The Role and Relevance of Exosomes in the Development and Progression of Prostate Cancer

by

ELHAM HOSSEINI BEHESHTI

M.Sc., The University of British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL

STUDIES

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2015

Abstract

Prostate cancer (PCa) is the leading diagnosed cancer in men. Prompt diagnosis of the disease can substantially improve its clinical outcome. Improving capability for early detection and developing new therapeutic targets in advanced disease are research priorities that will ultimately lead to better patient survival.

Eukaryotic cells secrete proteins via distinct regulated mechanisms, which are either ER/Golgi dependent or microvesicle-mediated. The release of microvesicles has been shown to provide a novel mechanism for intercellular communication. Exosomes are nanometer-sized membrane-vesicles, which are secreted from normal/cancerous cells. They are present in various biological fluids. Recent studies have demonstrated that cancerous cells secret exosomes, which may be differentiated from those, derived from normal cells based on their composition.

The main hypothesis for this Ph.D. thesis is **to assess exosomes as potential diagnostic biomarkers for PCa diagnosis and investigate the role of exosomes in PCa progression.**

In this study, exosomes were purified from the conditioned media of six different prostate cell lines and biological fluids obtained from PCa patients. Analysis using Nanosight^{TM}, western blot and transmission-electron-microscopy validated the size, purity and integrity of isolated exosomes. Uptake by different PCa cell lines, following exposure to exosomes, was confirmed using confocal microscopy. Proteomic analysis of isolated exosomes was performed using a Waters LC-QTOF/MS in conjunction with ProteinLynx and MASCOT software. In addition to possible underlying differences in protein profiles an additional part of this study investigated the lipid profiles and

cholesterol levels in exosomes as further potential diagnostic markers.

Our results have also confirmed the influence of PCa derived exosomes with different functional assays including apoptosis, proliferation and migration. Finally we demonstrated the effect of PCa cells derived exosomes *in vivo*, and their influence on tumor growth using a human xenograft animal model of PCa.

The results of this study have highlighted a potential for the differential protein/lipid composition of exosomes to be a source of diagnostic biomarkers for PCa amenable via non-invasive testing. Our experimental evidence also indicates that exosomes with different androgen receptor phenotypes attribute positively in many mechanisms that contribute to PCa progression presenting additional insight into exploratory research for novel therapeutic targets.

Preface

Several sections of chapter one are under preparation for a review paper describing the role of exosomes in prostate cancer diagnosis and progression.

I have designed all the experiments for this thesis and have conducted the majority of experiments under Dr. Guns' supervision.

Chapter Two: The transmission electron microscopy (TEM) experiment has run at the UBC Bio-image facility, where I received TEM training by Garnet Martens. Hans Adomat, has assisted me with Mass Spectrometry experiments performed in chapter two and Steven Pham (co-op student at Dr. Guns lab) has contributed in some of the experiments (mainly western blot and cell culture) and organizing mass spectrometry data in this chapter. This chapter has been published in the Journal of Molecular and Cellular Proteomics. Hosseini-Beheshti E, Pham S., Adomat H., Li N, Guns (Tomlinson) ES. Exosomes as Biomarker Enriched microvesicles: Characterization of Exosomal Proteins derived from a Panel of Prostate Cell Lines with Distinct androgen receptor (AR) Phenotypes. Mol Cell Proteomics. 2012 Jun 21.

Chapter Three: Clinical samples were collected in a collaboration with Dr. Anthony Michael Joshua, MBBS, PhD, FRACP, at the Princess Margaret Cancer Centre (UHN). Hans Adomat, has assisted with Mass Spectrometry experiments performed in this chapter and Wendy Choi (co-op student at Dr. Guns lab) has contributed in some of the experiments (mainly western blot and cell culture) and organizing mass spectrometry data in this chapter (UBC BCCA REB has approved the ethics application H09-01010 entitled Exosome isolation from human serum and urine). A version of this chapter has been submitted for publication.

Chapter Four: Dr. Louis-Bastien Weiswald (Ph.D.) from BC Cancer Agency has assisted with performing the 3D migration and cell motility assay. Mazyar Ghaffari (Ph.D. candidate in Vancouver Prostate Centre) and Mani Roshan-Moniri (Ph.D. student in Vancouver Prostate Centre) have contributed in wound healing, FACS analysis and real time cell migration assay respectively. Wendy Choi (co-op student at Dr. Guns lab) has contributed in cell culture, some western blot analysis and the DU145 exosomes *in vivo* study. Dr. Geetanjali Kharmate (Ph.D.) has assisted with some western blot analysis. Dr. Mohamed Hassona (Ph.D.), Leslie Chan and Mei Yieng Chin have contributed in the LNCaP exosome *in vivo* study. The Immunohistochemistry experiment was performed in collaboration with Dr. Ladan Fazli (M.D.) at The Vancouver Prostate Centre. A version of this chapter has been submitted for publication.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

IPA	Ingenuity Pathway Analysis
ADT	Androgen Deprivation Therapy
ANXA	Annexin
AR	Androgen Receptor
AUC	Area Under Curve
BPH	Benign Prostatic Hyperplasia
BRT	Brachytherapy
CD	Cluster of Differentiation
CLSTN1	Calsyntenin 1
CLU	Clusterin
CRPC	Castration Resistant Prostate Cancer
CYP	Cytochrome
D	Depth
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic Acid
DRE	Digital Rectal Examination
DTT	Dithiothreitol
EBRT	External Beam Radiation Therapy
ECE	Extra Capsular Extension
EE	Early Endosome

EGFR	Epidermal Growth Factor
EPCA	Early Prostate Cancer Antigen
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Extracellular Vesicle
FA	Formic Acid
FASN	Fatty Acid Synthesis
FLNC	Filamin C
FOLH	Folate Hydrolase
GDF15	Growth Differentiation Factor 15
GS	Gleason Score
HSP	Heat Shock Proteins
IGF-1	Insulin-like Growth Factor-1
IHC	Immunohistochemistry
IL-6	Interleukin 6
ILV	Intraluminal Vesicles
IPS	Ingenuity Pathway Software
KSFM	keratinocyte-SFM
L	Length
LAMP2	Lysosomal Associated Membrane Protein 2
LCMS	Liquid Chromatography Mass Spectrometry
LC-QTOF MS	Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone-Releasing Hormone

M	Metastasis
MHC	Major Histocompatibility Complex
miRNA	microRNA
mRNA	Messenger RNA
MTBE	Methyl-t-Butyl Ether
MVB/MVE	Multivesicular Bodies/Multivesicular Endosomes
N	Lymph Node
NK	Natural killer
Nm	Nanometer
NTA	NanoSight^{TM} Tracking Analysis
PAP	Prostatic Acid Phosphatase
PCa	Prostate Cancer
PLGS	Protein Lynx Global Server Software
PMV	Plasma Membrane Vesicle
PSA	Prostate Specific Antigen
RNA	Ribonucleic Acid
RT	Room Temperature
STAM1	Signal Transducing Adaptor Molecule 1
T	Tumor
TEM	Transmission Electron Microscopy
TfR	Transferrin Receptor
TNM	Tumour, Node, Metastasis
TSG101	Tumor Susceptibility Gene 101

UGM	Urogenital Sinus Mesenchyme
UGS	Urogenital Sinus
USA	United State of America
VLA-4	Very Late Antigen-4
W	Width
WB	Western Blot

Acknowledgments

Foremost, I would like to express my sincere gratitude to my dear supervisor, Dr. Emma S. (Tomlinson) Guns, for her endless support and wise supervision throughout this research project. Through the years of my Ph.D., I was very lucky to have you as a great role model in my professional and personal life. As I move towards next steps of my professional life, I will always remember our empowering meeting and your encouraging and calm personality. I am so thankful for your support at the time when I was going through rough times.

My sincere thanks are also due to the members of my supervisory committee, Dr. Aziz Ghahary and Dr. Christopher Ong for their constructive and valuable guidance. Their critical and constructive comments during this Ph.D. have improved the quality of my research and have made me a better scientist.

I am genuinely grateful to be surrounded by amazing friends, friendly staff and knowledgeable scientists at Vancouver Prostate Centre. I would like to specially extend my heartfelt thanks to, Hans Adomat, Dr. Jennifer Lock, Dr. Claudia Chavez-Munoz, Dr. Fan Zhang, Robert Bell, Dr. Ladan Fazli and Dr. Maryam Moussavi for their support and friendship within the past few years.

Many thanks to all my dear friends and talented colleague at Dr. Emma Guns' Lab and Vancouver Prostate Centre. Your support undoubtedly contributed to the quality of this work. The flaws that remain result from my own shortcoming.

Most importantly I would like to thank my family. My parents, Shahin and Mohammad Hashem and my dear brother Alireza. Words cannot describe how blessed I feel to be born in a family that is all about love and support. Last 10 years have been the best and

yet toughest years of my life and I would not be able to achieve all I had without your unending love, wisdom, patience and support.

Finally I want to thank the love of my life, Mazyar Ghaffari, for all the love, support and joy he has brought into my life. I have learnt a lot from you as an incredible human being and a knowledgeable scientist. This journey has been more fun only and only because of you.

To Science,

and

All whose lives have been affected by cancer

Chapter 1: Introduction

1.1 The Prostate Gland

1.1.1 Anatomy and Physiology

The human prostate is a small muscular gland, which is located anterior to the rectum and inferior to the urinary bladder in the pelvic body cavity. It is oval shaped with a rounded tip and surrounds the neck of the urethra as it exits the base of the bladder and merges with the ductal Vas deferens at the ejaculatory ducts (**Figure 1.1**). This male sex accessory gland weighs approximately 1-2 grams before puberty, however after puberty androgens act to mature the organ. The prostate measures approximately 2cm x 4cm x 3cm (D×W×L), the size of a walnut (Isaacs, 1994)(Cunha *et al.*, 2004), and typically weighs approximately 18 grams in males between the age of 20 and 50 years.

The anatomy of the prostate gland may be described in two different ways, using lobes or zones. In the 20^{th} century, based on the anatomy of laboratory animals, scientists suggested that the prostate gland was composed of different lobes (anterior lobe, median lobe, lateral lobe, and posterior lobe), despite the fact that there are no characteristic lobes in the human prostate (Lowsley, 1915)(Lowsley and Perez Venero, 1954)(Franks, 1954). Thereafter, during the 1980's McNeal established the concept of zones of the prostate rather than lobes (McNeal, 1980)(McNeal, 1981)(McNeal, 1988).

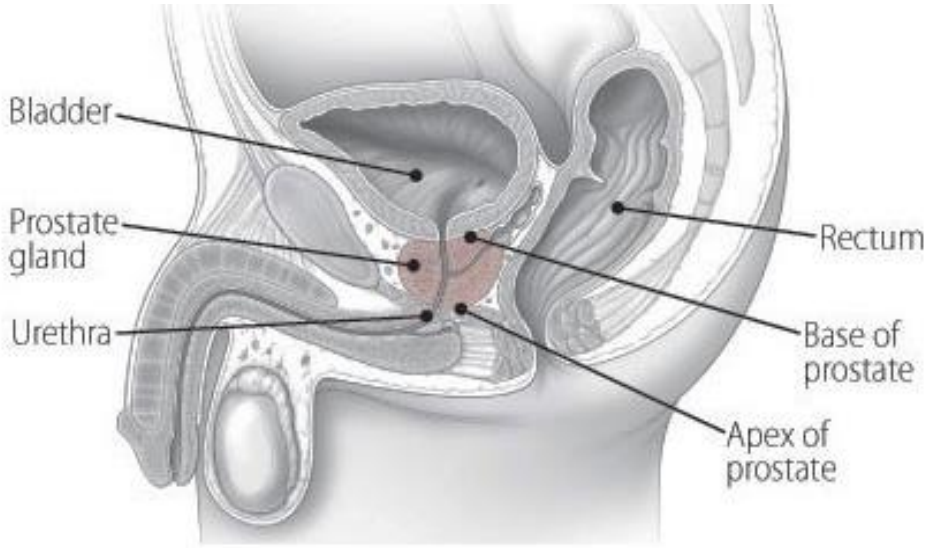


Figure 1.1 Location of the prostate gland.

The prostate gland is located anterior to the rectum and inferior to the urinary bladder in the pelvic body cavity.

© 2014 Annual Report on prostate Disease;

http://www.harvardprostateknowledge.org/prostate-basics.

As McNeal has described, an adult prostate is composed of three zones: peripheral zone (zone 1), central zone (zone 2) and transition zone (zone 3), enclosed by a capsule composed of collagen elastin and abundant smooth muscle (**Figure 1.2**).

The peripheral zone (zone 1), which makes up 70% of the prostate volume, comprises all the prostatic glandular tissue at the apex of the gland as well as all of the tissue located posteriorly near the capsule. It has been reported that the incidence of carcinoma, chronic prostatitis and post-inflammatory atrophy are relatively higher in the peripheral zone compared with the other zones (Hammerich *et al*., 2009).

The central zone (zone 2) is a cone-shaped area of the prostate gland which makes up 25% of the gland. The transition zone (zone 3), immediately surrounding the urethra,

is the innermost and smallest component of the prostate gland which is mainly involved in the development of Benign Prostatic Hyperplasia (BPH) and less commonly, adenocarcinoma (Hammerich *et al*., 2009)(Cuhna *et al*., 2004)(Hayward and Cunha, 2000).

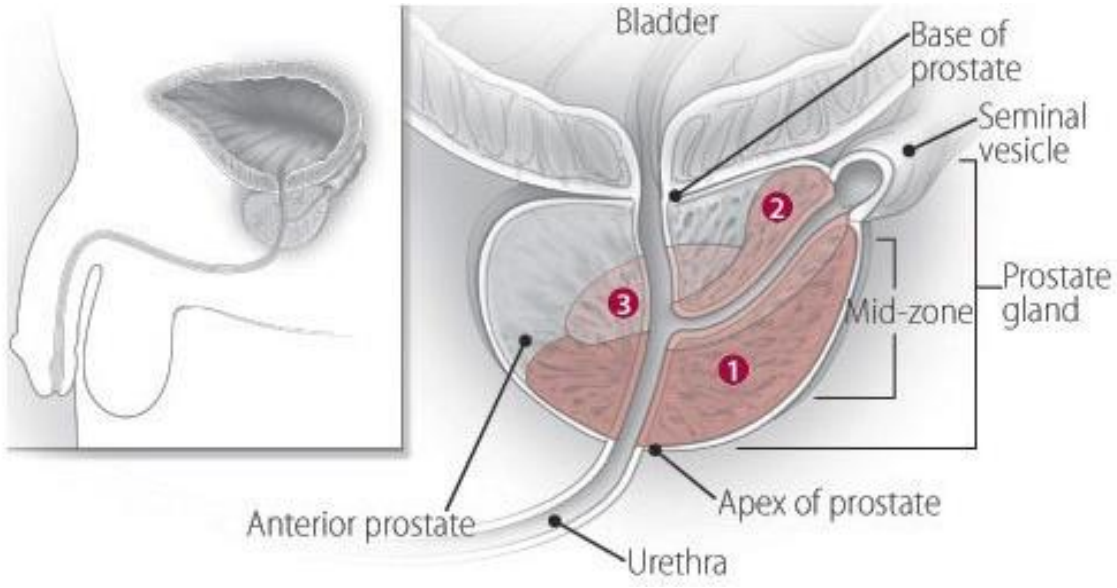


Figure 1.2 Zone of the prostate.

