiquitin- and ESCRT- independent, and other molecules such as CD63 may participate in this process (van Niel et al., 2011). Alternatively, another ESCRT-independent mechanism for EXO biogenesis dependent on the sphingolipid ceramide has been proposed (Trajkovic et al., 2008). Ceramide contributes to the inward budding of the plasma membrane generating another ILV population destined for secretion as EXOs (Trajkovic et al., 2008). However, the manner of cargo loading in the ceramide-dependent pathway is so far unknown.

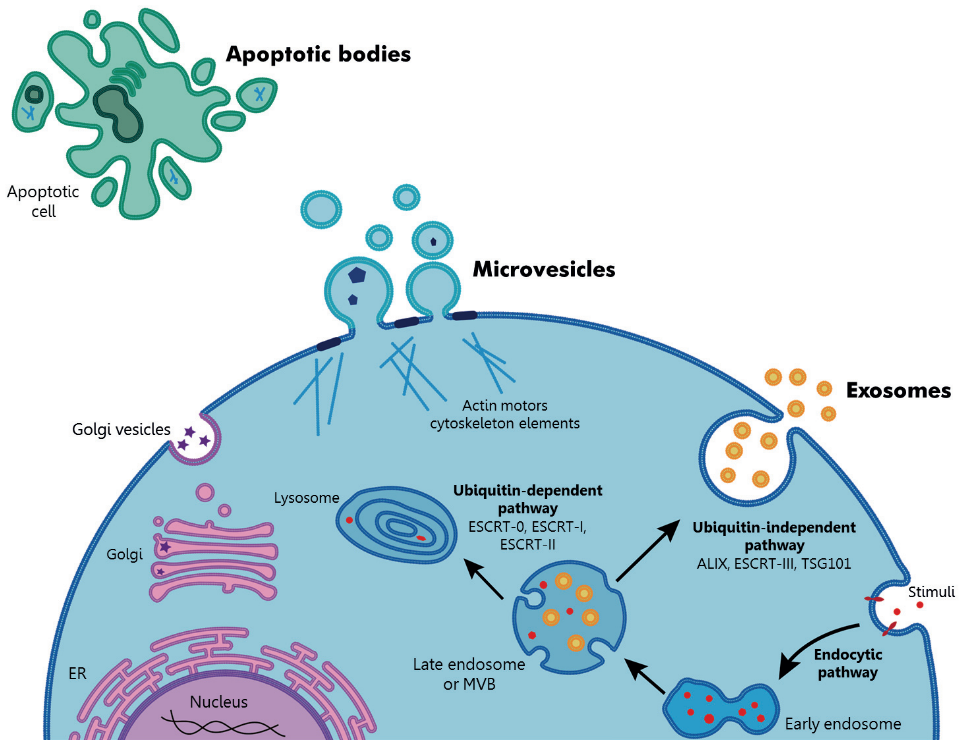

Figure 2. Schematic representation of the biogenesis of the EV subpopulations. **Exosome** formation is initiated by endocytosis. The endocytic vesicles first mature to early endosomes, and then into late endosomes or multivesicular bodies (MVBs). After interaction with components from the endosomal sorting complex (ESCRT), via the ubiquitin dependent pathway, MVB constituents can be sorted to lysosome for degradation. In the ubiquitin-independent pathway, other components such as ESCRT-III, ALG-2-interacting protein X (Alix), and tumor susceptibility gene 101 (TSG101) are involved. If Alix binds to the MVB components, Rab proteins will intervene and the cargo will be released by the fusion of MVBs with the plasma membrane. **Microvesicles** are formed by outward budding, fission, and shedding of the plasma membrane. The process is regulated by the processing of cytoskeleton as well as lipid dynamics, including lipid rafts that promote membrane curvature. **Apoptotic bodies** are EVs of varying size generated during programmed cell death after nuclear condensation, cell shrinkage, and fragmentation. Other vesicles such as Golgi transport vesicles contribute to the vesicular secretome of cells, but these are not EVs.

Outward budding or shedding and fission of the plasma membrane form blebs of varying size (100–1000 nm), termed MVs, which are released to the extracellular space (Théry et al., 2009) (**Figure 2**). During MV budding, lipid rafts including ceramide, regulatory proteins, and cytoskeleton elements, can promote membrane curvature and extensive cytoskeletal changes, promoting the formation of MVs (Bianco et al., 2009; McConnell and Tyska, 2007). Intracellular calcium changes and transporters are involved in the maintenance of membrane phospholipid asymmetry (Zwaal and Schroit, 1997), and so are likely involved in MV biogenesis (Piccin et al., 2007; Théry et al., 2009) although the regulatory mechanisms responsible for MV formation are still unknown. The formation of MVs has been speculated to share common features with the EXO biogenesis. For instance,

the ESCRT component TSG101 is known to interact with arrestin domain-containing protein 1, leading to the evagination of the plasma membrane and release of MVs (Kuo and Freed, 2012; Nabhan et al., 2012). Additionally, the interaction between actin-remodeling proteins such as the ARF6, and components of the Rho signaling pathway have been shown to participate in the MV formation (Li et al., 2012). Thus, the key molecules participating in the biogenesis of MVs and EXOs seem to be at least partially shared. Another category of EVs termed large oncosomes (LOs) was first described in relation to prostate cancer cells (Di Vizio et al., 2009). LOs are typically large EVs derived from cancer cells that have acquired a migratory and metastatic amoeboid phenotype. They usually range from 1-10 µm and contain oncogenic material (Minciacchi et al., 2015). The activation of protein kinase B (AKT) and epidermal growth factor receptor (EGFR) pathways, together with the silencing of the cytoskeletal regulator diaphanous related formin-3, promotes the release of LOs (Di Vizio et al., 2012; Di Vizio et al., 2009). Although MVs and LOs share some similarities, including the presence of molecules such as ARF6 involved in MV and LO biogenesis (Di Vizio et al., 2012; Muralidharan-Chari et al., 2009), it is not yet clear if LOs are a subtype of cancer-derived MVs or an entirely separate vesicle category.

During programmed cell death, after nuclear condensation, cell shrinkage, and fragmentation, cells generate variable sized ABs. They range from 50–5,000 nm and are packed with fragmented nuclear and cytoplasmic components from the dying cell (György et al., 2011; Nawaz et al., 2014), which explains their heterogeneity with regard to content, size, and morphology (**Figure 2**). The actin-myosin system has been proposed as the contractile force that drives the blebbing, and the Rho effector protein ROCK1, which participates in the phosphorylation of myosin, contributes to the formation of membrane blebs and ABs (Coleman et al., 2001). Caspase proteases are also involved in the externalization of phosphatidylserine that acts as an "eat me" signal, mediating the recognition of ABs by phagocytic cells (Fadok et al., 1992; Martin et al., 1995).

The complexity and overlap of pathways involved in EV biogenesis generates different explanations about the mechanisms involved in the formation and composition of EV subpopulations. However, while ABs are generated during programmed cell death, MVs and EXOs are formed from living cells during normal physiological and pathological processes.

2.1.3 Molecular composition of EVs

EVs carry a wide variety of molecules as their cargo and partly resemble their parent cells (Théry et al., 2009). EVs are composed of a lipid bilayer enclosing membrane-associated and soluble proteins, nucleic acids, lipids, and other metabolites (**Figure 3**). The specific composition of EVs protects their internal cargo from enzymatic degradation, and thus preserves them as a source of biological information. Three databases, including EVpedia "***evpedia**.info*" (Kim et al., 2015), Vesiclepedia "*www.**microvesicles**.org*" (Kalra et al., 2012), and Exocarta "*www.**exocarta**.org*" (Mathivanan et al., 2012), contain information about the currently known components of EVs.

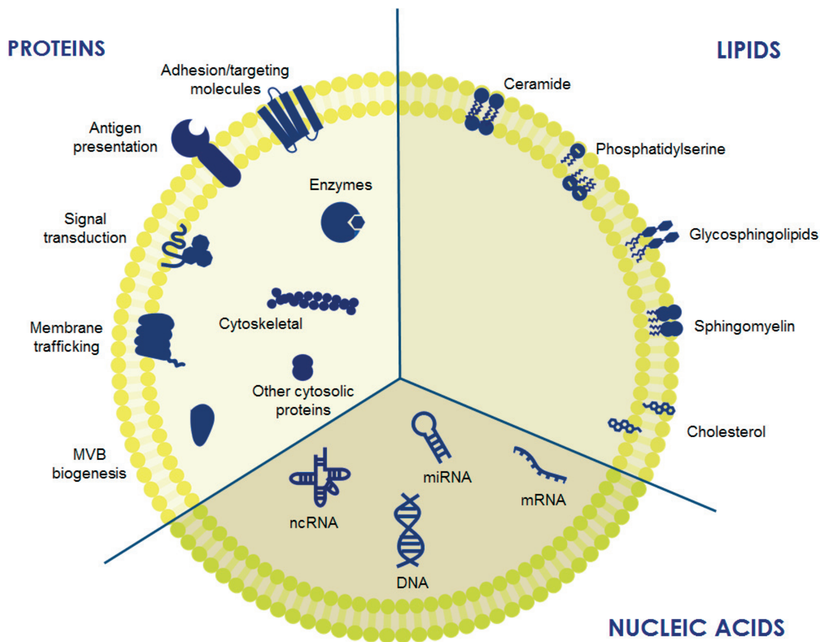

Figure 3. General representation of the EV composition. The molecular cargo of EVs consists of proteins, nucleic acids, lipids, and other metabolites. The EV composition widely varies based on the EV subpopulation and the cellular origin. The main subclasses of molecules identified in EVs based on their cellular location and function include: **proteins** e.g., adhesion or targeting molecules, enzymes, antigen presentation, signal transduction, cytoskeletal proteins, membrane trafficking, multivesicular bodies (MVB) biogenesis, and other cytosolic proteins; **lipids** e.g., ceramide, phosphatidylserine, glycosphingolipids, sphingomyelin, cholesterol; and **nucleic acids** e.g., DNAs, messenger RNAs (mRNAs), microRNAs (miRNAs), and other non-coding RNAs (ncRNAs).

Due to the endosomal and plasma membrane origin of EVs, they display within their cargo features of ILVs, MVBs, and cellular plasma membrane. EVs are enriched in several protein components, some of them known as "common EV markers". Tetraspanins such as CD63, CD9, CD81, CD82; membrane trafficking and lipid raft associated proteins such as annexin, flotillins, Rabs; and cytosolic proteins including heat shock proteins, TSG101, and Alix are some well-known examples of EV protein markers (Fevrier and Raposo, 2004; Mathivanan et al., 2010; Witwer et al., 2013; Zöller, 2009). Lipid-wise, EVs are known to be enriched in glycosphingolipids, sphingomyelin, cholesterol, phosphatidylserine, and ceramide compared to their parent cells (Llorente et al., 2013; Record et al., 2014; Trajkovic et al., 2008; Wubbolts et al., 2003). During the EV biogenesis, other metabolites such as sugars, nucleotides, amino acids, enzymatic cofactors, and regulatory molecules are also incorporated into the vesicles (Altadill et al., 2016; Mayr et al., 2009; Zhao et al., 2016), although only limited reports describing the metabolites present in different EVs are currently available.

A major discovery in the EV field was the finding that functional RNA species, including mRNAs and microRNAs (miRNAs) were present in EVs, and that the EV-associated RNA could be horizontally transferred to recipient cells and efficiently translated by them (Deregibus et al., 2007; Ratajczak et al., 2006; Valadi et al., 2007). Since these

discoveries, several other groups have identified RNA molecules in EVs isolated from cell cultures and biofluids (Quinn et al., 2015; Zhang et al., 2015a). It has also been shown by deep sequencing analysis that EVs contain additional non-coding RNAs molecules (ncRNAs) other than miRNAs. These RNA species include small interfering RNA (siRNA), vault RNA, transfer RNA, mitochondrial RNA, long non-coding RNA (lncRNA), Y RNA, piwi-interacting RNA, and small nucleolar RNA (Ahmed et al., 2014; Bellingham et al., 2012; Lunavat et al., 2015; Nolte-'t Hoen et al., 2012; Vojtech et al., 2014). However, the transfer and effect of these RNAs on recipient cells has not been investigated. A more recent breakthrough finding was the discovery of DNA molecules in EVs isolated from cell culture supernatants and biofluids. Several DNA molecules including mitochondrial DNA (mtDNA) (Guescini et al., 2010), single-stranded DNA (Balaj et al., 2011), and double-stranded DNA fragments (Cai et al., 2013; Waldenstrom et al., 2012) have been identified in various EVs.

Based on the simultaneous biogenesis of several EV subpopulations, and the limited efficacy of the currently available isolation methods to obtain pure EV subsets, the exact composition and characteristics of MVs and EXOs are not yet fully understood. The majority of protein markers are conserved across EV subsets as demonstrated by several quantitative proteomic studies (Aatonen et al., 2014; Clark et al., 2015; Keerthikumar et al., 2015; Kowal et al., 2016; Minciacchi et al., 2015; Turiák et al., 2011; Xu et al., 2015). However, their composition also varies based on their source of origin and the down-stream isolation method. To date, no specific markers are available to distinguish EV subsets, and most of the literature classifying MVs and EXOs based on their specific markers can be misleading. A recent study proposed a new way of categorizing EVs that could be implemented to any source of vesicles isolated from cell culture supernatants or biological fluids (Kowal et al., 2016). Based on the presence of protein markers, EVs can be classified as a) large EVs pelleted at low speeds, b) medium-sized EVs pelleted at intermediate speeds, and c) small EVs pelleted at high speeds. Among the small EVs, four subcategories emerged including: c1) small EVs co-enriched in CD63, CD9, and CD81 and endosomal markers; c2) small EVs devoid of CD63 and CD81, but enriched in CD9 and associated with endocytic and plasma membrane markers; c3) small EVs devoid of CD63, CD9, and CD81 not associated with endosomal signature; and c4) small EVs enriched in extracellular matrix proteins or serum-derived factors in the absence of endosomal signatures (Kowal et al., 2016).

2.1.4 EVs as mediators of cell-to-cell communication

Cell-to-cell communication is a key regulator of many biological and pathological processes. Cells communicate by secreting signaling molecules such as hormones, cytokines, growth factors, lipid mediators, and neurotransmitters that locally or remotely activate the target cells, inducing a broad range of responses. Part of the cellular secretome includes a heterogeneous mixture of EVs, which actively participate in the intercellular communication process. When EVs are released into the extracellular space, they can be eliminated from the body by secretion into biofluids e.g., urine, or be internalized by cells delivering their cargo and influencing the recipient's cells. The transfer of functional RNA species and proteins (Alvarez-Erviti et al., 2011; Montecalvo et al., 2012; Ratajczak et al., 2006; Valadi et al., 2007) was a concrete proof that EVs can effectively deliver their cargo to recipient cells. The induction of luciferase activity by luciferin-loaded EVs in luciferase transfected dendritic cells clearly supported the intercellular delivery of the EV cargo (Montecalvo et al., 2012). Additionally, the use of fluorescent membrane dyes e.g., PKH67, DiD, DiL; GFP-tagged EV proteins e.g., GFP-CD63; or fluorescent protein dyes e.g., carboxyfluorescein succinimidyl

ester (CFSE) and CFDA-SE, coupled with confocal microscopy has permitted a direct visualization of EV internalization and co-localization studies within cell organelles (Escrevente et al., 2011; Feng et al., 2010; Lai et al., 2015; Svensson et al., 2013; Tian et al., 2010). However, given the resolution limits of the current microscopes, the ability to detected single vesicles from clusters of EVs is still challenging.

The mechanisms by which EVs and their cargo are delivered into the cells have been subjected to continuous debate. EVs may become incorporated into the cells as a consequence of the continuous endocytosis of the cell membrane (Mulcahy et al., 2014). However, the specific protein contents of EVs such as tetraspanins, integrins, and immunoglobulins indicate an active EV internalization process that requires specialized interactions between EVs and cells. Currently, several mechanisms for EV uptake have been postulated and are discussed (Mulcahy et al., 2014). Briefly, EVs can either fuse with the cellular plasma membrane to deliver their cargo (Del Conde et al., 2005; Parolini et al., 2009), or be internalized by the cells as intact EVs. There is also evidence for alternative EV internalization mechanisms: energy-dependent receptor-mediated endocytosis, including clathrin-mediated endocytosis and caveolin-mediated endocytosis (Escrevente et al., 2011; Nanbo et al., 2013; Svensson et al., 2013), macropinocytosis (Fitzner et al., 2011; Tian et al., 2014b), and phagocytosis (Feng et al., 2010). However, the EV internalization process likely occurs via multiple mechanisms, and thus the existence of different internalization routes could reflect the simultaneous activation of uptake pathways based on the origin or the subpopulation of the EVs being internalized. Moreover, the degree to which some of these pathways represent EV clearing mechanisms rather than promoting cellular responses is far from clear. Although the responses caused by RNA molecules require EV internalization, phenotypic changes in recipient cells may be caused by receptor-ligand interactions without the need of EV uptake (Mulcahy et al., 2014). Indeed, the specificity between protein-to-protein interactions is likely what drives EV targeting to certain tissues (Hoshino et al., 2015; Svensson et al., 2013), although the exact mechanisms remain elusive.

2.1.5 Physiological and pathological functions of EVs

The specific physiological and pathological properties of EVs depend on the origin and characteristics of their parent cells (Kalluri, 2016; Théry et al., 2009). While recent research focuses on elucidating the roles of EVs in intercellular communication, the EV-mediated maintenance of homeostasis and regulation of physiological functions remains less explored. In healthy individuals, EVs participate in the regulation and maintenance of embryonic development, reproduction, coagulation, cell death, inflammation, angiogenesis, tissue repair, or act as immune modulators with either immune-activating or immune-suppressive effects (Yáñez-Mó, Siljander et al., 2015). On the other hand, in pathological settings such as cancer, EVs can contribute in major disease-related functions including epithelial-to-mesenchymal transition, inhibition of cell death, invasion, metastasis, tumor proliferation, stimulation of angiogenesis, immunosuppression, and eventually pre-metastatic niche formation (An et al., 2015; Azmi et al., 2013; Nawaz et al., 2014; Rak, 2013). Additionally, EVs actively participate in other pathologies such as viral and prion transmission (Nguyen et al., 2003; Pegtel et al., 2010). This review of literature will focus on the description of the potential role of EVs in cancer.

2.2 EVs in cancer

2.2.1 Role of EVs in the hallmark functions of cancer

Intercellular communication is increasingly distorted during tumor progression and several studies suggested that EVs are supportive of this process. Cancer-derived EVs can facilitate the intercellular exchange of bioactive cancer-related molecules contributing to the acquisition of the different hallmarks of cancer described by Hanahan and Weinberg (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). As summarized below and recently reviewed (Kanada et al., 2016; Meehan and Vella, 2016), there is now overwhelming evidences showing the participation of different cancer-derived EVs in the functional transfer of nucleic acids, proteins, and lipids to target cells promoting tumorigenesis. EVs enable cancer cell progression by, for instance, sustaining proliferative signaling, resisting cell death, promoting angiogenesis, increasing invasion and metastasis, and evading clearance by the immune system (**Figure 4**). Below, the currently available literature for the contribution of EVs to the acquisition of hallmark capabilities of cancer is reviewed.

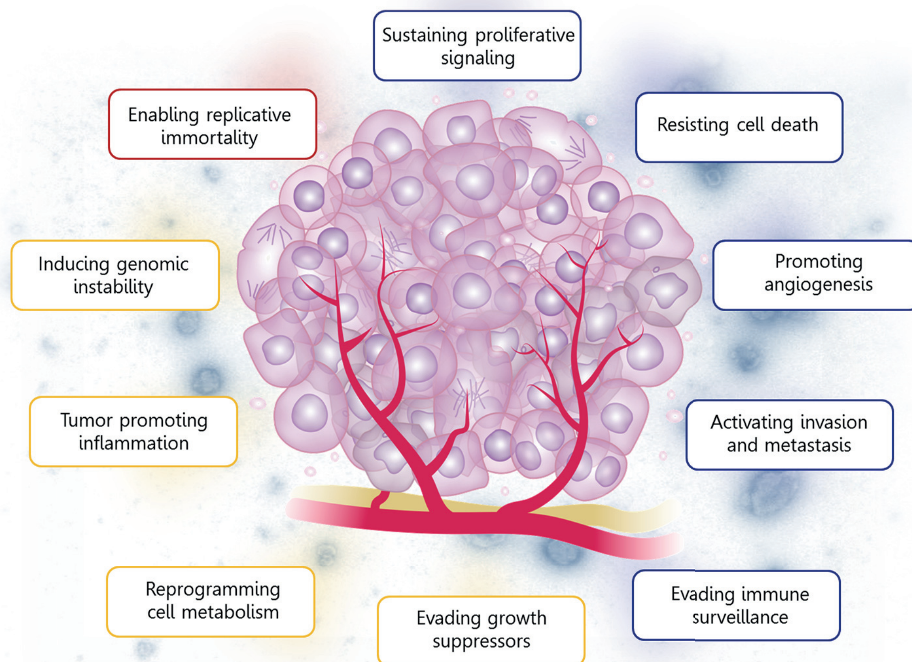

Figure 4. Schematic representation of the hallmarks of cancer acquired during tumor progression as described by Hanahan and Weinberg, Cell, 2011. The hallmark functions of cancer where EV participation has been shown are marked in blue, including sustaining proliferative signaling, resisting cell death, promoting angiogenesis, activating invasion and metastasis, and evading immune surveillance. The functions marked in yellow are those where the role of EVs is poorly understood, including evading growth suppressors, reprogramming cell metabolism, tumor-promoting inflammation, and inducing genomic instability. The participation of EVs enabling replicative immortality, marked in red, has not been so far reported.

Sustaining proliferative signaling

Cancer-derived EVs transfer proliferative signals to recipient cells contributing to their malignization by enhancing cellular growth and proliferation. EVs can participate in the activation of signaling pathways that are commonly dysregulated in cancer. For instance, cancer-derived EVs from glioma, gastric, prostate, bladder, and lung origin, participated in the activation of the phosphatidylinositide 3-kinases/AKT and/or MAPK/extracellular signal-regulated kinases pathways (Al-Nedawi et al., 2008; Choi et al., 2014; Qu et al., 2009; Yang et al., 2013). In addition, somatic mutations in cancer cells can activate down-stream signaling promoting tumor growth. The intercellular transference of the oncogenic form of EGFRvIII to cells lacking the isoform via glioma-derived EVs led to an increase in proliferation and survival of the glioma cells (Al-Nedawi et al., 2008). Similarly, the transfer of mutant KRAS-containing EVs to cells expressing only wild-type KRAS enhanced their cellular growth (Demory Beckler et al., 2013). In addition to oncogenic sequences, phosphorylated proteins, mRNAs, and miRNAs can also be transferred via EVs contributing to the progression of the tumors (Soldevilla et al., 2014; Yang et al., 2016). For example, EVs isolated from neuroblastoma, nasopharyngeal carcinoma, and thyroid cancer contained over-expressed levels of miRNAs that regulated cell proliferation and differentiation (Haug et al., 2015; Lee et al., 2015a; Ye et al., 2014). The transfer of EV-associated ΔNp73 mRNA to colon cancer cells has also been shown to provide proliferation potential and chemoresistance (Soldevilla et al., 2014).

Resisting cell death

Apoptosis-mediated programmed cell death is usually attenuated in certain tumor cells promoting tumorigenesis, and triggered in different cancer cells during tumor development or in response to therapy. Cancer-derived EVs have both direct and indirect roles in cell death. They have been implicated in the transfer of anti-apoptotic factors between cells, such as B-cell lymphoma (Bcl)-extra large, anchorage-independent growth and survival factors (Al-Nedawi et al., 2008; Antonyak et al., 2011). In contrast, EVs derived from gastric and bladder cancer cells were shown to suppress apoptosis by incrementing the expression of Bcl-2 and cyclin-D1, and reducing the levels of Bax and caspase-3 (Koga et al., 2005; Qu et al., 2009; Yang et al., 2013). Many different types of human cancers resulted in mutated or missing gene for tumor protein p53 (TP53), which then enabled resistance to apoptosis (Meek, 2009). Interestingly, TP53 mutations have been detected in EVs isolated from cancer patients, harboring the same TP53 mutations as the primary tumors (Kahlert et al., 2014; Thakur et al., 2014). However, how EVs are mechanistically involved in resisting cell death is not yet well understood.

Promoting Angiogenesis

The formation of new blood vessels is an essential requirement for tumor development and progression. EVs are active modulators of angiogenesis and endothelial cell activation, contributing to the formation of blood vessels within the tumors (Bian et al., 2014). EVs derived from platelets, leukocytes, and endothelial progenitor cells, have been shown to deliver pro-angiogenic factors and functional mRNAs promoting neo-angiogenesis both *in vivo* and *in vitro* (Deregibus et al., 2007; Rhee et al., 2004). Cancer-derived EVs expressing the tetraspanin Tspan8 were shown to promote angiogenic functions (Gesierich et al., 2006), in support of tumor growth, by elevating the levels of vascular endothelial growth factor (VEGF) and VEGR receptor 2 (Nazarenko et al., 2010). In the absence of pro-angiogenic signals, melanoma-derived EVs were also shown to promote tubule branching by modifying

the morphology of the endothelial tubule network (Hood et al., 2009). The increased vessel density and branching by the delivery of delta-like 4 has been reported in colorectal carcinoma (Jubb et al., 2009) and glioma-derived EVs (Sheldon et al., 2010). In line with those findings, cancer-derived EVs harboring the oncogenic form of EGFRvIII induced the activation of MAPK and AKT signaling pathways, which stimulated a complex angiogenic mechanism including the production of VEGF and signaling activation of VEGFR receptor 2 (Al-Nedawi et al., 2008; Al-Nedawi et al., 2009). Hypoxia is also closely related to tumor progression and aggressiveness, and it has been shown to promote the release of EVs by prostate, breast, glioma, and leukemic cells (King et al., 2012; Kucharzewska et al., 2013; Ramteke et al., 2015; Tadokoro et al., 2013). EV proteins and mRNAs derived from hypoxic glioblastoma cells and patient plasma were able to trigger angiogenesis and tumor growth (Kucharzewska et al., 2013; Skog et al., 2008). EVs can also promote angiogenesis with microRNAs (miR-214, miR-210) (Tadokoro et al., 2013; van Balkom et al., 2013), activate angiogenesis and suppress senescence (miR-92a) (Umezu et al., 2013), and also enhance cell migration, tube formation, and increase angiogenesis by targeting a factor that inhibits the hypoxia inducible factor-1 (HIF1) pathway (miR-135b) (Umezu et al., 2014). However, the molecular crosstalk during tumor development between hypoxic cells and EVs is only just been realized, and *in vivo* studies are required to discover the mechanisms used by hypoxic cells to communicate through EVs.

Activating invasion and metastasis

The crosstalk between the stroma- and cancer-derived EVs is relevant in tumor proliferation, and many studies are focusing on elucidating the role of EVs as mediators of cell invasion and metastases. EVs contribute to the epithelial-to-mesenchymal transition by the transference of EV-associated HIF1α (Aga et al., 2014) and miR-200 (Le et al., 2014). Significantly, EV-associated transforming growth factor beta (TGF-β) could trigger the differentiation of fibroblasts into myofibroblasts as characterized by the *de novo* expression of α-smooth muscle actin (Webber et al., 2015; Webber et al., 2010). Both stromal and mesenchymal cells secrete EVs with tumor promoting effects (Roccaro et al., 2013; Zhu et al., 2012). EVs secreted from cancer-associated fibroblasts (CAFs) have been shown to promote the migration and motility of breast cancer cells through Wnt-planar cell polarity signaling (Luga et al., 2012), and also the activation of key oncogenic pathways such as Notch and RhoA in cancer cells (Shimoda et al., 2014). In that line, EVs generated under hypoxic conditions were shown to promote invasiveness and stemness of prostate cancer cells, and the stimulation of a CAF phenotype in prostate stromal cells (Ramteke et al., 2015). Additionally, oncogenic protein tyrosine kinase- containing EVs increased gastrointestinal stromal tumor invasiveness (Atay et al., 2014). Similarly, mutant K-*ras* and H-*ras* and/or Rab proteins in cancer-derived EVs contributed to prostate cancer progression by neoplastic transformation of the phenotype of adipose-derived stem cells (Abd Elmageed et al., 2014).

EVs actively participate in the formation of the pre-metastatic niche, which is another fundamental phenomenon in the development of cancer. Firstly, melanoma-derived EVs have been shown to prepare sentinel lymph nodes for tumor metastasis (Hood et al., 2011). Metastatic melanoma-derived EVs were shown to reprogram bone marrow progenitor cells via the hepatocyte growth factor receptor, stimulating vasculogenic and pro-metastatic behavior (Peinado et al., 2012). Likewise, pancreatic cancer-derived EVs also participated in the preparation of the metastatic niche in liver (Costa-Silva et al., 2015). The presence of macrophage migration inhibitory factor in EVs induced the secretion of fibronectin by hepatic stellate cells and TGF-β production in liver Kupffer cells, contributing to the

remodeling of the extracellular matrix and promoting the metastatic niche formation (Costa-Silva et al., 2015). Additionally, several miRNAs within the EVs have been shown to influence the early phases of the niche formation; for instance, the transfer miRNAs such as miR-19a from brain astrocytes to cancer cells down-regulated the expression of phosphatase and tensin homolog (*PTEN*), which facilitated the proliferation of metastatic brain cells (Zhang et al., 2015b). Also, miR-122 in breast cancer-derived EVs reprogrammed the glucose metabolism, resulting in the suppression of glucose intake by normal cells and increased nutrient availability to cancer cells in support of metastasis (Fong et al., 2015). Likewise, EV-associated miR-200 showed altered gene expression and induction of local and distant breast cancer metastasis (Le et al., 2014). EV-derived miRNAs were also shown to regulate tight junction proteins; for instance, metastatic breast cancer cell-derived EVs secreted miR-105 thereby suppressing the expression of tight junction 1 protein in endothelial cells (Zhou et al., 2014). In addition, EVs derived from the metastatic brain cells carrying miR-181c were capable of disrupting tight junction proteins, contributing to cell extravasation and vessel leakiness, and promoting metastasis to brain and liver (Tominaga et al., 2015).

EV-regulation was recently reported to be involved in cell movement, with EVs coated with fibronectin-integrin complexes as critical motility-promoting cargo (Sung et al., 2015). Importantly, the specific integrin expression patterns in EVs may predetermine induction of organ-specific metastasis (Hoshino et al., 2015). However, it is possible that EVs alone cannot promote the metastatic niche formation, and due to the tumor complexity, other biomolecules from the tumor secretome are likely required to trigger this process (Bobrie et al., 2012; Jung et al., 2009).

Evading immune surveillance

Cancer cells must escape the immune system to survive and metastasize. Cancer cells have developed numerous mechanisms to evade the immune system in which EVs participate, although the contribution of EVs to the evasion of the immune surveillance and tumor immunoediting is not yet well understood. Cancer-derived EVs influence the immune evasion responses without direct interaction with immune cells, for instance by suppressing the anti-tumor T cell responses (Abusamra et al., 2005; Clayton et al., 2007; Huber et al., 2005; Taylor and Gercel-Taylor, 2005). EVs containing pro-apoptotic molecules, such as Fas-ligand and tumor necrosis factor-related apoptosis-inducing ligand, induce apoptosis of activated tumor-specific T cells inhibiting their cytotoxic effect towards the target tumor (Andreola et al., 2002; Kim et al., 2005). Cancer-derived EVs also induce immune suppression by promoting regulatory T cell expansion thereby contributing to the tumor escape from the immune system (Whiteside, 2005). Moreover, melanoma- and colorectal carcinoma-derived EVs inhibited monocyte differentiation towards dendritic cells by inducing the secretion of TGF-β (Valenti et al., 2006). Another immunosuppressive molecule, adenosine, present in tumor-derived EVs co-expressing CD39 and CD73, negatively regulated the local immune response by inhibiting T cell activation (Clayton et al., 2011). Lymphocyte cytotoxic functions were also impaired by circulating EVs in prostate cancer patients, promoting tumor escape (Lundholm et al., 2014). Additionally, EV-associated miRNAs such as let-7 and miR-155 were suggested to mediate the evasion of the immune response by regulating T-cell responses (Okoye et al., 2014).

Evading growth suppressors

To maintain tissue homeostasis, it is fundamentally important to eliminate any malfunctioning cells in the organism. Despite the clear evidence that cancer-derived EVs

sustain proliferative signals in cancer cells, the mechanisms of the evasion of cell growth suppression via EVs remains poorly understood, and only a few studies have assessed the effect of EVs evading growth suppressors. EVs have been suggested to harbor miRNAs with tumor suppressor targets, including miR-143, which can reduce the growth of prostate cancer cells (Kosaka et al., 2012) and miR-23b, which has been shown to reduce angiogenesis, invasion, and metastasis of bladder (Ostenfeld et al., 2014) and breast cancer cells (Ono et al., 2014).

Reprogramming cell metabolism

The acidification of the microenvironment during tumor formation occurs due to the up-regulation of the glycolytic pathway under hypoxic conditions. The acidic pH of tumors is essential for enhanced EV secretion and trafficking (Parolini et al., 2009). In addition, the secretion of HIFs can control EV formation in breast cancer cells (King et al., 2012), facilitating the acquisition of malignant properties by the cancer cells (Wang et al., 2014c). An outstanding study has recently demonstrated that CAF-derived EVs can reprogram the metabolic machinery of cancer cells by inhibiting mitochondrial oxidative phosphorylation, thereby increasing glycolysis and glutamine-dependent reductive carboxylation (Zhao et al., 2016). Hence, changes in the metabolic microenvironment of the tumor modulate intercellular communication via EVs, though the mechanisms are currently poorly understood.

Tumor-promoting inflammation

Cancer-associated inflammation responses usually involve the dysregulated activity of different immune cells. EVs have been reported to stimulate chronic inflammation by reducing the innate immune responses (Buzas et al., 2014; Fabbri et al., 2012). EVs can contribute to inflammation by carrying autoantigens, including heat-shock proteins, histones, α-enolase (Turiák et al., 2011); cytokines such as interleukin (IL)-1β (Boilard et al., 2010; Pizzirani et al., 2007), IL-18, (Gulinelli et al., 2012) and IL-8 (Baj-Krzyworzeka et al., 2011); lipid mediators (Barry et al., 1997; Esser et al., 2010); matrix metalloproteinases (Shimoda and Khokha, 2013); damage-associated molecular patterns including high mobility group protein B1 and S100 (Goh and Midwood, 2012; Schiller et al., 2013); and miRNAs such as let-7, miRNA-21, and miR-29a (Lehmann et al., 2012; Ohshima et al., 2010), some of which are ligands/activators of Toll-like receptors, such as toll-like receptor 7 (Ohshima et al., 2010). Despite the clear association of EVs and inflammation, the biological relevance during cancer progression has not yet been proven.

Inducing genomic instability

The role of EVs in prompting sustained genomic instability is currently unclear. It is also unknown whether the effect of EVs in the transformed cellular state is transient, permanent, or dependent on constant EV stimulation. A unique aspect of cancer EVs is that they contain activated oncoproteins, oncogenic DNA sequences, and oncomiRs that can be transferred between cells whereupon they prompt functional effects across cellular boundaries (Rak, 2013). Different oncogenic molecules such as *EGFR*, *Ras*, *Myc*, *LMP1*, *SV40 large T*, and latent membrane protein 1, have been identified in various types of EVs (Al-Nedawi et al., 2008; Balaj et al., 2011; Bergsmedh et al., 2001; Demory Beckler et al., 2013; Meckes et al., 2010; Skog et al., 2008). For instance, the internalization of ABs containing *H-ras*, *Myc* or *SV40 large T* caused a manifest tumorigenic conversion of immortalized fibroblast (Bergsmedh et al., 2001). Likewise, fibroblasts exposed to the EV-

mediated transfer of cancer-cell derived fibronectin and tissue transglutaminase showed permanent changes driving *in vivo* tumor cell growth (Antonyak et al., 2011). However, the intercellular transfer of oncogenic EVs containing mutant H-ras exerted transient regulatory effects that were unable to trigger a tumorigenic transformation in recipient cells (Lee et al., 2016). The transfer of oncogenes by EVs could support the notion that EVs might promote potent but transient effects in cancer promoting genomic instability, even though the exact mechanisms are yet to be discovered.

2.2.2 EVs in cancer diagnosis and prognosis

A major challenge of the cancer research field is the discovery of biomarkers with relevant diagnostic and prognostic value. Ideally, a good biomarker should be efficient, detecting the disease before it develops or at an early stage, and specific for each cancer type. A biomarker should also contribute to monitoring the disease progression and be informative of the response to a treatment (Schork, 2015). The analysis of cancer-derived EVs has opened new insights in the biomarker discovery field. Cancer cells usually display an altered vesiculation in comparison with normal cells, releasing more EVs into the biofluids (Al-Nedawi et al., 2008; Graves et al., 2004; Taylor and Gercel-Taylor, 2008). Additionally, EVs contain biomolecules within their cargo that reflect the content and pathological state of the parent cells, providing an enriched source of information (Kalluri, 2016). Because these molecules are contained within the EV membranes, they are also protected from degradation during transit. A number of these molecules, especially the EV-associated membrane proteins and nucleic acids, seem to show promise in cancer diagnosis and prognosis.

Currently, the majority of studies relate to the role of EV-associated RNAs as a possible source of biomarkers, with special emphasis in miRNAs (Yokoi et al., 2015). Circulatory miRNAs have shown remarkable stability in plasma and serum (Mitchell et al., 2008), and they have been reported to be enriched in EVs (Gallo et al., 2012). However, many studies do not adequately report if the identified miRNAs were EV-associated or not. Similarly, mRNAs have also been identified in cancer-derived EVs, but only a few studies have so far addressed the possible clinical relevance of mRNA molecules in EVs. A comprehensive summary of the RNA species found in cancer-derived EVs isolated from biofluids is presented in **Table I**.

Table I. EV-associated RNAs for diagnosis and prognosis of cancer.

Cancer type	EV cargo	Biofluid	Reference
Acute Leukemia	miR-92	plasma	(Tanaka et al., 2009)
Bladder	LASS2, GALNT1 mRNAs (present) ARHGEF39, FOX3 mRNAs (absent)	urine	(Perez et al., 2014)
Breast	miR-16, miR-1246, miR-451, miR-720	plasma, milk, ductal fluids	(Pigati et al., 2010)
	miR-101, miR-372, miR-373	serum	(Eichelser et al., 2014)
Cervical	miR-21, miR-146a	cervicovaginal lavage	(Liu et al., 2014)
Colorectal	let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a	serum	(Ogata-Kawata et al., 2014)
	miR-17-92a cluster		(Matsumura et al., 2015)
Esophageal	miR-21	serum	(Tanaka et al., 2013)

	miR-1246		(Takeshita et al., 2013)
Gastric	LINC00152	plasma	(Li et al., 2015)
	MAGE-1, HER-2/neu mRNAs		(Baran et al., 2010)
	miR-21, miR-1225-5p	peritoneal lavage	(Tokuhisa et al., 2015)
Glioblastoma	miR-21	cerebrospinal fluid	(Akers et al., 2013)
	RNU6-1, miR-320, miR-574-3p	serum	(Manterola et al., 2014)
	miR-21		(Mao et al., 2014)
	EGFRvIII mRNA		(Skog et al., 2008)
Glioma	IDH1 mRNA	cerebrospinal fluid	(Chen et al., 2013; Wang et al., 2014b)
Hepatocellular	miR-21	serum	(Wang et al., 2014b)
	miR-718		(Sugimachi et al., 2015)
Laryngeal	HOTAIR, miR-21	serum	(Wang et al., 2014a)
Lung	miR-17-3p, miR-21, miR-106a, miR-146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, miR-214	plasma	(Rabinowits et al., 2009)
	let-7f, miR-30e-3p		(Silva et al., 2011)
	miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100 and miR-154-3p		(Cazzoli et al., 2013)
	let-7f, miR-30e-3p, miR-223, miR-301		(Rodríguez et al., 2014)
	miR-486, miR-30d, miR-1, miR-499	serum	(Hu et al., 2010)
	miR-21 and miR-155		(Munagala et al., 2016)
Melanoma	miR-17, miR-19a, miR-21, miR-126, miR-149	plasma	(Pfeffer et al., 2015)
Nasopharyngeal	miR-24-3p, miR-891a, miR-106a-5p, miR-20a-5p, miR-1908	serum	(Ye et al., 2014)
Ovarian cancer	miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214	serum	(Taylor and Gercel-Taylor, 2008)
Pancreatic	miR-21, miR-17-5p	serum	(Que et al., 2013)
	miR-1246, miR-4644, miR-3976, miR-4306		(Madhavan et al., 2015)
	APBB1IP, ASPN, Daf2, FoxP1, Bc031781, Gng2 mRNAs	saliva	(Lau et al., 2013)
Prostate	miR-141	serum	(Mitchell et al., 2008)
	miR-107, miR-141, miR-375, miR-574-3p	serum/plasma	(Bryant et al., 2012)
	miR-1290, miR-375	plasma	(Huang et al., 2015)
	LncRNA-p21		(Işın et al., 2015)
	PCA-3, TMPRSS2 mRNAs	urine	(Dijkstra et al., 2014; Nilsson et al., 2009)
	AGR2 mRNA		(Neeb et al., 2014)
	CDH3 mRNA		(Royo et al., 2016)
	ERG, PCA-3, SPDEF mRNAs		(McKiernan et al., 2016)

LASS2: ceramide synthase 2; GALNT1: polypeptide N-acetylgalactosaminyltransferase 1; ARHGEF39: rho guanine nucleotide exchange factor 39; FOX3: RNA binding protein, fox-1 homolog 3; LINC00152: long non-

coding RNA Linc00152; MAGE-1: melanoma-associated antigen 1; HER-2/neu: human epidermal growth factor proto-oncogene neu; RNU6-1: RNA u6 small nuclear 1; EGFRvIII: epidermal growth factor receptor variant 3; IDH1: isocitrate dehydrogenase 1; HOTAIR: long-noncoding RNA; APBB1IP: Amyloid beta A4 precursor protein-binding family B member 1-interacting protein; ASPN: asporin; Daf-2: insulin-like receptor precursor in C elegans; FoxP1: forkhead box P1; Gng2: G protein subunit gamma 2; PCA-3: prostate cancer antigen-3; TMPRSS2: transmembrane protease serine 2; AGR2: anterior gradient 2; CDH3: cadherin 3; ERG: V-Ets avian erythroblastosis virus E26 oncogene homolog; SPDEF: SAM pointed domain containing Ets transcription factor.

In addition to RNA, EV-associated DNA may also hold prospective value for diagnostic purposes, since it can provide information about specific mutations present in the original tumors. DNA molecules exist in many different forms, including histone/DNA complexes or nucleosomes, EV-associated DNA, and virtosomes. These DNA forms can also be classified as circulating DNA, circulating-free DNA, cell-free DNA, and EV-DNA (Peters and Pretorius, 2011). In contrast to RNA, the presence of DNA in EVs have been much less studied.

In recent years, studies have focused on the EV-associated membrane proteins, since they may also serve as relevant EV-related diagnostic targets (Yokoi A et al., 2015). Particularly, cancer-derived EVs can be isolated using membrane specific proteins from cancer tissues. A comprehensive summary of the membrane proteins found in cancer-derived EVs isolated from biofluids is presented in **Table II**.

Table II. EV-associated proteins for diagnosis and prognosis of cancer.

Cancer type	EV cargo	Biofluid	Reference
Acute leukemia	CD34	plasma	(Hong et al., 2014)
Bladder	EPS812, mucin-4	urine	(Smalley et al., 2008)
	EDIL-3/Del1		(Beckham et al., 2014)
Breast	Fibronectin	plasma	(Moon et al., 2016)
	Periostin		(Vardaki et al., 2016)
	Glypican-1	serum	(Melo et al., 2015)
Colorectal	Claudin-3	ascites	(Choi et al., 2011)
	CD147, CD9	serum	(Yoshioka et al., 2014)
Gastric	CCR6, HER-2/neu, CD147, MAGE-1, C-MET	serum	(Baran et al., 2010)
Glioblastoma	EGFRvIII	serum	(Skog et al., 2008)
Lung	LRG1	urine	(Li et al., 2011)
	EGFR	serum	(Yamashita et al., 2013)
	SNX25, BTG1, PEDF, thrombospondin	pleural effusion	(Bard et al., 2004)
Melanoma	Caveolin-1, CD63	plasma	(Logozzi et al., 2009)
	TYRP2, VLA-4, Hsp70, MET		(Peinado et al., 2012)
Nasopharyngeal	Galectin-9	serum	(Klibi et al., 2009)
Ovarian	MMP2, MMP9, uPA	ascites	(Graves et al., 2004)
	CD24, EpCAM		(Runz et al., 2007)
	EpCAM	serum	(Taylor and Gercel-Taylor, 2008)
	L1CAM, CD24, ADAM10, CD147		(Keller et al., 2009)

	Claudin-4	plasma	(Li et al., 2009)
	TGFβ-1, MAGE3/6		(Szajnik et al., 2013)
Pancreatic	EGFR	plasma	(Arscott and Camphausen, 2011)
	Glypican-1	serum	(Melo et al., 2015)
Prostate	PSA, PSMA	urine	(Mitchell et al., 2009; Overbye et al., 2015)
	δ-catenin, caveolin-1, CD59		(Lu et al., 2009)
	ITGA3, ITGB1		(Bijnsdorp et al., 2013)
	TM256/C17orf61, LAMTOR1, ADIRF, TGM4, TMPRSS		(Overbye et al., 2015)
	CYP17A	serum	(Locke et al., 2009)
	Survivin	plasma	(Khan et al., 2011; Khan et al., 2012)
	PTEN		(Gabriel et al., 2013)
	PSA		(Gabriel et al., 2013)
	PSMA		(Mizutani et al., 2014; Park et al., 2016)
Renal carcinoma	MMP-9, DKP4, CD147, PODXL	urine	(Raimondo et al., 2013)

EDIL-3/Del1: epidermal growth factor like repeats and discoidin domains 3; CCR6: chemokine receptor 6; HER-2/neu: human epidermal growth factor proto-oncogene neu; MAGE-1: melanoma-associated antigen 1; C-MET: hepatocyte growth factor receptor; LRG1: leucine rich α2-glycoprotein 1; EGFR: epidermal growth factor receptor; SNX25: sorting nexin 25; BTG1: B-cell translocation gene 1; PEDF: pigment epithelium-derived factor; TYRP2: tyrosinase-related protein 2; VLA-4: very late antigen 4; Hsp-70: heat shock protein 70; MET: met proto-oncogene tyrosine kinase; LRG1: leucine-rich α2-glycoprotein; EGFRvIII: epidermal growth factor receptor variant III; MMP2: matrix metalloproteinase-2; MMp9: matrix metalloproteinase-9; uPA: plasminogen activator, urokinase; EpCAM: epithelial cell adhesion molecule; L1CAM: l1 cell adhesion molecule; ADAM10: a disintegrin and metalloproteinase domain 10; TGFβ-1: transforming growth factor beta 1; PSA: prostate-specific antigen; PSMA: prostate-specific membrane antigen; ITGA3: α3-integrin; ITGB1: ß1-integrin; TMEM265: transmembrane protein 256; LAMTOR1: late endosomal/lysosomal adaptor, MAPK and MTOR activator 1; ADIRF: adipogenesis regulatory factor; TGM4: transglutaminase 4; TMPRSS: transmembrane protease serine 2; CYP17A: cytochrome P450 17A1; PTEN: phosphatase and tensin homolog. MMP-9: matrix metallopeptidase 9; DKP4: dickkopf related protein 4; PODXL: podocalyxin like.

2.3 Prostate cancer

2.3.1 Current diagnosis and treatment

Prostate cancer is the second most commonly diagnosed cancer in men worldwide and accounts for 30.6% of all male cancers in Finnish men between the years 2010 and 2014 (Engholm et al., 2016). The probability of developing prostate cancer during a man´s lifetime is 15% (Siegel et al., 2015). Although prostate cancer has been extensively studied, so far there are only a few clinically used diagnostic and prognostic markers available. The heterogeneity of the tumors together with the variable clinical evolution of the patients, has led to a non-consensus of the causes behind prostate cancer progression (Tomlins et al., 2006). Currently, the most commonly used prognostic prostate cancer markers include the prostate-specific antigen (PSA), Gleason score, and clinical tumor stage (Catalona et al., 1991; Gittes, 1991; Gleason, 1966; Gleason and Mellinger, 1974; McNeal et al., 1986; Mellinger et al., 1967; Stamey et al., 1987). Thus, when prostate cancer is suspected due to elevated PSA levels in blood (4.0 ng/mL is the cut-off level for recommending a biopsy), the patient undergoes digital rectal examination to estimate the gland volume and anatomy of the tissue, followed by a local determination of the stage of the tumor (Cheng et al., 2012). Additionally,

a prostate biopsy confirms the diagnosis (Ukimura et al., 2013). Based on the results, the pathological stage and Gleason score, which grades the level of cancer cell differentiation and aggressiveness of the tumors (Epstein et al., 2016) are determined, and finally treatment options are evaluated.

Treatment strategies for prostate cancer depend on the stage and grade of the disease, in addition to the patient characteristics such as age, general condition, and additional treatments (Epstein et al., 1994; Heidenreich et al., 2014). Prostate tumors are often slow-growing, providing many opportunities for therapeutic intervention. The treatment options can be classified into two categories depending on the local or advance stage of the disease (Kirby et al., 2006). The majority of localized or regional cancers in early stages can be cured by radical prostatectomy or radiation therapy. These treatments significantly increase the patient's 5-year relative survival to nearly 100% (Jemal et al., 2010). If the disease has already spread outside the prostate, metastasizing, its progression can be delayed by surgery, androgen deprivation therapy, or a combination of both (Attard et al., 2016). Despite these treatments, a considerable proportion of patients will eventually develop castration-resistant prostate cancer, which at the moment is incurable (Scher and Sawyers, 2005), with a median overall survival of 18 months (James and Mason, 2015).

The currently used diagnostic markers for prostate cancer have limitations such as lack of specificity, accuracy, and predictive value, not being able to identify and stratify patients and generating false-positive results (Attard et al., 2016). Although the PSA test is considered to be organ-specific and has a prognostic value, the test is not truly prostate cancer-specific, which limits its usefulness. PSA fails to distinguish between indolent and aggressive prostate cancer as well as growth from benign prostate hyperplasia (Catalona, 1994; Collins et al., 1997; Thompson et al., 2005). Although the ratio of free *vs* total PSA and other parameters including PSA density, velocity, or doubling time of different PSA isoforms have been suggested to overcome these limitations, the use of these tests is not applicable in all cases (Artibani, 2012). Likewise, tests including the evaluation of PCA-3 in urine (Vlaeminck-Guillem et al., 2010), early prostate cancer antigen in serum (Dhir et al., 2004; Paul et al., 2005), and protein kinase C alpha in tissue (Perry A S et al., 2014), have not yet undergone systematic validation in large patients cohorts. Alternative strategies for biomarker discovery are clearly needed, and EVs could be considered as a new source for better predictive biomarkers. EVs have gained considerable attention as candidate biomarkers for diagnosis and prognosis of cancer, and multiple studies have documented their roles in different cancer types including prostate cancer.

2.3.2 EVs as prostate cancer biomarkers

Prostate cancer is one of the most studied cancer models, and hence research of prostate cancer-derived EVs has exponentially increased over the years. As previously summarized in **Tables I** and **II**, several EV molecules have been proposed as candidate markers for early diagnosis, prognosis, screening, efficacy, and response to therapy. In addition, markers to discriminate the pathological stages of the disease, including metastatic, localized, and castration-resistant prostate cancer have been suggested. Currently, prostate cancer EV research is mainly based on studies performed with clinical samples e.g., plasma-/serum-, urine-, semen- and prostatic secretions; tissue samples e.g., primary and metastatic tumors; cell lines e.g., DU-145, LNCaP, PC-3; and prostate cancer xenografts animal models.

Epithelial cells lining the prostate release vesicles termed prostasomes that belong to the EV family. Prostasomes are a very specific subset of neuroendocrine-like vesicles, of around 150 nm in average diameter, isolated from seminal and/or prostatic fluid (Duijvesz et al., 2011; Stridsberg et al., 1996). They are stored in the MVBs of acinar cells of the prostate gland and released after fusion with the plasma membrane (Ronquist et al., 2012). Prostasomes are positive for EV markers, including CD9 and CD63, but also other markers such as prostate-specific membrane antigen (PSMA), CD38, annexin A1, CD46, CD55, and CD59. The prostasome morphology is also thought to be different than that of other EVs (Poliakov et al., 2009), as well as their lipid composition (Arvidson et al., 1989; Duijvesz et al., 2011) and membrane structure (Stridsberg et al., 1996). In addition to the main role of prostasomes in human reproduction, they also appear to be involved in prostate cancer progression, for instance inducing invasion, migration, angiogenesis, and inhibition of the immune system (Aalberts et al., 2013). Overall, gaining knowledge about the EV content of prostate cancer tumors with different metastatic potential could be used for new diagnostic approaches. Proteomic and transcriptomic studies using prostate cancer-derived EVs are summarized here.

Proteomic analysis has been mainly based on studies of EVs from various prostate cancer cell lines and clinical samples. EVs isolated from PNT2, Du145, RWPE-1, PC-3, VCaP, and LNCaP cell lines have been extensively studied, identifying proteins linked to tumor invasiveness, diagnosis, and prognosis. These proteins include: CD151, disintegrin and metalloproteinase domain 10 (ADAM10), ADAM15, calmodulin-ubiquitin domain-containing protein 1, annexin A2, calsyntenin 1, filamin C, PSMA, growth differentiation factor 15, fatty acid synthase (FASN), and exportin 1 (Duijvesz et al., 2013; Hosseini-Beheshti et al., 2012; Jansen et al., 2009; Sandvig and Llorente, 2012). Using a novel aptamer platform, Notch3, l1 cell adhesion molecule, Ras-related C3 botulinum toxin substrate 1, ADAM9, milk fat globule-EGF factor 8 protein, and inter-α-trypsin inhibitor heavy chain family member 4, were identified as new cancer-associated proteins from prostate cancer-derived EVs (Webber et al., 2014). On a functional level, active packing of αvβ6 integrin in prostate cancer-derived EVs and its transfer to negative αvβ6 cells, was found to be able to promote cell migration (Fedele et al., 2015). In spite of the significance of these findings, only a few proteins have been positively validated in EVs isolated from patient samples. Among those proteins, several have been identified in EVs from the urine of prostate cancer patients. First, PSMA and PSA were detected in urine-derived EVs from prostate cancer patients (Mitchell et al., 2009). However, the presence of PSA in EVs could also be attributed to a possible contamination of soluble proteins in the EV preparation, as suggested by other groups (Jansen et al., 2009). Urine-derived EVs from prostate cancer patients have been found to contain increased amounts of certain proteins such as δ-catenin and FASN compared to controls without disease (Lu et al., 2009; Principe et al., 2013). Similarly, increased levels of α3-integrin and ß1-integrin were identified in the urine-derived EVs of metastatic prostate cancer patients compared to patients with a non-metastatic disease and/or benign prostate hyperplasia (Bijnsdorp et al., 2013). Detection of transmembrane protein 256 alone or in combination with late endosomal/lysosomal adaptor in urine EVs from prostate cancer patients has recently been proposed to increase the sensitivity and specificity of prostate cancer detection (Overbye et al., 2015). Moreover, detection of flotillin 2 and parkinsonism associated deglycase have been suggested to help distinguish between prostate cancer patients and healthy individuals (Wang et al., 2016). In line with those findings, a relevant discovery showed that EVs isolated from Du145 cells expressing PTEN protein could transfer their tumor suppressor cargo to PTEN negative prostate cells, thereby reducing proliferation and

apoptosis of cancer cells (Gabriel et al., 2013). Apparently, the transport of PTEN was an exclusive property of DU145 EVs, which contained an active form of the enzyme that phosphorylates the protein, which remained functional and protected from enzymatic degradation. Most importantly, these findings were further validated in EVs from plasma of prostate cancer patients compared to healthy volunteers, with a further correlation between PTEN expression in EVs and the Gleason Score of the patients (Gabriel et al., 2013). In addition to PTEN, the inhibitor of apoptosis, survivin, and PSMA have also been found to be differentially present in the plasma of prostate cancer patients compared to patients with benign prostate hyperplasia (Khan et al., 2012; Park et al., 2016). Likewise, the cytochrome P450 17A1 protein was highly expressed in the serum from prostate cancer patients compared to the serum of healthy controls, highlighting the potential use of EV-borne prostate cancer-specific proteins in the early detection of prostate cancer (Locke et al., 2009).

In addition to EV protein markers, comprehensive transcriptomic studies examining the role of EV-associated RNA species, including mRNAs, miRNAs, and lncRNAs have been performed and extensively reviewed (Hessvik et al., 2012; Soekmadji et al., 2013). In the context of urological cancers, EV-associated miRNAs have attracted the most attention. Many miRNAs isolated from plasma, serum, urine, prostatic secretions, and prostate cancer cell lines have been proposed as putative biomarkers for prostate cancer. However, the majority of the studies did not elucidate in which form the miRNAs were present, or if they were associated with EVs. Some examples include: miR-141, the first reported potential marker for prostate cancer diagnosis, progression, and metastatic staging (Brase et al., 2011; Mitchell et al., 2008); miR-1290 and miR-375 with survival prognosis in castration-resistant prostate cancer patients (Huang et al., 2015); miR-34a, for detecting the response to docetaxel treatment (Corcoran et al., 2014); miR-10a for the prediction of prostate cancer irrespective of androgen activity (Hessvik et al., 2012); and miR-141 and miR-375 for diagnosis (Bryant et al., 2012), although impure EVs were included in the study. On the other hand, the use of EV-associated mRNAs as possible biomarkers in prostate cancer has also been evaluated. Transmembrane serine protease fusion gene v-Ets avian erythroblastosis virus E26 oncogene homolog (*TMPRSS2-ERG)* mRNA transcripts were found in EVs isolated from prostate cancer cells (Jansen et al., 2009) and urine-derived EVs from prostate cancer patients. Urine-derived EVs from prostate cancer patients were also highly enriched in PCA-3, type II, *TMPSS2-ERG*, and anterior gradient 2 mRNAs (Dijkstra et al., 2014; Neeb et al., 2014; Nilsson et al., 2009). Moreover, mRNA transcripts of cadherin 3 (*CDH3*) were identified in urine-derived EVs from prostate cancer patients compared to patients with benign prostate hyperplasia. Genomic, transcriptional, and epigenetic alterations of the *CDH3* expression showed a correlation between the decrease in abundance of *CDH3* in EVs and the mRNA changes in the prostate tumor cells (Royo et al., 2016). Recently, a prostate cancer-screening test “ExoDx Prostate Intelliscore” from Exosome Diagnostics was proposed as a novel non-invasive test designed to predict the aggressiveness of prostate cancer. The test is based on the detection of ERG, PCA-3 and SAM pointed domain containing ETS transcription factor mRNAs in urine exosomes in combination with standard clinical parameters used to diagnose prostate cancer (McKiernan et al., 2016). Aside from the previously described molecules, DNA represents a more unexplored source of biomarkers. The first evidence of prostate vesicles carrying DNA was reported by Ronquist and collaborators in prostasomes (Ronquist et al., 2012; Ronquist et al., 2009), revealing the potential of EV-associated DNA in biomarker discovery.

2.4 Therapeutic potential of EVs

2.4.1 Harnessing the intrinsic functions of EVs for therapeutics

Based on the EV molecular composition and participation in intercellular communication processes, EVs from specific cell types may serve as novel tools for several therapeutic approaches, including immune-modulatory and regenerative therapies, anti-tumor therapy, pathogen vaccination, and drug delivery. Depending on their origin, EVs have both immune-activating and immune-suppressing properties. Immune activation can be relevant in immunotherapeutic applications, since EVs may promote the release of pro-inflammatory cytokines and the activation of immune cells (Théry et al., 2009). On the other hand, some EV-associated molecules such as IL-10, IL-12, TGF-β, and Fas-ligand have immune-suppressive properties, for instance inducing T cell apoptosis and inhibiting inflammation, which may be relevant in systemic inflammation as well as in neurodegenerative and autoimmune diseases (Andaloussi et al., 2013). The innate ability of EVs to modulate immune responses by, for instance, the participation of EVs derived from dendritic cells in antitumor responses, makes them especially good candidates to be used in cancer vaccination.

Immune-modulatory and regenerative therapies

The use of mesenchymal stem cells (MSC) as a cell therapy has intensively been investigated in many clinical trials for their immunosuppressive and regenerative effects (www.**ClinicalTrials**.gov database). It is now becoming evident that the biological effects of MSCs are caused by their secretome, including the EVs (Fais et al., 2016).

The use of MSC-derived EVs as a cell surrogate therapy has several advantages over therapies with stem cells, and MSC-derived EVs are under investigation for multiple inflammatory conditions as well as autoimmune and chronic diseases as recently reviewed (Lener et al., 2015). For instance, EVs from adipose-derived stem cells have shown therapeutic potential to treat Alzheimer's disease since they contain enzymatically active neprilysin, which lowered the levels of β-amyloid peptide secreted by neuroblastoma cells (Katsuda et al., 2013). Likewise, EVs derived from other cell types have also shown characteristics that could be exploited for therapeutic purposes. For instance, natural killer cell-derived EVs were shown to contain killer proteins that could induce tumor cell death (Lugini et al., 2012).