An adult prostate is composed of three zones: peripheral zone (1), central zone (2) and transition zone (3), enclosed by a capsule composed of collagen elastin and abundant smooth muscle.

© 2014 Annual Report on prostate Disease;

http://www.harvardprostateknowledge.org/prostate-basics.

1.1.2 Prostate Development

In all species the prostate gland is derived from the endodermal urogenital sinus (UGS), which is derived from the caudal terminus of the hindgut called the cloaca. The prostate gland is recognizable at 9 to 10 weeks of embryonic stage (Hayward and Cunha, 2000) and its development initiates as a response to androgen secreted from fetal testis.

Prostate tissue development is determined by androgen exposure rather than by genetically defined sex of the fetus. Therefore, the UGS of either sex fetus could develop into functional prostatic tissue if stimulated by androgen. (Cunha *et al*., 1980)(Takeda *et al*., 1986).

When androgen activates the AR in the surrounding embryonic connective tissue, urogenital sinus mesenchyme (UGM) induces the epithelial proliferation, ductal branching morphogenesis, and cyto-differentiation into basal and luminal epithelia subtype (Cunha *et al*., 1992)(Hayward and Cunha, 2000)(Cunha *et al*., 2004). As a result the prostatic epithelium directs the differentiation of smooth muscle in the prostate gland (Hayward *et al*., 1998).

1.1.3 Prostate Function

The main function of the prostate gland is to produce and secrete prostatic fluid, which is a thin, milky/white slightly alkaline fluid that constitutes roughly 30% of the volume of the semen, spermatozoa and seminal fluid (Huggins and Neal, 1942). Analysis of combined prostatic-vesicular secretions obtained by rectal massage was first reported by McCarthy et al. (1928)(McCarthy *et al*., 1928). The biochemistry and composition of prostatic fluid varies between species but primarily contains simple sugars (fructose and glucose), calcium, citrate ions, phosphate ions, a clotting enzyme, and a profibrinolysin and is mainly responsible for semen gelation, coagulation and liquefaction (Hayward and Cunha, 2000)(Stewart *et al*., 2004).

The sugar content of prostatic fluids acts as the nutrition source for sperm, while prostatic fluid enzymes work to break down proteins in semen after ejaculation to free sperm cells from the viscose semen. The protein content of prostatic fluid is involved in the coating

and un-coating of spermatozoa and in the interaction with cervical mucus (Hall and Guyton, 2010).

As mentioned above the prostatic fluid is slightly alkaline and its pH under normal conditions is around 7.31 (Fair and Cordonnier, 1977). This slightly alkaline characterization of prostatic fluid is essential to neutralize the acidity of seminal fluids during ejaculation as well as enhance the motility or fertility of the sperm (Hall and Guyton, 2010).

1.2 Prostate Cancer

1.2.1 Prostate Cancer Epidemiology

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-related death in men worldwide. In the 2014 Canadian Cancer Statistic annual report, it was indicated that about 2 in 5 Canadians will develop cancer in their lifetime and about 1 in 4 Canadians will die of cancer (1 in 8 males expected to be diagnosed with PCa in Canada). It also has been reported that more than half of new diagnosed cancer (52%) will be lung, breast, colorectal and PCa.

PCa has been reported to continue to be the leading diagnosed cancer in Canadian men and it has been estimated that 23,600 new cases will be diagnosed in 2014 (24% of all new male cases) and 4,000 will die from this disease (10% of all cancer death in men in 2014).

While the cause of PCa is still unknown, certain predictive risk factors have been linked to PCa onset including age, ethnicity, family history, genetic predisposition, environment, lifestyle, diet and obesity (Gronberg *et al.*, 1994)(Hayward and Cuhna, 2000).

PCa is recognised as a disease of older men, with less than 0.1% diagnosed in

men younger than 50 (Hayward and Cunha, 2000). However the disease incidence and mortality rate increase after 50. The mean age of PCa patients is reported to be between 72 to 74 years and more than 85% of patients are diagnosed after the age of 65 years (Hayward and Cunha, 2000). PCa incidence rates increase faster with age compared to any other cancer, therefore with a longer life expectancy and improved diagnostic methodologies PCa will continue to be a major health concern of the future (Gronberg *et al.,* 1994).

The incidence of PCa varies between different ethnic populations and countries. The highest rates of PCa occur in North America and Scandinavia, especially in the African-American population in the USA (137 per 100,000 per year) while Chinese people in Tianjin (1.9 per 100,000 per year) have the lowest rate of PCa. Although the reason behind the ethnical discrepancy remains unknown, genetic and environmental factors could play a major role in this observation (Williams and Powell, 2009)(Pinheiro *et al*., 2014).

Genetics based researchers have revealed that male relatives of PCa and breast cancer patients have a higher chance of developing the disease (Berry *et al.*, 2000)(Gronberg *et al.*, 1997)(Suarez *et al.*, 2000)(Xu *et al.*, 2001) this risk was also increased two- to three-fold for first degree relatives of PCa patients (Monroe *et al.*, 1995).

Results of ecological studies have revealed that PCa is also highly associated with factors contributing to lifestyle (diet, physical activity, environment, culture). In particular high intake of fat, red meat and dairy products seems to increase the incidence of PCa (Rodriguez *et al.*, 2003). Research has also revealed that while high intakes of α-linolenic acid (Giovanncci *et al.*, 1993) (Gann *et al.*, 1994)(Harvei *et al.*, 1997)(Godley *et al.*,1996)(De Stefani *et al.*, 2000)(Azrad *et al.*, 2012)(Pelser *et al.*, 2013) and calcium (Chan *et al.*, 2001)(Wilson *et al.*, 2015) have been associated with PCa development, high consumption of

tomato sauce (Giovannucci *et al.*, 2002) and Vitamin D (Chen and Holick, 2003) have been linked to lower incidence of PCa.

1.2.2 Prostate Cancer Development

Although the prostate gland was first described by Niccolo Massa in 1536, PCa was not identified until 1853 as a rare disease, most probably due to shorter lifespans and poor detection methods observed for the 19th century (Palmer, 1981). It is very well known that tumorigenesis in humans is a multistep process that results in the transformation of normal cells to highly malignant derivatives via a series of premalignant steps to invasive cancer (Hanahan andWeinberg, 2000)(Hanahan and Weinberg, 2011). Hanahan and Weinberg (2000) described six essential alterations in cell physiology, known as the "hallmarks of cancer" that direct the cell to evolve from normalcy to malignancy. A combination of these six major alterations, including:

a) sustaining proliferative signaling, b) evading growth suppressors, c) activating invasion and metastasis, d) enabling replicative immortality e) inducing angiogenesis and f) resisting cell death, have been proven to play a major role in the transformation of normal cells to cancer cells. In addition to these six "hallmarks of cancer" an increasing number of scientists have suggested that capability of cancer cells to reprogram cellular energy and metabolism as well as avoid immunological destruction are also essential factors required for cells to evolve from normalcy to malignancy (Hanahan and Weinberg, 2011).

Although the causes of PCa are not yet fully understood, PCa along with other kinds of malignancies may arise as a consequence of genetic alterations in stem cell populations. It is hypothesized that cancer stem-like cells are responsible for onset, progression and

relapse of malignancy. Normal epithelial cells retain cell-cell contacts which are responsible for homeostasis of cell renewal in the maintenance of healthy organs. Stem cells located in each organ are in charge of the steady state of self-renewal as well as repair following injury (Blanpain and Fuchs, 2009). During malignancy epithelial cells lose their epithelial-like characteristics and undergo differentiation into cancerous cells (Moltzahn *et al.*, 2008)(Ceppi and Peter, 2014). Alteration in mechanisms such as mesenchymal-epithelial equilibrium, stromal-epithelial interactions and differential regulation of growth factors as well as proteases have been reported to play a central role in PCa development (Cuhna *et al.*, 2003) (Nieto *et al.*, 2014).

1.2.3 Prostate Cancer Diagnosis

PCa diagnosis continues to be an area of enormous study, debate and controversy. Despite advances in diagnosis and treatment, PCa remain to be a major public health problem. As described earlier, during 2014, 23,600 men will be diagnosed with PCa, which represent 24% of all new cancers in men. In spite of extensive research on PCa, the natural history of this disease is poorly understood. As discussed above, the disease is remarkably heterogeneous and could vary from clinically silent, indolent and non-metastatic PCa to an aggressive and metastatic form of PCa, which causes morbidity and patient death. Early diagnosis of PCa, as well as our ability to distinguish the indolent PCa from aggressive forms would logically result in a huge benefit to patients as well as clinicians in helping to ultimately tailor treatment options.

The current routine screening test for PCa diagnosis in North America includes Digital Rectal Examination (DRE), measurement of Prostate Specific Antigen (PSA) in the

blood, and prostate biopsy (Lan *et al.*, 2008).

More than 100 years ago, Charles Childe established a hypothesis for cancer screening in his book "The control of a scourge, or How cancer is curable" arguing the importance of detection of subtle deviations from normal to identify asymptomatic latent cancer for curative treatment. Childe believed that a delay in cancer diagnosis is what makes it lethal (Childe, 1907). However with today's knowledge we all know that not all forms of cancer, especially PCas, will be destined to become aggressive or symptomatic, but the importance of early diagnosis of indolent and latent forms of PCa is clearly of benefit.

In 1905, Haugh Hampton Young was the first urologist to describe the DRE to screen for PCa. Since then the DRE has been mainly used to identify early palpable changes including nodules, asymmetry, or induration of the prostate. While this test was the only diagnostic tool for PCa, it is known to be very insensitive as a screening tool for detection of low-grade PCa (Hoffman, 2011) and no controlled studies have shown decreased morbidity or mortality of PCa when detected by DRE (Krahn *et al.,* 1994).

In the 1930's Gutman and Gutman reported that the level of Prostatic Acid Phosphatase (PAP) is elevated in men with metastatic PCa (Gutman and Gutman, 1938)(Lowe and Trauzzi, 1993). Subsequent to this Huggins and Hodges reported that PAP levels decreased significantly in PCa patients who have been treated with either orchiectomy or estrogen injection (Huggins and Hodges, 1972). Since then PAP has been the only useful serum tumor biomarker and its use has been wide spread for more than 50 years to detect, stage and monitor PCa treatment response (Heller, 1987). However, since PAP screening has limitations with regard to the detection of early stages of PCa, medical research scientists are continuously seeking new PCa biomarkers, which are more sensitive for the

detection of early stage PCa.

In 1970, Dr. Richard J. Ablin and his team discovered and characterized PSA in semen. It was not until the mid-1980s however that PSA was introduced into the clinic as a diagnostic tool for PCa (Stamey *et al.*, 1987). Since then, it has been broadly used for PCa detection, staging, monitoring and response to therapy. Although PSA has always been a valuable tool, in the absence of a better non-invasive diagnostic tool, due to the lack of specificity there has been a lot of controversy surrounding this test.

Over-reaction to elevated PSA tests has resulted in a high negative biopsy rate (Roddam *et al.*, 2005). Numerous reports and studies confirmed that PSA could be elevated in response to a variety of factors as well as certain activities unrelated to PCa such as e.g. sex, ejaculation, bicycle riding as well as some diseases and associated medical procedures including BPH, prostatitis, urinary retention and catheter placement (Croswell *et al*., 2011). The very first prostate biopsy was performed in 1922 by Barringer who adapted Martine and Ellis' technique of needle puncture to obtain tissue for histological analysis (Barringer, 1931). Several years later in the 1930's, needle aspiration biopsy was developed by Ferguson. In this modified technique Ferguson used an 18-gauge needle via a transperineal approach and was able to remove adequate tissue in 78-86% of his cases (Ferguson, 1933). The history of prostate biopsy has changed significantly since the prostate punctures that were carried out in the 1920s and the inclusion of Magnetic resonance imaging (MRI)-targeted biopsy is now in use in some clinics for PCa diagnosis (since 2005). However, biopsy techniques have improved significantly over the years significant procedural risks remain and include rectal pain and discomfort, difficulty and burning sensation during urination, and bloody urine and semen. In some rare cases skin and urine infections are also

encountered. Therefore, efforts to find new biomarkers which are more specific to PCa and could be used for not only diagnosing PCa but also its management and treatment, are clearly important.

1.2.4 New Biomarkers in Prostate Cancer Diagnosis

After the successful completion of the Human Genome Project which was followed by the discovery of a variety of biomarkers for the early detection of numerous pathological disorders, prediction of disease progression and response to therapy, with the support of Human Proteome Organization, a global effort was focused on mapping the human proteome using a variety of proteomic tools in biomarker research. While proteomic analysis of biological fluids presents with significant challenges, this type of analysis has the potential to provide an overview of protein changes in different organs that could ultimately lead to biomarker discovery as well as a better understanding of cancer development and progression (Wood *et al.*, 2013)(Honda *et al.*, 2013). The identification of proteins and their associated post-translational modifications via proteomic analysis of most biological fluids is obtainable via relatively non-invasive procedures.

Three main groups of cancer biomarkers are classified as follows:

a)Diagnostic biomarkers; to detect the absence or presence of cancer

b)Prognostic biomarkers; to predict the probable clinical course or recurrence of cancer

c) Stratification biomarkers; to select the suitable treatment strategies to which the patient will be most responsive to (Hilton *et al*., 2013).

The ideal biomarker should be easily sampled through a non-invasive procedure and have a high sensitivity and specificity to the disease. PCa biomarkers can be identified in

blood, urine, prostatic fluids or prostate tissue. DNA, RNA, proteins, metabolites or even cellular process such as angiogenesis or proliferation/apoptosis could potentially be used as biomarkers for PCa diagnosis (Hays *et al.*, 1996).

To this date PSA is the sole PCa biomarker widely adopted and routinely in use clinically, however due to its limitations, PCa researchers continue their search for alternatives with better and higher specificity for PCa diagnosis and prognosis.

In addition to what has been discussed above, PSA density, PSA velocity, PSA doubling time, Free PSA, Pro-PSA, Prostate Cancer Antigen 3 (PCA3), Early Prostate Cancer Antigen (EPCA), TMPRSS2-EST gene fusion are some potential biomarkers sometimes used clinically but have been mainly adopted for PCa research and are still undergoing validation (Prensner *et al.*, 2012).

1.2.5 Prostate Cancer Grading and Staging

Urologists and urological scientists have designed several staging and grading classification systems to categorize PCa with respect to size, localization, degree of extra capsular extension (ECE) and presence or absence of metastasis. The goal of this classification is mainly to help the urologist plan the treatment and predict prognosis (Greene, 2002). The first staging system was developed in 1956 and was mainly based on the DRE and radiographic studies (Hilton *et al.*, 2013). This system categorized the PCa to 4 different stages A to D, where the presence of subclinical disease is classified as stage A, Stage B being the presence of tumor confined to the gland with no evidence of metastasis, stage C is local extra-prostatic invasion of surrounding tissues and stage D is PCa that has metastasized to distant sites in the body. This system was the most common staging system

used in the United States for 20 years. In 1975 Jewett modified this system and sub classified stage A to A1 and A2 based on whether the disease was histologically focal (A1) or diffused (A2). With a similar approach he subcategorized the stage B and C to two classes of B1 and B2, C1 and C2. In which B1 was identified with focal unilateral involvement of the prostate, B2 was with involvement of both lobes without capsular involvement, C1 was when the capsule was minimally involved and C2 was when the disease involved the capsule as well as more extensive local tissue invasion ECE–producing bladder outlet or Ureteral obstruction (van den *et al.*, 1993) (Bostwick *et al.*, 1994).

The Tumour, Node, Metastasis (TNM) system was developed by the American Joint Committee in 1975 and has been mainly used by urologists to stage PCa. The TNM system sub- classifies PCa using three categories of tumor (T), lymph node (N) and metastasis (M), in which T measures the primary tumor and the extent that it has spread into the neighboring tissue, N will measure the tumour involvement with the regional or nearby lymph node and M gauges whether the malignancy has metastasized to distant organs of the body (Greene, 2002).

Table 1.1 is adopted from the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) 2002 manual and is widely used in clinical practice, teaching and medical literature.

Table 1.1 TNM staging of prostate cancer.

TNM Staging of Prostate Cancer	
Stage	Description
T Stage	
TX	Primary tumor can be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor neither palpable nor vissible imaging
T1a	Tumor incidental histologic findings in ≤ 5% of tissue resected
T1b	Tumor incidental histologic findings in > 5% of tissue resected
T1c	Tumor identified by needle biopsy (eg, because of elevated PSA level)
T2	Tumor confined within prostate
T2a	Tumor invloves no more than half of one lobe
T2b	Tumor involves more than half of one lobe but not boh lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostatic capsule**
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades the seminal vesicle(s)
T4	Tumor is fixed to or invades adjacent structures other than seminal vesicles (eg, bladder, levator animuscle, pelvic wall)
N Stage	
NX	Regional lymph nodes not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
M Stage***	
M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

* Tumor found in one or both lobes with needle biopsy but not palpable or reliably visible at imaging is classified as T1c disease

** Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is classified as T2 disease, not T3.

*** When more than one site of metastasis is present, the most advanced category is used

In addition to the TNM staging system, the histologic grade of the PCa is often evaluated by the pathologist using a different grading system called Gleason Score (GS). This widely used system is entirely based on the histological pattern of the arrangement of carcinoma cells in haematoxylin and eosin (H&E) stained prostatic tissue sections and dictates the degree of differentiation of the neoplastic cells (Huggins and Hodges, 2002).This score is the sum of twonumbers which represent the two most common types of glandular growth pattern within the prostate tumor. GS scales from 1 to 5 (**Table 1.2**), in which 5 is assigned to cancer cells with a very poor differentiation pattern (Gleason, 1992)(Humphery, 2004).

Table 1.2 TNM classification for prostate cancer.

Anatomic Stage/Prognostic Groups					
Group	**T**	**N**	**M**	**PSA**	**Gleason**
Stage I	T1a-c	N0	M0	PSA<10	Gleason≤6
	T2a	N0	M0	PSA<10	Gleason≤6
	T1-2a	N0	M0	PSA X	Gleason X
Stage IIA	T1a-c	N0	M0	PSA<20	Gleason 7
	T1a-c	N0	M0	PSA≥10 <20	Gleason≤6
	T2a	N0	M0	PSA<20	Gleason≤7
	T2b	N0	M0	PSA<20	Gleason≤7
	T2b	N0	M0	PSA X	Gleason X
Stage IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1-2	N0	M0	PSA≥20	Any Gleason
	T1-2	N0	M0	Any PSA	Gleason≥8
Stage III	T31-b	N0	M0	Any PSA	Any Gleason
Stage IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

1.2.6 Role of Androgen in the Development of Prostate Cancer

PCa may also be classified into different phases associated with Androgen Dependence, including Androgen Dependent (AD) phase, Regression and Castration Resistant Prostate Cancer (CRPC).

Androgens are essential for the development of male sexual organs as well as secondary sex characteristics. In particular androgens play a major role in regulating the development, growth, and survival of prostatic tissues (Janster, 1999)(Balk, 2002)(Culig and Bartsch, 2006)(Koltz, 2006). As depicted in Figure 1.3.**,** when PCa initially develops growth of the epithelial cells is androgen-dependent. In 1941 Huggins and Hodges suggested a potential role of androgens and the AR in PCa. Their studies concluded that androgen ablation causes regression of primary and metastatic androgen-dependent tumours (Huggins and Hodges, 1972). In 1966 Dr. Huggins was awarded the Nobel Prize for Physiology or Medicine for this discovery. In 1967 Huggins went on to publish an article discussing the role of hormones in the development of cancer. During his initial research he reported that estrogen treatment in dogs results in shrinkage of the prostate gland. In this article he also mentioned that serum proteolytic enzyme content decreased or was eliminated as a result of removing circulating androgens by gonadectomy (Tagnon *et al*., 1952)(Huggins, 1967). These findings confirmed the critical significance of hormones in survival of hormone responsive cancers. Later it became clear that inhibition of androgen production could also be procured by shutting down the hypothalamus-pituitary-gonadal axis using chemical Luteinizing hormone-releasing hormone (LHRH) agonists (Cox and Crawford, 1995).

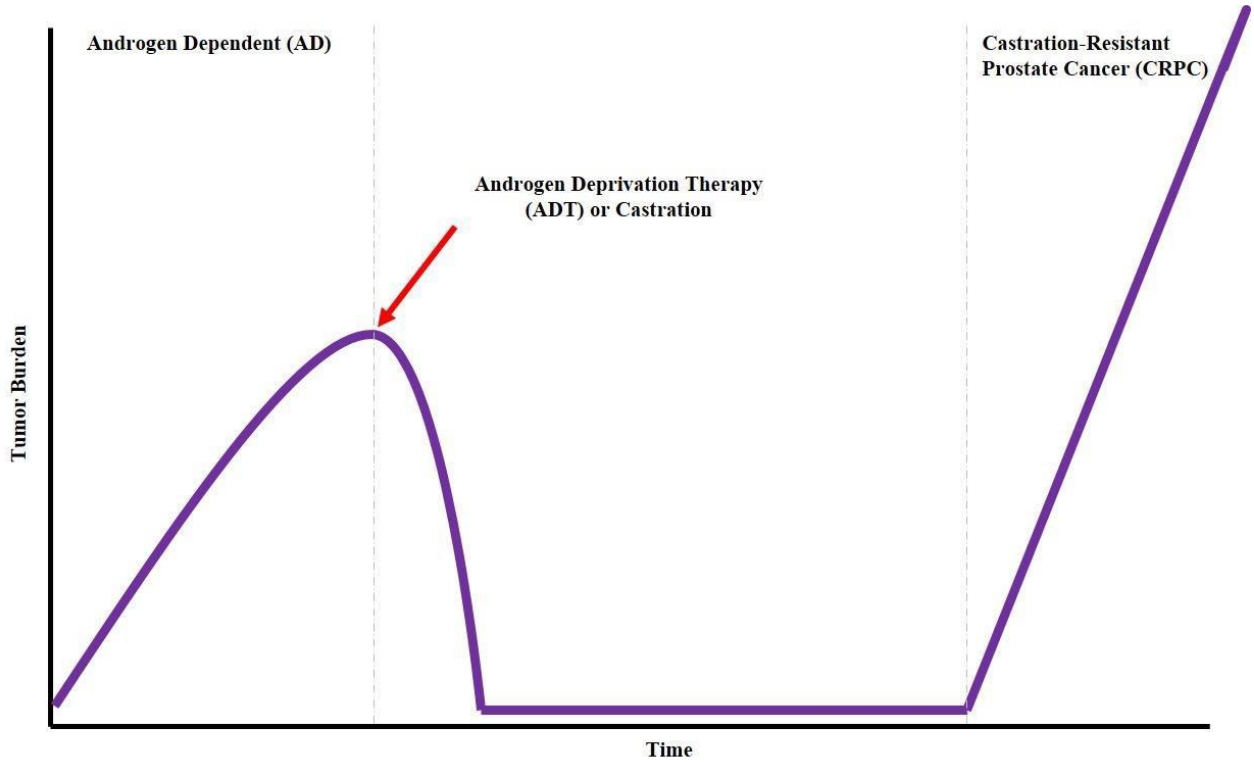


Figure 1.3 Schematic of clinical PCa progression.

Despite the fact that PCa tends to progress slowly and often with no symptoms, over time, which varies from months to years most PCa cases will develop to CRPC and can metastasize, mainly to the bones or brain via the lymph nodes and seminal vesicles.

Current understanding of CRPC development explains that genetic modification is a key player in tumorigenesis as well as CRPC development (Ruijter *et al.*, 1999). Although the development of CRPC and PCa metastasis is still under investigation, Feldman and Feldman (2001) discuss five potential mechanisms involved in CRPC development (Feldman and Feldman, 2001).

The first mechanism described is one in which PCa cells avoid the effects of Androgen Deprivation Therapy (ADT) by lowering the threshold of androgen levels, as a

requirement for growth. There are several potential mechanisms that would lead to the induction of tumor cell proliferation despite a low level of androgen procured as a result of ADT. Amplification in the expression of AR, which leads to enhanced ligand-occupied receptor content, is one of the mechanisms that play a role in the hypersensitization of this pathway. The second hypersensitization mechanism to circumvent ADT is the increase in tumor cell sensitivity of the AR ligand, dihydrotestosterone (DHT) (Rahman *et al.*, 2004)(Mostaghel *et al*., 2012). Amplification of this pathway can result from increased expression of AR, enhanced AR stability and AR nuclear localization. The third mechanism which leads to CRPC development is the local production in androgens enabled via the enhanced activity of steroidogenesis enzymes such as cytochrome P450 17A1 (CYP17) and 5α-reductase which allow local production of androgens, as well as enhanced conversion of testosterone to DHT. CRPC development via alternative AR ligand binding, sometimes referred to 'AR promiscuity' can occur via 3 mechanisms, AR mutation, co-regulator alteration and growth-factor activated outlaw pathways (Feldman and Feldman, 2001)(Umar *et al*., 2012).

Increases in steroidogenic precursors and activation of AR via outlaw pathways is another major pathway contributing to CRPC development (Saraon *et al*., 2011). The outlaw pathway which has already been described in breast cancer (McGuire *et al.*, 1991) could play a role in CRPC development. The premise of this being that AR could be activated by certain growth factors such as Insulin-like Growth Factor-1 (IGF-1), Keratinocyte Growth Factor (KGF) and Epidermal Growth Factor (EGFR), creating an outlaw receptor to activate AR in the absence of androgen (Saraon *et al*., 2011). Overexpression of Her-2/Neu could activate AR-dependent genes in the absence of AR ligands

(Hu *et al.*, 2010) The AR can be transformed into an outlaw receptor via increased activity of AKT, which mediates outlaw AR activation (Taichman *et al.*, 2007)(Mediwala *et al.*, 2011). All of the mechanisms discussed above require the presence of AR and its signalling pathway for CRPC development however when targeted therapies inhibit crucial survival signalling pathways, cancer cells adapt to facilitate proliferation or inhibition of apoptosis via bypass mechanisms. Mutations of targets alongside the upregulation of parallel survival pathways facilitate evasion of therapeutic strategies, which ultimately lead to the development of CRPC (Taichman *et al.*, 2007)(Mediwala *et al.*, 2013). Finally, PCa may also circumvent the effects of ADT via the lurker pathway. During this phenomenon epithelial androgen independent stem cells continue to proliferate following ADT resulting in clonal expansion of castration resistant tumors (Feldman and Feldman, 2001)(Li *et al.*, 2014).

1.2.7 Prostate Cancer Treatment

PCa patients are presented with several different therapeutic options depending on the grade and stage of cancer at diagnosis.

1.2.7.1 Localized Prostate Cancer Treatment

As discussed above localized PCa is characteristic in that PCa hasn't escaped the confinement of the prostate capsule, however it is important to identify the subset of men with aggressive localized PCa or localized intermediate and/or high risk PCa when treatment options are being assessed. Localized PCa could be categorized to three different groups;

i) Confined or low risk PCa with a PSA <10 ng/ml and GS 6 with TNM staging of cT1c–cT2a.

ii) Intermediate risk PCa presenting with a PSA between 10.1 to 20 ng/ml or biopsy GS 7 or TNM staging of cT2b–c.

iii) High risk PCa which has been defined as a non-metastatic disease which extends out beyond the prostate capsule and manifests with local tissue invasion and a combined GS of at least 8 with a PSA level greater than 20 ng/ml (D'Amico *et al*., 1998)(Mohler *et al*., 2014)(Heidenreich *et al*., 2014).

The primary course of treatment for locally confined PCa includes Radio-Therapy (RT), Radical Prostatectomy (RP), transperineal brachytherapy, cryotherapy, High-Intensity Focused Ultrasound (HIFU), ADT and in some cases active surveillance (Hammad, 2008)(Gomella *et al*., 2009).

The first option for very low risk confined PCa is active surveillance. Active surveillance or watchful waiting define a treatment plan that involves watching a patient's progress closely without giving any treatment unless the disease prognosis changes. Evidence suggests that PCa patients with low risk localized and well-differentiated PCa have a 20-year PCa specific survival rate of 80-90% (Chodak *et al*., 1994)(Albertsen *et al*., 1998). While no treatment will be prescribed, these PCa patients will be regularly checked for disease progression or a likelihood of rapid progression of PCa.