Cancer vaccines

Another area of EV research focuses on the use of EVs as cancer vaccines. EVs fulfill the entire antigen presenting requirements for a cell-free vaccine. Completed and still running clinical trials of EV-based therapies have been recently summarized (Ohno et al., 2016). The first study of EVs as cancer vaccines in 1998 showed the suppression of murine tumor growth in a T cell-dependent manner by dendritic cell-derived EVs pulsed with tumor peptides (Zitvogel et al., 1998). This discovery led to two phase I clinical trials where antigen-loaded EVs derived from immature dendritic cells were used to treat metastatic melanoma patients (Escudier et al., 2005) and advanced non-small cell lung cancer patients (Morse et al., 2005). However, although in both studies EVs showed a low cytotoxic effect, no specific response against the tumor was detected, and only a small number of patients benefited from the therapies. As a result, a phase II study based on EVs isolated from mature dendritic cells was used to treat advanced non-small cell lung cancer patients (Viaud et al., 2011). The study showed that EVs alone or EVs co-injected with immune-stimulatory adjuvants efficiently

promoted naïve T cell priming. Using a different approach, a phase I clinical trial based on the combination of ascites tumor-derived EVs with granulocyte-macrophage colony-stimulating factor as an immune-stimulatory adjuvant, resulted in beneficial antitumor dendritic cell activity in patients with advanced colorectal cancer (Dai et al., 2008). Recently, results obtained in preclinical studies showed improved anti-tumor effect of dendritic cell-derived EVs by using natural killer T cell activating agents (Gehrmann et al., 2013). Also, new vaccination strategies have been developed based on EVs from non-mammalian cells, including bacterial outer membrane vesicles and plant vesicles as therapeutic carriers against cancer and infectious diseases (Ohno et al., 2016). These approaches have entered phase I clinical trials and are currently under evaluation. For example, the use of outer membrane vesicles as a vaccine to treat meningitis recently concluded the phase II clinical trials (Findlow et al., 2010), with a highly promising protection of infants from meningitis. Taken together, these studies open new and exciting possibilities for EVs as immunotherapeutic agents.

2.4.2 EVs in drug delivery

Current challenges in drug delivery

The most extensively used drug delivery platforms are lipid-based nanoparticles, including solid lipid nanoparticles and liposomes. Liposomes are considered to be the most successful drug-carrier systems to date (Felice et al., 2014). They are artificial uni-lamellar or multi-lamellar spherical vesicles of 20 nm to more than 1 μm, primarily comprising phospholipids, either from plant or animal sources (Felice et al., 2014; Maximilien et al., 2015). Due to their resemblance to cellular membranes and their ability to incorporate drugs, liposomes have been widely studied in the delivery of pharmaceutical compounds for more than 50 years. They possess several benefits such as biodegradability, biocompatibility, and they are easily modifiable, facilitating specific cell targeting, which makes them valued as optimal delivery systems (Bozzuto and Molinari, 2015). Currently, several liposomal formulations have been used as medicines in the clinics, including the first FDA-approved Doxil®, a PEGylated doxorubicin liposome formulation for the treatment of solid tumors, and Marqibo®, the latest FDA approved liposome formulation which is a sphingosomal/cholesterol encapsulation of vincristine sulphate for the treatment of Philadelphia chromosome-negative acute lymphoblastic leukaemia (Allen and Cullis, 2013; Maximilien et al., 2015). Despite the extensive study of liposomes for more than five decades, there are still unresolved problems, and only a limited amount of liposomal drugs are approved for clinical use (Allen and Cullis, 2013). Important limitations are the toxicity at repeated administration, immunostimulation, complement activation, and the lack of batch-to-batch reproducibility (Bozzuto and Molinari, 2015; Fais et al., 2016). Notably, lipid- and polymer-based nanoparticles have limited specificity and do not exhibit sufficient targeting at the cellular level to extrahepatic tissues, failing to combine intracellular drug delivery efficacy with biocompatibility (Stremersch et al., 2016a). Their limited ability to penetrate organs and tissues outside the reticuloendothelial system is also a major downside (Bozzuto and Molinari, 2015; Fais et al., 2016). Therefore, efforts are now focused on new therapeutic possibilities to overcome the current liposomal formulation drawbacks.

Advantages of EVs as drug carriers

As outlined in section 2.1.4, EVs mediate signaling responses between cells and their surroundings by delivering a wide variety of active biomolecules. EVs possess several interesting features that make them ideal carriers of therapeutic drugs (**Table III**). Due to their natural origin, they surpass the stability, toxicity, and immunostimulation problems of current liposomal formulations. Other advantages also include their efficient internalization by recipient cells, and their natural ability and tropism to target specific cells or organs (Ha et al., 2016; Stremersch et al., 2016a).

Table III. Advantages of EVs in drug delivery

Characteristics	Function
Protein/lipid architecture	Protect encapsulated cargo
	Contribute to the stabilization in blood
	Modify the pharmacokinetics and biodistribution of the cargo
	Stimulates uptake
	Intrinsic cell tissue and targeting properties
Nano size	Minimizes the recognition by the reticuloendothelial system
	Suitable administration via various routes
Natural origin	Reduce activation of adaptive immune system
	Biocompatibility and biodegradability

Adapted from (Fais et al., 2016; Ha et al., 2016; Stremersch et al., 2016a).

The prospective use of EVs as customizable drug carriers and delivery vehicles has appealing potential, and several studies have unraveled their role in different aspects of their therapeutic use, as discussed below.

Applications of EVs as drug delivery vehicles

Due to their numerous advantages, EVs are now being explored as new therapeutic opportunities to carry and deliver several drug types, and these applications have been reviewed by Johnsen et al., 2014 and Stremersch et al., 2016a. EVs can carry a wide variety of molecules, including macromolecules such as DNA, RNA, and proteins, and small molecules e.g., doxorubicin, curcumin, or paclitaxel (Fais et al., 2016), improving the pharmacokinetic profile of the encapsulated therapeutic compounds by protecting the drug from degradation and/or early release. Currently, different strategies exist to load the therapeutic cargo within the EVs. These approaches can be based on pre-EV formation, where the therapeutic cargo is first loaded into EV producing cells packing the cargo during their biogenesis, or post-EV formation, where isolated EVs are loaded with the cargo via electroporation, passive incubation, or destabilizing of the EV membrane (Stremersch et al., 2016a; Vader et al., 2014). The therapeutic cargo delivered by EVs can be classified into macromolecular drugs, including nucleic acids and proteins, and small molecular drugs.

- *Nucleic acids and proteins*

Plasmid vectors expressing the therapeutic cargo in cells such as mRNA (Zomer et al., 2015), miRNA (Kosaka et al., 2010), siRNA (Liu et al., 2015), and the transfection of the producer cell with the cargo (Zhang et al., 2010), are some of the commonly used approaches to load nucleic acids in EVs before their formation. Proteins can be specifically sorted into EVs by creating fusion constructs which contain the protein of interest bound to EV-associated proteins such as platelet-derived growth factor receptor (Ohno et al., 2012), lactadherin (Estelles et al., 2007; Rountree et al., 2011), or lysosomal associated membrane protein 2b (LAMP-2b) (Alvarez-Erviti et al., 2011; Tian et al., 2014a). Post-EV formation loading approaches have also been used for nucleic acids. A pioneering study reported the electroporation of siRNA into dendritic cell-derived EVs, and its functional delivery through the blood-brain barrier in a murine model of Alzheimer's disease (Alvarez-Erviti et al., 2011). Since then, several reports have shown successful loading of nucleic acids into EVs and their efficacy in targeted cells (Chen et al., 2015; Cooper et al., 2014; Lamichhane et al., 2015; Shtam et al., 2013; Wahlgren et al., 2012). Novel drug delivery strategies such as decorating EVs with therapeutic nucleic acids, including cholesterol-conjugated siRNA (Stremersch et al., 2016c) and hydrophobically modified siRNAs (Didiot et al., 2016) are now also being explored.

- *Small molecular drugs*

The use of EVs as carriers of small molecular drugs could be of a great advantage, since EV-loading can significantly reduce the dose requirement and increase the specificity of targeting, thereby decreasing the side effects of toxic cancer treatments. The loading of drugs into EVs has been accomplished using both pre- and post-EV formation methods. Using the pre-EV formation approach, MSCs incubated with free paclitaxel produced paclitaxel-loaded EVs with anti-proliferative effects *in vitro* (Pascucci et al., 2014). Similarly, EVs secreted by cisplatin-treated tumor cells carried cisplatin within their cargo (Federici et al., 2014). The simple incubation of EVs at ambient or high temperatures with hydrophobic membrane-permeable drugs, such as curcumin and doxorubicin, exhibited a high drug loading efficiency and an increase in the cytotoxic effect of the loaded EVs compared with the free drugs (Sun et al., 2010; Yang et al., 2015). Interestingly, plant-derived EVs loaded with curcumin are now under clinical examination to treat colon cancer (Ohno et al., 2016), Membrane-destabilizing methods have also been used to lead drugs into EVs. For instance, EVs bearing an iRGD targeting peptide were loaded with doxorubicin by electroporation and then administered to mice harboring αv integrin-positive tumors, which led to a significantly reduced tumor size (Tian et al., 2014a). Different loading strategies including permeabilization with saponin, sonication, freeze-thaw cycles, incubation, or extrusion were used to compare the loading of small molecules such as porphyrins (Fuhrmann et al., 2015) and macromolecules like catalases (Haney et al., 2015) and their phototoxic and neuroprotective effects in recipient cells, respectively.

2.5 Prospects of EVs as cancer biomarkers and drug delivery carriers

Based on the above evidences, EVs have a promising future as both prospetive biomarkers for different diseases including cancer, as well as therapeutic tools for personalized medicine (**Figure 5**). The presence of EVs in the circulation of both healthy and sick individuals reveals the role of EVs as messengers to transport and deliver information to different cells in the body, contributing not only to the regulation of normal physiological

body functions, but also to the spreading of the diseases. The review of the literature presented above showed striking evidences supporting the use of EV content in the context of biomarker discovery, early diagnosis, disease monitoring, and treatment. The personalized information of EVs isolated from individual patients, including EV proteome, metabolome, transcriptome, and genome profile, and the ability of EVs to influence cell transformation, will help to tailor specific integrated diagnostics platforms and treatment strategies. Despite these evidences, a more comprehensive understanding of the role of individual EV subpopulations in the heterogeneous mix of EVs constituting the tumor secretome will undoubtedly provide new perspectives on using EVs as diagnostic tools.

Taking advantage of the self-derived nature of patient EVs, they could also be used to treat previously diagnosed diseases by tailoring an individual and more specific treatment plan with reduced side effects. The future of the clinical use of EVs depends upon further investigation and interdisciplinary collaboration among researchers, since EV-mediated diagnostics and therapeutics are still in their early stages, and further investigation is needed before it can be translated into the clinics.

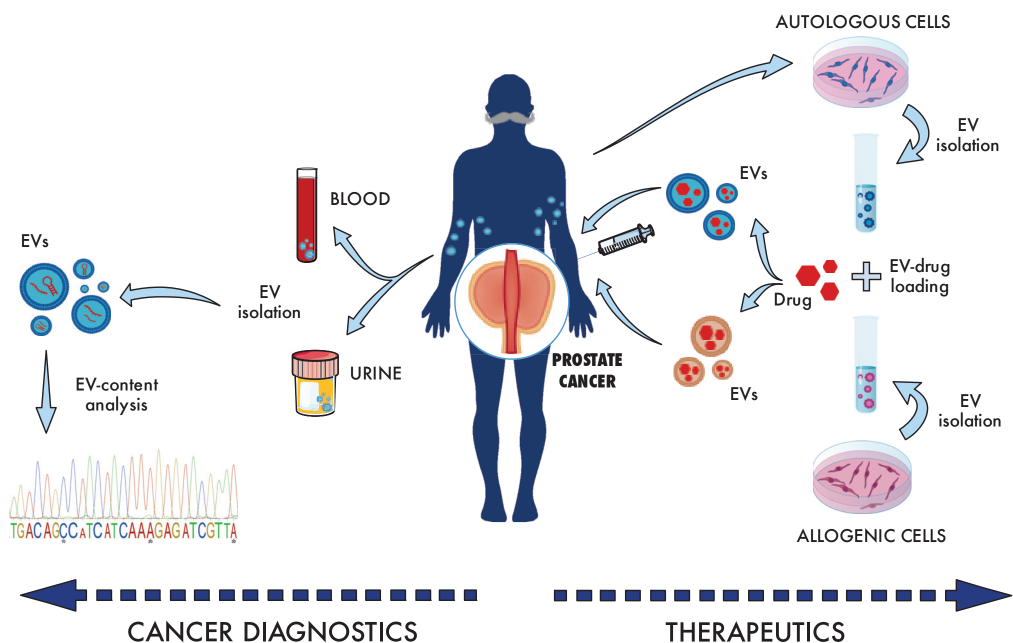

Figure 5. Schematic representation of personalized approaches for prostate cancer diagnostics and therapeutics using EVs. Cancer-derived EVs present in blood and other fluids represent a potential source of cancer markers, containing specific information of the tumors. Screening platforms based on the analysis of the composition of EVs isolated from biofluids of individual cancer patients, the so-called “liquid biopsy”, will open up new possibilities in the fields of cancer diagnosis and treatment. Alternatively, EVs could potentially be harvested from exogenous sources (allogenic) or from the own patient (autologous) and exploited as therapeutics or for the delivery of drugs. The figure was produced party using Servier Medical Art.

3 Aims of the study

The overall aim of this thesis was to evaluate the applicability and functional relevance of EVs as a source of biomarkers and as therapeutic drug carriers using prostate cancer as a disease model.

The detailed aims of this thesis were:

- To investigate the nucleic acid cargo of EV subpopulations, particularly gDNA and mRNA, as a possible source of prostate cancer biomarkers (**I**, **II**).
- To evaluate the role of prostate cancer cell-derived EVs in cell-to-cell communication (**III**).
- To assess the usability of EV subpopulations as drug delivery carriers (**IV**).

4 Materials and Methods

The materials and methods used are described in this chapter. The more detailed description of the experimental procedures can be found in the original publications **I**–**IV**.

4.1 Cell lines and culture conditions (I, II, III, IV)

Table IV. Human cell lines used in the studies.

Cell line	Cell type	Characteristics	Source	Culture media	Publication
LNCaP (CRL-1740)	prostate cancer epithelial	derived from metastatic site: supraclavicular lymph node	ATCC[2]	RPMI 1640 medium supplemented with 10% EV-depleted fetal bovine serum	I, II, III, IV
PC-3 (CRL-1435)	prostate cancer epithelial	derived from metastatic site: bone	ATCC[2]	DMEM/F12 medium supplemented with 10% EV-depleted fetal bovine serum	I, II, III, IV
PNT2	normal prostate epithelial	immortalized with SV40	ECACC[3]	Keratinocyte-SFM with bovine pituitary extract and human recombinant epidermal growth factor	II, III
RC92a/hTERT[1]	prostate cancer epithelial	primary cells immortalized with hTERT	Dr. Rhim[4]	Keratinocyte-SFM with bovine pituitary extract and human recombinant epidermal growth factor	I, III

[1]RC-92a/hTERT cells were obtained from radical prostatectomy specimen according to Water Reed Medical Center and Uniformed Service University of the Health Sciences Internal Review Board Protocol. RC-92a tumor cells were obtained from a 57-year-old Caucasian patient who had clinical stage B0 and Gleason 3+3.
[2]American Type Culture Collection (ATCC), Masanasas, VA, USA
[3]European Collection of Authenticated Cell Cultures (ECACC), Salisbury, UK
[4]Provided by Dr. Rhim JS, Center for Prostate Disease Research, University of the Health Sciences, Bethesda, MD, USA

For publication **III**, cell index-doubling times of PC-3 and PNT2 cells were analyzed using RTCA iCELLigence machine (ACEA Biosciences). Prostate cells were seeded on E L8 PET-plates in 2-fold dilution series (40,000 to 5,000 cells/well) as recommended by the manufacturer. After cell attachment, the plates were analyzed using the proliferation program of the iCELLigence instrument. RPMI 1640 and DMEM/F12 cell culture media were supplemented with 10% EV-depleted fetal bovine serum (FBS). The FBS was EV-depleted by centrifugation at 110,000 x g_{avg} for 16–18 h using 50.2 Ti or 45 Ti rotors, followed by filtration through a 0.22 µm filter.

4.2 Patient samples (I)

EDTA-blood samples were obtained from prostate cancer patients (for patient characteristics see publication **I**), and healthy male donors after receiving written consent

(ethical permission number R03203 from the Tampere University Hospital). Plasma samples were obtained after centrifugation of the whole blood two times at 2,500 x *g* for 15 min.

4.3 Antibodies (II, III, IV)

The primary antibodies used in publication **II**, **III,** and **IV** are summarized in **Table V**. For a more complete description of the concentrations and conditions used refer to the publications.

Table V. List of antibodies used in the studies.

<table>
<tr><th>Antibody anti-</th><th>Cellular localization</th><th>Application</th><th>Source</th><th>Publication</th></tr>
<tr><td>GM130</td><td>Golgi apparatus stack membrane</td><td rowspan="4">Immuno-fluorescence</td><td rowspan="2">Cell Signaling MA, USA</td><td rowspan="4">III</td></tr>
<tr><td>EEA1</td><td>Cytoplasm and early endosome membrane</td></tr>
<tr><td>Lamp1</td><td>Cell, endosome and lysosome membranes</td><td>IOWA University USA</td></tr>
<tr><td>CD44</td><td rowspan="3">Membrane</td><td>Dr. Jalkanen Turku, Finland</td></tr>
<tr><td>CD9</td><td rowspan="9">Western blotting</td><td rowspan="5">Santa Cruz Biotechnology CA, USA</td><td>III, IV</td></tr>
<tr><td>CD81</td><td>II, IV</td></tr>
<tr><td>Calnexin</td><td>Endoplasmic reticulum membrane</td><td>II</td></tr>
<tr><td>Flotillin-I</td><td>Cell membrane. Membrane-associated protein of caveolae. Endosome</td><td>II</td></tr>
<tr><td>GAPDH</td><td>Cytoplasm, cytosol, perinuclear region. Membrane</td><td>III, IV</td></tr>
<tr><td>CD63</td><td>Lysosomal associated membrane protein</td><td rowspan="4">BD Biosciences USA</td><td>III, IV</td></tr>
<tr><td>HSP70</td><td>Cytoplasm. Localized in cytoplasmic mRNP granules</td><td>III</td></tr>
<tr><td>Tubulin</td><td>Major constituent of microtubules</td><td rowspan="2">IV</td></tr>
<tr><td>TSG101</td><td>Mainly cytoplasm, membrane, and late endosome membrane</td></tr>
</table>

mRPN: messenger ribonucleoprotein

4.4 EV isolation (I, II, III, IV)

4.4.1 Differential centrifugation

Cell-conditioned media from the above mentioned cell lines and plasma samples were subjected to differential ultracentrifugation to isolate EV-enriched subpopulations (**Figure 6**). Cell conditioned media was collected when the cells reached 80% confluence, and centrifuged at 1,000 x *g* for 10 min to remove cell debris. The remaining supernatant was carefully collected and centrifuged at 2,500 x *g* for 25 min (1,200 x *g* in publication **I**) to pellet ABs. The supernatant was then centrifuged at 20,000 x g_{avg} for 60 min (SLA 1500 rotor, Sorvall, in publications **I, III,** and **IV**) or 25 min (45 Ti rotor, Beckmann Coulter, in

publication **II**) to pellet MVs. The remaining supernatant was filtered through 0.22 μm filter (only publication **I**) and centrifuged at 110,000 x g_{avg} for 1 h (publication **I** and **IV**) or 2 h (publications **II** and **III**) using 50.2 Ti or 45 Ti rotors to pellet EXOs. The centrifugation time was increased from 1 h to 2 h in order to increase the yield of EXOs recovered during isolation. The completed cell media that has not been in contact with cells was also subjected to EV isolation and used as a control. Particle counts close to background levels of buffers were identified, and the remaining particle counts were at least 100-fold lower compared to EV samples.

In these four studies I will refer to EVs as a general term that encompasses all EV subtypes. The term EXOs will be used when referring to enrich vesicles of endosomal origin pelleted at 110,000 x *g*. The term MVs will refer to enriched vesicles pelleted at 20,000 x g, that are formed by outward budding of the cellular plasma membrane. The term ABs will refer to vesicles generated from apoptotic cells, pelleted at lower speeds 1,000–2,500 x *g*.

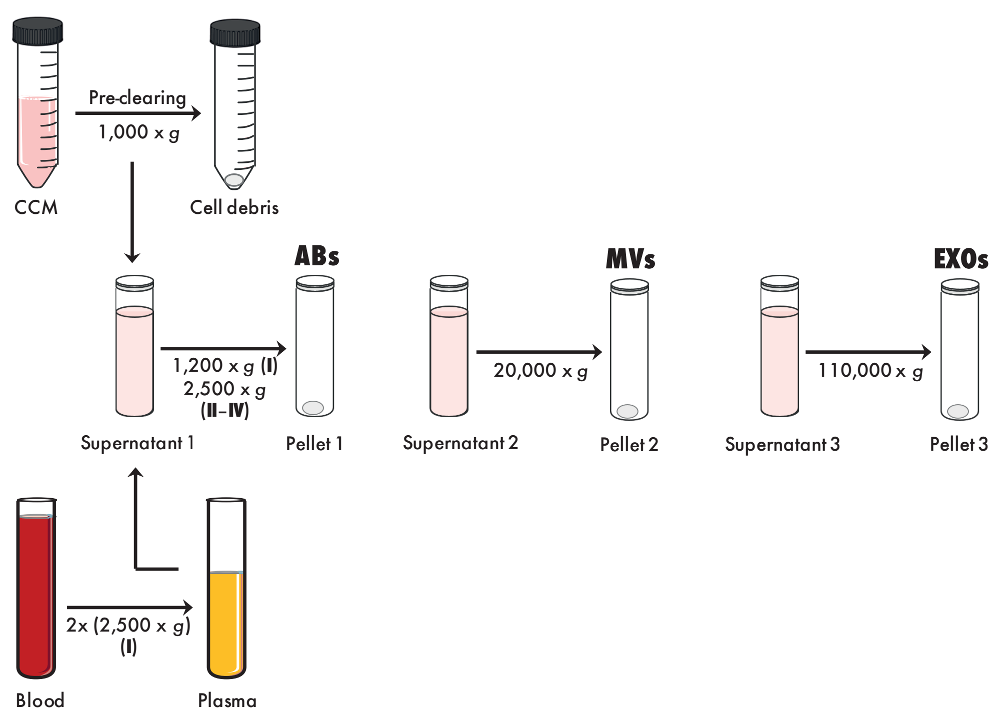

Figure 6. Experimental flow-chart of the isolation of prostate cell-derived and plasma-derived EV subpopulations. After cell debris removal or plasma separation from the whole blood, the samples were subjected to a differential centrifugation protocol, to obtain **ABs**: apoptotic bodies, **MVs**: microvesicles, and **EXOs**: exosomes. EV pellets were immediately stored at -80 °C or alternatively were freshly used, as indicated in the studies. The publication numbers are displayed in parenthesis. CCM: cell-conditioned media.

4.5 EV characterization (I, II, III, IV)

4.5.1 *Transmission electron microscopy (I, II, III, IV)*

For transmission electron microscopy (TEM) analysis, EV samples were incubated onto formvar glow-discharged copper grids for 2 min at 4 °C. Grids were washed, blotted dry with filter paper, and negatively stained with 2% aqueous uranyl acetate (Sigma-Aldrich). Following washes, samples were dried in darkness for 20 min and analyzed using FEI Tecnai Spirit G2 (Soft Image System Morada camera) and Tecnai 12 (Gatan Orius SC 1000B bottom mounted CCD-camera) operated at 80 kV.

For Cryo-TEM analysis (publication **IV**), glow-discharged carbon grids were first purified with the Solarus plasma cleaning system (Gatan) and blotted with 3 µL of EV samples. Samples were vitrified in liquid nitrogen using Vitrobot (FEI) and imaged using a JEOL JEM-3200FSC instrument operated at 200 kV.

4.5.2 Nanoparticle tracking analysis and zeta potential (I, III, IV)

The size distribution and particle concentration of the EV preparations were analyzed using the Nanoparticle Tracking Analyzer (NTA) instrument (Malvern Instruments Ltd, Malvern, UK) with LM10 view unit and red laser (638 nm, 40mW) (publication **I**) or with a LM14 view unit, blue laser (405 nm, 70mW) (publications **III** and **IV**) and a CMOS camera (Hamamatsu Photonics, Hamamatsu, Japan). Equipment settings for data acquisition, which were optimized based on the EV source, were kept constant between measurements: camera level 13–14, auto settings off, polydispersity and reproducibility high with 40–100 particles per image, acquisition time three 90 s videos, screen gain 10 and threshold 10. Data analysis was performed with NTA 2.3 software (NanoSight, Amesbury, UK).

For zeta potential measurements (publication **IV**), EV samples were diluted with DPBS and the pH was adjusted to 7.4. Samples were transferred to the capillary cell DTS1070 (Malvern, Worcestershire, UK) and measured at 25 °C using a Zetasizer Nano ZS (Malvern).

4.5.3 Protein content and Western blotting (I, II, III, IV)

EVs and cellular protein samples were lysed in RIPA lysis buffer (Thermo Fisher Scientific) supplemented with a protease inhibitor mixture (Sigma-Aldrich), and analyzed in triplicates using the BCA or MicroBCA protein assay kit (Pierce, Thermo Fisher Scientific) and Varioskan Flash multireader (v.2.4.3) with a 562 nm absorbance filter (Thermo Fisher Scientific). Western blotting samples were prepared with 2x Laemmli buffer (Bio-Rad) and heated at 95 °C for 5 min. 50–25 µg of protein from EVs and cells were loaded onto 12 % Mini-PROTEAN TGX™ gels (Bio-Rad) and transferred to Protran nitrocellulose membrane (Whatman International Ltd). Membranes were blocked in 5% (w/v) skim milk powder in Tris-buffered saline-0.1% Tween 20 (TBST) for 1 h to overnight. Blocked membranes were incubated with the primary antibodies diluted in 0.25–2.5% milk-TBS presented in **Table V**. The membranes were washed with TBST, and incubated for 45 min to 1 h at room temperature (RT) with the goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) or the ECL donkey anti-rabbit IgG-HRP secondary antibodies diluted in 0.25–2.5% milk-TBST. Membranes were washed and incubated with Luminata Crescendo Western HRP Substrate (Millipore) or ECL Prime Western Blotting Detection (GE Healthcare Limited). Membranes were visualized on VersaDoc 4000 MP (Bio-Rad) or Amersham Hyperfilm ECL (GE Healthcare Limited).

4.5.4 Fluorescent labeling and flow cytometry (III, IV)

EVs were labeled using 1–2 μg/mL of the following lipophilic aminostyryl dyes: $DiIC_{18}(5)$-DS or SP-$DiOC_{18}(3)$ (Molecular Probes, Thermo Fisher Scientific). In publication **IV**, paclitaxel-loaded EV samples were double-labeled with DiD (Biotium) and OregonGreen-paclitaxel (Invitrogen, Thermo Fisher Scientific) to study the cellular distribution of the drug -loaded EVs. The EVs were incubated with the dyes for 20–60 min at RT and the free dye was removed by size exclusion chromatography or ultracentrifugation at 110,000 x g. In publication **III**, samples were measured with Apogee A50micro for 120 sec with optimized settings. To demonstrate the specificity of the labeling protocol, labeled EVs were treated with 0.15% SDS and analyzed using FlowJo 10.0 software (Treestar, USA).

4.5.5 DNA analysis (I)

DNA isolation, profiling and pre-amplification

To remove possible external EV-associated nucleic acids, EV samples were treated with 100 mg/mL of RNase A (Thermo Fisher Scientific) for 2 min at RT and 27 Kunitz U/mL of DNase I (Qiagen, Hilden, Germany) for 30 min at 37 °C followed by enzyme inactivation. Total DNA was extracted from EV and cell samples using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer´s instructions. The quality, profile, size, and concentration of the DNA samples was analyzed by capillary electrophoresis using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with a High Sensitivity DNA kit and confirmed by SPECTROstar Nano (BMG Labtech). The total DNA was pre-amplified using the GenomePlex Complete WGA2 kit (Sigma-Aldrich, St Louis, MO, USA) following manufacturer's recommendations.

PCR and sequencing

Conventional and quantitative PCR, together with Sanger sequencing were used to confirm and validate the presence of *MLH1*, *PTEN,* and *TP53* genomic DNA sequences in EV subpopulations and cells. Specific primers (Sigma-Aldrich) were design using Primer 3 and nucleotide Blast (BLASTN, NCBI) tools, and the PCR conditions were optimized for each sample (**I**, Table II). Briefly, for conventional PCR, 10 ng of DNA was used per reaction and samples were run in a T100 Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). For quantitative PCR assays, 50 ng of DNA mixed with the Fast SYBR Green Master Mix (Applied Biosystems) was run using a Lightcycler 480 (Roche Diagnostics). The melting curves and cycle threshold (Ct) values were analyzed with Lightcycler 480, v.1.5 software, and manually reviewed. Samples, external standards, and non-template controls were run in triplicate and the resulting PCR products were validated by electrophoresis in 1% agarose gels. The PCR products were extracted and purified using Gel extraction kit and QIAqick PCR purification kit (Qiagen) and sequenced using a BigDye Terminator v.3.1. Cycle Sequencing kit and ABI 3730 DNA analyzer automated sequencer (Applied Biosystems). Sequencing results were analyzed using Geneious v 4.8.5 software.

4.5.6 mRNA analysis (II)

RNA isolation, profiling and pre-amplification

Total RNA was extracted from EVs and cell samples using a miRNAeasy micro kit (Qiagen) as recommended by the manufacturer. The concentration, quality, and size of the RNA was measured by capillary electrophoresis after sample denaturation at 72 °C for 2 min, using RNA 6000 Nano and Pico total RNA kits and 2100 Bioanalyzer instrument (Agilent

Technologies). 500 ng of RNA were subjected to DNA removal, cDNA synthesis, and instant pre-amplification using the RT2 PCR System PreAMP and Human Prostate Cancer Pathway mix (Qiagen).

Reverse transcription quantitative PCR (RT-qPCR)

For RT-qPCR analysis, PAHS-135Z Human Prostate Cancer Pathway RT^2 Profiler PCR Arrays (SaBiosciences, Qiagen) were used as described by manufacturer. 100 ng/µL of EV-RNA samples mixed with the RT^2 SYBR Green Master Mix were run in biological triplicates in a CFX96 thermocycler (Bio-rad). The 3.5 PCR Array Data Analysis software (SaBiosciences) was utilized to calculate the mRNA levels of each gene and compared to all the genes across the array (global normalization). A stringent fold-change threshold of 10 was used for the mRNA analysis.

4.6 EV drug-loading and delivery (IV)

EVs were loaded with 5 µM paclitaxel-DPBS solution by passive incubation at 22 °C for 1 h. The unbound drug was removed by pelleting the EVs at 170,000 x g for 2 h, washing with DPBS, and pelleting them again at 170,000 x g for 2 h. Prior to the measurement, the acetonitrile extraction method was confirmed by spiking non-loaded EVs and 1.8 µM paclitaxel. To measure the loading efficiency of the EV-associated paclitaxel, samples were dissolved in acetonitrile, centrifuged at 10,000 x g to remove precipitates, and the supernatant was measured in the Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters). The *in vitro* drug release of the paclitaxel from EVs was analyzed in DPBS, pH 7.4, at 37 °C for 24 h and 48 h. Half of the volume of the release medium was withdrawn at each time point by ultracentrifugation of the EV samples at 170,000 x g for 2 h and collection of the supernatant, and the withdrawn medium was then replaced. The drug concentration of the supernatant was analyzed by UPLC.

4.7 *In vitro* cell-based assays (III, IV)

4.7.1 EV uptake and intracellular trafficking (III, IV)

Automated cellular imaging system (III)

Prostate cells were seeded in 96 well plates at a density of 3,000–4,000 cells/well. Cytoplasmic membranes were labeled with CellBrite Green Cytoplasmic Dye (Biotium), according to manufacturer´s instructions followed by incubation with 10^9 particles/mL of $DiIC_{18}(5)$-DS labeled -EVs for different times. Prior to the analysis, cell samples were washed with DPBS, fixed with 4% paraformaldehyde at RT for 10 min, and analyzed by IN Cell Analyzer 1000 high-content system (GE Healthcare, Life Sciences) configured with a CCD camera and a 20×/0.45 NA objective. The percentage of cells with EVs was quantified using segmentation algorithms and normalized to the total number of cells per image.

Flow cytometry (III, IV)

Prostate cells were seeded in 6 well plates at 300,000–500,000 cells/well and incubated with $DiIC_{18}(5)$-DS-labeled EVs. After incubation for different times, cells were washed twice with DPBS, harvested, fixed using high-grade cold 99% ethanol, and stored at -20 °C overnight. After ethanol removal and re-hydration, samples were run in a Gallios flow cytometer (Beckman Coulter), collecting 20,000–100,000 events/sample and analyzed with

FlowJo. Unstained cells with and without EVs were used as negative controls to gate the positive populations based on forward- and size- scatter profiles.

Confocal laser scanning microscopy (III, IV)

Prostate cells were seeded in chamber slides and incubated with EVs for 16 h and 24 h. For the co-localization experiments in publication **III**, cells were fixed with 4% paraformaldehyde in 0.1 M DPBS for 20 min at RT, permeabilized using 0.1% Triton X-100 in 1% BSA-DPBS for 10 min and blocked with 1% BSA-DPBS for 20 min. The cells were incubated overnight in blocking solution at 4 °C with the antibodies described in **Table V**. After washing, cells were incubated with the secondary antibody Texas red anti-mouse (1:100) or Texas red anti-rabbit (1:500) (Vector laboratories, CA, USA) for 1 h at RT. Nuclei were stained with DAPI (1 µg/mL, Sigma), and the samples were imaged with Zeiss Axio Observer inverted microscope (63×NA 1.4 oil immersion objective) equipped with Zeiss LSM 800 confocal module (Carl Zeiss Microimaging GmbH, Jena, Germany). Image acquisition was done with ZEN2 software (Carl Zeiss Microimaging GmbH). Co-localization analyses were conducted using Imaris 7.7.2 software, by selecting $DiIC_{18}$-EVs channel as region of interest (ROI). Thresholds were auto adjusted by the software within the ROI. The percentage of ROI material of DiI- co-localizing with CD44, EEA1, Lamp-1, GM130 markers was separately analyzed from at least eight images containing multiple cells.

For the co-localization studies (publication **IV**), prostate cancer cells were incubated with free Oregon Green paclitaxel, DiO-labeled EVs or double-labeled paclitaxel-loaded EVs (as described in section 4.4.5) for 24 h or 48 h. Live cells were labeled with 70 nM of LysoTracker Red (Invitrogen, Thermo Fisher Scientific) for 2 h, followed by washes for dye removal. Image and video acquisitions were conducted using a High Content Screening Automation Leica TCS SP5II (Leica Microsystems) or a 3I Marianas (3I Intelligent Imaging Innovations) with a 63×NA 1.46 oil immersion objective. Confocal images were processed using Fiji Image J 1.49 software.

4.7.2 Cell viability (III, IV)

The cytotoxic effect of the different concentrations of free paclitaxel and paclitaxel-loaded EVs in prostate cancer cells (publication **IV**) was assessed using the AlamarBlue assay (Life Technologies, Thermo Fisher Scientific), measuring the fluorescent intensity with excitation at 560 nm and emission at 590 nm using Varioskan Flash Multimode Reader (Thermo Fisher Scientific). AlamarBlue uses the reducing power of living cells to quantitatively measure cell proliferation. The active ingredient resazurin is reduced to resofurin, which is red in color and highly fluorescent.

For viability analysis by flow cytometry (publication **III**), cell samples were harvested in 2% FBS-DPBS solution, labeled with 7-Aminoactinomycin D (BD Pharmingen), which is a fluorescent intercalator that undergoes spectral shift upon association with DNA, and immediately analyzed.

4.7.3 Cell cycle (III)

Prostate cancer cells were incubated with 10^9 particles/mL of SP-$DiOC_{18}(3)$-labeled EVs. Cells were washed, harvested, fixed using high-grade cold 99% ethanol, and stored at -20 °C overnight. After ethanol removal and rehydration, samples were incubated with 3 µM propidium iodine (Molecular Probes, Thermo Fisher Scientific) in 4% FBS-DPBS for 15 min

at RT. Samples were run in a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo.

4.7.4 Proliferation (III)

For analysis of proliferation of prostate cancer cells treated and non-treated with EVs (10^9 particles/mL), cells were seeded in 6 well plates at 300,000–500,000 cells/well and incubated with 5 μM CellTrace CFSE dye for 20 min at 37 °C. The residual dye was removed from the cultures by washing three times with DPBS, cell samples were harvested in 2% FBS-DPBS solution and immediately run with a FACS Verse flow cytometer (BD Biosciences) and analyzed with FlowJo.

4.7.5 Migration (III)

Prostate cancer cell migration was determined using a Radius fibronectin coated kit 24 well assay (Cell Biolabs). Cells were seeded at 200,000 cells/mL and incubated overnight. After gel removal, 10^9 particles/mL of EVs were added, and the gap closure was monitored using a phase-contrast inverted EVOS XL microscope (Thermo Fisher Scientific) at 10× magnification. Cell migration images were analyzed using ImageJ 1.49v software.

4.8 Statistical analysis (I, II, III, IV)

For publication **I**, statistical analysis was performed using R-project 3.0.2 software. Student’s t-test with a Shapiro-Wilk test or non-parametric Wilcoxon signed ranked test were used. For publication **II** and **III**, statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Unpaired student’s t-test, one-way or two-way ANOVA with Tukey's multiple comparisons test or Dunnett's multiple comparisons test were used. For publication **IV**, statistical analysis was carried out using SPSS 22.0 and SigmaPlot 11.0. Paired and unpaired student’s t-test, one-way ANOVA with Tamhane’s T2 post hoc t test, and Mann-Whitney Rank Sum test were used. The results were considered statistically significant as follow: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, $^{****}P<0.0001$. All results are presented as mean ± standard error of the mean (SEM).

5 Results

This chapter summarizes the main results of this thesis and the new results obtained after combining the studies. More details can be found in the original publications (**I-IV**).

5.1 Prostate cancer cells release distinct EV subpopulations (I, II, III, IV)

5.1.1 Characteristics of prostate EVs

EV subpopulations from various prostate cell lines and human plasma samples were isolated by differential centrifugation. The protocol used in these studies (**Figure 6**) allowed the separation of three major enriched but heterogeneous EV subsets, namely ABs, MVs, and EXOs (**I-IV,** Fig. 1). ABs pelleted at the lower centrifugation speeds (1,200–2,500 x g), were usually the less abundant EV subsets as shown by TEM analysis. MVs, isolated at 20,000 x g, were heterogeneous lipid membrane particles with very diverse sizes and morphologies when analyzed by TEM. MVs had an average size of ~ 200–250 nm as shown by NTA analysis, with vesicles ranging from ~ 100–800 nm in diameter dependent on their source of origin. EXOs, obtained after ultracentrifugation at 110,000 x g, had a “cup-shaped” morphology when analyzed by TEM, which is now known to be an artificial artifact of the technique, and variable sizes ranging from ~ 50–200 nm when analyzed by NTA. When techniques that preserve the EV structure, such as Cryo-TEM, were used to characterize the EVs, a large diversity of sizes and morphologies were observed in the MV and EXO enriched subpopulations (IV, Fig. 2). Cryo-TEM analysis revealed highly electron dense vesicles with lipid bilayers and remarkable multi-structural vesicles, such as double vesicles, triple to sextuple vesicles, and vesicle-containing sacks. The amount of EVs released from LNCaP, PC-3, RC92a/hTERT, and PNT2 cell lines was similar, around 5–50 x 10^9 particles/mL per million cells when NTA 2.3 software and LM14 view unit with blue laser were used to measure the samples. The variation of the EV amount was dependent on the cell source of the EVs, the incubation time of the culture media with the cells, and the EV subpopulation analyzed. In contrast, the total protein contents of the different cell line-derived EVs were different, with a possible influence of the protein carryover from the cell media. For instance, LNCaP, PC-3, and RC92a/hTERT MVs and EXOs had differences in their total protein content, depending on the cellular origin of the EVs and the vesicle subpopulation analyzed (**I**, Fig. 3), with MVs typically having a higher particle to protein ratio than EXOs. LNCaP and RC92a/hTERT EV particle numbers measured by NTA correlated well with the total protein content of the vesicles, whereas no correlation was apparent for PC-3 EVs. When prostate cancer patient samples were analyzed, the total concentration of EVs isolated from plasma samples of cancer patients was higher than that from healthy donors (**I**, Fig. 2). Analysis of the typical “EV-markers” from the prostate cancer cell-derived MVs and EXOs by western blotting showed the presence of protein markers such as CD9, CD63, CD81, and TSG101, common to both MVs and EXOs, but with enriched expression levels in comparison to the respective cell lysates when equal protein amount was analyzed (**II-IV,** Fig. 1). This data revealed qualitative differences of the markers, but it did not provide quantitative comparisons of EV numbers or their cargo between the cell lines due to the different media composition.

5.1.2 Differential nucleic acid cargo of EVs

Next, the nucleic acid contents of these same prostate cell-derived EV subpopulations were investigated (**I**, **II**). ABs, MVs, and EXOs isolated from LNCaP, PC-3, PNT2, and RC92a/hTERT cells, had very similar total DNA and RNA nucleic acid profiles between different cell types, but these profiles were very different among the different subpopulations (**Figure** 7). Additionally, MVs and EXOs derived from the benign prostate epithelial PNT2 cells displayed different RNA profiles from the EVs derived from prostate cancer cells of primary or metastatic origin (**II**, Fig. 1). Prostate cancer and non-cancer cell-derived EVs carried DNA and RNA fragments of variable sizes. DNA molecules were largely protected from enzymatic degradation, suggesting that the nucleic acid cargo analyzed resided mostly within the internal vesicle cargo. However, the RNA content of the EV subsets was comprised of both adsorbed RNA molecules to the EV surface and RNAs packed with the EV internal cargo.

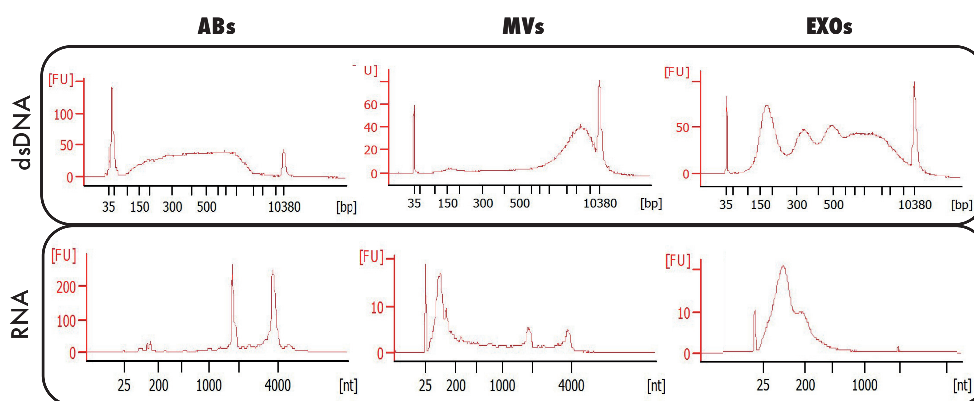

Figure 7. Characterization of the nucleic acid content of prostate cancer cell-derived EV subpopulations. Representative electropherograms for apoptotic bodies (ABs), microvesicles (MVs), and exosomes (EXOs) displaying the total double stranded (ds)DNA and RNA profiles analyzed by capillary electrophoresis using 2100 Bioanalyzer. The y-axes show fluorescence units (FU) and the x-axes represents base pairs (bp) for DNA and nucleotide length (nt) for RNA. The DNA peaks at 35 and 10,000 bp represent the lower and the upper internal DNA markers. The RNA peaks at 25 nt represent the internal standards used, and peaks around 2,000 nt and 4,000 nt represent ribosomal RNA 18 and 28 subunits, respectively.

Prostate cancer-derived ABs contained large and abundant fragments of double-stranded DNA up to 10,000 bp, and were enriched in ribosomal RNA (rRNA) with a relatively low amount of small RNA molecules. MVs shared similar DNA and RNA profiles with the ABs. MVs contained smaller amounts of double-stranded DNA in comparison to EXOs, with DNA fragments ranging from 1,000–10,000 bp. The RNA profile of MVs fractions showed enrichment in rRNA, which was visible as 18S and 28S peaks, whereas small RNAs were less abundant compared to EXOs. In contrast, EXOs were enriched in both DNA and small RNA molecules. The DNA amount present in prostate cancer EXOs varied among samples. EXOs from prostate cell lines showed a distinctive DNA profile with a range of fragment sizes between 50–10,000 bp, with characteristic DNA peaks around 150, 360, and 450 bps.

5.2 EVs contain double-stranded gDNA harboring prostate cancer mutations (I)

One of the main aims of this thesis was to characterize the DNA content of prostate cancer-derived EV subpopulations and assess their possible relevance as cancer biomarkers, since so far, the presence of DNA in EVs has not been deeply investigated. Thus, the DNA content of ABs, MVs, and EXOs from LNCaP, PC-3, and RC92a/hTERT prostate cancer cell lines and plasma samples from prostate cancer patients and healthy donors were analyzed (**I**, Fig. 4, 5). Specifically, the presence of gDNA fragments of MutL homolog 1 (*MLH1*), *PTEN*, and *TP53* genes and their mutational status was investigated (**Figure 8**). By conventional PCR and qPCR analysis, *MLH1* gDNA fragments of 108 bp were detected in all the EV subpopulations except RC92a/hTERT EXOs. *PTEN* gDNA fragments of 225 bp were only detected in LNCaP ABs and EXOs, and they contained a frame-shift mutation in the codon 6 (K6fs*4) by a deletion of two adenines. Additionally, a 316 bp gDNA fragment of the *TP53* gene was confirmed only in LNCaP ABs, which also harbored a polymorphism (P72R) detected by the sequencing analysis (**Figure 8. A, B**).

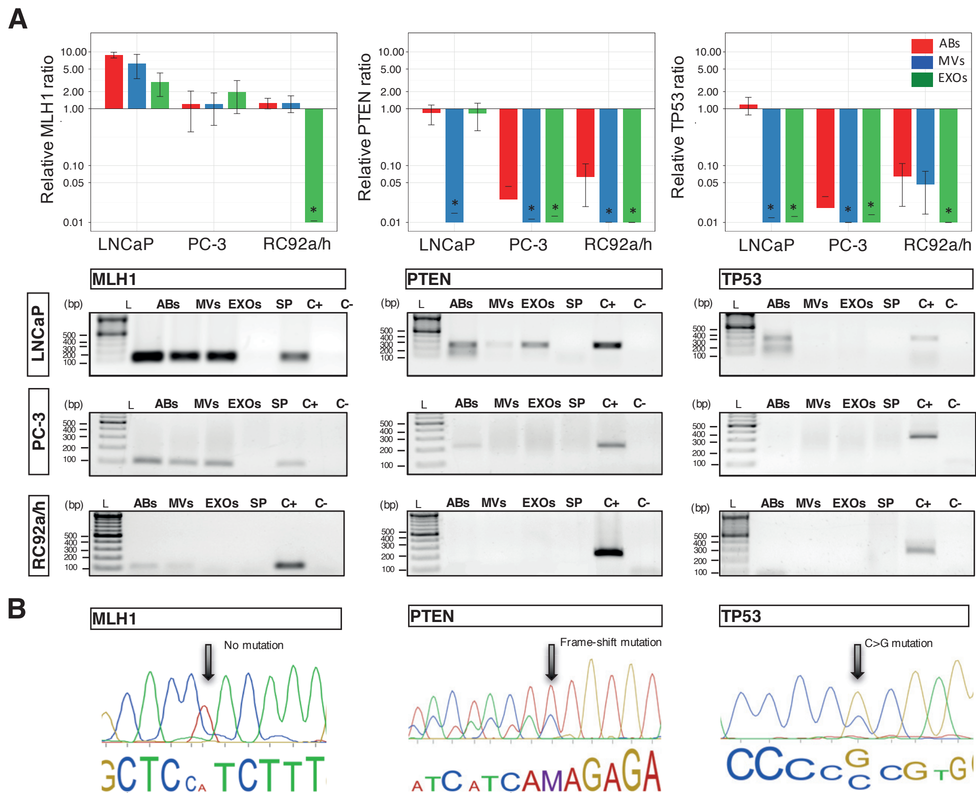

Figure 8. Presence of mutated genomic DNA fragments in EV subpopulations. A) Relative ratio of genomic DNA (gDNA) fragments of *MLH1*, *PTEN*, and *TP53* assessed by quantitative PCR (qPCR) in LNCaP, PC-3 and RC92a/hTERT EVs using GAPDH as a reference gene. Asterisk (*) represents that the gene was not detectable by qPCR or not present in the samples analyzed. Apoptotic bodies (ABs): red; microvesicles (MVs): blue, and exosomes (EXOs): green. Bar graphs represent the average value of three independent experiments ± SEM. Representative agarose gels of *MLH1*, *PTEN*, and *TP53*

gDNA fragments of ABs, MVs, and EXOs from LNCaP, PC-3 and RC92a/hTERT cells. Ladder (L), supernatant from the last ultracentrifugation as an internal control (SP), positive control (C+), and negative control (C-). B) Chromatograms displaying mutations in *PTEN* and *TP53* fragments in ABs and EXOs. The arrows mark the position of the no *MLH1* mutation (left), *PTEN* mutation in codon 6 (delAA) (center), and *TP53* mutation in codon 215 (CCC to CGC) (right). Publication **I**, Fig. 4.

5.3 Specific prostate cancer mRNA signatures of EVs (II)

The mRNA content of MVs and EXOs enriched fractions isolated from LNCaP and PC-3 prostate cancer cells and benign PNT2 prostate epithelial cells was evaluated for their putative use in future clinical diagnosis in prostate cancer. MVs and EXOs derived from LNCaP and PC-3 cells were shown to have their own distinct prostate cancer-specific mRNA signatures based on their different cellular origin when mRNA transcripts of 84 genes related to prostate cancer were analyzed by RT-qPCR -based mRNA arrays (**Figure 9**).

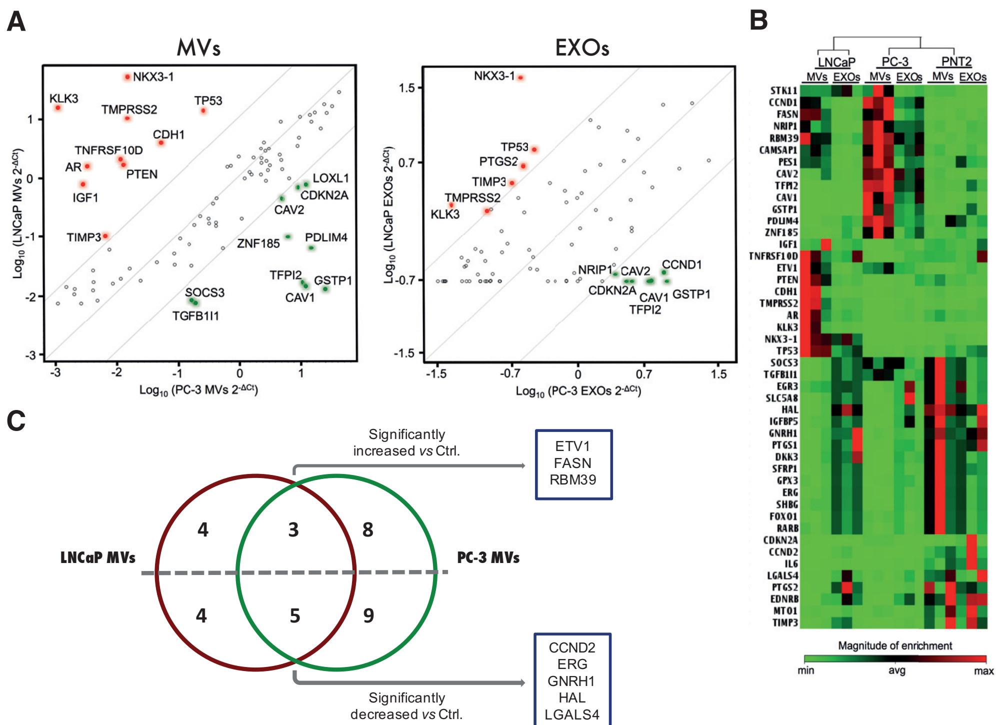

Figure 9. Prostate cancer-derived microvesicles (MVs) and exosomes (EXOs) have unique mRNA signatures resembling their parent cells. A) Scatter plots showing a log transformation of the relative mRNA level of each gene ($2^{-\Delta Ct}$) between: LNCaP MVs (y-axis) and PC-3 MVs (x-axis), and LNCaP EXOs (y-axis) and PC-3 EXOs (x-axis). The gray lines indicate a boundary of 10. Genes at prominent coordinates are annotated. All data are representative of three independent experiments per group. Fold-change cut-off =10. B) Non-supervised hierarchical clustering indicating normalized enrichment of the mRNA levels of the most significant genes detected in the LNCaP and PC-3 MVs and EXOs in comparison with the PNT2 MVs or PNT2 EXOs. C) Venn diagram representing the statistically significant common and unique mRNAs for LNCaP MVs (red) and PC-3 MVs (green) compared with PNT2 MVs (Ctrl). The genes displayed in the boxes are common mRNAs for both LNCaP and PC-3 MVs which are differentially increased or decreased vs Ctrl. Publication **II**, Fig. 2, 3 and Table 1.

MVs and EXOs from LNCaP cells contained characteristic mRNA transcripts of genes with documented androgen regulated functions. These include kallikrein-related peptidase 3 (*KLK3),* also known as *PSA*; NK3 Homeobox 1 (*NKX3-1)* and *TMPRSS2*, genes related to prostate cancer progression; tissue inhibitor of metalloproteinase-3 (*TIMP3)* and *TP53* genes (**Figure 9. A**). On the other hand, in PC3-derived MVs and EXOs, genes reported to be associated with advanced and metastatic stages of prostate cancer were detected, including mRNAs of glutathione S-transferase pi 1 (*GSTP1)*, caveolin-1 (*CAV1)*, *CAV2*, cyclin-dependent kinase inhibitor 2A (*CDKN2A),* and *TFPI2* (**Figure 9. A**). A cluster analysis of the gene patterns revealed differences in the specific mRNA cargo of EVs (**Figure 9. B**). This allowed the separation of the EVs based on either their benign epithelial or cancer origin. Importantly, the mRNAs of the analyzed genes were more abundant in LNCaP and PC-3 cell-derived MVs compared to EXOs (**II,** Fig. 3), and all the transcripts present in EXOs were also found in MVs.

To evaluate the usability of the mRNA content of LNCaP and PC-3 cell-derived MVs in the prediction of the cancerous status of the parent cells, the mRNA transcripts of prostate cancer-derived MVs were compared with the mRNAs of the benign control PNT2 MVs (**Figure 9. C**). Around 55.8% of the mRNAs were found to be common across the samples, whereas 44.2% were unique. Sixteen mRNAs from LNCaP MVs and 32 mRNAs from PC-3 MVs, were statistically significantly different from mRNAs in PNT2 MVs. From the analysis of the statistical changes in the mRNA abundance and the overlap between the normalized samples, eight mRNAs were found to be unique for LNCaP MVs [increased (*CDH1*, *NKX3-1*, *TMPRSS2*, *TP53*), decreased (*CAV1*, *GSTP1*, *SOCS3*, *TFPI2*)], and 17 for PC-3 MVs [increased (*CAMSAP1*, *CAV1*, *CAV2*, *GSTP1*, *NRIP1*, *PES1*, *TFPI2*, *ZNF185*), decreased (*AR*, *EGR3*, *IGF1*, *PTEN*, *RARB*, *SFRP1*, *SLC5A8*, *TMPRSS2*, *TNFRSF10D*)] (**II,** Fig. 4 or Supplementary Tables 2-3).

After determining the genes with statistically significant mRNA levels in prostate cancer MVs compared to the non-cancer MVs, the common mRNAs for both LNCaP and PC-3 MVs were determined. Increased mRNA levels of several genes were detected, including: Ets variant 1 (*ETV1*), a gene that directs androgen metabolism and confers aggressive prostate cancer; *FASN*, a fatty acid metabolism gene highly up-regulated in prostate cancer; and RNA binding protein 39 (*RBM39*), a gene implicated in cancer progression. On the other hand, a decrease in the mRNA levels in prostate cancer MVs of genes such as cyclin D2 (*CCND2*), a crucial cell cycle-regulatory gene down-regulated in prostate cancer cells and the transcription factor *ERG*, were observed (**II,** Table 1). These results were compared with studies based on microarray analysis of EV-associated mRNAs collected in the EVpedia database. The common mRNAs identified in prostate cancer MVs were compared with the same mRNAs reported in EVs from different sources (**II,** Fig. 5). With the exception of FASN mRNAs, which were detected in 6 out of 10 studies, few reports found the studied mRNAs, and none of them in prostate cancer EVs. Finally, the common mRNAs detected in prostate cancer MVs were compared with the clinical microarray datasets of benign and malignant prostate tissues publically available in the GEO database (GSE55945). Some of the mRNAs discovered in prostate cancer MVs, including *ETV1* and *FASN* genes, were shown to be differentially over-expressed in malignant prostate tissues in comparison to benign controls (**II,** Fig. 5).

5.4 Uptake and functionality of prostate cancer EVs depends on the metastatic stage of the parent cells (III, IV)

Next, the differences in the internalization and functional effects of prostate EVs and their contribution to cancer progression were analyzed. MVs and EXOs from various prostate cell lines of metastatic (PC-3 and LNCaP cells), primary (RC92a/hTERT cells), and benign (PNT2 cells) origin were compared to study how the cancer status of the parent cell affected the EV uptake.

EV internalization was shown to be an active process dependent on both temperature and EV concentration. EV uptake was disrupted by incubation of the samples at 4°C, and an EV concentration of 10^9 particles/mL was found to be the optimal vesicle amount to study EV uptake by flow cytometry (**III**, Fig. 1). To test whether cancer and non-cancer cells could differentially internalize EVs, the amount of cells containing EVs at several time points was analyzed by three different approaches; an automated microscopic imaging system, confocal microscopy, and flow cytometry (**Figure 10**). EV uptake began immediately after the EV addition and continued at least up to 24 h in a time-dependent manner (**III**, Fig. 2, 3; **IV**, Fig. 3). The uptake of metastatic site-derived EVs (PC-3, LNCaP) was clearly more efficient than the uptake of EVs from benign or primary cancer cells (PNT2, RC92a/hTERT). This phenomenon was detected both in the benign and metastatic recipient cells (PNT2 and PC-3) (**III**, Fig. 2). No statistically differences in the internalization kinetics were found between MVs and EXOs (**III**, Fig. 2; **IV**, Fig. 3). Consequently, MVs and EXOs were combined as a total EV pool to further analyze the differences between the EVs based on their origin.

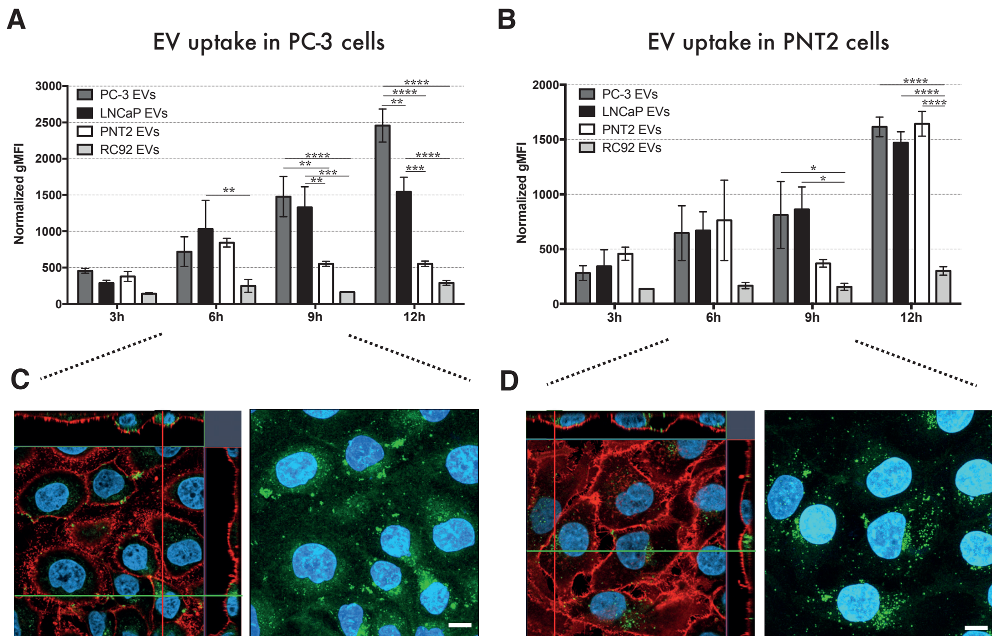

Figure 10. Uptake kinetics and internalization of prostate cancer cell-derived EVs. EVs isolated from prostate cancer cell lines of metastatic origin (PC-3 and LNCaP); a primary prostate cancer cell line (RC92a/hTERT); and normal prostate epithelial cells (PNT2) were incubated with cancer and non-cancer cells. A, B) EV internalization analysis was performed by flow cytometry in PC-3 cells and PNT2 cells. Results are plotted as normalized geometric mean of fluorescent intensity (gMFI) of EV

fluorescence in cells. Bars represent mean ± SEM of three independent experiments per group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, two-way ANOVA with Tukey's multiple comparisons test. C, D) Representative confocal images depicting internalization of $DiIC_{18}(5)$-DS-labeled EVs (pseudo-colored green) by PC-3 and PNT2 cells (CD44 plasma membrane marker pseudo-colored red and nuclei colored blue). 3D projections of z-stacks are also shown. Scale bars, 5 µm. Publication **III**, Fig. 3.