RP, has been the most commonly used treatment for healthy men younger than 70 with localized PCa. Patients with confined PCa or low to intermediate risk localized PCa are the best candidates for RP. While the goals of patients who undergo this procedure is to be cancer free and nerve sparing surgical techniques are globally observed, the main complication of concern for these patients are urinary incontinence and erectile dysfunction (Keyes *et al*., 2013)(Akduman and Crawford, 2006)(Heidenreich *et al*., 2014).

Another option for localized PCa includes radiation therapy. RT or the use of ionizing radiation to destroy cancer cells, has been a line of therapy for many cancers including PCa. RT could be delivered by two different modalities; External Beam Radiation Therapy (EBRT) and Brachytherapy (BRT). RT could be used alone or in combination with other treatment options for low to high risk localized PCa (Heidenreich *et al*., 2014)(Kollmeier and Zelefsky, 2012). EBRT is the most common form of RT in which an external source of radiation is directed to the prostate in short bursts and this is repeated daily for an extensive time period. In contrast, during BRT the radiation is delivered internally via implanted radioactive seeds into the prostate in low or high doses for up to 6 month (Chang *et al*., 1988). Treatment response rate for the two delivery techniques are similar.

1.2.7.2 Castration Resistant Prostate Cancer Treatments

If the PCa spreads beyond the prostate capsule or it fails to respond to non-surgical localized therapies, then ADT is the most common line of treatment for these patients. It has been reported that approximately 80% of metastatic PCa patients will respond to ADT with a median progression-free survival of 1-2 years and overall survival of 2-4 years (Rennie *et al*., 2013).

In 1940 Huggins and Hodges pioneered the use of ADT by estrogen treatment and orchiectomy for the treatment of PCa (Huggins and Hodges, 1972). Bilateral orchiectomy is a surgical procedure essentially describing castration in which both testis are removed to directly block androgen production and subsequent stimulation of AR by androgens in the prostate. This has several advantages including low cost and morbidity and high compliance however the psychological problems associated with this favors other

chemical methods of castration which have more recently been adopted. LHRH agonists including Goserelin, Leuprolide and Buserelin can competitively bind LHRH receptor in the pituitary gland and suppress the secretion of luteinizing hormone (LH) in the blood stream and therefore inhibit testosterone synthesis by the testis (Labrie *et al*., 1980)(Msaouel *et al*., 2007).

Unfortunately LHRH agonist treatments cause an unanticipated initial increase in testosterone termed "Flare" response, which may produce or exacerbate symptoms such as urinary obstruction, bone pain and spinal cord compression (Knudsen and Scher, 2009). Subsequently LHRH antagonists, (e.g. Degarelix) were developed to avoid the flare response and inhibit LHRH binding to its receptor in order to inhibit its action (Msaouel *et al*., 2007)(Labrie, 2004).

Anti-androgens or androgen-antagonists are another class of drugs for the treatment of metastatic PCa, which act competitively with testicular or adrenal androgen to block AR ligand binding and subsequent nuclear translocation and transcriptional activation in prostate cells (Koltz 2008). In addition, to avoid the androgen flare response of LHRH agonists, this class of PCa drugs are usually added as a second line of hormonal therapy at the time of PCa progression. Anti-androgen drugs are classified in two groups: non-steroidal anti-androgens (e.g. Flutamide, Bicalutamide, nilutamide, MDV3100) and steroidal anti-androgens (e.g. cyproterone acetate). While non-steroidal anti-androgens have no gonadotropic or hypothalamic feedback effects to suppress the circulating levels of testosterone, steroidal anti-androgens which directly influence the gene expression due to their fat-soluble nature, do possess this feedback activity (Rennie *et al*., 2013).

The third line of treatment for metastatic PCa includes the use of steroidogenesis

enzyme inhibitors (e.g. Ketoconazole, Abiraterone acetate) which inhibit the broad spectrum production of steroids (Ketoconazole) or the testicular and/or tumor-tissue associated androgen synthesis enzymes such as CYP17, in a more specific manner (Abiraterone acetate). Abiraterone acetate in particular has shown a great deal of promise for extending the prolonged survival of late stagemetastatic PCa, in individuals who have progressed after receiving chemotherapy (Klotz, 2008)(Harshman and Taplin, 2013).

1.3 Extracellular Vesicles

It has been more than forty years since Anderson reported the membrane-enclosed vesicles in the matrix of epiphyseal cartilage (Anderson, 1969). Since then an enormous number of studies have been focused on characterizing different Extracellular Vesicles (EVs) and understanding their role in biological and pathological processes as communication vesicles, or biological messengers/transporters.

Studies have shown that these vesicles could be released from almost all cell types and are found in different body fluids such plasma (Caby *et al*., 2005), serum (Taylor *et al*., 2006) (Taylor and Gercel-Taylor, 2008), malignant ascites (Andre *et al*., 2002)(Brad *et al*., 2004), urine (Pisitkun *et al.*, 2004), amniotic fluid (Asea *et al*., 2008), bronchoalveolar lavage fluid (Admyre *et al*., 2003)(Hawari *et al*., 2004) and breast milk (Admyre *et al*., 2007). Differences in the array of microvesicles found in the extracellular matrix depend on cellular origin, biogenesis as well as the mechanism of formation. Different nomenclature have been used to characterize or describe different vesicles that are released from cells. Exosomes, microvesicles and apoptotic vesicles seem to be the three major types of vesicles that have been discussed and studied by different groups (Thery *et al.*, 2009)(Beyer *et al*.,

2010)(Mathivanan *et al*., 2010). Physiological characteristics of these vesicles including size, density, and appearance under microscopy, sedimentation, lipid composition, main protein markers and cellular origin are the criteria for classification of these vesicles. For the purpose of clarity in this thesis any membrane enclosed vesicles that are released into the extracellular matrix are called Extracellular Vesicles (EVs), and since EVs are a heterogeneous mixture of vesicles with a size range of 30-1000 nm they could include (but are not limited to) Plasma Membrane Vesicles (PMV/MV) as well as Exosomes **(Table 1.3)**.

Table 1.3 Secreted vesicles by prostate or PCa cells*

Vesicle	Size (nm)	Putative Protein Marker	RNA Marker examples	reference
Exosomes	30-200	CD9, CD63, CD81, CD82, Annexins, RAB proteins, Alix, TSG101	PCA3, TMPRSS:ERG	Thery et al., 2006; Simpson et al., 2008; Jansen et al.,2009; Nilsson et al., 2009;; Yoshioka et al.,2013
Prostasomes	50-500	CD13, CD46, CD55, CD59, Annexins, RAB proteins		Ronquist et al., 1985; Ronquist et al., 1986; Rooney et al., 1993; Arienti et al., 1997
Oncosomes	500-10000	Signal Transductionm Proteins, Integrins, Selectins, CD40 ligand, ARF6	DIAPH3	Di vizio et al., 2009, 2012
Microvesicles	200-1000	ARF6, Rho family memebers	EGFRvIII	Lehmann et al., 2006; Skog et al., 2008; Muralidharan et al.,2009; Lima et al., 2009; Shao et al.,2012
Ectosomes (microparticles)	50-1000	CR1, Protelytic enzyme		Haubold et al., 2009; Dashevsky et al., 2009

**modified version of the table in (Duijvesz et al., 2011)*

On the other hand vesicles that are directly produced and released through the outward budding of the plasma membrane are called Plasma Membrane Vesicles (PMVs). PMVs are a heterogeneous mixture of vesicles with different size and shapes; their sizes vary between 200- 1000 nm and they have an irregular round shape under Transmission Electron Microscopy (TEM). Research confirms the presence of different classes of

proteins, mRNA, miRNA and lipids in these vesicles. As a result the PMV surface markers are mainly dependent on the composition of the membrane area that they are originated from.

When viewed under TEM, exosomes are 30-200 nm in diameter and appear to be cup- shaped. The characteristic shape occurs as a result of the vacuum, fixation and dehydration during sample preparation. They are cholesterol-rich nanovesicles encapsulated by a lipid bi-layer membrane and are rich in a plethora of various proteins (Thery *et al*., 2001)(van der Pol *et al*., 2012), an array of lipids and nucleic acids (DNA (Thakur *et al*., 2014)(Kahlert *et al*., 2014) and RNA (Lotvall and Valadi, 2007)(Huang *et al*., 2013)(De Smaele *et al*., , 2010)).

While the main focus of this thesis is to understand the role and relevance of exosomes in PCa growth and progression, it is important to understand that all cells (normal and cancerous) release several different classes of vesicles (PMVs, Platelet derived Microparticles and Exosomes) into the extracellular matrix at the same time, therefore these vesicles coexist simultaneously and the current vesicle isolation and purification methodology will only enrich one population/type over another and not necessarily separate the other classes of EVs from exosomes completely. Therefore in the absence of a validated exosomal marker, most of the vesicles research has been historically carried out on an enriched sup-population of EVs rather than a homogenous population of pure class/type of vesicles.

1.3.1 Exosomes

The Exosome story, describing a non-plasma membrane derived vesicle with

endosomal origin began ~30 years ago when Stahl and Jonhstone (published within a week of each other) described Transferrin Receptor (TfR) shedding during reticulocyte maturation (Harding and Sathl, 1982)(Pan and Johnstone, 1983).

The term "exosome" was first used to describe intracellular particles that are involved in RNA editing and then again by Trams et al. in 1981 to describe membrane fragments isolated from biological fluids. However it was Rose Johnstone who coined the term "exosomes" for non- plasma membrane derived nanovesicles that are released into the extracellular space during reticulocyte maturation (Johnstone *et al.*, 1987)(Trams, 1981). As discussed, by definition exosomes are 30-200 nm nano-vesicles with 1.13-1.19 g/ml density that are (van der Pol *et al*., 2012)(Thery *et al*., 2009) encapsulated by a bi-layer phospholipid membrane (Laulagnier *et al*., 2004). These nanovesicles are rich in cholesterol, sphingomyelin and ceramide (van der Pol *et al*., 2012) and are rich in a plethora of various proteins, an array of lipids and nucleic acids (DNA and RNA) (**Figure 1.4**).

Similar to all other vesicles exosomes are spherical vesicles which have been described as cup-shaped after fixation, dehydration and negative staining and visualization by TEM. Exosomes density usually varies between 1.13 to 1.19 g/ml and they are isolated by differential centrifugation followed by an ultracentrifugation step in sucrose cushion (200-500g to remove cell and cellular debris, 10,000-20,000g to pellet vesicles larger than 200nm and 100,000- 200,000g to isolate vesicles smaller than 200nm).

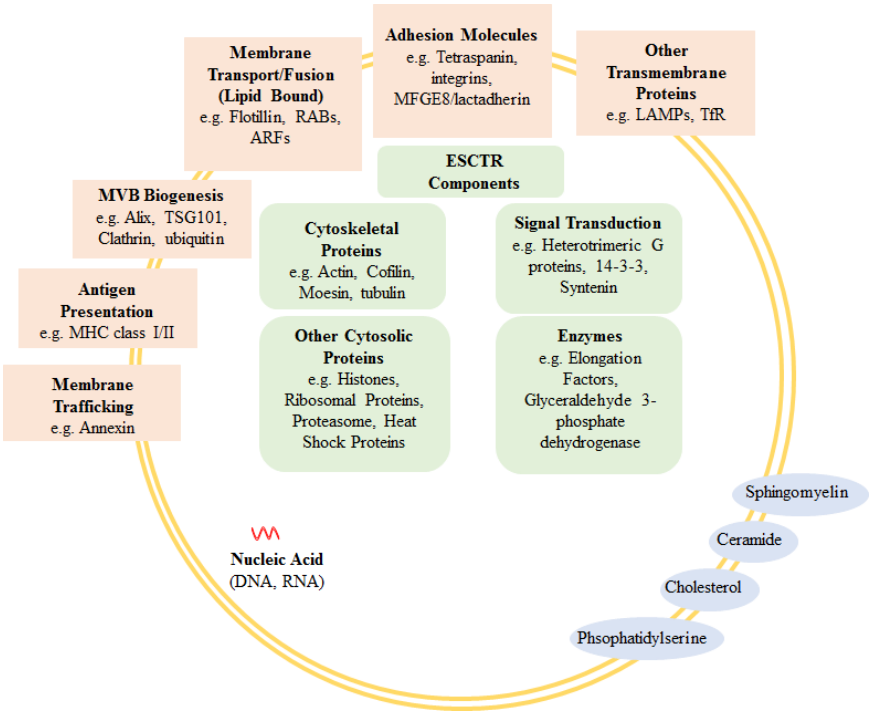


Figure 1.4 A Summary of Exosome content.

Exosomes contain and protect the integrity of various proteins and an array of lipids, mRNA and miRNA which would otherwise be hydrolytically or enzymatically broken down if they existed as free soluble molecules in the extracellular microenvironment.

However according to the current literature no exosomal protein markers have been reported for these vesicles. As a consequence of their endosomal origin, and independent of their cell type, all exosomes share some common proteins involved in membrane transport and fusion (e.g. Annexins and Flotillin), cytoskeletal proteins (e.g. Actin and Tubulin), adhesion molecules (e.g. Integrins and Tetraspanins), antigen presentation (e.g. MHC I, II), signal transduction (e.g. 14-3-3 and Syntenin) and ESCRT (Endosomal Sorting Complexes Required for Transport) components. While some of the proteins found in exosomes derived from different cell lines are the same, cellular origin of exosomes is thought to be recognizable based on their protein content (Stoorvogel *et al.*, 2002). For example,

intestinal cell exosomes have the transmembrane protein A33 expressed on their surface, T-cell derived exosomes bear CD3 (Cluster of Differentiation 3) and, similarly, prostate cell derived exosomes may be recognizable based on the presence of membrane antigen folate hydrolase 1 (FOLH1; Folate Hydrolase 1)(Ronquist *et al*., 2010).

These vesicles are released from a variety of different cells including normal cells and tumor cells (Thery *et al.*, 2002) and although the underlying mechanisms of exosome function is not fully understood it is very well known that exosomes are formed in the endosomal compartment of cells and are released upon fusion of Multivesicular Bodies/Multivesicular Endosomes (MVB/MVE) with the plasma membrane (Thery *et al*., 2002). The schematic cartoon in **Figure 1.5.** depicts Early Endosome (EE) formation as a result of the invagination of specific regions of the plasma membrane. In addition, endocytotic cargo transported out of the cell is sorted from EE into Intraluminal Vesicles (ILV). Mechanisms involved in protein sorting into ILVs are still under investigation however there is evidence supporting the involvement of ubiquitin and Endosomal Sorting Complex Required for Transport (ESCRT machinery) in this process. Finally, fusion of late endosome or MVB with plasma membrane releases ILVs into the extracellular matrix or the tissue microenvironment.

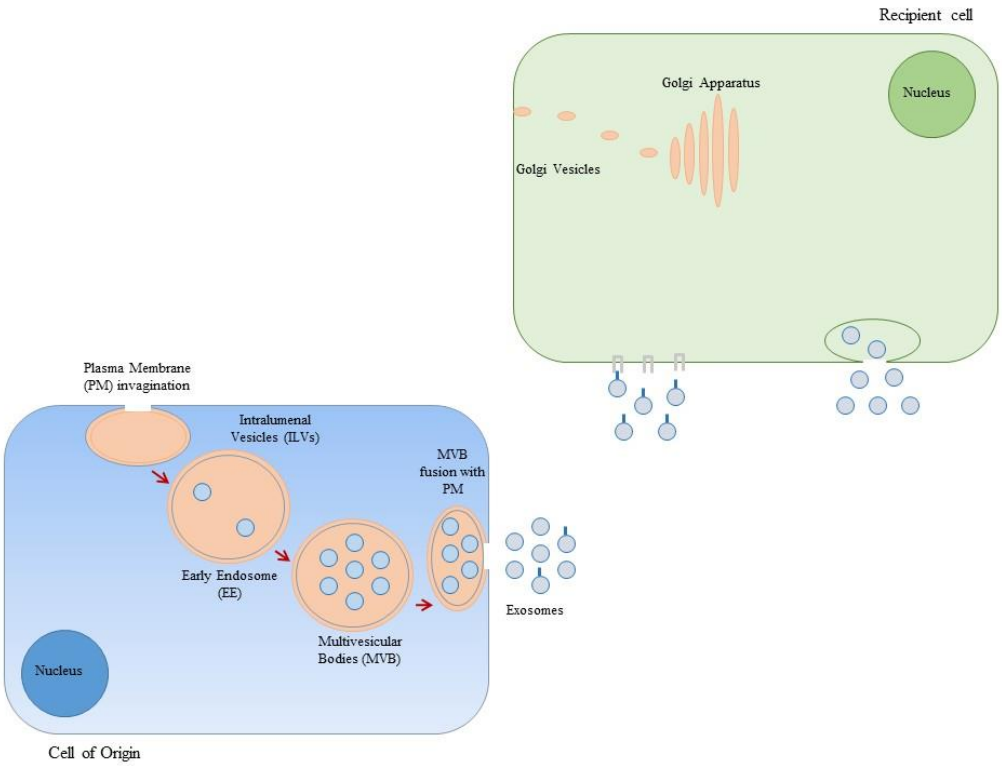


Figure 1.5 Mechanism involved in exosome formation and trafficking into the microenvironment.

Accumulating evidence suggests that induction of intracellular calcium (Jaiswal *et al*., 2002)(Stoeck *et al*., 2006)(Kramer-Albers *et al*., 2007)(Lakkaraju and Rodriguez-Boulan, 2008) overexpression of Rab7, Rab11 and Rab35 or citron kinase (Savina *et al*., 2002)(Colombo *et al*., 2014) as well as a reduction in membrane cholesterol, or inhibition of cholesterol biosynthesis (Llorente *et al*., 2007), could stimulate the release of exosomes into the microenvironment.

As shown in **Figure 1.5.**, once released, exosomes will interact with recipient target cells via different mechanisms such as fusion with the plasma membrane or adhesion to corresponding receptors on the plasma membrane (Lakkaraju and Rodriguez-Boulan, 2008). Regardless of their mode of interaction with the recipient cell, exosomes have been shown to have an enormous impact on their recipients. From embryonic development to physiological condition, and from tumor growth (D'Souza-Schorey and Clancy, 2012) to

tumor vaccine (Hsu *et al*., 2003) exosomes are proving to be unique as highly mobile, discrete packages of protein and nucleic acids that are essential for intercellular communications.

1.3.2 Exosomes in Cancer

Although, the mechanisms underlying exosome formation and secretion are still under investigation, studies have revealed that exosomes contribute to tumorigenesis primarily via modulation and restructuring of the cellular microenvironment and can generate the metastatic niche as well as attenuate of tumor immune responses (Whiteside, 2013)(Zhang *et al*., 2011).

It is well-known that release of EVs is triggered by conditions such as hypoxia (King *et al*., 2012), radiation (Arscott *et al*., 2013), injury (Broges *et al*., 2013), in different diseases such as cancer as a general response to cellular stress (Ratajczak *et al*., 2006). In addition to that different factors such as cell type, cell cycle, cell activation and stage of cancer, contribute to the amount and composition of exosomes formed and released from various cells (Thery *et al*., 2009).

Studies on tumor-derived microvesicles suggest that exosomes play a significant role in cell communication thus potentially influencing cancer progression via different mechanisms (Abusamra *et al*., 2005).

EVs could transfer oncoproteins or oncogenes to neighboring cells and facilitate tumor development and progression via different mechanisms. There are several studies that have reported EVs as active contributors to the tumor microenvironment demonstrating the influence of tumor-derived MVs in their surrounding microenvironment. Effects of these secreted vesicles has been implied in angiogenesis and metastasis in lung and breast

cancer (Ratajczak *et al*., 2006)(Baj-Krzyworzeka *et al*., 2006)(Janowska-Wieczorek *et al*., 2006).

The ability of EVs to promote cancer metastasis or angiogenesis have been studied by different researchers. Hao et al. in 2006 demonstrated the effect of highly metastatic B16-10 derived exosomes in metastatic lung tumor development. Mice injected with B16-10 exosomes develop metastatic colonies when compared to a control group (Hao *et al*., 2006). Interestingly EVs released under hypoxia conditions, exhibited enhanced metastatic properties, enhanced angiogenesis, increased invasion and mediated loss of cell adhesiveness (Park *et al*., 2010).

Several studies have reported that EVs shape the tumor microenvironment by influencing the behavior of bone marrow derived cells. Specifically, breast cancer derived exosomes have been shown to target the $CD11b^{+}$ myeloid precursor cells in the bone marrow. Moreover this study demonstrates that the differentiations of murine myeloid precursor as well as human monocytes into dendritic cells were suppressed due to the induction of IL-6 in bone marrow following exosome treatment (Yu *et al*., 2007).

Other studies have shown that in addition to the role of tumor derived exosomes in differentiation and function of innate immune system cells, these vesicles could also influence the adaptive immune response by interfering with T-cell survival, proliferation and function mimicking the effect of myeloid-derived suppressor cells during tumor progression (Andreola *et al*., 2002)(Huber *et al*., 2005)(Kim *et al*., 2005).

On the other hand "tumor-exosome driven education" is another major consequence of cancer-derived exosome exposure to neighboring cells. During this process exosomes serve as active entities, which deliver functional oncogenes and oncoproteins (EGFRvIII) to

target cells and influence and define the future behavior of recipient cells. The transferred vesicles have been reported to impart transfer of the oncogene or oncoprotein activity such as activation of transforming signaling pathways or morphological transformations (Al-Nedawi *et al*., 2008)(Skog *et al*., 2008).

Tumor derived EVs have been shown to contribute to the creation of an adaptive environment for PCa and Pancreatic Cancer, support the establishment of a favorable prostate tumoral niche in a process dependent on CD44v6 and support melanoma cancer metastasis in a phosphatidylserine-dependent manner (Castellana *et al*., 2009)(Jung *et al*., 2009)(Lima *et al*., 2009)(Peinado *et al*., 2012).

In addition, cancer cells could lose proteins such as Fas-associated death domain protein via EV secretion, which would result in tumor progression or enhanced aggressiveness. Fas-associated death domain protein is a key adaptor protein transmitting the apoptotic signal mediated by death receptors which is lost in many different types of cancer cells and can therefore be used as prognostic factor (Tourneur *et al*., 2008).

In a similar process EVs could also protect the targeting of tumor cells by drugs via sequestration, transport and expulsion of chemotherapeutic drugs to/from tumor cells (Safaei *et al*., 2005)(Federici *et al*., 2014) as well as delivering hepatic enzymes throughout the body (Conde-Vancells *et al*., 2010).

As it has been discussed above, exosomes are now recognized as novel communication vesicles that provide the tumor microenvironment with cargo needed to induce a phenotype that supports tumor cell metastasis or to condition the metastatic niche to regulate immune responses targeted toward tumor cells. In addition to be a point of intervention, or biological target in cancer treatment and/or prevention of chemotherapeutic

resistance (Tickner *et al*., 2014), exosomes could also be used as potential biomarkers for cancer diagnosis and prognosis (Hosseini-Beheshti *et al*., 2012).

1.3.2.1 Exosomes as a Source of Potential Diagnostic Cancer Biomarkers

Current biomarkers for cancer screening and diagnosis suffer from either low levels of sensitivity, which causes the diagnostic process to miss patients in early stages of cancer (false negative) and/or display a low level of specificity which results in diagnosing patients with no cancer (false positive).

EVs are currently undergoing scrutiny as a major potential biomarker source for many different pathological disorders including cancer and PCa. The accessibility of these vesicles in biological fluids (such as blood, urine, milk, saliva, malignant ascites, amniotic fluid and bronchoalveolar lavage fluid), along with inherent cargo protection from the attack of nucleases and proteases allowing for longer biomarker half-life, have prompted intensive investigation into their use for many recent biomarker discovery studies (Arroyo *et al*., 2011).

Their accessibility through minimally invasive procedures as well as the presence of different classes of proteins (Thery *et al*., 2001) and genetic material (DNA and RNA) (Lotvall and Valadi, 2007)(Huang *et al*., 2013)(Kahlert *et al*., 2014)(Thakur *et al*., 2014) in these vesicles make them a better representative source of biomarkers for intra-tumoral heterogeneity than fine needle biopsy would afford.

In addition to their diagnostic potential, the possibility of the real-time monitoring of therapeutic response and development of chemoresistant mechanisms to anti-cancer drugs via EVs analysis make them an interesting avenue for diagnostic and prognostic biomarker researchers to explore.

Pre-clinical and clinical studies on biological fluid derived EVs confirmed the presence of more than forty proteins or miRNA in EVs which could be used for screening, early diagnosis, prognosis, therapy monitoring and personalized medicine approaches (Wood *et al*., 2013) (Honda *et al.*, 2013). The presence of p-Met, Caveolin-1, TYRP2, HSP70, HSC70, VLA-4 and EGFR inEVs derived from melanoma patients(Logozzi *et al*., 2009)(Peinado *et al*., 2012)(Thakur *et al*., 2014), miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214, TGFB1, MAGE3/6 in EVs derived from ovarian cancer patients (Taylor and Gercel-Taylor 2008)(Szajnik *et al*., 2013), miR-151a, miR-30a-3p, miR-200b-5p, miR-629, miR-100, miR-154- 3p and BRAFV600E in EVs derived from lung cancer patients (Cazzoli *et al*., 2013)(Thakur *et al*., 2014) and PSA, survivin, TMPRSS2:ERG2 and PCA3 mRNAs in EVs derived from PCa patients (Mitchell *et al*., 2009) (Khan *et al*., 2012)(Ogata-Kwata *et al*., 2014) (Mizutani *et al*., 2014) are examples that demonstrate the potential of EVs for exploitation as biomarker shuttles. The specific identification of survivin in plasma derived EVs, TMPRSS2:ERG and PCA3 mRNA in urine derived EVs and PSA and Prostate-Specific Membrane Antigen (PSMA) in urine and plasma derived EVs of PCa patients introduces a promising tool for early diagnosis of PCa via a relatively non-invasive procedure. While these findings need to be validated in large clinical studies there is a strong indication that they could present a platform for the future with respect to the clinical use of exosomes as biomarkers for PCa diagnosis. A summary of potential diagnostic/prognostic biomarkers in PCa has been reported in **Table 1.4.**

Table 1.4 PCa extracellular protein/genetic biomarker signatures.

Extracellular Cargo	Sample	Clinical Implication	Reference
Protein Content			
Prostatic acid phosphatase	EPS exosomes/urine exosome/Prostasomes	Diagnosis of PCa	Kovak et al., 2013, Principe et al., 2013
Lactotransferrin	EPS exosomes/urine exosome/	Diagnosis of PCa	Principe et al., 2013
Dipeptidyl peptidase 4	EPS exosomes/urine exosome/Prostasomes	Diagnosis of PCa	Kovak et al., 2013; Principe et al., 2013
Protein-glutamine g-glutamyltransferase 4	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Sorbitol dehydrogenase	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Neprilysin	EPS exosomes/urine exosome/Prostasomes	Diagnosis of PCa	Kovak et al., 2013; Principe et al., 2013
Semenogelin-1	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Zinc-a-2-glycoprotein	EPS exosomes/urine exosome	Diagnosis of PCa	Kovak et al., 2013; Principe et al., 2013
Prostate-specific antigen	EPS exosomes/urine exosome	Diagnosis of PCa	Dijkstra et al., 2014; Principe et al., 2013
Aminopeptidase N	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Galectin-3-binding protein	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Glutamate carboxypeptidase 2 (PSMA)	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013

Extracellular Cargo	Sample	Clinical Implication	Reference
Transmembrane protease serine 2	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Fatty acid synthase	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Galectin-3	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Prostate stem cell antigen	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Growth/differentiation factor 15	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Metalloproteinase inhibitor 1	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
PTEN	Plasma/Plasma exosome	Diagnosis of PCa	Gabriel et al., 2013
Survivin	Plasma/Plasma exosome	Diagnosis of PCa	Khan et al., 2013
Kallikrein-2	EPS exosomes/urine exosome	Diagnosis of PCa	Principe et al., 2013
Genetic Content			
PCA3	Urine exosome	Diagnosis of PCa	Dijkstra et al., 2013
TMPRSS2-ERG	Urine exosome	Diagnosis of PCa	Dijkstra et al., 2013
miR-141	serum	Diagnosis of advanced PCa	Mitchell et al., 2008
miRNA-375 and miRNA-141	Serum/Serum exosome	Diagnosis of advanced PCa	Brase et al., 2011; ; Li et al., 2015

Extracellular Cargo	Sample	Clinical Implication	Reference
miR-let-7e, miR-let-7c, and miR-30c, miR-622 and miR-1285	Plasma	Diagnosis of PCa	Chen et al., 2012
miR-24, miR-26b, miR-30c, and miR-223, miR-874, miR-1274a, miR-1207-5p, miR-93, and miR-106a	Serum	Diagnosis of PCa and prognosis of disease progression	Moltzahn et al., 2011
miR-107, miR-141, miR-375, and miR-574-3p	Plasma/Serum MVs Urine cell pellets/serum exosome	miR-375 :metastasis and prognostic marker for CRPC, miR-141: metastasis; miR-107, miR-574-3p: diagnosis	Bryant et al., 2011; Li et al., 2015; Huang et al., 2015
miR-26a, miR-195, and miR-let-7i	Serum	miR-26a for the discrimination of PCA and BPH patients	Mahn et al., 2011
miR-141, miR-298, miR-346, and miR-375	Serum	Prediction of clinical outcome	Selth et al., 2012
miR-375, miR-378a-5p, and miR-141 miR-409-3p	Serum	Diagnosis of metastatic CRPC from low-risk, localized PCa	Nguyen et al., 2013
miR-16, miR-126, miR-141,	Plasma	Diagnosis of metastatic CRPC from localized PCa	Watahiki et al., 2013
miR-141	Plasma	Prediction of clinical course and response to therapy	Gonzales et al., 2011
miR-21 and miR-221	Plasma/serum exosomes	Diagnosis of intermediate risk from low risk CAPRA scores, but not eligible to predict PCa aggressiveness	Shen et al., 2012; ; Li et al., 2015
miR-21	Serum	Not significant for diagnosis	Sanders et al., 2012
miR-1290	Plasma exosome	and prognostic marker for CRPC	Huang et al., 2015

In particular urine is an ideal source for urological cancer diagnosis, not only can it be easily sampled in high volume via a non-invasive procedure but it also contains a variety of proteins originating from the bladder, prostate and kidney which could reflect the physiological and pathological changes that would be occurring during cancer development and progression (Wood *et al*., 2013).