Additionally, when the uptake efficiency of EVs of cancer and non-cancer origin was compared using flow cytometry, a similar trend for EV uptake kinetics was observed in both cell types. Metastatic site-derived PC-3 and LNCaP EVs were overall more efficiently internalized than EVs derived from RC92a/TERT prostate cancer primary cells, or from PNT2 benign prostate epithelial cells by PC-3 cells after 9 h and 12 h incubations (**Figure 10. A**). The benign PNT2 cells significantly internalized PC-3 and LNCaP EVs as well as PNT2 EVs after 9 h and 12 h, but almost no RC92a/hTERT EV internalization was observed (**Figure 10. B**). Overall, no significant differences in the total EV uptake between the cells was observed (**III,** Fig. 3). Confocal microscopy analysis confirmed the intracellular presence of EVs in both PNT2 and PC-3 cells (**Figure 10. C**, **D**). Independently of the EV subpopulation analyzed, the majority of the EVs were inside the cells and not only adhering to the cell surface, as depicted by the low 5% co-localization of labeled EVs with the cellular plasma membrane (**III,** Fig. 4). Moreover, around 30% of the EVs co-localized with the endolysosomal compartments (**III,** Fig. 4), suggesting that EV internalization process may occur through endocytosis. PNT2 EVs co-localized significantly more with early endosomes compared to PC-3 EVs, which was in line with the slower uptake of PNT2 EVs (**III,** Fig. 3). No other significant differences were detected in the co-localization of cancer *vs* non-cancer derived EVs with the cell organelles. Since EVs can also have protein motifs on their surface that could be used to mimic a possible targeted delivery to their autologous cells, the effect of the removal of the membrane surface proteins of EVs on the overall vesicle uptake was also assessed. Trypsin treatment did not alter the uptake of EXOs. However, after 24 h incubation, a decrease could be observed in the internalization of trypsin-treated MVs (**IV**, Fig. 3).

High-resolution confocal microscopy images showed that EV internalization was not a constant and uniform process for all cells cultured under the same conditions (**Figure 11. 9A**). When the labeled EVs were added to the cells, some of them internalized the EVs whereas other cells did not. Therefore, the influence of the cellular phase [resting phase/Gap1 (G0/G1), DNA synthesis (S), and Gap2 (G2) and mitosis (M)] on EV internalization was addressed by using PC-3 cells incubated with LNCaP EVs. Although a trend of increased EV uptake of cells in G2/M compared to G0/G1 or S phases was observed, no significant differences in the cell cycle phases were shown for the total EV population at the early time points. Interestingly, after 24 h and 48 h incubation with the EVs, a statistically significant increase in the EV concentration was observed in cells that were in G2/M phase (**III,** Fig. 5). Results also showed that EV uptake did not increase the total amount of cells in G2/M phase (**III,** Fig. 5). When MVs and EXOs were incubated separately, PC-3 cells in G2/M phase displayed a significantly higher MV count already evident after 3 h and 6 h incubation compared to cells in S or G0/G1 phases. The cellular concentration of EVs could be roughly classified according to the cell cycle phase G2/M > S > G0/G1 (**III,** Fig. 5).

The ability of prostate-derived EVs to promote functional changes in recipient cells was next evaluated. The proliferation of cancer and non-cancer cells was analyzed after incubation with EVs derived from LNCaP, PC-3, RC92a/hTERT, and PNT2 cells (**III,** Fig. 6).

For PC-3 cells (with a doubling time of approx. 38 h), an increase in proliferation of the EV-treated cells in comparison to the control became statistically significant only after 72 h incubation.

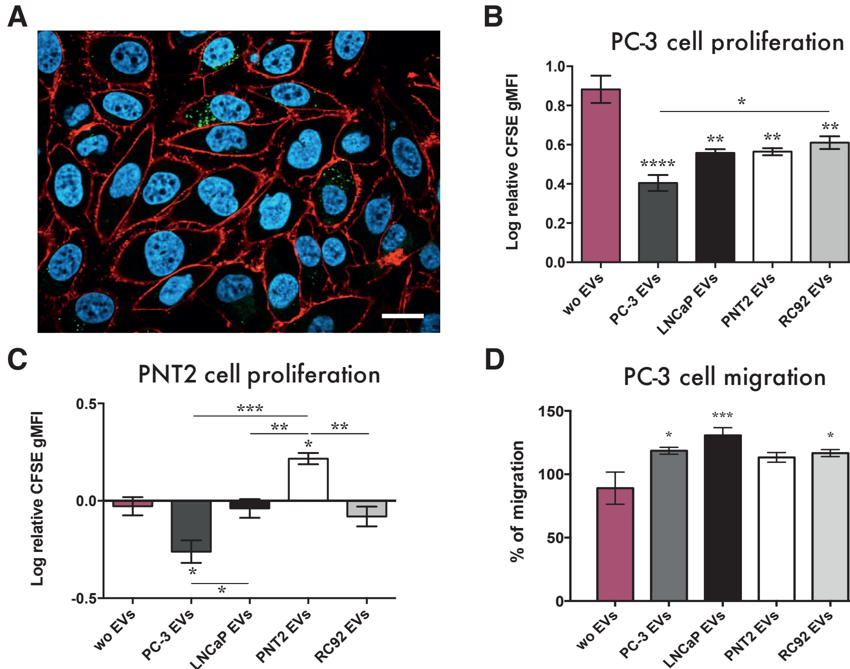

Figure 11. Metastatic EVs promote cellular proliferation and migration of prostate cells. A) PC-3 cells were incubated for 12 h with DiIC18(5)-DS EVs (pseudo-colored green) and cells were immunostained with CD44 for plasma membrane (red) and DAPI for nuclei (blue). Scale bars 20 µm. Proliferation of B) PC-3 cells and C) PNT2 cells after 72 h incubation with EVs derived from PC-3, LNCaP, RC92a/hTERT, and PNT2 cells or without (wo) EVs. Cellular proliferation was measured by flow cytometry using CFSE label. The results are presented as the Log_{10} of the relative CFSE geometric mean fluorescent intensity (gMFI). Small or negative values indicate less CFSE label and consequently more cellular proliferation. Bars are mean ± SEM of three independent experiments per group. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001, one-way ANOVA with Tukey's multiple comparisons test. D) PC-3 cell migration was monitored during the incubation with EVs for up to 6 h. Graph showing the percentage (%) of total migration of PC-3 cells after addition of EVs or control without (wo) EVs. Error bars are mean ± SEM of six independent experiments per group. **P* <0.05, ***P* <0.01, one-way ANOVA with Dunnett's multiple comparisons test. Publication **III**, Fig. 6.

All the EVs increased the cellular proliferation of PC-3 cells (**Figure 11. B**). However, the metastatic PC-3 EVs increased the cell proliferation significantly more than the other EV types. When PC-3 cell-derived MVs and EXOs were compared for their capacity to enhance cell viability, EXOs had a stronger viability enhancing effect than MVs, although both subpopulations enhanced cell viability (**IV**, Fig. 4). The effect was EV concentration dependent, achieving an enhanced effect with 10^8 particles/mL or higher concentrations. In contrast, an EV-induced increase of the proliferation of the benign slow-growing PNT2 cells was already apparent after 48 h incubation (**III**, Fig. 6). EVs from both cells of metastatic origin showed increased PNT2 cell proliferation compared to the primary and benign cell-

derived EVs. This effect became more notable after 72 h incubation, with PC-3 EVs significantly increasing the cell growth compared to the other EVs and control (**Figure 11. C**). Remarkably, PNT2 EVs did not induce proliferation of PNT2 cells, but showed a slight growth reduction compared to the control. All prostate cancer cell-derived EVs, including primary and metastatic EVs, significantly enhanced migration of PC-3 prostate cancer cells, whereas EVs isolated from benign cells had no effect (**Figure 11. D**). Overall, the EV internalization efficiency and stimulation of malignant transition in recipient cells could be classified according to the metastatic potential of the EV parent cells PC-3 > LNCaP > RC92a/hTERT > PNT2.

5.5 EV-mediated paclitaxel delivery enhances the cytotoxicity of the drug (IV)

To evaluate the usability of EV subpopulations as drug delivery vehicles, LNCaP and PC-3 MVs and EXOs were tested in the *in vitro* delivery of paclitaxel, a pharmacological antimitotic cancer drug. Here, cancer-derived EVs were used as a proof of concept model for autologous cancer treatment, since cancer cell-derived EVs were efficiently internalized by cells as proven above (**Figure 10**). EVs were loaded by passive diffusion since paclitaxel is a small and highly hydrophobic drug with a log P value of 3.96 and an aqueous solubility of less than 0.01 mg/mL (Surapaneni et al., 2012). By using 5 μM paclitaxel, the average loading efficiency of MVs and EXOs, independent of the particle concentration, was around 10.3% and 7.5%, respectively (**IV**, Table 1), with no apparent release of paclitaxel from EVs to the media after 24 h and 48 h in the study.

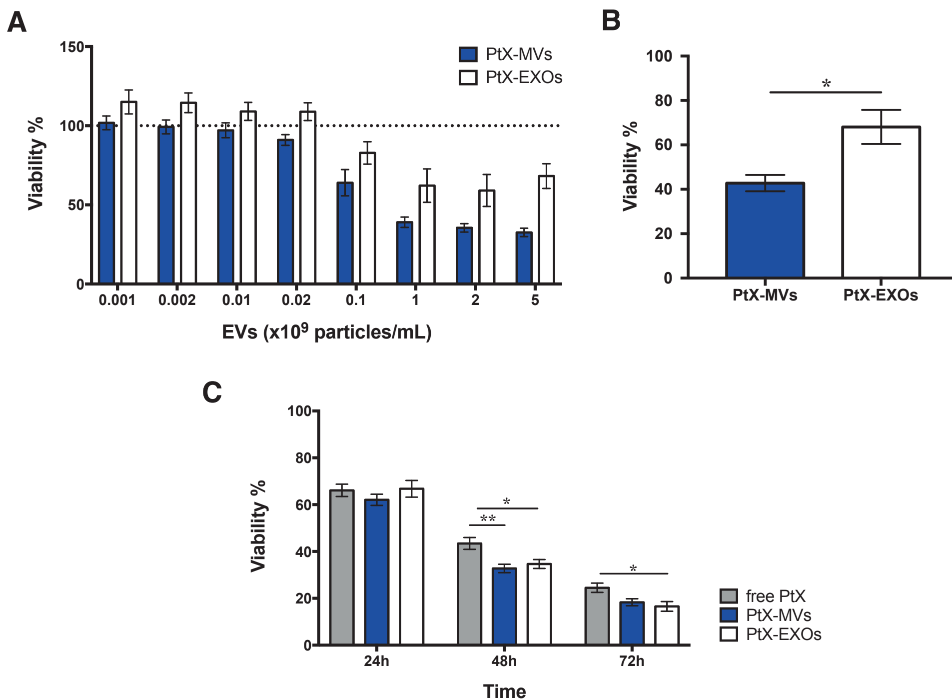

Figure 12. Enhanced cytotoxic effect of EV-mediated delivery of paclitaxel. Cell viability experiments were conducted to analyze the effect of free 5 μM paclitaxel (free PtX) and paclitaxel-loaded EVs (PtX-EVs) in prostate cancer cells. A) PC-3 cells were incubated with different

concentrations of paclitaxel-loaded microvesicles (PtX-MVs) and paclitaxel-loaded exosomes (PtX-EXOs) during 48 h to establish the dose-dependency effect of the PtX-EVs. MVs and EXOs were loaded with 1 nmol of PtX per 10^9 vesicles and dilution series were established to evaluate the dose dependency of PtX-EVs. Bars are mean ± SEM of three independent experiments per group. B) Overall comparison of the cytotoxic effect of the different concentrations of PtX-MVs and PtX-EXOs which had a cytotoxic effect in PC-3 cells after 48 h incubation. Bars represent mean ± SEM of four independent experiments per group. *P <0.05, unpaired t test. C) Cytotoxic effect of MVs and EXOs loaded with 5 nmol PtX per 10^8 vesicles at 24 h, 48 h, and 72 h. Bars represent mean ± SEM of five independent experiments per group. *P <0.05, **P <0.01, two-way ANOVA with Dunnett´s multiple comparison test. The presented results are re-analysis of publication **IV**, Fig. 5.

Paclitaxel-loaded MVs had a stronger cytotoxic effect than paclitaxel-loaded EXOs, reducing the viability of PC-3 cells regardless of the EV concentration used (**Figure 12. A**). A possible saturation point for paclitaxel-loaded MVs and EXOs was apparent at around 10^9 particles/mL, at which point the maximal cytotoxic effect of paclitaxel-loaded EVs was observed. When the overall cytotoxic effects of concentrations of paclitaxel-loaded MVs and EXOs below the saturation point were compared, MVs had a statistically significant cytotoxic effect compared to EXOs, reducing the viability of cells by 60% after 48 h incubation (**Figure 12. B**). Lower concentrations of EVs loaded with the same amount of drug, corresponding to a ratio of paclitaxel/EV 50 times higher than in the previous settings, induced a statistically significant increase in the cytotoxicity of both paclitaxel-loaded MVs and EXOs compared with the free drug (5 µM) after 48 h and 72 h (**Figure 12. C**). These results indicate that the combination of high amounts of paclitaxel per vesicle and a number of EVs below the saturation point produce the maximum cytotoxic effect.

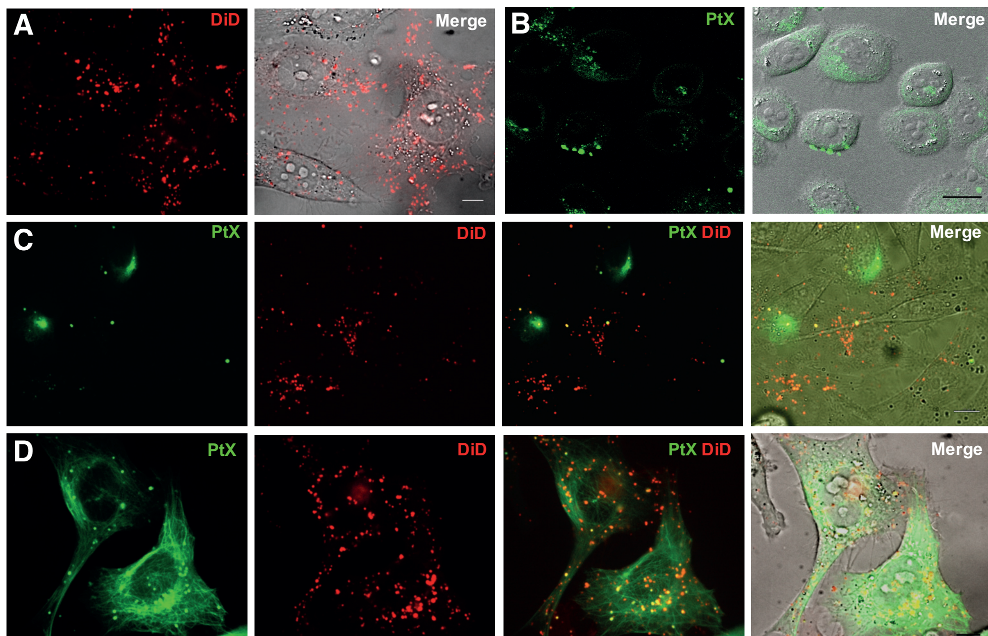

Figure 13. Internalization of paclitaxel (PtX) and DiD-labeled EVs by prostate cancer cells. LNCaP cells were incubated during 24 h with A) DiD-labeled EVs (red), B) Free 5 µM PtX (green), C)

PtX-loaded DiD-labeled EVs, and D) combination of free PtX and DiD-labeled EVs. Representative images are shown. Scale bars 10 µm. Publication **IV**, Fig. 7.

The increased cytotoxic effect of paclitaxel-loaded EVs was not caused by the presence of free paclitaxel in solution, since no detectable leaking of paclitaxel was detected, and incubation of cells with a combination of free drug and EVs resulted in a lag, but not enhanced, cytotoxic effect of paclitaxel (**IV**. Fig. S2).

In further analysis of the effect of paclitaxel-loaded EVs in LNCaP and PC-3 cancer cells, an overlap in the labeled EVs and the fluorescent paclitaxel showed that drug was delivered to the cells via EVs (**IV,** Fig. 6, 7). While the internalization of DiD-labeled EVs did not have any negative effect in terms of the cell viability (**Figure 13. A**), the incubation of prostate cancer cells with free 5 µM paclitaxel caused cell shrikage and death (**Figure 13. B**). When paclitaxel was delivered to the cells by EVs, drug/EV complexes were internalized by the cells through endocytosis, as shown by the co-localization of the labeled EVs with lysosomes (**IV,** Fig. 6). In the endosomal network, the fluorescent paclitaxel seemed to separate from the EV-label, binding to the microtubules and eventually spreading within the cell causing death (**IV,** Fig. 7) (**Figure 13. C**). The EV-mediated effect was similar to that of the free paclitaxel, albeit weaker most likely due to the smaller amount of paclitaxel. Finally, free paclitaxel was added to the cells together with the DiD-labeled EVs without the drug (**Figure 13. C**). Cells that internalized notable amount of EVs were still alive after 24 h incubation, counteracting the effect of free paclitaxel. Although EVs were not loaded with paclitaxel, the drug could partially bind EVs in the cell media due to its high hydrophobicity, as could be qualitatively shown by the co-localization of paclitaxel with the endolysosomal cell compartment.

6 Discussion

6.1 EV-nucleic acid cargo as an emerging source of prostate cancer biomarkers

When it comes to treating cancer, a more specific diagnosis is needed to identify the presence of tumors in early stages, increasing the number of treatment options. New non-invasive and more efficient sources of biomarkers are required to stratify patients harboring e.g., different stages of prostate cancer. The discovery that the content of EVs is tissue/cell-specific has underlined a particular interest in the use of EVs as a potential source of cancer biomarkers. One of the primary aims of this thesis was to assess the nucleic acid cargo of EVs as a possible source of prostate cancer biomarkers, particularly the gDNA content (publication **I**) and mRNA content (publication **II**) of different EV subpopulations.

The RNA cargo of EVs has been widely investigated in cancer. miRNAs have attracted the most attention, while far fewer studies have evaluated the use of EV-associated mRNAs as a possible source of biomarkers (**Table I**). In contrast to RNA, EV-associated DNA represents a more unexplored source of information that, in the context of prostate cancer, was first discovered in prostasomes (Ronquist et al., 2012; Ronquist et al., 2009). The results presented in this thesis showed that, despite the notable overlap in size distribution and protein markers of the different EV subpopulations obtained by differential centrifugations, prostate cell-derived EV subpopulations had distinct DNA and RNA contents (**Figure 7**). DNA fragments were first found in ABs, which was an expected finding, as during cell death by apoptosis DNA is cleaved into different sized fragments and secreted to the extracellular space, partly associated to apoptotic blebs as previously shown (Halicka et al., 2000; Holmgren et al., 1999; Schiller et al., 2008). ABs and MVs were also enriched in rRNA, with relatively low amounts of small RNA molecules, as also described in ABs and MVs from the mast, erythroleukemic, and melanoma cell lines (Crescitelli et al., 2013; Lunavat et al., 2015; Willms et al., 2016). On the other hand, EXOs contained increased amounts of DNA compared to other EVs, and mainly small RNA molecules within their cargo. The same RNA pattern has previously been observed in EXOs from other cellular sources (Crescitelli et al., 2013; Lunavat et al., 2015; Willms et al., 2016), but in some of these studies, EXOs were also shown to contain a broad spectrum of fragmented and intact RNA molecules, including mRNAs, miRNAs, and other ncRNA species (Ahmed et al., 2014; Bellingham et al., 2012; Lunavat et al., 2015; Nolte-'t Hoen et al., 2012; Vojtech et al., 2014). It is also plausible that certain RNAs detected in our study might be located outside the EV lumen associated with the outer membrane. Currently, the use of RNAse treatment prior to RNA extraction of EVs is rather controversial. The enzymatic treatment would be of benefit, as it will remove all possible RNA contaminants from cell lysis, allowing the analysis of the RNA molecules within EVs. However, RNAses can be extremely difficult to dose and difficult to inactivate, which could compromise the integrity of the RNA in the EVs. For instance, no major differences were reported in the total RNA content of ABs, MVs, and EXOs enriched fractions before and after RNAse treatment (Lunavat et al., 2015), indicating that EVs confer a certain degree of RNA protection. On the other hand, EXOs had a variable amount of DNA, with different length DNA molecules identified in both prostate cancer cell line derived- and plasma-derived EXOs after DNAse treatment. Similar results have also been shown in other cancer cell-derived and plasma-derived EXOs, with protected DNA fragments ranging from 100 bp to 17 kbp after enzymatic treatment (Cai et al., 2013; Kahlert et al., 2014; Thakur et al., 2014). Interestingly, the DNA profile traces of prostate cancer EXOs suggest substantial

fragmentation of the EXO-DNA (**Figure 7**). The pattern of DNA size in EXOs was found to be similar to that of the DNA wrapped around histone proteins (Olins and Olins, 2003). Therefore, we hypothesize that the DNA found in EXOs may partly be protected from degradation by histone proteins either inside the vesicles or in association with them.

Although different DNA and RNA content in prostate cancer EV subpopulations was shown by these studies, improvements in the EV isolation and characterization will contribute to a better understanding of the variability of EVs in isolates. Currently, there are no methods that allow the exact separation of EV subpopulations (Witwer et al., 2013). New isolation procedures including affinity-based isolations e.g., microfluidic devices, photosensitizer, and immunomagnetic beads (Im et al., 2014; Jørgensen et al., 2013; Kanwar et al., 2014; Kim et al., 2012a; Tauro et al., 2012), size-based isolations e.g., acoustic waves and micro pillar-based methods (Lee et al., 2015b; Santana et al., 2014; Wang et al., 2013), and novel characterization techniques as Raman spectroscopy (Smith et al., 2015; Stremersch et al., 2016b), Surface Plasmon Resonance (Rupert et al., 2014; Suutari et al., 2016; Zhu et al., 2014), and new generation flow cytometers fine-tuned to measure particles below 400 nm (Headland et al., 2014; Pol et al., 2014), will contribute to a deeper characterization of more "pure" EV subpopulations. The implementation of these techniques in combination with genomics, transcriptomics, proteomics, and metabolomics of specific EV subsets will allow a broader perspective of the utility and functional relevance of the unique cargo responsible for driving cancer progression.

6.1.1 Oncogenic DNA content of EVs

EV-associated DNA may hold remarkable value in clinical diagnostics as candidate for cancer biomarkers, since it can provide information about the characteristics of the tumors, including their mutation pattern. The results presented in the first study aimed to determine the gDNA content of prostate cancer-derived EVs and its utility to evaluate mutations (publication **I**).

The presence of mutated gDNA fragments of *MLH1*, *PTEN*, and *TP53* genes was confirmed in ABs and EXOs isolated from prostate cancer cell lines (**Figure 8**). The presence of mutated DNA content within EVs from pancreatic cancer, melanoma, and lung cancer origin was simultaneously reported by others (Kahlert et al., 2014; Lee et al., 2014; Thakur et al., 2014). The detection of oncogenic mutations in EVs from different origins, including *RAS*, *BRAF*, and *EGFR* genes, was in line with our findings in prostate cancer cell-derived EVs. The fact that gDNA is found in EVs reflecting the mutational status of the parent tumor cells supports the concept of EV-associated DNA as a new effluent biomarker type. By secreting EVs, part of the gDNA content of the cells can be specifically circulated as protected DNA, which might contribute to genomic instability as a result of the functional transfer of oncogenic sequences, as previously reported for ABs (Bergsmedh et al., 2001), and also to the preparation of the pre-metastatic niche of the tumors. However, it is far from clear if the protected DNA from prostate cancer cell line-derived EVs represents the whole genome, as previously claimed by the genome sequencing analyses of other EV subtypes (Kahlert et al., 2014; Lee et al., 2014; Ronquist et al., 2009; Thakur et al., 2014) or if the cells can specifically pack certain kinds of DNA in vesicles, such as mtDNA (Guescini et al., 2010), for instance during the advanced stages of prostate cancer. Moreover, what the function of this DNA is and how it is packed into the EVs remains unclear.

In the prostate cancer field, this study was the first to show that double-stranded gDNA molecules were detected in EVs isolated from plasma samples of prostate cancer patients.

Prostate cancer patients showed an increased number of EVs compared to healthy controls. Increased EV concentrations in the systemic circulation of patients with other cancers have also been reported, with an estimated 4,000 trillion EVs in the blood circulation, which is approximately double the number found in blood from healthy individuals (Kalluri, 2016). However, none of the mutations present in the cell line-derived EVs could be detected in the plasma EVs from the patient cohort analyzed. This could be due to the too small number of cancer-originating EVs in the diverse collection of EVs in the circulation, or due to the absence of these mutations in these small patient cohort. In future studies, the analysis of bigger patient cohorts and the use of tissue- or cancer-specific markers to enrich the number of cancer EVs from the heterogeneous EV pool, as shown by immunoaffinity capture of colon cancer A33-EXOs and EpCAM-EXOs (Tauro et al., 2013), could increase the probability of detecting oncogenic mutations. Moreover, patient's tumor biopsies should be analyzed in order to validate the presence or absence of the mutations found in EVs. The use of more sensitive methods such as co-amplification-at-lower denaturation-temperature PCR, which selectively amplifies and identifies minority alleles and low-level somatic mutations, will surely contribute to the identification of EV-associated oncogenic sequences reflecting the original tumors as recently shown (García-Romero et al., 2016).

The comparison between cell lines and patient's plasma samples from the same disease model puts in perspective cell lines as a mimic of pure cancer cell populations, limiting the translation of the results to *in vivo* settings. While established cancer cell lines generate a wide diversity of EV subsets arising from the same cell type, plasma from cancer patients is heterogeneous, where EVs from many different sources, both from cancer and all normal cells, including immune cells, co-exist. This is likely one of the reasons why biomarker discovery with EVs isolated from biofluids is most challenging, and why it is relevant to isolate and study the correct type of EVs as the source of biomarkers. Additionally, no biomarker based on EV analysis has yet been realized, partially due to the lack of standardization and reference materials (Valkonen et al., 2016), which complicates the analysis of the EVs and the comparison of results between patients.

Clearly, studying the mutation signature of DNA in the prostate cancer EVs and correlating these findings with clinical parameters of the individual patients, including the tumor stage, PSA levels, and treatment response, could contribute to an earlier diagnosis of the disease and maybe even help to further tailor specific treatments for prostate cancer patients.

6.1.2 EV-associated mRNA in the detection of prostate cancer

RNA signatures of EVs have been reported to be specific for their cells of origin, discriminating, for instance, the transformation status of cancer cells (Ahadi et al., 2016; Jenjaroenpun et al., 2013). In the second study of this thesis, mRNA transcripts of genes known to be involved in prostate cancer were identified in prostate cancer cell-derived MVs and EXOs from LNCaP and PC-3 cells (publication **II**), the two major prostate cancer cell models for low and high metastatic potential, respectively (Dozmorov et al., 2009). The results showed that MVs rather than EXOs were enriched in the mRNAs analyzed by RT-qPCR. Based on these findings, MVs rather than EXOs would represent the optimal EV subpopulation for biomarker mining. Since most of the EVs in the MV population are usually larger than the majority of EVs in the EXO population, they might be able to carry more bioactive molecules, including mRNAs. So far, the EV field has held the EXOs in superior position as cancer biomarker discovery (An et al., 2015; Kalluri, 2016), which makes this a

significant observation. Other than in thrombosis and hemostasis, where microparticles have been extensively studied (Aatonen et al., 2012), the advantageous properties of the larger vesicles have been less explored, and only a few studies have focused on larger cancer vesicles, such as LOs (Minciacchi et al., 2015). Additionally, it is postulated that EV contents are not randomly packed, raising the possibility of selective export of certain molecules within EVs (Yáñez-Mó, Siljander et al., 2015). This cargo specificity was shown by the presence of certain miRNAs within EXOs and their absence in the parent tumor cells (Nolte-'t Hoen et al., 2012; Ohshima et al., 2010; Zhang et al., 2010). When prostate cancer EVs were analyzed, the RNA profile of MVs resembled more the RNA profile of the parent cells compared to EXOs, thereby providing a more accurate snapshot of the tumor cell transcriptome. Additionally, the unique mRNA signatures of LNCaP and PC-3 MVs facilitated the distinction of the characteristics of their parent cells. MVs and EXOs from androgen sensitive LNCaP cells contained characteristic mRNA transcripts of genes with documented androgen regulated functions, including for instance *TMPRSS2*, previously identified as a fusion gene *TMPRSS2-ERG* in EVs isolated from prostate cancer cells and urine from prostate cancer patients (Dijkstra et al., 2014; Jansen et al., 2009; Nilsson et al., 2009). On the other hand, the majority of the mRNAs associated with PC-3 MVs were related to advanced and metastatic stages of the prostate cancer (Jarrard et al., 1997; Meiers et al., 2009; Yang et al., 1999), as reflected by the high metastatic potential of PC-3 cells (Dozmorov et al., 2009). This argues that different cancer cell lines from the same cancer type, such as prostate cancer, can release an EV secretome carrying essentially different RNA molecules. It also proves that the features of the parent cells can be detected and analyzed within MVs, supporting the concept of liquid biopsy analysis of circulating EVs (Moon et al., 2016; Yoshioka et al., 2014). However, the possibility that some of the EV-associated RNA molecules were adsorbed to EVs cannot be excluded, complicating the comparison of the relative amount of EV-associated RNAs among cell lines. This is of particular importance when trying to compare EV-associated RNAs from different cellular sources as cancer-specific biomarkers. To reduce this possibility and to avoid considering subtle differences, a high fold-change threshold limit was chosen in order to study the significant changes in the EV-associated mRNAs.