1.3.2.2 Exosomes in Cancer Therapy

The importance of EV in intercellular communication and cancer development/progression renders EVs as potential novel therapeutic targets. EVs could be used as targets in cancer therapy via two major avenues. The first would involve elimination of EVs derived from cancer cells from the circulation and could be realized by the inhibition of EVs formation, blocking EVs release and inhabitation of EVs uptake by target cells

(Bobrie *et al.*,2012)(Peinado *et al*., 2012)(Verderio *et al.*, 2012). The second avenue, which has been studied in recent years, is the use of EVs as a drug delivery component (El Andaloussi *et al*., 2013)(Yang *et al*., 2015).

Recent studies have started to shed light on the molecular mechanism involved in the biogenesis of exosomes and their internalization by recipient cells. Even though the whole process of exosome formation, release and uptake is not fully elucidated yet, a few promising key proteins have been identified that play an important role in different stages of this process. Therefore inhibition of proteins involved these key processes may be effective and novel cancer therapy targets (Colombo *et al*., 2013).

ESCRT machinery is required to facilitate the trafficking of ubiquitinated cargo into MVBs and ILVs. Therefore targeting the ESCRT family and/or the associated proteins could influence exosomes formation, the key challenge being target specificity. Colombo et al. (2013) demonstrated that silencing of genes for ESCRT-0; hepatocyte growth factor regulated tyrosine kinase substrate (HRS), Signal Transducing Adaptor Molecule 1 (STAM1) and ESCRT-I; TSG101, decreased the secretion of EV-associated CD63 and MHCII. In addition to reducing the number of released exosomes these three genes seem to also modulate the size and/or protein content of these vesicles (Colombo *et al*., 2013). Rab27a/b are two other key proteins involved in intercellular trafficking of MVBs, its docking to plasma membrane and subsequent exosomes release. Silencing of these two proteins or their effectors Slp4 and Slac2b has been shown to lead to reduced exosome secretion (Ostrowski *et al*., 2010). Inhibition of Rab27a in melanoma cells has also been shown to contribute to the reduction in tumor growth and development of lung metastasis in human xenograft models in mice (Peinado *et al*., 2012).

Moreover factors such as overexpression of Rab11, citron kinase, calcium ionophore and cholesterol concentration are known to stimulate MVB exocytosis and exosome secretion. Intriguing work by Trajkovic et al. (2008) demonstrated that ceramide, a cone-shaped lipid generated upon hydrolysis of sphingomyelin by sphingomyelinase trigger budding of ILVs into MVBs (Trajkovic *et al*., 2008). It is also known that sphingolipid ceramide is required for exosome secretion in some cells. In particular, activation of sphingomyelinase 2 and elevation of ceramide level have been shown to induce exosome secretion (Wang *et al.*, 2012)(Kong *et al.*, 2015) . Therefore, wild type mice treated with GW4869 produced a significantly lower number of lung multiplicities after injection of Lewis lung carcinoma cells when compared to control (Fabbri *et al*., 2012).

Calcium ionophores have been shown to stimulate exosome release in many cell types including epithelial cells and neurons (Savina *et al*., 2003)(Faure *et al*., 2006)(Kramer-Albers *et al*., 2007) and interestingly, inhibition of Na^+/H^+ and Na^+/Ca^{2+} exchangers by dimethyl amiloride (DMA) results in the reduction of exosome secretion from the erythroleukaemia cell line K562 as well as in mice bearing EL4 lymphoma tumors (Savina *et al.*, 2003).

As part of an alternative mechanism, the formation of EVs can be independent of ESCRT machinery and occur via direct shedding from the plasma membrane therefore, targeting the proteins such as Ras homolog family member (RhoaA) and/or ADP-ribosylation factor 6 (Arf6) could be another strategy for inhibiting/reducing EVs release (Muralidharan-Chari *et al*., 2009)(Li *et al*., 2012). Preliminary results have suggested that targeting these pathways may have direct influence on cancer therapy.

Finally, a novel and different strategy is elimination of cancer derived EVs from the

entire circulation system as a therapeutic adjuvant in cancer.

While the biogenesis and internalisation of EVs are poorly understood, there is actually a better understanding in the field of mechanisms involved in EV formation and release compared to their uptake. With respect to the inhibition of EVs uptake by target cells, Christianson et al. (2013) have reported that Heparin Sulfate Proteo-Glycans (HSPGs) present on target cells play a pivotal role in exosomes internalization based on their observation that the uptake of glioblastoma derived EVs was especially inhibited by free heparin sulfate chain (Christianson *et al*., 2013).

Recently, using a different approach Aethlon Medical Inc. (San Diego, USA) have proposed that therapeutic filtration of cancer-derived EVs from the entire circulatory system on an antibody-coated matrix could remove immunosuppressive proteins and improve treatment outcomes. Evidently having knowledge of specific biomarkers representative of different types of cancer derived EVs would play a major role in the success of this approach.

Lastly, in recent years, several studies have focused on the importance of exosome-based drug delivery, especially in cancer models. Scientists have recently established the role of exosomes as therapeutic carriers for both interfering RNAs (Wahlgren *et al*., 2012) and/or chemotherapeutic drugs (Tian *et al*., 2014) and have been successful in achieving significant cell apoptosis and tumor regression (Ju *et al*., 2013)(Bryniarski *et al*., 2013)(Mizrak *et al*., 2013). However, different shortcomings and obstacles including suitable isolation protocols required for clinical scale, choice of exosomes donor cells, type of loading procedures, route of exosomes administration and use of targeting peptides on the exosome surface, exist in this approach. Regardless, emerging research is very promising

for the potential use of exosomes in targeting cancer and personalizing therapy (Maguire *et al*., 2012)(Pan *et al*., 2012)(Yin *et al*., 2013)(Katakowski *et al*., 2013)(Ju *et al*., 2013) (Hood *et al*., 2014)(Gong *et al*., 2015).

1.3.2.3 Exosomes in Prostate Cancer

A PubMed database search on exosomes and PCa (input: "Exosome AND Prostate Cancer") performed (search entry dates: October 17th, 2009 and November 17th, 2014) yielded 11 papers (1 review paper) on the initial search and 73 papers (7 review papers) for the second search. The exponential growth in the number of research articles on this topic during last 5 years is evidence in itself of the importance of these vesicles in cancer and especially PCa.

All of the papers identified as part of the database search were used herein to evaluate the advancement of the exosomes field in the context of PCa. While the majority of these papers are focused on understanding the role of exosomes in PCa development and progression in order to translate this knowledge into novel potential therapeutic targets for PCa in the future, a quarter of these articles have studied the potential of these nanovesicles as a source of biomarkers for use in PCa diagnosis and treatment response.

In general as discussed above, in addition to the type of EVs and their molecular cargo, which is influenced by various factors including cell status, cell cycle and physiological and pathological condition of the parental cells (Minciacchi *et al*., 2015), the cell type and status of EV recipient cells could further play a pivotal role in the mode of action of these vesicles. Since the initial report of Laurence Zitvogel in 1998, which described the immune-protective/immune-therapeutic role of dendritic cell derived exosomes (Zitvogel

et al., 1999) and Clotilde Thery's work in 1999 which also defined the protein/peptide composition of these vesicles and the rationale behind their immune stimulation functions (Thery *et al*., 1999), more than a thousand papers have been published on this topic. These have typically attempted to explain the role of exosomes/their cargo/donor or recipient cell status/external factors in induction or suppression of the immune system as well as activation/inhibition of different pathways, which are shown to play a pivotal role in progression/delay of different physiological and pathological disorders.

The first paper on this topic, which was published by Yang et al. (2002) confirmed that CML28, immunogenic antigen, is identical to hRrp46p, which was reported as a component of human exosomes. Furthermore, their results suggested that immunogenicity of CML28 occurs as a result of its overexpression in tumor cells (Yang *et al*., 2002).

Three years later Abusamra et al. (2005) demonstrated that LNCaP derived FasL-expressing exosomes inhibit T-cell proliferation, may target circulating $CD8^+$ T-cell and suppressed T-cell response via induction of apoptosis (Abusamra *et al*., 2005).

A couple of years after that, effects of cancer cell derived exosomes on immune-evasion were studied by Aled Clayton. Their study demonstrated the impairment of IL-2 mediated lymphocyte proliferation as well as inhibition of NK cell killing function in a T-cell independent manner that was observed in response to human tumor-derived exosomes (Clayton *et al*., 2007).

The role of exosomes in PCa progression independent from the immune system response was first described by our group in 2009, when the presence of CYP17, a crucial enzyme for *de novo* androgen synthesis within the tumor microenvironment after removal of testicular androgens by castration, was confirmed in PCa derived exosomes (Locke *et al*.,

2009).

In 2011 Saraswati et al indicated that Galectin-3, a multivalent carbohydrate-binding protein involved in cell adhesion, cell cycle control, immunomodulation, and cancer progression, is associated with prostasomes and exosome-like vesicles secreted by the prostate. In addition their results demonstrated that PSA may regulate Galectin-3 during PCa progression (Saraswati *et al*., 2011).

In one of the most recent studies on the role of PCa derived exosomes on PCa progression, Lundholm et al. (2014) have reported that PCa derived exosomes are responsible for down- regulation of NKG2D receptor on natural killer cells as well as $CD8^+$ T cells which impairs lymphocyte cytotoxic function and promotes tumor escape (Lundholm *et al.*, 2014).

Taken together all these published reports provide evidence that cancer-derived exosomes represent a pivotal mechanism which enables either immune evasion in cancer or plays a direct role in promoting the mechanisms involved in cancer progression.

Since 2010, an exponential increase in the number of EV and PCa related articles was observed and more than 40 research articles have been published which describe the role and relevance of these nanovesicles and/or their protein/genetic content in PCa development and progression (many of these have been cited in Chapter Four).

Specifically, Lehmann et al. (2008) was the first to describe exosomes as potential diagnostic tools for non-invasive PCa detection. Their data suggested that delta-catenin co- localization and co-isolation with caveolin-1 and CD59 could be due to its release into the extracellular milieu through exosome/prostasome associated pathways. They also demonstrated that delta-catenin can be detected in PCa cell culture media, human PCa

stroma tissue and PCa patients' urine and hence could be a potential PCa biomarker (Lehmann *et al*., 2008).

Lehmann's findings were supported by other impactful research which confirmed the presence of PSA, PSMA (Mitchell *et al*., 2009) and TMPRSS2-ERG (Jansen *et al*., 2009) on PCa derived exosomes to prove that exosome oriented diagnostic research holds promise as a potential source of clinical cancer biomarkers. More than 20 research articles have been published since these three first seminal reports supporting the likelihood of exosomes as potential biomarkers (Nilsoon *et al*., 2009)(Ronquist *et al*., 2010)(Duijvesz *et al*., 2011)(Principe *et al*., 2013)(Huang *et al*., 2013)(Drake *et al*., 2014).

In 2012 we published the first comprehensive proteomic and lipidomic analysis on six different prostate cell derived exosomes with different AR phenotypes (including: DU145, PC3, LNCaP, VCaP, C4-2 and RWPE-1) using Liquid Chromatography Mass Spectrometry. Our proteomic results yielded 50 candidate protein biomarkers which have been previously reported to have potential in diagnosis, prognosis, disease progression, efficacy and response to therapy for a variety of pathological diseases. Specifically, our proteomic data confirmed the presence of ANXA2, CLSTN1, FASN, FLNC, FOLH1 and GDF15 in PCa-derived exosomes. These proteins have all been previously reported as candidate biomarkers in PCa diagnosis (Hosseini-Beheshti *et al*., 2012).

Khan et al. (2012) report that survivin, an inhibitor of apoptosis, which is associated with PCa development, progression and drug resistance, exists in plasma-derived exosomes of normal, BPH and PCa subjects. The exosomal survivin levels were reported to be significantly higher in both low and high-grade PCa patient plasma derived exosomes when compared to BPH and/or control. This presents potential in that the elevated levels of this

protein in PCa could therefore be used as an early diagnostic biomarker for PCa (Khan *et al*., 2012).

The discovery of different potential biomarkers in cancer-derived exosomes and the presence and accessibility of these nanovesicles in almost all the biological fluids via minimally invasive procedures offers promise for better, easier and earlier detection of PCa. To this date there are more than 20 research articles that have published descriptions of protein profiling (Ronquist *et al.*, 2010)(Park *et al*., 2013)(Principe *et al*., 2013)(Wood *et al*., 2013) or genomic content analysis (Chen *et al*., 2011)(Bryant *et al*., 2012)(Hessvik *et al*., 2013) of cancer derived exosomes in the hopes of discovering novel biomarkers for PCa diagnosis and/or prognosis.