The common mRNAs for both LNCaP and PC-3 prostate cancer MVs were first identified in our study, and only a few reports have examined the presence of mRNA transcripts of these genes in other cancer-derived EVs (Kim et al., 2015). Another interesting finding was the possible clinical relevance of the candidate mRNAs. Comparative analysis of these mRNAs with a publically available datasets of mRNAs from malignant and benign prostate tissue samples showed the over-expression of e.g., *ETV1* and *FASN* mRNAs in patient samples, which were also abundant in the prostate cancer EVs. In future studies, a validation of these results from EVs isolated from blood or urine of prostate cancer patients compared to patients with benign prostate hyperplasia will elucidate the significance of these findings and their utility in future biomarker research.

Furthermore, we also believe that a good candidate marker should be based on the expression ratio of mRNA transcripts of several genes dysregulated in cancer rather than on the expression of a single gene. The use of mRNAs of genes that are differentially increased and decreased in prostate cancer-derived EV samples compared to controls would be rather more informative, as proposed by the results of this study. Overall, these findings indicate that the MV-associated mRNAs could be of potential use to assess disease progression, highlighting the possible relevance of examining the mRNA cargo in MVs as a partial representation of the tumor transcriptome.

Together, presented studies (publication **I** and **II)** provide further insights into the proportion of cell genome and transcriptome that can be identified in EVs and highlight the relevance of the nucleic acid cargo of EVs in cancer diagnostics. The different cargo characteristics of the analyzed EV subpopulations demonstrate that EVs represent the dynamic changes in the tumor and its progression. Based on the presented results, the combination of DNA and RNA analysis from EVs is also suggested as a more robust and sensitive cancer biomarker.

6.2 Towards elucidating the role of EV-mediated cell-to-cell communication

In cancer, EVs facilitate tumor progression by supplying biomolecules to the tumor niche in support of oncogenic processes towards tumor development and dissemination (**Figure 4**). Based on that, the next point addressed in this thesis was the evaluation of the role of prostate cancer cell-derived EVs in cell-to-cell communication (publication **III**).

6.2.1 Contribution of EVs to prostate cancer progression

To efficiently deliver their cargo, EVs can either fuse with the plasma membrane or be internalized as intact EVs by the cells. The studies presented in the thesis (publication **III** and **IV**) compared the uptake efficiencies and the effects on the internalization of different EVs on prostate cancer and benign cells. Results showed that EV uptake by cancer and non-cancer cells was a constant and active process as previously reported (Christianson et al., 2013; Feng et al., 2010; Svensson et al., 2013). No apparent differences were shown in the overall internalization kinetics of MVs and EXOs when subpopulations were compared. This was a relevant finding, since the vast majority of the studies investigating the role of EVs in intercellular communication have not compared the different EV subsets (Mulcahy et al., 2014), and only a few reports so far have depicted the differential roles of MVs and EXOs e.g., in HIV-1 infection (Kadiu et al., 2012). Importantly, prostate cancer and non-cancer cells internalized EVs with similar efficiencies. Several studies have reported changes in the EV uptake based on the phagocytic or non-phagocytic status of the EV acceptor cells and their cancer or non-cancerous origin (Feng et al., 2010; Rana et al., 2012). Interestingly, in publication **III**, metastatic site-derived EVs were more efficiently internalized than those from benign or primary malignant cells (**Figure 10**). The co-localization of EVs with endolysosomal compartments supports the evidence that EVs are preferentially internalized through endocytosis, in agreement with other reports (Escrevente et al., 2011; Nanbo et al., 2013; Svensson et al., 2013). However, the possible fusion of EVs with the plasma membrane cannot be excluded. Higher co-localization of PNT2 EVs with the early endosomes as compared to PC-3 EVs is likely to reflect the slower uptake of non-cancer vesicles. However, the faster internalization kinetics and intercellular trafficking of cancer EVs may depend, for instance, on the specific protein-to-protein interactions between EVs and acceptor cells, as speculated by other groups (Christianson et al., 2013; Raposo et al., 1996; Svensson et al., 2013). The enzymatic treatment of EVs with proteinase K to remove EV surface protein was shown to reduce the uptake of EVs by ovarian cancer cells (Escrevente et al., 2011). The uptake efficiency of EXOs after protein removal was not altered for the present study, but a decrease in the MV internalization was apparent for trypsin treated MVs (publication **IV**). However, besides membrane proteins, other EV components such as lipid rafts may also have a relevant role in the EV uptake (Svensson et al., 2013). Several studies evidenced the

participation of lipid raft-associated proteins in EV uptake, such as e.g., caveolin and flotillins, supporting the hypothesis that lipid rafts are partly involved in the EV internalization mechanism (Mulcahy et al., 2014). Additionally, certain pathways like macropinocytosis may not require any specific interaction between the EVs and cells (Nakase et al., 2015), and could simultaneously occur with other internalization routes when the cell recognizes specific EV subsets. Although exciting, these observations must be interpreted cautiously. Despite the fact that confocal microscopy analysis evidenced EV internalization and co-localization with cellular organelles (**Figure 10**) (**III**, Fig. 3, 4), lipophilic dyes cannot be used for long-term tracking of the EV cargo. New labeling strategies such as fluorescently tagged molecules bound to the EV-membrane proteins or lipids have to be implemented to track the delivery of the EV cargo while EVs are trafficking in the cells.

The cell cycle analysis performed in this study showed that prostate cancer cells internalized EVs more efficiently during G2/M phases (**III**, Fig. 5). To our knowledge, this is the first time that the influence of cell cycle on EV uptake has been described. These results are consistent with a previous finding examining the role of cell cycle in the cellular uptake of synthetic nanoparticles (Kim et al., 2012b). This study revealed an increased concentration of nanoparticles when cells are in G2/M, in support of the results presented in publication **III**. However, the possibility that the higher EV counts in the mitotic cells were due to the stimulation of mitosis by the EVs cannot be excluded, since cancer EVs support cell viability and proliferation, as demonstrated in publications **III** and **IV** and also shown by others (Haga et al., 2015; Lindoso et al., 2015; O'Brien et al., 2013; Yang et al., 2013). However, a trend of higher EV counts in the mitotic cells was already apparent after a short incubation (3 h to 6 h), when the cells had not had sufficient time to proliferate, indicating that EVs might promote cell division.

Enhanced EV uptake by actively dividing cancer cells could also stimulate cancer cell spreading. For this reason, the ability of prostate-derived EVs to induce functional changes in the recipient cells was evaluated. In contrast to prostate benign and primary malignant cells, metastatic site-derived EVs promoted a more proliferative and migratory behavior in the recipient cancer and non-cancerous cells. Other reports have also demonstrated that malignant metastatic and non-metastatic cell line-derived EVs induced a differential complement activation and markedly reduced stiffness and adhesion in recipient cells compared to non-malignant cell line-derived EVs (Whitehead et al., 2015). Metastatic breast cancer cells also secreted EVs with distinct protein signatures, which increased cancer cell movement (Harris et al., 2015), complementing these results. However, the proof that the increase in prostate cancer cell proliferation and migration was related to the metastatic potential of the parent cells of EVs was first demonstrated in this study. These findings also support the concept that EVs derived from cells of metastatic origin harbor the potential to support the malignant transition in recipient cells (**Figure 11**). Remarkably, PNT2 EVs did not induce proliferation of PNT2 cells, but slightly showed a growth reduction compared to the control and other EV treatments. This feature of non-malignant EVs could be of advantage for instance in the delivery of therapeutic molecules.

Despite the extensive research on intercellular communication, the role of EVs in cellular signaling has only started to become clear. Overall, the results presented here strongly support the hypothesis that prostate cancer EVs have an active role in stimulating cancer progression and malignization compared to non-malignant cell-derived EVs. However, considering the importance of EV-mediated cellular communication and the limited knowledge available regarding the molecular mechanisms that drive those changes, it is highly relevant to investigate the differences in the EV characteristics and cargo to

understand their role in heterogeneous cancers. The study of disease-promoting EV, as well as the current mechanisms of EV internalization into cells, could reveal new ways to specifically target diseases by blocking communication between healthy and disease tissues. In addition, analysis of the uptake kinetics of different EVs may help to select the most appropriate EV source for optimized entry. This may be a key feature for using EVs as novel nanocarriers for the delivery of therapeutic cargo.

6.3 EVs as emerging targets for drug delivery

Understanding EV-mediated communication in cancer progression and the effects of different EV subpopulations is a key feature for designing novel strategies to use EVs as carriers for therapeutic cargo. The last study of this thesis addressed the suitability of EV subpopulations, including MVs and EXOs, as cancer drug carriers and in the *in vitro* delivery of paclitaxel, an antimitotic cancer drug (publication **IV**).

The drug loading efficiency of EVs has been shown to be dependent on the hydrophobic nature of the drugs (Fuhrmann et al., 2015). In publication **IV**, by using passive diffusion, MVs and EXOs were loaded with paclitaxel, with an average loading degree of ~ 9.2%, independent of the EV concentration used. The paclitaxel loading yield of ~ 21 mg/g EV in our study is in accordance with the paclitaxel yield of ~ 7.3 mg/g EV using a similar EV-loading strategy (Yang et al., 2015). On the other hand, using pre-EV formation methods, the paclitaxel-loading yield has been reported to be ~ 2 µg/g EV (Pascucci et al., 2014), which is significantly less compared to the drug/EV incubation reported in our study. Similar EV-loading strategies have been used with other hydrophobic and small therapeutic compounds, such as curcumin (Zhuang et al., 2011) and doxorubicin (Jang et al., 2013; Smyth et al., 2015), facilitating its passage across the membrane. However, these same advantageous properties that benefit loading may contribute to the leakage of the drug out of EVs (Smyth et al., 2015). Similar amounts of paclitaxel could bind MVs and EXOs in the high or low EV-concentration containing conditions, with no apparent release of paclitaxel from EVs to the media. To date, a limited number of studies have compared the efficiencies of EV subsets as drug carriers (Zhuang et al., 2011), and the majority of the studies rely on the delivery of drugs within EXOs (Johnsen et al., 2014).

Interestingly, the EV-mediated cytotoxic effect of paclitaxel was significantly higher compared to the effect of the free drug (**Figure 12**). Although both EV subpopulations enhanced the cytotoxic effect of the drug, MVs seemed to be slightly more effective carriers of paclitaxel than EXOs, as EXOs required a higher paclitaxel/EXO ratio, whereas MVs had comparable cytotoxicity enhancing effect regardless of the paclitaxel/MV ratio used (**IV**, Fig. 5). A possible explanation for this could be that the EXOs induced more proliferation of cancer cells than MVs, maybe partially counteracting the effect of paclitaxel. Since the exact mechanisms are unknown, there is currently no explanation how EVs can enhance the cytotoxic effect of paclitaxel. One possible reason could be that EVs can carry more drug into the cells than what they can gather from the surrounding media, as shown earlier (Tang et al., 2012). An alternative explanation could be that EVs deliver the drug into an optimal subcellular location, such as the second paclitaxel binding target Bcl-2, promoting apoptosis (Ferlini et al., 2009) and thereby enhancing the cytotoxic effect of the drug.

Importantly, this study showed that the use of MVs and EXOs as drug carriers and delivery systems could be advantageous for the administration of chemotherapeutic treatments. A lower amount of the drug will be needed in order to achieve the same cytotoxic effect, thereby reducing the adverse effects of the treatments. This is a highly relevant aspect,

as EV-mediated cancer cell targeting could contribute to decreasing the side effects of the drugs (Stremersch et al., 2016a; Tang et al., 2012). Cancer-derived EVs were used as a proof of concept model for autologous cancer treatment, since cancer-derived EVs were efficiently internalized by cancer cells (**Figure 10**) and functional studies have demonstrated that cancer EVs have a specific cell tropism to their own tumors (Hoshino et al., 2015). While exploiting the intrinsic cell targeting properties of cancer-derived EVs could enhance the effect of paclitaxel, there may be risks and important limitations to consider before their use in *in vivo* models. As shown in publications **III** and **IV**, EVs might prompt increased cell viability and proliferation of cells. In the case of cancer treatment, this effect could be compensated for by delivering the cytotoxic drug. Hence, the use of EVs from non-cancer sources could be of benefit, since they might be capable of reducing the cellular proliferation of the tumors while preserving their targeting capabilities (Ohno et al., 2016). In addition, the design of artificial nano-size vesicles using the specific targeting moieties of cancer EVs as a blueprint will be of benefit in the delivery of therapeutics. Overall, the observations presented here suggest that autologous EVs may have potential for the effective delivery of chemotherapeutics to cancer cells. However, *in vivo* models to analyze the targeting and pharmacokinetics of the cancer-derived EVs have to be developed in order to evaluate their usability in drug delivery.

7 Conclusions

In this thesis, the prospects of cancer-derived EVs in biomarker discovery and drug delivery were explored using prostate cancer as a model.

First, cancer-derived EV subpopulations were shown to have different nucleic acid contents, particularly gDNA and mRNA, correlating to specific cellular signatures of their parent cells. This work was the first to report that EV subpopulations from prostate cancer cells and plasma samples of prostate cancer patients contained double-stranded gDNA fragments and that this DNA could harbor differential mutations present in the parent cancer cells, emphasizing their potential as future cancer markers. However, the ability to detect mutations from biofluids-derived EVs together with the correlation to the patient's clinical parameters needs to be established properly before EVs can be utilized in cancer diagnosis. Additionally, differential mRNA levels were found between distinct prostate cancer cell-derived EV subpopulations, with a unique and distinctive cancer signature identified based on the ratio of increased *vs* decreased mRNAs. Together these findings illustrate the relevant and significant translational potential of the combined nucleic acid analysis of EVs to better understand and contribute to the diagnosis of prostate cancer.

Next, the transfer of functional aspects related to the hallmarks of cancer through EVs was demonstrated, highlighting the relevant role of EVs in cell-to-cell communication and cancer malignancy. The cancer status of the cellular source of the vesicles influences both the recognition and internalization capabilities of EVs by cells, promoting alterations of the cellular functions towards a more malignant state.

Finally, cancer cell-derived EV subpopulations were shown to effectively carry and deliver a small molecular drug, and to mediate an enhanced cytotoxic effect with a reduced drug amount when delivered by EVs. These results showcase the potential for the development of a new generation of EV-based drugs, and for the development of new drug delivery systems utilizing the beneficial characteristics of EVs.

Taken together, the results presented in this thesis provide a broad view of the possibilities and applications of EVs in cancer research. This thesis will contribute to a better understanding of the roles of EVs in intercellular communication and cancer progression, and suggests novel diagnostic and therapeutic opportunities for future clinical translation.

8 Future prospects

The extracellular vesicle field has rapidly and exponentially grown in recent years. The promising and breakthrough results of multiple studies using EVs in different areas of biomedical and translational research have revealed a totally new field in which the multiple functions of EVs are now being investigated. Exciting attempts are being made to determine the biochemical and biophysical properties of EVs to further improve their detection and classification and to deepen the overall understanding of their roles in diseases such as cancer. However, several limitations have to be overcome before EVs use can be translated from the bench to the clinics. Some technical aspects remain to be clarified, including the EV classification and standardized protocols for their isolation, together with reference materials for their reliable measurements in clinical and pharmaceutical settings. The developments of new technologies that allow the reproducible isolation of large amounts of pure EV samples are a crucial need. Another major issue to improve the understanding of the particular functions of EVs is the separation of pure and different EV subpopulations of similar size, density or composition. New devices or techniques have to be developed to specifically allow the capture of a particular kind of EV and to implement single-component EV analysis.

Despite the remarkable findings in the EV field, the question still remains as to whether it is necessary to generate EVs to survive and what is the function of a particular EV subset within the heterogeneous EV population. Many of the assumptions regarding the functional and mechanistic roles of EVs in intercellular communication need to be further validated using *in vivo* models and well-characterized EV subpopulations. Although the use of established cell lines is of great advantage for long-term studies to standardize the EV isolation and characterization protocols, the implementation and use of patient's own primary cells and biofluids, although challenging, will provide new insights. Parallel comparisons of enzymatically treated *vs* untreated EVs prior nucleic acid extraction is of great importance to better characterize the specificity of the EV cargo and its possible roles and function. The combinations of new and traditional "omic" approaches will stratify EV subsets based on their unique characteristics, providing new hints about their functionality. Additionally, DNA- and RNA-based next generation sequencing studies of EVs isolated from the patient's own biofluids will likely contribute to an earlier diagnosis of various diseases such as cancer, by avoiding all the unnecessary invasive procedures and by complementing the already existing diagnostic tests.

Future studies will likely explore in more depth the delivery of therapeutic molecules using EVs because of their efficient transfer of proteins, mRNAs, miRNAs, and therapeutic drugs into selective targets, which could contribute to the creation of new treatment modalities. Improved knowledge on the EV uptake mechanisms and the EV proteins responsible for the organ tropism will hence benefit the design of innovative and sophisticated drug delivery systems. However, due to the lack of efficient methods for drug loading, up-scalable EV production, and bulk preparation, and feasible targeting of selected tissues, new EV studies addressing these challenges and questions have to be carried out. The increased understanding of the EV biogenesis and processing machinery can be used to improve the loading efficiency by engineering the cargo. Additionally, bioinspired EV-mimicking nanovesicles or similar approaches may represent improved ways to ensure sufficient amounts of EVs with efficient drug-loading capacities for clinical use, and a way of moving forward in the theranostic field. It is possible that e.g., plant-derived EVs may solve the above-mentioned restrictions, since plant-derived EVs may represent a potentially unlimited source of EVs for drug delivery.

EVs are rapidly rising as a new independent era in cancer diagnostics and therapies with special relevance in personalized medicine. The increasing evidence of the specific cargo composition of EVs opens a wide range of new prospects and applications of EVs. With the insight gained by standardized and sensitive technologies, many currently unknown functions of EV in health and disease will be resolved.

9 References

Aalberts, M., Stout, T.A., and Stoorvogel, W. (2013). Prostasomes: extracellular vesicles from the prostate. Reproduction *147,* R1-14.

Aatonen, M.T., Öhman, T., Nyman, T.A., Laitinen, S., *et al.* (2014). Isolation and characterization of platelet-derived extracellular vesicles. J. Extracell Vesicles. *3.* 24692

Aatonen, M., Grönholm, M., and Siljander, P.R. (2012). Semin. Thromb. Hemost. 38, 102-113.

Abd Elmageed, Z.Y., Yang, Y., Thomas, R., Ranjan, M., *et al.* (2014). Neoplastic Reprogramming of Patient-Derived Adipose Stem Cells by Prostate Cancer Cell-Associated Exosomes. Stem Cells *32,* 983-997.

Abusamra, A.J., Zhong, Z., Zheng, X., Li, M., *et al.* (2005). Tumor exosomes expressing Fas ligand mediate CD8 T-cell apoptosis. Blood Cells, Molecules, and Diseases *35,* 169-173.

Aga, M., Bentz, G.L., Raffa, S., Torrisi, M.R., *et al.* (2014). Exosomal HIF1α supports invasive potential of nasopharyngeal carcinoma-associated LMP1-positive exosomes. Oncogene *33,* 4613-4622.

Ahadi, A., Brennan, S., Kennedy, P.J., Hutvagner, G., and Tran, N. (2016). Long non-coding RNAs harboring miRNA seed regions are enriched in prostate cancer exosomes. Sci. Rep. *6,* 24922.

Ahmed, W., Philip, P.S., Tariq, S., and Khan, G. (2014). Epstein-Barr virus-encoded small RNAs (EBERs) are present in fractions related to exosomes released by EBV-transformed cells. PloS One *9,* e99163.

Akers, J.C., Ramakrishnan, V., Kim, R., Skog, J., *et al.* (2013). MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. PloS One *8,* e78115.

Allen, T.M., and Cullis, P.R. (2013). Liposomal drug delivery systems: from concept to clinical applications. Adv. Drug Deliv. Rev. *65,* 36-48.

Allen, T.M., and Cullis, P.R. (2004). Drug delivery systems: entering the mainstream. Science *303,* 1818-1822.

Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., *et al.* (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat. Cell Biol. *10,* 619-624.

Al-Nedawi, K., Meehan, B., Kerbel, R.S., Allison, A.C., and Rak, J. (2009). Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. Proc. Natl. Acad. Sci. U. S. A. *106,* 3794-3799.

Altadill, T., Campoy, I., Lanau, L., Gill, K., *et al.* (2016). Enabling Metabolomics Based Biomarker Discovery Studies Using Molecular Phenotyping of Exosome-Like Vesicles. PloS One *11,* e0151339.

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., *et al.* (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat. Biotechnol. *29,* 341-345.

An, T., Qin, S., Xu, Y., Tang, Y., *et al.* (2015). Exosomes serve as tumour markers for personalized diagnostics owing to their important role in cancer metastasis. J J. Extracell Vesicles. *4,* 27522.

Andaloussi, S.E., Mäger, I., Breakefield, X.O., and Wood, M.J. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. Nature Reviews Drug Discovery *12,* 347-357.

Anderson, H.C. (1969). Vesicles associated with calcification in the matrix of epiphyseal cartilage. J. Cell Biol. *41,* 59-72.

Andreola, G., Rivoltini, L., Castelli, C., Huber, V., *et al.* (2002). Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J. Exp. Med. *195,* 1303-1316.

Antonyak, M.A., Li, B., Boroughs, L.K., Johnson, J.L., *et al.* (2011). Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. Proc. Natl. Acad. Sci. U. S. A. *108,* 4852-4857.

Arscott, W.T., and Camphausen, K.A. (2011). EGFR isoforms in exosomes as a novel method for biomarker discovery in pancreatic cancer. Biomark Med. *5,* 821.

Artibani, W. (2012). Landmarks in prostate cancer diagnosis: the biomarkers. BJU Int. *110,* 8-13.

Arvidson, G., Ronquist, G., Wikander, G., and Öjteg, A. (1989). Human prostasome membranes exhibit very high cholesterol/phospholipid ratios

yielding high molecular ordering. Biochimica Et Biophysica Acta (BBA)-Biomembranes *984,* 167-173.

Atay, S., Banskota, S., Crow, J., Sethi, G.*, et al.* (2014). Oncogenic KIT-containing exosomes increase gastrointestinal stromal tumor cell invasion. Proc. Natl. Acad. Sci. U. S. A. *111,* 711-716.

Attard, G., Parker, C., Eeles, R.A., Schröder, F.*, et al.* (2016). Prostate cancer. The Lancet *387,* 70-82.

Azmi, A.S., Bao, B., and Sarkar, F.H. (2013). Exosomes in cancer development, metastasis, and drug resistance: a comprehensive review. Cancer Metastasis Rev. *32,* 623-642.

Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A.*, et al.* (2012). Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat. Cell Biol. *14,* 677-685.

Baj-Krzyworzeka, M., Weglarczyk, K., Mytar, B., Szatanek, R.*, et al.* (2011). Tumour-derived microvesicles contain interleukin-8 and modulate production of chemokines by human monocytes. Anticancer Res. *31,* 1329-1335.

Balaj, L., Lessard, R., Dai, L., Cho, Y.*, et al.* (2011). Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. Nature Communications *2,* 180.

Baran, J., Baj-Krzyworzeka, M., Weglarczyk, K., Szatanek, R.*, et al.* (2010). Circulating tumour-derived microvesicles in plasma of gastric cancer patients. Cancer Immunology, Immunotherapy *59,* 841-850.

Bard, M.P., Hegmans, J.P., Hemmes, A., Luider, T.M.*, et al.* (2004). Proteomic analysis of exosomes isolated from human malignant pleural effusions. American Journal of Respiratory Cell and Molecular Biology *31,* 114-121.

Barry, O.P., Pratico, D., Lawson, J.A., and FitzGerald, G.A. (1997). Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. J. Clin. Invest. *99,* 2118-2127.

Beckham, C.J., Olsen, J., Yin, P., Wu, C.*, et al.* (2014). Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression. J. Urol. *192,* 583-592.

Bellingham, S.A., Coleman, B.M., and Hill, A.F. (2012). Small RNA deep sequencing reveals a distinct miRNA signature released in exosomes from prion-infected neuronal cells. Nucleic Acids Res. *40,* 10937-10949.

Bergsmedh, A., Szeles, A., Henriksson, M., Bratt, A.*, et al.* (2001). Horizontal transfer of oncogenes by uptake of apoptotic bodies. Proc. Natl. Acad. Sci. U. S. A. *98,* 6407-6411.

Bian, S., Zhang, L., Duan, L., Wang, X.*, et al.* (2014). Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. Journal of Molecular Medicine *92,* 387-397.

Bianco, F., Perrotta, C., Novellino, L., Francolini, M.*, et al.* (2009). Acid sphingomyelinase activity triggers microparticle release from glial cells. Embo J. *28,* 1043-1054.

Bijnsdorp, I.V., Geldof, A.A., Lavaei, M., Piersma, S.R.*, et al.* (2013). Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients. J. Extracell Vesicles. *2.* 22097.

Bishop, N., and Woodman, P. (2000). ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. Mol. Biol. Cell *11,* 227-239.

Bobrie, A., Krumeich, S., Reyal, F., Recchi, C.*, et al.* (2012). Rab27a supports exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can promote tumor progression. Cancer Res. *72,* 4920-4930.

Boilard, E., Nigrovic, P.A., Larabee, K., Watts, G.F.*, et al.* (2010). Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science *327,* 580-583.

Bozzuto, G., and Molinari, A. (2015). Liposomes as nanomedical devices. International Journal of Nanomedicine *10,* 975-999.

Brase, J.C., Johannes, M., Schlomm, T., Fälth, M.*, et al.* (2011). Circulating miRNAs are correlated with tumor progression in prostate cancer. International Journal of Cancer *128,* 608-616.

Bryant, R.J., Pawlowski, T., Catto, J.W.F., Marsden, G.*, et al.* (2012). Changes in circulating microRNA levels associated with prostate cancer. Br. J. Cancer *106,* 768-774.

Buschow, S.I., Liefhebber, J.M., Wubbolts, R., and Stoorvogel, W. (2005). Exosomes contain

ubiquitinated proteins. Blood Cells Mol. Dis. *35,* 398-403.

Buzas, E.I., György, B., Nagy, G., Falus, A., and Gay, S. (2014). Emerging role of extracellular vesicles in inflammatory diseases. Nature Reviews Rheumatology *10,* 356-364.

Cai, J., Han, Y., Ren, H., Chen, C.*, et al.* (2013). Extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells is a novel mechanism for genetic influence between cells. Journal of Molecular Cell Biology *5,* 227-238.

Catalona, W.J. (1994). Management of cancer of the prostate. N. Engl. J. Med. *331,* 996-1004.

Catalona, W.J., Smith, D.S., Ratliff, T.L., Dodds, K.M.*, et al.* (1991). Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. N. Engl. J. Med. *324,* 1156-1161.

Cazzoli, R., Buttitta, F., Di Nicola, M., Malatesta, S.*, et al.* (2013). microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer. Journal of Thoracic Oncology *8,* 1156-1162.

Chargaff, E., and West, R. (1946). The biological significance of the thromboplastic protein of blood. J. Biol. Chem. *166,* 189-197.

Chen, G., Zhu, J., Zhang, Z., Zhang, W.*, et al.* (2015). Transformation of Cell-Derived Microparticles into Quantum-Dot-Labeled Nanovectors for Antitumor siRNA Delivery. Angewandte Chemie International Edition *54,* 1036-1040.

Chen, W.W., Balaj, L., Liau, L.M., Samuels, M.L.*, et al.* (2013). BEAMing and droplet digital PCR analysis of mutant IDH1 mRNA in glioma patient serum and cerebrospinal fluid extracellular vesicles. Molecular Therapy—Nucleic Acids *2,* e109.

Chen, Y.A., and Scheller, R.H. (2001). SNARE-mediated membrane fusion. Nature Reviews Molecular Cell Biology *2,* 98-106.

Cheng, L., Montironi, R., Bostwick, D.G., Lopez-Beltran, A., and Berney, D.M. (2012). Staging of prostate cancer. Histopathology *60,* 87-117.

Choi, D., You, S., Jung, J.H., Lee, J.C.*, et al.* (2014). Extracellular vesicles shed from gefitinib-resistant nonsmall cell lung cancer regulate the tumor microenvironment. Proteomics *14,* 1845-1856.

Choi, D., Park, J.O., Jang, S.C., Yoon, Y.J.*, et al.* (2011). Proteomic analysis of microvesicles derived from human colorectal cancer ascites. Proteomics *11,* 2745-2751.

Christianson, H.C., Svensson, K.J., van Kuppevelt, T.H., Li, J.P., and Belting, M. (2013). Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. Proc. Natl. Acad. Sci. U. S. A. *110,* 17380-17385.

Clark, D.J., Fondrie, W.E., Liao, Z., Hanson, P.I.*, et al.* (2015). Redefining the breast cancer exosome proteome by tandem mass tag quantitative proteomics and multivariate cluster analysis. Anal. Chem. *87,* 10462-10469.

Clayton, A., Mitchell, J.P., Court, J., Mason, M.D., and Tabi, Z. (2007). Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. Cancer Res. *67,* 7458-7466.

Clayton, A., Al-Taei, S., Webber, J., Mason, M.D., and Tabi, Z. (2011). Cancer Exosomes Express CD39 and CD73, Which Suppress T Cells through Adenosine Production. Journal of Immunology *187,* 676-683.

Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M.*, et al.* (2001). Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. *3,* 339-345.

Collins, M., Ransohoff, D., and Barry, M. (1997). Early detection of prostate cancer - Serendipity strikes again. Jama-Journal of the American Medical Association *278,* 1516-1519.

Colombo, M., Raposo, G., and Théry, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu. Rev. Cell Dev. Biol. *30,* 255-289.

Cooper, J.M., Wiklander, P., Nordin, J.Z., Al-Shawi, R.*, et al.* (2014). Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. Movement Disorders *29,* 1476-1485.

Corcoran, C., Rani, S., and O'Driscoll, L. (2014). miR-34a is an intracellular and exosomal predictive biomarker for response to docetaxel with clinical relevance to prostate cancer progression. Prostate *74,* 1320-1334.

Costa-Silva, B., Aiello, N.M., Ocean, A.J., Singh, S.*, et al.* (2015). Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat. Cell Biol. *17,* 816-826.

Crescitelli, R., Lasser, C., Szabo, T.G., Kittel, A., *et al.* (2013). Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. J. Extracell Vesicles. *2*. 20677.

Dai, S., Wei, D., Wu, Z., Zhou, X., *et al.* (2008). Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. Molecular Therapy *16,* 782-790.

Del Conde, I., Shrimpton, C.N., Thiagarajan, P., and Lopez, J.A. (2005). Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood *106,* 1604-1611.

Demory Beckler, M., Higginbotham, J.N., Franklin, J.L., Ham, A.J., *et al.* (2013). Proteomic analysis of exosomes from mutant KRAS colon cancer cells identifies intercellular transfer of mutant KRAS. Mol. Cell. Proteomics *12,* 343-355.

Deregibus, M.C., Cantaluppi, V., Calogero, R., Lo Iacono, M., *et al.* (2007). Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood *110,* 2440-2448.

Dhir, R., Vietmeier, B., Arlotti, J., Acquafondata, M., *et al.* (2004). Early identification of individuals with prostate cancer in negative biopsies. J. Urol. *171,* 1419-1423.

Di Vizio, D., Morello, M., Dudley, A.C., Schow, P.W., *et al.* (2012). Large oncosomes in human prostate cancer tissues and in the circulation of mice with metastatic disease. The American Journal of Pathology *181,* 1573-1584.

Di Vizio, D., Kim, J., Hager, M.H., Morello, M., *et al.* (2009). Oncosome Formation in Prostate Cancer: Association with a Region of Frequent Chromosomal Deletion in Metastatic Disease. Cancer Res. *69,* 5601-5609.

Didiot, M., Hall, L.M., Coles, A.H., Haraszti, R.A., *et al.* (2016). Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. Molecular Therapy. *24,* 1836-1847.

Dijkstra, S., Birker, I.L., Smit, F.P., Leyten, G.H.J.M., *et al.* (2014). Prostate Cancer Biomarker Profiles in Urinary Sediments and Exosomes. J. Urol. *191,* 1132-1138.

Dozmorov, M.G., Hurst, R.E., Culkin, D.J., Kropp, B.P., *et al.* (2009). Unique patterns of molecular profiling between human prostate cancer LNCaP and PC-3 cells. Prostate *69,* 1077-1090.

Duijvesz, D., Burnum-Johnson, K.E., Gritsenko, M.A., Hoogland, A.M., *et al.* (2013). Proteomic profiling of exosomes leads to the identification of novel biomarkers for prostate cancer. PloS One *8,* e82589.

Duijvesz, D., Luider, T., Bangma, C.H., and Jenster, G. (2011). Exosomes as biomarker treasure chests for prostate cancer. Eur. Urol. *59,* 823-831.

Eichelser, C., Stuckrath, I., Muller, V., Milde-Langosch, K., *et al.* (2014). Increased serum levels of circulating exosomal microRNA-373 in receptor-negative breast cancer patients. Oncotarget *5,* 9650-9663.

Engholm, G., Ferlay, J., Christensen, N., Kejs, A., *et al.* (2016). NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 7.3 (08.07.2016). Association of the Nordic Cancer Registries. Danish Cancer Society. *11/16*.

Epstein, J.I., Zelefsky, M.J., Sjoberg, D.D., Nelson, J.B., *et al.* (2016). A Contemporary Prostate Cancer Grading System: A Validated Alternative to the Gleason Score. Eur. Urol. *69,* 428-435.

Epstein, J., Walsh, P., Carmichael, M., and Brendler, C. (1994). Pathological and Clinical Findings to Predict Tumor Extent of Nonpalpable (Stage-T1c) Prostate-Cancer. Jama-Journal of the American Medical Association *271,* 368-374.

Escrevente, C., Keller, S., Altevogt, P., and Costa, J. (2011). Interaction and uptake of exosomes by ovarian cancer cells. BMC Cancer *11,* 1.

Escudier, B., Dorval, T., Chaput, N., André, F., *et al.* (2005). Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. Journal of Translational Medicine *3,* 1.

Esser, J., Gehrmann, U., D'Alexandri, F.L., Hidalgo-Estévez, A.M., *et al.* (2010). Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration. J. Allergy Clin. Immunol. *126,* 1032-1040. e4.

Estelles, A., Sperinde, J., Roulon, T., Aguilar, B., *et al.* (2007). Exosome nanovesicles displaying G protein-coupled receptors for drug discovery. International Journal of Nanomedicine *2,* 751.

Fabbri, M., Paone, A., Calore, F., Galli, R., *et al.* (2012). MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. Proc. Natl. Acad. Sci. U. S. A. *109,* E2110-6.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., *et al.* (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. *148,* 2207-2216.

Fais, S., O'Driscoll, L., Borras, F.E., Buzas, E., *et al.* (2016). Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine. ACS Nano *10,* 3886-3899.

Fedele, C., Singh, A., Zerlanko, B.J., Iozzo, R.V., and Languino, L.R. (2015). The alphavbeta6 integrin is transferred intercellularly via exosomes. J. Biol. Chem. *290,* 4545-4551.

Federici, C., Petrucci, F., Caimi, S., Cesolini, A., *et al.* (2014). Exosome release and low pH belong to a framework of resistance of human melanoma cells to cisplatin. PloS One *9,* e88193.

Felice, B., Prabhakaran, M.P., Rodríguez, A.P., and Ramakrishna, S. (2014). Drug delivery vehicles on a nano-engineering perspective. Materials Science and Engineering: C *41,* 178-195.

Feng, D., Zhao, W., Ye, Y., Bai, X., *et al.* (2010). Cellular internalization of exosomes occurs through phagocytosis. Traffic *11,* 675-687.

Ferlini, C., Cicchillitti, L., Raspaglio, G., Bartollino, S., *et al.* (2009). Paclitaxel directly binds to Bcl-2 and functionally mimics activity of Nur77. Cancer Res. *69,* 6906-6914.

Fevrier, B., and Raposo, G. (2004). Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr. Opin. Cell Biol. *16,* 415-421.

Findlow, J., Borrow, R., Snape, M.D., Dawson, T., *et al.* (2010). Multicenter, open-label, randomized phase II controlled trial of an investigational recombinant Meningococcal serogroup B vaccine with and without outer membrane vesicles, administered in infancy. Clin. Infect. Dis. *51,* 1127-1137.

Fitzner, D., Schnaars, M., van Rossum, D., Krishnamoorthy, G., *et al.* (2011). Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J. Cell. Sci. *124,* 447-458.

Fong, M.Y., Zhou, W., Liu, L., Alontaga, A.Y., *et al.* (2015). Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. Nat. Cell Biol. *17,* 183-194.

Fuhrmann, G., Serio, A., Mazo, M., Nair, R., and Stevens, M.M. (2015). Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. J. Controlled Release *205,* 35-44.

Gabriel, K., Ingram, A., Austin, R., Kapoor, A., *et al.* (2013). Regulation of the tumor suppressor PTEN through exosomes: a diagnostic potential for prostate cancer. PloS One *8,* e70047.

Gallo, A., Tandon, M., Alevizos, I., and Illei, G.G. (2012). The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PloS One *7,* e30679.

García-Romero, N., Carrión-Navarro, J., Esteban-Rubio, S., Lázaro-Ibáñez, E., *et al.* (2016). DNA sequences within glioma-derived extracellular vesicles can cross the intact Blood-Brain Barrier and be detected in peripheral blood of patients. Oncotarget *8*, 1416-1428-

Gehrmann, U., Hiltbrunner, S., Georgoudaki, A.M., Karlsson, M.C., *et al.* (2013). Synergistic induction of adaptive antitumor immunity by codelivery of antigen with alpha-galactosylceramide on exosomes. Cancer Res. *73,* 3865-3876.

Gesierich, S., Berezovskiy, I., Ryschich, E., and Zoller, M. (2006). Systemic induction of the angiogenesis switch by the tetraspanin D6.1A/CO-029. Cancer Res. *66,* 7083-7094.

Ghossoub, R., Lembo, F., Rubio, A., Gaillard, C.B., *et al.* (2014). Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. Nature Communications *5*.

Gittes, R.F. (1991). Carcinoma of the prostate. N. Engl. J. Med. *324,* 236-245.

Gleason, D.F. (1966). Classification of prostatic carcinomas. Cancer Chemother. Rep. *50,* 125-128.

Gleason, D.F., and Mellinger, G.T. (1974). Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. J. Urol. *111,* 58-64.

Goh, F.G., and Midwood, K.S. (2012). Intrinsic danger: activation of Toll-like receptors in rheumatoid arthritis. Rheumatology *51,* 7-23.

Goossens, N., Nakagawa, S., Sun, X., and Hoshida, Y. (2015). Cancer biomarker discovery and validation. Transl. Cancer. Res. *4,* 256-269.

Gould, S.J., and Raposo, G. (2013). As we wait: coping with an imperfect nomenclature for extracellular vesicles. J. Extracell Vesicles. *2*. 20389.

Graves, L.E., Ariztia, E.V., Navari, J.R., Matzel, H.J., *et al.* (2004). Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Res. *64,* 7045-7049.

Guescini, M., Genedani, S., Stocchi, V., and Agnati, L.F. (2010). Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. J. Neural Transm. *117,* 1-4.

Gulinelli, S., Salaro, E., Vuerich, M., Bozzato, D., *et al.* (2012). IL-18 associates to microvesicles shed from human macrophages by a LPS/TLR-4 independent mechanism in response to P2X receptor stimulation. Eur. J. Immunol. *42,* 3334-3345.

György, B., Szabó, T.G., Pásztói, M., Pál, Z., *et al.* (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cellular and Molecular Life Sciences *68,* 2667-2688.

Ha, D., Yang, N., and Nadithe, V. (2016). Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. Acta Pharmaceutica Sinica B *6*, 287-296.

Haga, H., Yan, I.K., Takahashi, K., Wood, J., *et al.* (2015). Tumour cellderived extracellular vesicles interact with mesenchymal stem cells to modulate the microenvironment and enhance cholangiocarcinoma growth. J. Extracell Vesicles. *4*. 24900.

Halicka, H.D., Bedner, E., and Darzynkiewicz, Z. (2000). Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp. Cell Res. *260,* 248-256.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144,* 646-674.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100,* 57-70.

Haney, M.J., Klyachko, N.L., Zhao, Y., Gupta, R., *et al.* (2015). Exosomes as drug delivery vehicles for Parkinson's disease therapy. J. Controlled Release *207,* 18-30.

Harding, C., and Stahl, P. (1983). Transferrin recycling in reticulocytes: pH and iron are important determinants of ligand binding and processing. Biochem. Biophys. Res. Commun. *113,* 650-658.

Harris, D.A., Patel, S.H., Gucek, M., Hendrix, A., *et al.* (2015). Exosomes released from breast cancer carcinomas stimulate cell movement. PloS One *10,* e0117495.

Haug, B.H., Hald, O.H., Utnes, P., Roth, S.A., *et al.* (2015). Exosome-like Extracellular Vesicles from MYCN-amplified Neuroblastoma Cells Contain Oncogenic miRNAs. Anticancer Res. *35,* 2521-2530.

Headland, S.E., Jones, H.R., D'Sa, A.S., Perretti, M., and Norling, L.V. (2014). Cutting-edge analysis of extracellular microparticles using ImageStreamX imaging flow cytometry. Scientific Reports *4*. 5237.

Heidenreich, A., Bastian, P.J., Bellmunt, J., Bolla, M., *et al.* (2014). EAU guidelines on prostate cancer. part 1: screening, diagnosis, and local treatment with curative intent-update 2013. Eur. Urol. *65,* 124-137.

Hessvik, N.P., Phuyal, S., Brech, A., Sandvig, K., and Llorente, A. (2012). Profiling of microRNAs in exosomes released from PC-3 prostate cancer cells. Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms *1819,* 1154-1163.

Holmgren, L., Szeles, A., Rajnavolgyi, E., Folkman, J., *et al.* (1999). Horizontal transfer of DNA by the uptake of apoptotic bodies. Blood *93,* 3956-3963.

Hong, C.S., Muller, L., Boyiadzis, M., and Whiteside, T.L. (2014). Isolation and characterization of CD34 blast-derived exosomes in acute myeloid leukemia. PLoS One *9,* e103310.

Hood, J.L., Pan, H., Lanza, G.M., and Wickline, S.A. (2009). Paracrine induction of endothelium by tumor exosomes. Laboratory Investigation *89,* 1317-1328.

Hood, J.L., San, R.S., and Wickline, S.A. (2011). Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer Res. *71,* 3792-3801.

Hoshino, A., Costa-Silva, B., Shen, T., Rodrigues, G., *et al.* (2015). Tumour exosome integrins determine organotropic metastasis. Nature *527,* 329-335.

Hosseini-Beheshti, E., Pham, S., Adomat, H., Li, N., and Guns, E.S.T. (2012). Exosomes as Biomarker Enriched Microvesicles: Characterization of Exosomal Proteins Derived from a Panel of Prostate Cell Lines with Distinct AR Phenotypes. Mol. Cell. Proteomics *11,* 863-885.

Hsu, C., Morohashi, Y., Yoshimura, S., Manrique-Hoyos, N.*, et al.* (2010). Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. J. Cell Biol. *189,* 223-232.

Hu, Z., Chen, X., Zhao, Y., Tian, T.*, et al.* (2010). Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J. Clin. Oncol. *28,* 1721-1726.

Huang, X., Yuan, T., Liang, M., Du, M.*, et al.* (2015). Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer. Eur. Urol. *67,* 33-41.

Huber, V., Fais, S., Iero, M., Lugini, L.*, et al.* (2005). Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. Gastroenterology *128,* 1796-1804.

Huotari, J., and Helenius, A. (2011). Endosome maturation. Embo J. *30,* 3481-3500.

Hurley, J.H., and Hanson, P.I. (2010). Membrane budding and scission by the ESCRT machinery: it's all in the neck. Nature Reviews Molecular Cell Biology *11,* 556-566.

Hurley, J.H., and Odorizzi, G. (2012). Get on the exosome bus with ALIX. Nat. Cell Biol. *14,* 654-655.

Im, H., Shao, H., Park, Y.I., Peterson, V.M.*, et al.* (2014). Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat. Biotechnol. *32,* 490-495.

Işın, M., Uysaler, E., Özgür, E., Köseoğlu, H.*, et al.* (2015). Exosomal lncRNA-p21 levels may help to distinguish prostate cancer from benign disease. Frontiers in Genetics *6,* 168.

James, N., and Mason, M. (2015). Docetaxel and/or zoledronic acid for hormone-naïve prostate cancer: First survival results from STAMPEDE. J. Clin. Oncol. *33*.

Jang, S.C., Kim, O.Y., Yoon, C.M., Choi, D.*, et al.* (2013). Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. ACS Nano *7,* 7698-7710.

Jansen, F.H., Krijgsveld, J., van Rijswijk, A., van den Bemd, G.*, et al.* (2009). Exosomal Secretion of Cytoplasmic Prostate Cancer Xenograft-derived Proteins. Mol. Cell. Proteomics *8,* 1192-1205.

Jarrard, D.F., Bova, G.S., Ewing, C.M., Pin, S.S.*, et al.* (1997). Deletional, mutational, and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer. Genes, Chromosomes and Cancer *19,* 90-96.

Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010). Cancer statistics, 2010. CA: A Cancer Journal for Clinicians *60,* 277-300.

Jenjaroenpun, P., Kremenska, Y., Nair, V.M., Kremenskoy, M.*, et al.* (2013). Characterization of RNA in exosomes secreted by human breast cancer cell lines using next-generation sequencing. PeerJ *1,* e201.

Johnsen, K.B., Gudbergsson, J.M., Skov, M.N., Pilgaard, L.*, et al.* (2014). A comprehensive overview of exosomes as drug delivery vehicles—endogenous nanocarriers for targeted cancer therapy. Biochimica Et Biophysica Acta (BBA)-Reviews on Cancer *1846,* 75-87.

Johnstone, R.M., Adam, M., Hammond, J.R., Orr, L., and Turbide, C. (1987). Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J. Biol. Chem. *262,* 9412-9420.

Jørgensen, M., Bæk, R., Pedersen, S., Søndergaard, E.K.*, et al.* (2013). Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. J. Extracell Vesicles. *2*. 20920.

Jubb, A., Turley, H., Moeller, H., Steers, G.*, et al.* (2009). Expression of delta-like ligand 4 (Dll4) and markers of hypoxia in colon cancer. Br. J. Cancer *101,* 1749-1757.

Jung, T., Castellana, D., Klingbeil, P., Hernández, I.C.*, et al.* (2009). CD44v6 dependence of premetastatic niche preparation by exosomes. Neoplasia *11,* 1093-IN17.

Kadiu, I., Narayanasamy, P., Dash, P.K., Zhang, W., and Gendelman, H.E. (2012). Biochemical and biologic characterization of exosomes and microvesicles as facilitators of HIV-1 infection in macrophages. J. Immunol. *189,* 744-754.

Kahlert, C., Melo, S.A., Protopopov, A., Tang, J., *et al.* (2014). Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. J. Biol. Chem. *289,* 3869-3875.

Kalluri, R. (2016). The biology and function of exosomes in cancer. J. Clin. Invest. *126,* 1208.

Kalra, H., Simpson, R.J., Ji, H., Aikawa, E., *et al.* (2012). Vesiclepedia: A Compendium for Extracellular Vesicles with Continuous Community Annotation. PLoS. Biol. *10,* e1001450.

Kanada, M., Bachmann, M.H., and Contag, C.H. (2016). Signaling by Extracellular Vesicles Advances Cancer Hallmarks. Trends in Cancer *2,* 84-94.

Kanwar, S.S., Dunlay, C.J., Simeone, D.M., and Nagrath, S. (2014). Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes. Lab on a Chip *14,* 1891-1900.

Katsuda, T., Tsuchiya, R., Kosaka, N., Yoshioka, Y., *et al.* (2013). Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. Scientific Reports *3.* 1197.

Keerthikumar, S., Gangoda, L., Liem, M., Fonseka, P., *et al.* (2015). Proteogenomic analysis reveals exosomes are more oncogenic than ectosomes. Oncotarget *6,* 15375-15396.

Keller, S., Konig, A.K., Marme, F., Runz, S., *et al.* (2009). Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. Cancer Lett. *278,* 73-81.

Khan, S., Jutzy, J.M., Aspe, J.R., McGregor, D.W., *et al.* (2011). Survivin is released from cancer cells via exosomes. Apoptosis *16,* 1-12.

Khan, S., Jutzy, J.M., Valenzuela, M.M.A., Turay, D., *et al.* (2012). Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer. PloS One *7,* e46737.

Kim, G., Yoo, C.E., Kim, M., Kang, H.J., *et al.* (2012a). Noble polymeric surface conjugated with zwitterionic moieties and antibodies for the isolation of exosomes from human serum. Bioconjug. Chem. *23,* 2114-2120.

Kim, J.A., Åberg, C., Salvati, A., and Dawson, K.A. (2012b). Role of cell cycle on the cellular uptake and dilution of nanoparticles in a cell population. Nature Nanotechnology *7,* 62-68.

Kim, D.K., Lee, J., Kim, S.R., Choi, D.S., *et al.* (2015). EVpedia: a community web portal for extracellular vesicles research. Bioinformatics *31,* 933-939.

Kim, J.W., Wieckowski, E., Taylor, D.D., Reichert, T.E., *et al.* (2005). Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. Clin. Cancer Res. *11,* 1010-1020.

King, H.W., Michael, M.Z., and Gleadle, J.M. (2012). Hypoxic enhancement of exosome release by breast cancer cells. BMC Cancer *12,* 1.

Kirby, R.S., Partin, A.W., Feneley, M.R., and Parsons, J.K. (2006). Prostate cancer: principles and practice (Taylor & Francis).

Klibi, J., Niki, T., Riedel, A., Pioche-Durieu, C., *et al.* (2009). Blood diffusion and Th1-suppressive effects of galectin-9-containing exosomes released by Epstein-Barr virus-infected nasopharyngeal carcinoma cells. Blood *113,* 1957-1966.

Koga, K., Matsumoto, K., Akiyoshi, T., Kubo, M., *et al.* (2005). Purification, characterization and biological significance of tumor-derived exosomes. Anticancer Res. *25,* 3703-3707.

Kosaka, N., Iguchi, H., Yoshioka, Y., Hagiwara, K., *et al.* (2012). Competitive interactions of cancer cells and normal cells via secretory microRNAs. J. Biol. Chem. *287,* 1397-1405.

Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., *et al.* (2010). Secretory mechanisms and intercellular transfer of microRNAs in living cells. J. Biol. Chem. *285,* 17442-17452.

Kowal, J., Arras, G., Colombo, M., Jouve, M., *et al.* (2016). Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc. Natl. Acad. Sci. U. S. A. *113,* E968-77.

Kucharzewska, P., Christianson, H.C., Welch, J.E., Svensson, K.J., *et al.* (2013). Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. Proc. Natl. Acad. Sci. U. S. A. *110,* 7312-7317.

Kuo, L., and Freed, E.O. (2012). ARRDC1 as a mediator of microvesicle budding. Proc. Natl. Acad. Sci. U. S. A. *109,* 4025-4026.

Lai, C.P., Kim, E.Y., Badr, C.E., Weissleder, R., *et al.* (2015). Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. Nature Communications *6*. 7029.

Lamichhane, T.N., Raiker, R.S., and Jay, S.M. (2015). Exogenous DNA loading into extracellular vesicles via electroporation is size-dependent and enables limited gene delivery. Molecular Pharmaceutics *12,* 3650-3657.

Lau, C., Kim, Y., Chia, D., Spielmann, N., *et al.* (2013). Role of pancreatic cancer-derived exosomes in salivary biomarker development. J. Biol. Chem. *288,* 26888-26897.

Le, M.T., Hamar, P., Guo, C., Basar, E., *et al.* (2014). miR-200–containing extracellular vesicles promote breast cancer cell metastasis. J. Clin. Invest. *124,* 5109-5128.

Lee, J.C., Zhao, J., Gundara, J., Serpell, J., *et al.* (2015a). Papillary thyroid cancer–derived exosomes contain miRNA-146b and miRNA-222. J. Surg. Res. *196,* 39-48.

Lee, K., Shao, H., Weissleder, R., and Lee, H. (2015b). Acoustic purification of extracellular microvesicles. ACS Nano *9,* 2321-2327.

Lee, T.H., Chennakrishnaiah, S., Audemard, E., Montermini, L., *et al.* (2014). Oncogenic ras-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells. Biochem. Biophys. Res. Commun. *451,* 295-301.

Lee, T.H., Chennakrishnaiah, S., Meehan, B., Montermini, L., *et al.* (2016). Barriers to horizontal cell transformation by extracellular vesicles containing oncogenic H-ras. Oncotarget *7,* 51991-52002.

Lehmann, S.M., Krüger, C., Park, B., Derkow, K., *et al.* (2012). An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. Nat. Neurosci. *15,* 827-835.

Lener, T., Gimona, M., Aigner, L., Borger, V., *et al.* (2015). Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. J. Extracell Vesicles *4,* 30087.

Li, B., Antonyak, M.A., Zhang, J., and Cerione, R.A. (2012). RhoA triggers a specific signaling pathway that generates transforming microvesicles in cancer cells. Oncogene *31,* 4740-4749.

Li, J., Sherman-Baust, C.A., Tsai-Turton, M., Bristow, R.E., *et al.* (2009). Claudin-containing exosomes in the peripheral circulation of women with ovarian cancer. BMC Cancer *9,* 1.

Li, Q., Shao, Y., Zhang, X., Zheng, T., *et al.* (2015). Plasma long noncoding RNA protected by exosomes as a potential stable biomarker for gastric cancer. Tumor Biol. *36,* 2007-2012.

Li, Y., Zhang, Y., Qiu, F., and Qiu, Z. (2011). Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC. Electrophoresis *32,* 1976-1983.

Lindoso, R.S., Collino, F., and Camussi, G. (2015). Extracellular vesicles derived from renal cancer stem cells induce a pro-tumorigenic phenotype in mesenchymal stromal cells. Oncotarget *6,* 7959-7969.

Liu, J., Sun, H., Wang, X., Yu, Q., *et al.* (2014). Increased exosomal microRNA-21 and microRNA-146a levels in the cervicovaginal lavage specimens of patients with cervical cancer. International Journal of Molecular Sciences *15,* 758-773.

Liu, Y., Li, D., Liu, Z., Zhou, Y., *et al.* (2015). Targeted exosome-mediated delivery of opioid receptor Mu siRNA for the treatment of morphine relapse. Sci. Rep. *5,* 17543.

Llorente, A., Skotland, T., Sylvänne, T., Kauhanen, D., *et al.* (2013). Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids *1831,* 1302-1309.

Locke, J.A., Fazli, L., Adomat, H., Smyl, J., *et al.* (2009). A novel communication role for CYP17A1 in the progression of castration-resistant prostate cancer. Prostate *69,* 928-937.

Logozzi, M., De Milito, A., Lugini, L., Borghi, M., *et al.* (2009). High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PloS One *4,* e5219.

Lu, Q., Zhang, J., Allison, R., Gay, H., *et al.* (2009). Identification of extracellular δ-catenin accumulation for prostate cancer detection. Prostate *69,* 411-418.

Luga, V., Zhang, L., Viloria-Petit, A.M., Ogunjimi, A.A., *et al.* (2012). Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell *151,* 1542-1556.

Lugini, L., Cecchetti, S., Huber, V., Luciani, F., *et al.* (2012). Immune surveillance properties of human NK cell-derived exosomes. The Journal of Immunology *189,* 2833-2842.

Lunavat, T.R., Cheng, L., Kim, D., Bhadury, J., *et al.* (2015). Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells–Evidence of unique microRNA cargos. RNA Biology *12,* 810-823.

Lundholm, M., Schröder, M., Nagaeva, O., Baranov, V., *et al.* (2014). Prostate tumor-derived exosomes down-regulate NKG2D expression on natural killer cells and CD8 T cells: mechanism of immune evasion. PLoS One *9,* e108925.

Madhavan, B., Yue, S., Galli, U., Rana, S., *et al.* (2015). Combined evaluation of a panel of protein and miRNA serum-exosome biomarkers for pancreatic cancer diagnosis increases sensitivity and specificity. International Journal of Cancer *136,* 2616-2627.

Manterola, L., Guruceaga, E., Gallego Perez-Larraya, J., Gonzalez-Huarriz, M., *et al.* (2014). A small noncoding RNA signature found in exosomes of GBM patient serum as a diagnostic tool. Neuro Oncol. *16,* 520-527.

Mao, X., Sun, Y., and Tang, J. (2014). Serum miR-21 is a diagnostic and prognostic marker of primary central nervous system lymphoma. Neurological Sciences *35,* 233-238.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., *et al.* (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. *182,* 1545-1556.

Mathivanan, S., Ji, H., and Simpson, R.J. (2010). Exosomes: extracellular organelles important in intercellular communication. Journal of Proteomics *73,* 1907-1920.

Mathivanan, S., Fahner, C.J., Reid, G.E., and Simpson, R.J. (2012). ExoCarta 2012: database of exosomal proteins, RNA and lipids. Nucleic Acids Res. *40,* D1241-4.

Matsumura, T., Sugimachi, K., Iinuma, H., Takahashi, Y., *et al.* (2015). Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. Br. J. Cancer *113,* 275-281.

Maximilien, J., Beyazit, S., Rossi, C., Haupt, K., and Bui, B.T.S. (2015). Nanoparticles in Biomedical Applications. In Measuring Biological Impacts of Nanomaterials, Springer) pp. 177-210.

Mayr, M., Grainger, D., Mayr, U., Leroyer, A.S., *et al.* (2009). Proteomics, metabolomics, and immunomics on microparticles derived from human atherosclerotic plaques. Circ. Cardiovasc. Genet. *2,* 379-388.

McConnell, R.E., and Tyska, M.J. (2007). Myosin-1a powers the sliding of apical membrane along microvillar actin bundles. J. Cell Biol. *177,* 671-681.

McKiernan, J., Donovan, M.J., O'Neill, V., Bentink, S., *et al.* (2016). A novel urine exosome gene expression assay to predict high-grade prostate cancer at initial biopsy. JAMA Oncol. *7,* 882-889.

McNeal, J.E., Bostwick, D.G., Kindrachuk, R.A., Redwine, E.A., *et al.* (1986). Patterns of progression in prostate cancer. Lancet *1,* 60-63.

Meckes, D.G.,Jr, Shair, K.H., Marquitz, A.R., Kung, C.P., *et al.* (2010). Human tumor virus utilizes exosomes for intercellular communication. Proc. Natl. Acad. Sci. U. S. A. *107,* 20370-20375.

Meehan, K., and Vella, L.J. (2016). The contribution of tumour-derived exosomes to the hallmarks of cancer. Crit. Rev. Clin. Lab. Sci. *53,* 121-131.

Meek, D.W. (2009). Tumour suppression by p53: a role for the DNA damage response? Nature Reviews Cancer *9,* 714-723.

Meiers, I., Shanks, J.H., and Bostwick, D.G. (2007). Glutathione S-transferase pi (GSTP1) hypermethylation in prostate cancer: review 2007. Pathology *3*, 299-304.

Mellinger, G.T., Gleason, D., and Bailar, J.,3rd. (1967). The histology and prognosis of prostatic cancer. J. Urol. *97,* 331-337.

Melo, S.A., Luecke, L.B., Kahlert, C., Fernandez, A.F., *et al.* (2015). Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature *523,* 177-182.

Minciacchi, V.R., You, S., Spinelli, C., Morley, S., *et al.* (2015). Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles. Oncotarget *6,* 11327-11341.

Mitchell, P.J., Welton, J., Staffurth, J., Mason, M.D., *et al.* (2009). Can urinary exosomes act as

treatment response markers in prostate cancer? Journal of Translational Medicine *7,* 1.

Mitchell, P.S., Parkin, R.K., Kroh, E.M., Fritz, B.R.*, et al.* (2008). Circulating microRNAs as stable blood-based markers for cancer detection. Proc. Natl. Acad. Sci. U. S. A. *105,* 10513-10518.

Mizutani, K., Terazawa, R., Kameyama, K., Kato, T.*, et al.* (2014). Isolation of prostate cancer-related exosomes. Anticancer Res. *34,* 3419-3423.

Montecalvo, A., Larregina, A.T., Shufesky, W.J., Stolz, D.B.*, et al.* (2012). Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood *119,* 756-766.

Moon, P.G., Lee, J.E., Cho, Y.E., Lee, S.J.*, et al.* (2016). Fibronectin on circulating extracellular vesicles as a liquid biopsy to detect breast cancer. Oncotarget *7*, 40189-40199.

Morse, M.A., Clay, T.M., Hobeika, A.C., Osada, T.*, et al.* (2005). Phase I study of immunization with dendritic cells modified with fowlpox encoding carcinoembryonic antigen and costimulatory molecules. Clin. Cancer Res. *11,* 3017-3024.

Mulcahy, L.A., Pink, R.C., and Carter, D.R.F. (2014). Routes and mechanisms of extracellular vesicle uptake. J. Extracell Vesicles. *3*. 24641.