Recently in 2014, Corcoran et al. performed a global miRNA profiling of both PCa cell lines (PC3, DU145 and 22RV1 and their respective age matched docetaxel-resistant) and their exosomes. The clinical evaluation of their candidate miRNA supports the importance of miR-34a with PCa incidence and progression. Furthermore the expression of miR-34a seems to be decreased with biochemical recurrence making this miRNA an indicator of potential early treatment failure. The decreased level of miR-34a in PCa patient derived urinary exosomes compared to BPH or control donors suggests its potential as a predictive biomarker for response to Docetaxel (Corcoran *et al*., 2014). In a similar attempt Huang et al. (2014) and Neeb et al. (2014) identified two miRNA candidates, miR-1290 and miR-375 as well as two splice variant transcripts of the anterior gradient 2 gene AGR2- SV-G and SV-H as prognostic biomarkers of survival in CRPC patients and potential PCa diagnostic biomarkers, respectively (Huang *et al*., 2014)(Neeb *et al*., 2014).

In addition to their role in PCa progression and as potential biomarkers, exosomes

have been studied as novel cancer therapeutic delivery systems or therapeutic targets in PCa (Kooijmans *et al.,* 2012). In 2006, Goyal et al. reported exosomes as a delivery system for lycopene, a lipophilic antioxidant, which is thought to have a chemopreventive role in PCa. Specifically, their data showed that exosomes could provide a protective shield against lycopene degradation hence maximizing the effectiveness of its delivery to the site of action (Goyal *et al*., 2006).

MVA-BN-PRO (BN ImmunoTherapeutics) is a next-generation immunotherapeutic that may provide a higher frequency of protection or more prolonged survival by encoding 2 TAAPSA, and PAP product and is in a phase I clinical trial for the treatment of PCa. Rountree et al. (2011) have demonstrated that exosome targeting, by fusion of the antigen to ClC2 domain of lactadherin, improves the antigenicity of poorly immunogenic proteins and enhances therapeutic efficacy (Rountree *et al*., 2011).

Exosomes have also been studied as future nanoscale and cell-free cancer vaccines. Recent evidence suggests that dendritic-cell derived exosomes and tumor-cell derived exosomes have potential as novel immunotherapy treatments for cancer via their interaction with cells involved in the immune system, thereby priming the immune system to recognize and destroy cancer cells. As an example, engineered tumor-derived exosomes, which contain auto and allo-genic human MUC1 were shown to be immunologically effective and suppressed growth of human MUC1-expressing tumors in an MHC-independent manner (Cho *et al*., 2005).

In agreement with the examples described above, similar studies have revealed exciting results confirming the potential of these nanovesicles for use as cancer vaccines or potential new targets for cancer therapy.

1.4 Hypothesis and Specific Aims

The main hypothesis for this Ph.D. thesis is: **To assess exosomes as a source of potential future biomarkers for PCa diagnosis and investigate the role of exosomes in prostate cancer progression.**

In the second chapter of this Ph.D. thesis, I have hypothesized that exosomes derived from PCa cells contain a different cargo (protein and lipid) than normal or benign prostate cell lines and could therefore potentially be used as biomarkers in PCa diagnosis. We also speculate that these potential differences could be exploited in targeting strategies as a futuristic therapeuticapproach. Therefore the specific aims for this chapter was to first, isolate and purify exosomes from five different PCa cell lines with different AR phenotype including PC3 and DU145 cells which are AR –ve and represent the more aggressive type of PCa and LNCaP, VCaP and C4-2 cells which are AR +ve and represent earlier stages of PCa, as well as one benign epithelial prostate cell line, RWPE-1 (AR +ve). Secondly we characterize all of the isolated exosomes using different techniques and confirm their uptake by different PCa cell lines using confocal microscopy. For the third specific aim of this chapter we performed a comprehensive proteomic analysis of all six different prostate cell lines using mass spectrometry to understand differences between the protein profiles released via exosome externalization in different prostate cell lines. The final part of this study was to investigate the difference in broad classes of lipids and cholesterol as constituents of different prostate cell lines and their exosomes.

Taken together our findings in this chapter form a platform for future clinical validation research using exosomes as biomarkers for PCa diagnosis as well as potential therapeutic targets, which could be important in the treatment of CRPC.

The third chapter of this thesis was built upon our proteomic and cholesterol findings in the second chapter. Based on what we observed in our *in vitro* data we speculated that similar phenomena should be seen in exosomes derived from PCa patients when compared to healthy donors. Therefore, the first step and one of the main challenges for the research described in this chapter, as well as in the EVs field, was to develop methodology and protocols for exosome isolation and purification from biofluid components (blood and urine) of normal healthy controls and cancer patients, while removing unwanted housekeeping proteins during the isolation/purification step. The second part of Chapter Three was focused on characterizing isolated exosomes using TEM, WB and NTA. In follow up to the second chapter we performed a comprehensive MS-based proteomic analysis on exosome samples to understand the major differences between exosomes derived from blood vs. urine in healthy controls and cancer patient groups. As well as possible underlying differences in protein profiles an additional part of this study was to investigate the cholesterol level in exosome isolates and explore this as a potential differential diagnostic tool.

Taken together this study examines the potential of direct MS based proteomic and/or cholesterol profiling of exosomes derived from different biological fluids and its use for biomarker analysis in biological fluids.

In Chapter Four of this Ph.D. thesis we focus on the pivotal role of exosomes in tumorigenesis. We speculated that exosomes confer phenotypic changes in surrounding cells, which impact PCa progression, treatment resistance and metastasis. In order to address our overarching hypothesis in this chapter we treated three different prostate cell lines (LNCaP, DU145 and RWPE-1) with exosomes derived from two different PCa cell lines, LNCaP and DU145. We then studied the influence of these exosomes on different

functional assays designed to evaluate cellular processes including apoptosis, proliferation and migration. Lastly we studied the effect of exosomes derived from PCa cells on PSA level and tumor growth using the LNCaP human xenograft model representing hormone sensitive PCa. Our results fuel excitement for future research and we anticipate our work will be contributing significantly to current exosome research in the field of PCa.

Chapter 2: Exosomes as Biomarker Enriched Microvesicles: Characterization of Exosomal Proteins Derived from a Panel of Prostate Cell Lines with Distinct AR Phenotypes

2.1 Introduction

PCa is the leading type of cancer diagnosed in men. Prompt diagnosis of the disease can substantially improve its clinical outcome. Improving capability for early detection, as well as developing new therapeutic targets in advanced disease are research priorities that will ultimately lead to better patient survival.

The routine screening test for PCa diagnosis in North America includes measurement of PSA in the blood, DRE and a prostate biopsy (Lan *et al*., 2008). PSA screening for PCa detection is controversial because certain activities can induce the production of PSA, unrelated to the presence of cancer (Croswell *et al*., 2011). Consequently prostate biopsy, albeit an invasive procedure, remains the only definitive diagnostic test for PCa. There is an urgent current need, therefore, for the discovery of relevant biomarkers to replace the existing diagnostic tests for better, easier and earlier detection of PCa (Croswell *et al*., 2011).

One possible source of biomarkers, which could be used as part of a diagnostic test are exosomes. All cells produce and release exosomes which are often found in different body fluids such as plasma (Caby *et al*., 2005), serum (Taylor *et al*., 2006)(Taylor-Gercel-taylor *et al*., 2008), malignant ascites (Andre *et al*., 2002)(Brad *et al*., 2004), urine (Pisitkun *et al*., 2004), amniotic fluid (Asea *et al*., 2008), bronchoalveolar lavage fluid (Admyre *et al*., 2003)(Hawari *et al*., 2004) and breast milk (Admyre *et al*., 2007). Recent studies suggest however that cancer cells produce exosomes, which may be differentiated from those derived from normal cells primarily based upon their cargo. Exosomes are cup-shaped

(Thery *et al*., 2009) encapsulated by a bi-layer lipid membrane (Laulagnier *et al*., 2004) with a membrane-bound compartment varying between 30- 200 nm in size (Thery *et al*., 2009). As mentioned above, they are secreted from both normal cells and tumour cells and while the underlying mechanism of exosome function is not fully understood it is known that exosomes are formed in the endosomal compartment of cells and are secreted upon fusion of MVB with the plasma membrane (Thery *et al*., 2002).

Therefore, the main purpose of this chapter was to observe the release of exosomes by prostate cells, and determine characteristic differences between exosomes released by parent cells with different characteristic and AR phenotypes. In order to answer this question, in addition to one non-malignant cell line, we used five different PCa cell lines which contain/lack AR and were representative of different stages of PCa. We then confirmed the transfer of exosomes to target cells in culture using confocal microscopy of fluorescence labeled exosomes. We subsequently performed a comprehensive proteomic analysis of all six different prostate cell lines using mass spectrometry to understand differences between the protein profiles released via exosome externalization in different prostate cell lines. The final part of this study was to investigate the difference in broad classes of lipids and cholesterol as constituents of different prostate cell lines and their exosomes.

Taken together the comprehensive characterization of exosomes derived from prostate cell lines which have distinct AR +/-ve expression phenotypes, provides a basis for evaluating transfer of identified composite exosome proteins between different PCa cells as part of a recognized cell communication phenomenon. In addition this study forms a platform for future clinical validation research using exosomes as biomarkers for PCa diagnosis as well as potential therapeutic targets, which could be important in the treatment of

CRPC.

2.2 Materials and Methods

2.2.1 Cell Culture

PC3, DU145 and VCaP human PCa cells (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) while LNCaP and C4-2 cells (ATCC) were cultured in RPMI 1640, supplemented with 5% FBS (Invitrogen) and antibiotic, at 37°C in 5% CO_2. RWPE-1 (ATCC) cells also were grown in keratinocyte-SFM (KSFM) with growth supplement (GIBCO) and 1% penicillin-streptomycin (Invitrogen). Cells were grown to 60-70% confluency were washed with sterile PBS buffer and removed from serum and incubated in culture media for 72 hours for exosome purification and collection.

CLUGFP stably over-expressing LNCaP cells were maintained in 200 mg/ml G418 (Invitrogen) containing RPMI medium supplemented with 10% FBS (Invitrogen) and antibiotic, at 37°C in 5% CO_2.

2.2.2 Exosome Isolation

Exosomes were purified from the serum free media following exposure to different PCa cell lines including PC3, DU145, VCaP, LNCaP, C4-2 and benign epithelial prostate cell line, RWPE-1, as well as a CLUGFP stably over-expressing LNCaP cell line. For exosome purification, 200 ml of each cell line's conditioned medium was cleared by centrifugation at 6,000 g at 4°C for 10 minutes to remove protein aggregates and cell debris. The pre-cleared medium was concentrated to 2 ml using a 100 kDa MWCO Centricon Plus-20 filter capsule (Millipore, Billerica, MA). Samples were transferred to

ultracentrifuge tubes containing 300 µl of 30% sucrose-deuterium oxide (D_2O). Sample tubes were then ultracentrifuged using a fixed angel 70.1 Ti rotor (Beckman Coulter) at 100,000g for 1 hour at 4°C. Purified exosomes (350µl) were collected off the cushion of sucrose (350 µl exosomes were isolated from 10,000,000 originally seeded cells) (**Figure 2.1**).

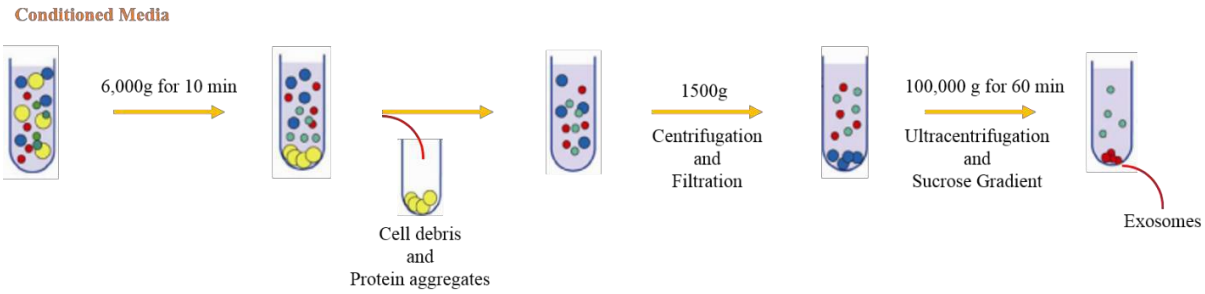


Figure 2.1 Exosomes enrichment workflow in conditioned media.

2.2.3 Transmission Electron Microscopy of Exosomes

Isolated exosomes (2.5 µl) were dried onto freshly glow discharged 300 mesh formvar/carbon-coated TEM grids (Ted Pella, Redding California, USA), negatively stained with 2% aqueous uranyl acetate and observed with a Hitachi H7600 TEM (Hitachi High-Technologies Corp., Tokyo, Japan) operated at 80kV. Images were captured with a side mounted 1K AMT Advantage digital camera (Advanced Microscopy Techniques, Corp. Woburn, MA, USA).

2.2.4 Western Blot Analysis

All samples of exosomes and cell lysates were analyzed for total protein concentration using the BCA protein determination kit (Sigma, Oakville, Ontario,

Canada). Twenty-five micrograms of total protein associated with purified exosomes were loaded on 12% acrylamaide gel. Relative enzyme levels were quantified using antibodies specific for exosome markers; mouse monoclonal β-Actin (1:1000 Sigma), rabbit polyclonal Lysosomal Associated Membrane Protein 2 (LAMP2) (1:1000 Abcam) rabbit monoclonal Tubulin, mouse monoclonal HSP70, goat polyclonal HSP90, mouse monoclonal Rab5, mouse monoclonal CD9 and mouse polyclonal CD63, (1:1000 Santa Cruz Biotechnology, Inc., Santa Cruz, CA). In order to evaluate the purity of the exosome preparations, all exosomes samples were also blotted against GRP94 (1:1000 Cell Signaling) to demonstrate the absence of cellular contaminants from cell lysate in our exosome preparation.

2.2.5 Exosome Labelling

PC3, VCaP, LNCaP, C4-2 and RWPE-1 cells were seeded (10,000 cells per chamber) in each chamber slide (Lab-Tek II chamber slide with cover, Thermo Fisher scientific) one day prior to exosome isolation. Fresh exosomes purified from DU145 cells (described above) were labeled with Cell Tracker^{TM} Orange CMTMR teramethylrhodamine (0.5 µM, Invitrogen) and incubated with different prostate cell lines for 12 hours at 37°C and 5% CO_2. After 12 hours prostate cells were washed twice with PBS and fixed with 4% paraformaldehyde. Finally all slide chambers were mounted with Vectashield H-1200 (Vector Laboratories Inc.) containing DAPI for nuclei staining. Internalization of fluorescent DU145 exosomes by other cancerous and benign prostate cell lines was monitored under Zeiss (LSR780) confocal microscope (Carl Zeiss, Thornwood, NY). The pinhole was set at 1 Airy unit.