Munagala, R., Aqil, F., and Gupta, R.C. (2016). Exosomal miRNAs as biomarkers of recurrent lung cancer. Tumor Biol. 1-12.

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M.*, et al.* (2009). ARF6-Regulated Shedding of Tumor Cell-Derived Plasma Membrane Microvesicles. Current Biology *19,* 1875-1885.

Nabhan, J.F., Hu, R., Oh, R.S., Cohen, S.N., and Lu, Q. (2012). Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. Proc. Natl. Acad. Sci. U. S. A. *109,* 4146-4151.

Nakase, I., Kobayashi, N.B., Takatani-Nakase, T., and Yoshida, T. (2015). Active macropinocytosis induction by stimulation of epidermal growth factor receptor and oncogenic Ras expression potentiates cellular uptake efficacy of exosomes. Scientific Reports *5*.

Nanbo, A., Kawanishi, E., Yoshida, R., and Yoshiyama, H. (2013). Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells. J. Virol. *87,* 10334-10347.

Nawaz, M., Camussi, G., Valadi, H., Nazarenko, I.*, et al.* (2014). The emerging role of extracellular vesicles as biomarkers for urogenital cancers. Nature Reviews Urology *11,* 688-701.

Nazarenko, I., Rana, S., Baumann, A., McAlear, J.*, et al.* (2010). Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. Cancer Res. *70,* 1668-1678.

Neeb, A., Hefele, S., Bormann, S., Parson, W.*, et al.* (2014). Splice variant transcripts of the anterior gradient 2 gene as a marker of prostate cancer. Oncotarget *5,* 8681-8689.

Nguyen, D.G., Booth, A., Gould, S.J., and Hildreth, J.E.K. (2003). Evidence That HIV Budding in Primary Macrophages Occurs through the Exosome Release Pathway. J. Biol. Chem. *278,* 52347-52354.

Nilsson, J., Skog, J., Nordstrand, A., Baranov, V.*, et al.* (2009). Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. Br. J. Cancer *100,* 1603-1607.

Nolte-'t Hoen, E.N., Buermans, H.P., Waasdorp, M., Stoorvogel, W.*, et al.* (2012). Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions. Nucleic Acids Res. *40,* 9272-9285.

O'Brien, K., Rani, S., Corcoran, C., Wallace, R.*, et al.* (2013). Exosomes from triple-negative breast cancer cells can transfer phenotypic traits representing their cells of origin to secondary cells. Eur. J. Cancer *49,* 1845-1859.

Ogata-Kawata, H., Izumiya, M., Kurioka, D., Honma, Y.*, et al.* (2014). Circulating exosomal microRNAs as biomarkers of colon cancer. PloS One *9,* e92921.

Ohno, S., Drummen, G.P., and Kuroda, M. (2016). Focus on Extracellular Vesicles: Development of Extracellular Vesicle-Based Therapeutic Systems. International Journal of Molecular Sciences *17,* 172.

Ohno, S., Takanashi, M., Sudo, K., Ueda, S.*, et al.* (2012). Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. Molecular Therapy *1*, 185-191.

Ohshima, K., Inoue, K., Fujiwara, A., Hatakeyama, K., *et al.* (2010). Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. PloS One *5,* e13247.

Okoye, I.S., Coomes, S.M., Pelly, V.S., Czieso, S., *et al.* (2014). MicroRNA-containing T-regulatory-cell-derived exosomes suppress pathogenic T helper 1 cells. Immunity *41,* 89-103.

Olins, D.E., and Olins, A.L. (2003). Chromatin history: our view from the bridge. Nature Reviews Molecular Cell Biology *4,* 809-814.

Ono, M., Kosaka, N., Tominaga, N., Yoshioka, Y., *et al.* (2014). Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. Sci Signal *7,* 63.

Ostenfeld, M.S., Jeppesen, D.K., Laurberg, J.R., Boysen, A.T., *et al.* (2014). Cellular disposal of miR23b by RAB27-dependent exosome release is linked to acquisition of metastatic properties. Cancer Res. *74,* 5758-5771.

Overbye, A., Skotland, T., Koehler, C.J., Thiede, B., *et al.* (2015). Identification of prostate cancer biomarkers in urinary exosomes. Oncotarget *6,* 30357-30376.

Pan, B., and Johnstone, R.M. (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell *33,* 967-978.

Park, Y.H., Shin, H.W., Jung, A.R., Kwon, O.S., *et al.* (2016). Prostate-specific extracellular vesicles as a novel biomarker in human prostate cancer. Sci. Rep. *6,* 30386.

Parolini, I., Federici, C., Raggi, C., Lugini, L., *et al.* (2009). Microenvironmental pH is a key factor for exosome traffic in tumor cells. J. Biol. Chem. *284,* 34211-34222.

Pascucci, L., Coccè, V., Bonomi, A., Ami, D., *et al.* (2014). Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: a new approach for drug delivery. J. Controlled Release *192,* 262-270.

Paul, B., Dhir, R., Landsittel, D., Hitchens, M.R., and Getzenberg, R.H. (2005). Detection of prostate cancer with a blood-based assay for early prostate cancer antigen. Cancer Res. *65,* 4097-4100.

Pegtel, D.M., Cosmopoulos, K., Thorley-Lawson, D.A., van Eijndhoven, M.A., *et al.* (2010). Functional delivery of viral miRNAs via exosomes. Proc. Natl. Acad. Sci. U. S. A. *107,* 6328-6333.

Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., *et al.* (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat. Med. *18,* 883-891.

Perez, A., Loizaga, A., Arceo, R., Lacasa, I., *et al.* (2014). A pilot study on the potential of RNA-associated to urinary vesicles as a suitable non-invasive source for diagnostic purposes in bladder cancer. Cancers *6,* 179-192.

Peters, D.L., and Pretorius, P.J. (2011). Origin, translocation and destination of extracellular occurring DNA—a new paradigm in genetic behaviour. Clinica Chimica Acta *412,* 806-811.

Pfeffer, S.R., Grossmann, K.F., Cassidy, P.B., Yang, C.H., *et al.* (2015). Detection of Exosomal miRNAs in the Plasma of Melanoma Patients. Journal of Clinical Medicine *4,* 2012-2027.

Piccin, A., Murphy, W.G., and Smith, O.P. (2007). Circulating microparticles: pathophysiology and clinical implications. Blood Rev. *21,* 157-171.

Pigati, L., Yaddanapudi, S.C., Iyengar, R., Kim, D., *et al.* (2010). Selective release of microRNA species from normal and malignant mammary epithelial cells. PloS One *5,* e13515.

Pizzirani, C., Ferrari, D., Chiozzi, P., Adinolfi, E., *et al.* (2007). Stimulation of P2 receptors causes release of IL-1beta-loaded microvesicles from human dendritic cells. Blood *109,* 3856-3864.

Pol, E., Coumans, F., Grootemaat, A., Gardiner, C., *et al.* (2014). Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. Journal of Thrombosis and Haemostasis *12,* 1182-1192.

Poliakov, A., Spilman, M., Dokland, T., Amling, C.L., and Mobley, J.A. (2009). Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen. Prostate *69,* 159-167.

Principe, S., Jones, E.E., Kim, Y., Sinha, A., *et al.* (2013). In-depth proteomic analyses of exosomes isolated from expressed prostatic secretions in urine. Proteomics *13,* 1667-1671.

Qu, J., Qu, X., Zhao, M., Teng, Y., *et al.* (2009). Gastric cancer exosomes promote tumour cell proliferation through PI3K/Akt and MAPK/ERK activation. Digestive and Liver Disease *41,* 875-880.

Que, R., Ding, G., Chen, J., and Cao, L. (2013). Analysis of serum exosomal microRNAs and clinicopathologic features of patients with pancreatic adenocarcinoma. World Journal of Surgical Oncology *11,* 1.

Quinn, J.F., Patel, T., Wong, D., Das, S., *et al.* (2015). Extracellular RNAs: development as biomarkers of human disease. J. Extracell Vesicles *4,* 27495.

Rabinowits, G., Gerçel-Taylor, C., Day, J.M., Taylor, D.D., and Kloecker, G.H. (2009). Exosomal microRNA: a diagnostic marker for lung cancer. Clinical Lung Cancer *10,* 42-46.

Raiborg, C., and Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature *458,* 445-452.

Raimondo, F., Morosi, L., Corbetta, S., Chinello, C., *et al.* (2013). Differential protein profiling of renal cell carcinoma urinary exosomes. Molecular BioSystems *9,* 1220-1233.

Rak, J. (2013). Extracellular vesicles - biomarkers and effectors of the cellular interactome in cancer. Front. Pharmacol. *4,* 21.

Ramteke, A., Ting, H., Agarwal, C., Mateen, S., *et al.* (2015). Exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules. Mol. Carcinog. *54,* 554-565.

Rana, S., Yue, S., Stadel, D., and Zoeller, M. (2012). Toward tailored exosomes: The exosomal tetraspanin web contributes to target cell selection. Int. J. Biochem. Cell Biol. *44,* 1574-1584.

Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., *et al.* (1996). B lymphocytes secrete antigen-presenting vesicles. J. Exp. Med. *183,* 1161-1172.

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. J. Cell Biol. *200,* 373-383.

Ratajczak, J., Miekus, K., Kucia, M., Zhang, J., *et al.* (2006). Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia *20,* 847-856.

Record, M., Carayon, K., Poirot, M., and Silvente-Poirot, S. (2014). Exosomes as new vesicular lipid transporters involved in cell–cell communication and various pathophysiologies. Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids *1841,* 108-120.

Rhee, J., Black, M., Schubert, U., Fischer, S., *et al.* (2004). The functional role of blood platelet components in angiogenesis. Thromb. Haemost. *92,* 394-402.

Roccaro, A.M., Sacco, A., Maiso, P., Azab, A.K., *et al.* (2013). BM mesenchymal stromal cell–derived exosomes facilitate multiple myeloma progression. J. Clin. Invest. *123,* 1542-1555.

Rodríguez, M., Silva, J., López-Alfonso, A., López-Muñiz, M.B., *et al.* (2014). Different exosome cargo from plasma/bronchoalveolar lavage in non-small-cell lung cancer. Genes, Chromosomes and Cancer *53,* 713-724.

Ronquist, G.K., Larsson, A., Stavreus-Evers, A., and Ronquist, G. (2012). Prostasomes are heterogeneous regarding size and appearance but affiliated to one DNA-containing exosome family. Prostate *72,* 1736-1745.

Ronquist, K.G., Ronquist, G., Carlsson, L., and Larsson, A. (2009). Human prostasomes contain chromosomal DNA. Prostate *69,* 737-743.

Rountree, R.B., Mandl, S.J., Nachtwey, J.M., Dalpozzo, K., *et al.* (2011). Exosome targeting of tumor antigens expressed by cancer vaccines can improve antigen immunogenicity and therapeutic efficacy. Cancer Res. *71,* 5235-5244.

Royo, F., Zuniga-Garcia, P., Torrano, V., Loizaga, A., *et al.* (2016). Transcriptomic profiling of urine extracellular vesicles reveals alterations of CDH3 in prostate cancer. Oncotarget *7,* 6835-6846.

Runz, S., Keller, S., Rupp, C., Stoeck, A., *et al.* (2007). Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM. Gynecol. Oncol. *107,* 563-571.

Rupert, D.L., Lässer, C., Eldh, M., Block, S., *et al.* (2014). Determination of exosome concentration in solution using surface plasmon resonance spectroscopy. Anal. Chem. *86,* 5929-5936.

Sandvig, K., and Llorente, A. (2012). Proteomic analysis of microvesicles released by the human prostate cancer cell line PC-3. Mol. Cell. Proteomics *11,* M111.012914.

Santana, S.M., Antonyak, M.A., Cerione, R.A., and Kirby, B.J. (2014). Microfluidic isolation of cancer-cell-derived microvesicles from hetergeneous extracellular shed vesicle populations. Biomed. Microdevices *16,* 869-877.

Savina, A., Vidal, M., and Colombo, M.I. (2002). The exosome pathway in K562 cells is regulated by Rab11. J. Cell. Sci. *115,* 2505-2515.

Scher, H.I., and Sawyers, C.L. (2005). Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. J. Clin. Oncol. *23,* 8253-8261.

Schiller, M., Bekeredjian-Ding, I., Heyder, P., Blank, N., *et al.* (2008). Autoantigens are translocated into small apoptotic bodies during early stages of apoptosis. Cell Death & Differentiation *15,* 183-191.

Schiller, M., Heyder, P., Ziegler, S., Niessen, A., *et al.* (2013). During apoptosis HMGB1 is translocated into apoptotic cell-derived membraneous vesicles. Autoimmunity *46,* 342-346.

Schork, N.J. (2015). Personalized medicine: time for one-person trials. Nature *520,* 609-611.

Sheldon, H., Heikamp, E., Turley, H., Dragovic, R., *et al.* (2010). New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. Blood *116,* 2385-2394.

Shimoda, M., and Khokha, R. (2013). Proteolytic factors in exosomes. Proteomics *13,* 1624-1636.

Shimoda, M., Principe, S., Jackson, H.W., Luga, V., *et al.* (2014). Loss of the Timp gene family is sufficient for the acquisition of the CAF-like cell state. Nat. Cell Biol. *16,* 889-901.

Shtam, T.A., Kovalev, R.A., Varfolomeeva, E.Y., Makarov, E.M., *et al.* (2013). Exosomes are natural carriers of exogenous siRNA to human cells in vitro. Cell Communication and Signaling *11,* 1.

Siegel, R.L., Miller, K.D., and Jemal, A. (2015). Cancer statistics, 2015. CA: A Cancer Journal for Clinicians *65,* 5-29.

Silva, J., Garcia, V., Zaballos, A., Provencio, M., *et al.* (2011). Vesicle-related microRNAs in plasma of nonsmall cell lung cancer patients and correlation with survival. Eur. Respir. J. *37,* 617-623.

Skog, J., Würdinger, T., van Rijn, S., Meijer, D.H., *et al.* (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat. Cell Biol. *10,* 1470-1476.

Smalley, D.M., Sheman, N.E., Nelson, K., and Theodorescu, D. (2008). Isolation and identification of potential urinary microparticle biomarkers of bladder cancer. Journal of Proteome Research *7,* 2088-2096.

Smith, Z.J., Lee, C., Rojalin, T., Carney, R.P., *et al.* (2015). Single exosome study reveals subpopulations distributed among cell lines with variability related to membrane content. J. Extracell Vesicles *4,* 28533.

Smyth, T., Kullberg, M., Malik, N., Smith-Jones, P., *et al.* (2015). Biodistribution and delivery efficiency of unmodified tumor-derived exosomes. J. Controlled Release *199,* 145-155.

Soekmadji, C., Russell, P.J., and Nelson, C.C. (2013). Exosomes in prostate cancer: putting together the pieces of a puzzle. Cancers *5,* 1522-1544.

Soldevilla, B., Rodriguez, M., San Millan, C., Garcia, V., *et al.* (2014). Tumor-derived exosomes are enriched in DeltaNp73, which promotes oncogenic potential in acceptor cells and correlates with patient survival. Hum. Mol. Genet. *23,* 467-478.

Stamey, T.A., Yang, N., Hay, A.R., McNeal, J.E., *et al.* (1987). Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N. Engl. J. Med. *317,* 909-916.

Stremersch, S., De Smedt, S.C., and Raemdonck, K. (2016a). Therapeutic and diagnostic applications of extracellular vesicles. J. Controlled Release *244*, 167-183.

Stremersch, S., Marro, M., Pinchasik, B., Baatsen, P., *et al.* (2016b). Identification of Individual Exosome-Like Vesicles by Surface Enhanced Raman Spectroscopy. Small *24*, 3292-32301.

Stremersch, S., Vandenbroucke, R.E., Van Wonterghem, E., Hendrix, A., *et al.* (2016c). Comparing exosome-like vesicles with liposomes for the functional cellular delivery of small RNAs. J. Controlled Release *232,* 51-61.

Stridsberg, M., Fabiani, R., Lukinius, A., and Ronquist, G. (1996). Prostasomes are neuroendocrine-like vesicles in human semen. Prostate *29,* 287-295.

Sugimachi, K., Matsumura, T., Hirata, H., Uchi, R., *et al.* (2015). Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. Br. J. Cancer *112,* 532-538.

Sun, D., Zhuang, X., Xiang, X., Liu, Y., *et al.* (2010). A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. Molecular Therapy *18,* 1606-1614.

Sung, B.H., Ketova, T., Hoshino, D., Zijlstra, A., and Weaver, A.M. (2015). Directional cell movement through tissues is controlled by exosome secretion. Nature Communications *6.* 7164.

Surapaneni, M.S., Das, S.K., and Das, N.G. (2012). Designing paclitaxel drug delivery systems aimed at improved patient outcomes: current status and challenges. ISRN Pharmacol.623139.

Suutari, T., Silen, T., Saari, H., Desai, D., *et al.* (2016). Real-Time Label-Free Monitoring of Nanoparticle Cell Uptake. Small *45*, 6289-6300.

Svensson, K.J., Christianson, H.C., Wittrup, A., Bourseau-Guilmain, E., *et al.* (2013). Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. J. Biol. Chem. *288,* 17713-17724.

Szajnik, M., Derbis, M., Lach, M., Patalas, P., *et al.* (2013). Exosomes in Plasma of Patients with Ovarian Carcinoma: Potential Biomarkers of Tumor Progression and Response to Therapy. Gynecol. Obstet. (Sunnyvale) *Suppl 4,* 3.

Tadokoro, H., Umezu, T., Ohyashiki, K., Hirano, T., and Ohyashiki, J.H. (2013). Exosomes derived from hypoxic leukemia cells enhance tube formation in endothelial cells. J. Biol. Chem. *288,* 34343-34351.

Takeshita, N., Hoshino, I., Mori, M., Akutsu, Y., *et al.* (2013). Serum microRNA expression profile: miR-1246 as a novel diagnostic and prognostic biomarker for oesophageal squamous cell carcinoma. Br. J. Cancer *108,* 644-652.

Tanaka, M., Oikawa, K., Takanashi, M., Kudo, M., *et al.* (2009). Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. PloS One *4,* e5532.

Tanaka, Y., Kamohara, H., Kinoshita, K., Kurashige, J., *et al.* (2013). Clinical impact of serum exosomal microRNA-21 as a clinical biomarker in human esophageal squamous cell carcinoma. Cancer *119,* 1159-1167.

Tang, K., Zhang, Y., Zhang, H., Xu, P., *et al.* (2012). Delivery of chemotherapeutic drugs in tumour cell-derived microparticles. Nature Communications *3,* 1282.

Tauro, B.J., Greening, D.W., Mathias, R.A., Ji, H., *et al.* (2012). Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. Methods *56,* 293-304.

Tauro, B.J., Greening, D.W., Mathias, R.A., Mathivanan, S., *et al.* (2013). Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. Mol. Cell. Proteomics *12,* 587-598.

Taylor, D., and Gercel-Taylor, C. (2005). Tumour-derived exosomes and their role in cancer-associated T-cell signalling defects. Br. J. Cancer *92,* 305-311.

Taylor, D.D., and Gercel-Taylor, C. (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol. Oncol. *110,* 13-21.

Thakur, B.K., Zhang, H., Becker, A., Matei, I., *et al.* (2014). Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res. *24,* 766-769.

Théry, C., Ostrowski, M., and Segura, E. (2009). Membrane vesicles as conveyors of immune responses. Nature Reviews Immunology *9,* 581-593.

Théry, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. Nature Reviews Immunology *2,* 569-579.

Thery, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., *et al.* (2001). Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J. Immunol. *166,* 7309-7318.

Thompson, I.M., Ankerst, D.P., Chi, C., Lucia, M.S., *et al.* (2005). Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. Jama *294,* 66-70.

Tian, T., Wang, Y., Wang, H., Zhu, Z., and Xiao, Z. (2010). Visualizing of the cellular uptake and

intracellular trafficking of exosomes by live-cell microscopy. J. Cell. Biochem. *111,* 488-496.

Tian, Y., Li, S., Song, J., Ji, T.*, et al.* (2014a). A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. Biomaterials *35,* 2383-2390.

Tian, T., Zhu, Y.L., Zhou, Y.Y., Liang, G.F., *et al.* (2014b). Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. J. Biol. Chem. *289,* 22258-22267.

Tokuhisa, M., Ichikawa, Y., Kosaka, N., Ochiya, T.*, et al.* (2015). Exosomal miRNAs from peritoneum lavage fluid as potential prognostic biomarkers of peritoneal metastasis in gastric cancer. PloS One *10,* e0130472.

Tominaga, N., Kosaka, N., Ono, M., Katsuda, T.*, et al.* (2015). Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood–brain barrier. Nature Communications *6*. 6716.

Tomlins, S.A., Rubin, M.A., and Chinnaiyan, A.M. (2006). Integrative biology of prostate cancer progression. Annu.Rev.Pathol.Mech.Dis. *1,* 243-271.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L.*, et al.* (2008). Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science *319,* 1244-1247.

Trams, E.G., Lauter, C.J., Salem, J.N., and Heine, U. (1981). Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochimica Et Biophysica Acta (BBA)-Biomembranes *645,* 63-70.

Turiák, L., Misják, P., Szabó, T.G., Aradi, B.*, et al.* (2011). Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in BALB/c mice. Journal of Proteomics *74,* 2025-2033.

Ukimura, O., Coleman, J.A., de la Taille, A., Emberton, M.*, et al.* (2013). Contemporary role of systematic prostate biopsies: indications, techniques, and implications for patient care. Eur. Urol. *63,* 214-230.

Umezu, T., Ohyashiki, K., Kuroda, M., and Ohyashiki, J. (2013). Leukemia cell to endothelial cell communication via exosomal miRNAs. Oncogene *32,* 2747-2755.

Umezu, T., Tadokoro, H., Azuma, K., Yoshizawa, S.*, et al.* (2014). Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1. Blood *124,* 3748-3757.

Vader, P., Breakefield, X.O., and Wood, M.J. (2014). Extracellular vesicles: emerging targets for cancer therapy. Trends Mol. Med. *20,* 385-393.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M.*, et al.* (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. *9,* 654-659.

Valenti, R., Huber, V., Filipazzi, P., Pilla, L.*, et al.* (2006). Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. Cancer Res. *66,* 9290-9298.

Valkonen, S., van der Pol, E., Böing, A., Yuana, Y.*, et al.* (2016). Biological reference materials for extracellular vesicle studies. European Journal of Pharmaceutical Sciences *98*, 4-16.

van Balkom, B.W., de Jong, O.G., Smits, M., Brummelman, J.*, et al.* (2013). Endothelial cells require miR-214 to secrete exosomes that suppress senescence and induce angiogenesis in human and mouse endothelial cells. Blood *121,* 3997-4006, S1-15.

van Niel, G., Charrin, S., Simoes, S., Romao, M.*, et al.* (2011). The tetraspanin CD63 regulates ESCRT-independent and-dependent endosomal sorting during melanogenesis. Developmental Cell *21,* 708-721.

Vanlandingham, P.A., and Ceresa, B.P. (2009). Rab7 regulates late endocytic trafficking downstream of multivesicular body biogenesis and cargo sequestration. J. Biol. Chem. *284,* 12110-12124.

Vardaki, I., Ceder, S., Rutishauser, D., Baltatzis, G.*, et al.* (2016). Periostin is identified as a putative metastatic marker in breast cancer-derived exosomes. Oncotarget *7,* 74966- 74972.

Viaud, S., Ploix, S., Lapierre, V., Thery, C.*, et al.* (2011). Updated technology to produce highly immunogenic dendritic cell-derived exosomes of clinical grade: a critical role of interferon-gamma. J. Immunother. *34,* 65-75.

Vlaeminck-Guillem, V., Ruffion, A., André, J., Devonec, M., and Paparel, P. (2010). Urinary prostate cancer 3 test: toward the age of reason? Urology *75,* 447-453.

Vojtech, L., Woo, S., Hughes, S., Levy, C., *et al.* (2014). Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. Nucleic Acids Res. *42,* 7290-7304.

Wahlgren, J., De L Karlson, T., Brisslert, M., Vaziri Sani, F., *et al.* (2012). Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. Nucleic Acids Res. *40,* e130.

Waldenstrom, A., Genneback, N., Hellman, U., and Ronquist, G. (2012). Cardiomyocyte Microvesicles Contain DNA/RNA and Convey Biological Messages to Target Cells. Plos One *7,* e34653.

Wang, J., Zhou, Y., Lu, J., Sun, Y., *et al.* (2014a). Combined detection of serum exosomal miR-21 and HOTAIR as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma. Medical Oncology *31,* 1-8.

Wang, L., Skotland, T., Berge, V., Sandvig, K., and Llorente, A. (2016). Exosomal proteins as prostate cancer biomarkers in urine: From mass spectrometry discovery to immunoassay-based validation. European Journal of Pharmaceutical Sciences *98*, 80-85.

Wang, Z., Wu, H., Fine, D., Schmulen, J., *et al.* (2013). Ciliated micropillars for the microfluidic-based isolation of nanoscale lipid vesicles. Lab on a Chip *13,* 2879-2882.

Wang, H., Hou, L., Li, A., Duan, Y., *et al.* (2014b). Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. Biomed. Res. Int. *2014,* 864894.

Wang, T., Gilkes, D.M., Takano, N., Xiang, L., *et al.* (2014c). Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. Proc. Natl. Acad. Sci. U. S. A. *111,* E3234-42.

Webber, J.P., Spary, L.K., Sanders, A.J., Chowdhury, R., *et al.* (2015). Differentiation of tumour-promoting stromal myofibroblasts by cancer exosomes. Oncogene *34,* 290-302.

Webber, J., Steadman, R., Mason, M.D., Tabi, Z., and Clayton, A. (2010). Cancer exosomes trigger fibroblast to myofibroblast differentiation. Cancer Res. *70,* 9621-9630.

Webber, J., Stone, T.C., Katilius, E., Smith, B.C., *et al.* (2014). Proteomics analysis of cancer exosomes using a novel modified aptamer-based array (SOMAscan) platform. Mol. Cell. Proteomics *13,* 1050-1064.

Whitehead, B., Wu, L., Hvam, M.L., Aslan, H., *et al.* (2015). Tumour exosomes display differential mechanical and complement activation properties dependent on malignant state: implications in endothelial leakiness. J. Extracell Vesicles *4,* 29685.

Whiteside, T. (2005). Tumour-derived exosomes or microvesicles: another mechanism of tumour escape from the host immune system? Br. J. Cancer *92,* 209-211.

Willms, E., Johansson, H.J., Mager, I., Lee, Y., *et al.* (2016). Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci. Rep. *6,* 22519.

Witwer, K.W., Buzas, E.I., Bemis, L.T., Bora, A., *et al.* (2013). Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J. Extracell Vesicles. *2*. 20360.

Wolf, P. (1967). The nature and significance of platelet products in human plasma. Br. J. Haematol. *13,* 269-288.

Wubbolts, R., Leckie, R.S., Veenhuizen, P.T., Schwarzmann, G., *et al.* (2003). Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J. Biol. Chem. *278,* 10963-10972.

Xu, R., Greening, D.W., Rai, A., Ji, H., and Simpson, R.J. (2015). Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct. Methods *87,* 11-25.

Yamashita, T., Kamada, H., Kanasaki, S., Maeda, Y., *et al.* (2013). Epidermal growth factor receptor localized to exosome membranes as a possible biomarker for lung cancer diagnosis. Die Pharmazie-an International Journal of Pharmaceutical Sciences *68,* 969-973.

Yáñez-Mó, M., Siljander, P.R., Andreu, Z., Zavec, A.B., *et al.* (2015). Biological properties of extracellular vesicles and their physiological functions. J. Extracell Vesicles. *4*. 27066.

Yang, L., Wu, X., Wang, D., Luo, C., and Chen, L. (2013). Bladder cancer cell-derived exosomes inhibit tumor cell apoptosis and induce cell proliferation in vitro. Molecular Medicine Reports *8,* 1272-1278.

Yang, Q., Diamond, M.P., and Al-Hendy, A. (2016). The emerging role of extracellular vesicle-derived miRNAs: implication in cancer progression and stem cell related diseases. Journal of Clinical Epigenetics *2, 1*.

Yang, T., Martin, P., Fogarty, B., Brown, A., *et al.* (2015). Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio. Pharm. Res. *32,* 2003-2014.

Yang, G., Truong, L.D., Wheeler, T.M., and Thompson, T.C. (1999). Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. Cancer Res. *59,* 5719-5723.

Ye, S., Li, Z., Luo, D., Huang, B., *et al.* (2014). Tumor-derived exosomes promote tumor progression and T-cell dysfunction through the regulation of enriched exosomal microRNAs in human nasopharyngeal carcinoma. Oncotarget *5,* 5439-5452.

Yokoi, A., Yoshioka, Y., and Ochiya, T. (2015). Towards the realization of clinical extracellular vesicle diagnostics: challenges and opportunities. Expert Review of Molecular Diagnostics *15,* 1555-1566.

Yoshioka, Y., Kosaka, N., Konishi, Y., Ohta, H., *et al.* (2014). Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. Nature Communications *5,* 2591.

Zhang, J., Li, S., Li, L., Li, M., *et al.* (2015a). Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics, Proteomics & Bioinformatics *13,* 17-24.

Zhang, L., Zhang, S., Yao, J., Lowery, F.J., *et al.* (2015b). Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. Nature *527,* 100-104.

Zhang, Y., Liu, D., Chen, X., Li, J., *et al.* (2010). Secreted monocytic miR-150 enhances targeted endothelial cell migration. Mol. Cell *39,* 133-144.

Zhao, H., Yang, L., Baddour, J., Achreja, A., *et al.* (2016). Tumor microenvironment derived exosomes pleiotropically modulate cancer cell metabolism. Elife *5,* e10250.

Zhou, W., Fong, M.Y., Min, Y., Somlo, G., *et al.* (2014). Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. Cancer Cell *25,* 501-515.

Zhu, L., Wang, K., Cui, J., Liu, H., *et al.* (2014). Label-free quantitative detection of tumor-derived exosomes through surface plasmon resonance imaging. Anal. Chem. *86,* 8857-8864.

Zhu, W., Huang, L., Li, Y., Zhang, X., *et al.* (2012). Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth in vivo. Cancer Lett. *315,* 28-37.

Zhuang, X., Xiang, X., Grizzle, W., Sun, D., *et al.* (2011). Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. Molecular Therapy *19,* 1769-1779.

Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., *et al.* (1998). Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. Nat. Med. *4,* 594-600.

Zöller, M. (2009). Tetraspanins: push and pull in suppressing and promoting metastasis. Nature Reviews Cancer *9,* 40-55.

Zomer, A., Maynard, C., Verweij, F.J., Kamermans, A., *et al.* (2015). In vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. Cell *161,* 1046-1057.

Zwaal, R.F., and Schroit, A.J. (1997). Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood *89,* 1121-1132.

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