In an independent experiment and in order to confirm the uptake of a tagged protein from exosomes, fresh isolated exosomes derived from CLUGFP stably over-expressing

LNCaP cell line were incubated with PC3 (AR-ve) and LNCaP (AR+ve) for 12 hours at 37°C and 5% CO_2. After removal of media, cells were fixed in ice-cold MeOH/Acetone (3:1) for 10 minutes, and then washed in TBS buffer and permeabilized in 0.1% Triton X-100 in TBS for 15 minutes at room temperature (RT). Non-specific binding was avoided by blocking in odyssey solution for 30 minutes at RT. Primary purified mouse anti E-Cadherin was diluted (1:250 BD Transduction Laboratories™) in blocking agent and incubated with cells for 1 hr at RT. Secondary antibody Alexa Fluor 568 goat antimouse IgG (2 mg/ml, Invitrogen) was incubated with cells for 30 minutes at RT. Finally, as described above, all slide chambers were mounted and monitored using confocal microscopy.

2.2.6 Proteomic Analysis of Exosomes

An in solution trypsin digestion protocol was used to generate peptides for Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry (LC-QTOF MS) analysis. The isolated exosomes were initially sonicated for 5-10 minutes to disrupt the structures and expose proteins. An aliquot of extracted exosomes (~2µg/µl protein) was diluted with 8 µl 50 mM ammonium bicarbonate, 1 µl of 100 mM dithiothreitol (DTT) was added and reduction carried out at 65°C for 35 minutes. This was followed by alkylation using 2 µl of 100 mM iodoacetamide and incubation for 30 minutes at RT in the dark. 1 µl of 100 ng/µl trypsin was then added and the sample incubated overnight at 37°C. The resulting peptides were separated using a 75 µm x 100 mm 1.7µm BEH130 C18 column using a 3-40% linear acetonitrile gradient, with 0.1% Formic Acid (FA) present, at 0.3 µl/min over 40 minutes using a NanoAcquity™ Liquid Chromatography (LC) (Waters). The column was re-equilibrated for 20 minutes between runs. Column elute was directed into a Synapt™ mass

spectrometer through a 20 μm capillary held at 3.2 kV. Instrument calibration was carried out using glu-fibrinogen fragments and Glu-fib was also used as a lock mass to compensate for any calibration drift. The instrument was run in V-mode with a mass resolution of approximately 10,000. A data dependent method was used with a 1 second scan followed by up to 3 fragment scans, using ion intensity and charge state as the main selection criteria. The accumulated data was analysed using ProteinLynx Global Server software (PLGS2.3) using peptide and fragment mass accuracies of 25 ppm and 0.1 Da respectively. Uniform carbamido methyl C and variable N-terminal acetyl, M oxidation, N deamidation and C pripionamide were selected as permitted modifications, up to one missed cleavage allowed and a maximum protein MW of 250 K. This search engine was applied to the full Uniprot 15.0 database, human species. A search of SwissProt 57.1, Homo Sapiens (human; 20401 sequences) was also carried out using Mascot (2.3) search using the pkl peak list files generated in PLGS. An ion score cutoff of 32 is specified as identical or highly homologous according to Mascot outputs. A score ≥40 is typical in many reports and we similarly considered peptides above this cutoff as positive hits. Only proteins with ≥2 positive peptide hits are considered statistically significant. As well, no proteins containing a subset of peptides within our proteins hits were considered in our evaluation. Spectral counting could be used for a semi-quantitative analysis however only the presence of particular proteins is reported here.

2.2.7 Pathway Analysis

Data generated from proteomic mass spectrometric analysis were analyzed using Ingenuity Pathway Analysis (IPA; Ingenuity System, Redwood city, CA,

www.ingenuity.com) to identify potential biomarker signatures and their application in different pathological conditions, in particular cancer and specifically PCa. In addition, we randomly selected 220 proteins from 827109 human proteins and used IPA to identify general biomarker signature as well as PCa specific biomarkers. This was compared with exosomal proteins and biomarker data to increase confidence in conclusions drawn from the IPA data. Ingenuity Knowledge Base tool was also used to identify biological function and canonical pathways that identified exosomal proteins are involved in. IPA is a regularly updated database, which uses the current knowledge available on genes, proteins, normal cellular and pathological processes, signaling and metabolic pathways, needed for pathway construction.

2.2.8 Lipidomic Analysis

Isolated exosomes and cell pellets were extracted using a protocol adapted from that published by Matyash et al. (2008) which is equivalent to the Folch and Bligh and Dyer recipes (Matyash *et al.*, 2008)(Bligh and Dyer, 1959) in terms of efficiency. Isolated exosomes (30 µl) or vortexed cell pellets (10 µl) were extracted by addition of 200 µl of methanol followed by 900 µl of MTBE (methyl-t-butyl ether), vortexing well and rotating at room temperature for 30 minutes. 500 µl of water was then added and the sample rotated for an additional 10 minutes. The phases were separated by centrifugation (10 min) using a Centrivap (~1000g) and the upper organic layer transferred to a fresh tube. A second extraction with an additional 500 µl of MTBE was similarly carried out and pooled with the initial fraction. The pooled extracts were dried in a Centrivap, redissolved in 60 µl of 70/30 methanol/chloroform, further diluted with 140 µl methanol and centrifuged at

15,000 g for 5 min before transferring to LC vials.

The extracts were separated with an Acquity™ LC (Waters) using a 2.1 mm x 100 mm 1.7 µm BEH C8 column with 20 mM ammonium acetate (A) and 80/20 methanol/isopropanol (B) using the following gradient: 0 min, 25%; 0.2 min, 25%; 3 min, 65%; 25 min, 95%; 40 min, 95% (%B) and a 5 min re-equilibration to initial conditions. The LC was coupled to a Synapt™ mass spectrometer run in W-mode with a mass resolution of approximately 18,000. Instrument calibration was carried out using sodium formate ion clusters and leucine-enkaphalin was usedas a lock mass to compensate for any calibration drift. Data was accumulated sequentially as 0.5 slow collision energy scans followed by 0.2 s higher collision scans. Masses unique to extracts, i.e. not present in blank extracts, were submitted to Lipid Maps and correlated with bulk glycerophospholipid and glycerolipid, sphingolipid and glycosphingolipid class using a 0.01 m/z cut-off. A comparative quantitative assessment of the content of each lipid class present in the samples was accomplished by obtaining extracted ion chromatograms for each m/z, retention time combination using Quanlynx. While differences in ionization efficiency are expected, the area under curve (AUC) for each peak thus obtained still provides a reasonable quantitative estimate for the particular lipid present. The AUC data was exported to Excel and the relative amount of each bulk lipid group calculated as (sum of all AUC's for a particular bulk lipid group)÷ (sum of AUC's for all bulk lipid groups) x 100%.

2.2.9 Cholesterol Analysis

The extraction for cholesterol analysis was similar to that used for lipid analysis except the samples were each spiked with 200 ng of deuterated (d6) cholesterol internal

standard and were derivatization carried out according to the method of Liebisch et al. (2006) prior to analysis. Dried extracts were dissolved in 400 µl of acetyl chloride:chloroform 1:5 and incubated at room temperature for 1 hour. Samples were placed on ice, 500 µl of water added to each and then vortexed several times for 30 minutes. The bottom organic layer was then transferred to a fresh tube and the sample extracted with an additional 0.5 ml of chloroform for 30minutes. The extracts were pooled, dried in a Centrivap, redissolved in 60 µl of 70:30 methanol:chloroform, further diluted with 140 µl methanol and centrifuged at 15,000 g for 5 min before transferring to LC sample vials. LC-MS analysis was carried out with a Waters Acquity UPLC coupled to a Quattro Premier XE using a 2.1x50 mm, BEH 1.7µM C18 column. Mobile phase consisted of acetonitrile/0.1M ammonium acetate 9/1 (A) and isopropanol (B) with the following gradient: 0.2 min, 25%; 5-8 min, 70%, 8.1 min, 25% (%B) with a 10 min run length. Instrument parameters were optimized for the m/z's of ammonium adducts of acetate derivatized cholesterol and the m/z 369 fragment was used for MRM quantitation. AUC's for cholesterol acetate and d6 cholesterol acetate were obtained using Quanlynx and a linear calibration curve from 0.2-10µg/ml, R^2>0.99, was generated using AUC ratios. Recoveries and conversions to derivatized species was greater than 90% for cholesterol.

2.2.10 Statistical Analysis

Unless indicated, analyses were performed on data generated from triplicate exosome preparations per cell line and their cell lysates. Results were expressed as mean ± standard deviation. Statistical significance for differences between exosomes derived from different prostate cell lines and their corresponding cell lysate samples were evaluated by Student's t-test ($p<0.05$). In order to compare more than two groups of cell lines or exosomes (e.g. AR+ve PCa cell line exosomes/cell lysate versus all other PCa cell lines) data were analyzed using one-way analysis of variance (ANOVA)(parametric) or Kruskal-Wallis one-way ANOVA (nonparametric), followed by Student Newman Keuls multiple comparison test (SigmaStat™ Statistical Software, Version 3.1, SPSS Inc., Chicago, IL, USA). Biomarker analyses were statistically analyzed using Fisher's Exact Test.

2.3 Results

2.3.1 Purification and Characterization of Exosomes Derived from Different Prostate Cells

The production of exosomes from PC3, DU145, VCaP, LNCaP, C4-2 PCa and RWPE-1 benign prostate cells was examined. To validate exosome isolation and purification, transmission electron microscopies as well as WB analysis were utilized. Isolated exosomes from all six different prostate cell lines were fixed onto formvar-coated carbon EM grids for visualization by TEM. TEM data revealed that all six prostate cell lines released a homogenous mixture of cup- shaped, rounded vesicles with diameters varying between 30-200 nm (**Figure 2.2**).

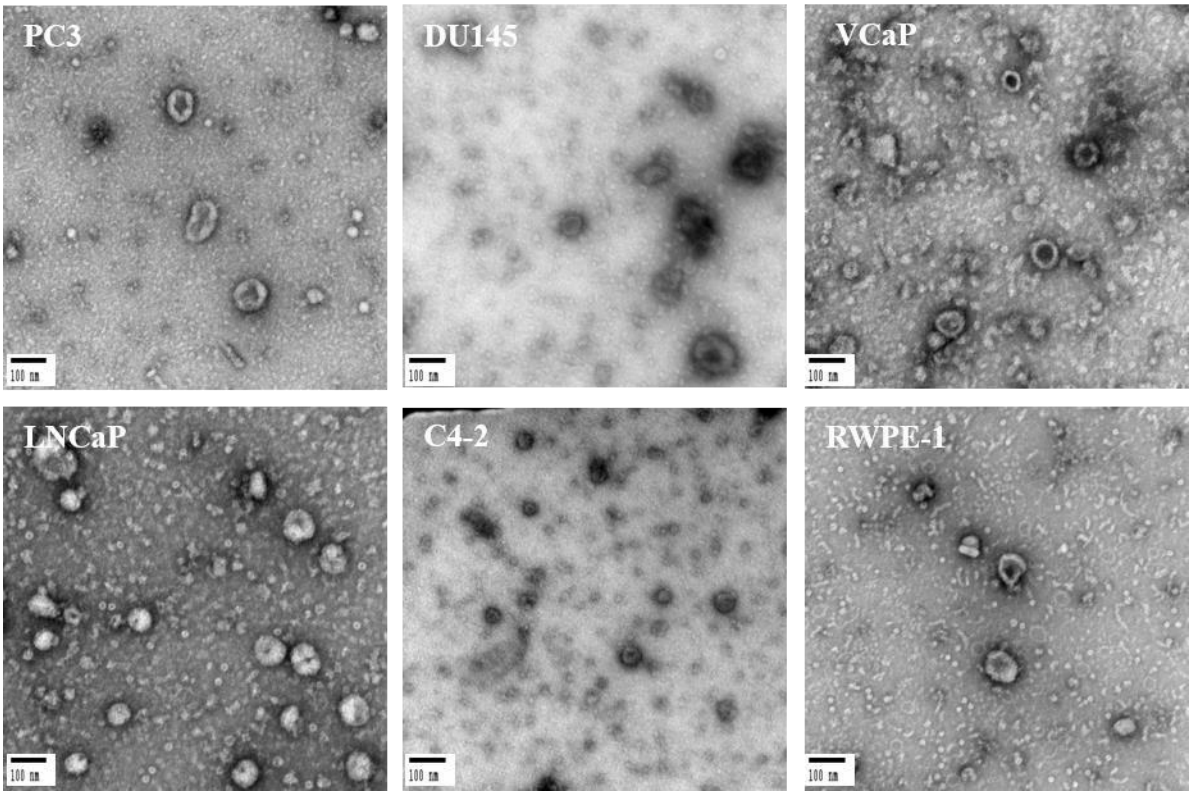


Figure 2.2 Transmission Electron Microscopy.

TEM images of exosomes derived from different androgen independent and androgen sensitive PCa cell lines including PC3, DU154, VCaP, LNCaP and C4-2 versus benign epithelial prostate cell line RWPE-1. Exosomes were negatively stained with 2% uracyl acetate after removing the extra moisture. Cup-shaped structures, with 30-200 nm size were identified as being exosomes. Protein concentration of vesicles was quantified using a BCA assay. WB analysis was performed to examine the expression of different exosomal markers in whole cell lysate as compared with derived exosomes of various prostate cell lines. Different exosomal markers were identified in exosomes derived from all six prostate cell lines.

As shown in **Figure 2.3 A.**, exosome samples contained at least four or more of the following markers: Actin, Tubulin (cytoskeletal protein), HSP70, HSP90 (Heat Shock Protein), CD9, CD63 (Tetraspanin) Rab5 (small GTPase) and LAMP2. Further, we investigated the presence of the above proteins in the corresponding cell lysate. As anticipated, HSP90, a stress marker often associated with cancer (McCready *et al.*, 2010),

was not enriched in either cell lysate or exosomes from the benign epithelial prostate cell line RWPE-1 however, substantial amounts of HSP90 were observed in the PCa cell lines. Interestingly, while the exosomes contained CD9 and CD63 (**data not shown**) we found little or no detectable band for these proteins in their parent cell lines (**Figure 2.3 B.**).

In order to confirm the purification of our exosome isolate, we analysed all exosome samples for the presence of GRP94 (ER marker). Our data indicates the absence of GRP94 in five of six exosome samples, with a very faint band seen in exosomes derived from PC3 cell line. Taken together, the WB (molecular) and TEM (biophysical) data demonstrate that both cancerous and benign prostate cells produce exosomes that have similar characteristics to those secreted from other cells. Our preparation technique, which consisted of several centrifugation steps along with filtration, and a final ultracentrifugation step using a sucrose cushion, decreases contamination with cell debris and produces high quality purified exosome free from other membrane vesicles or protein aggregates.

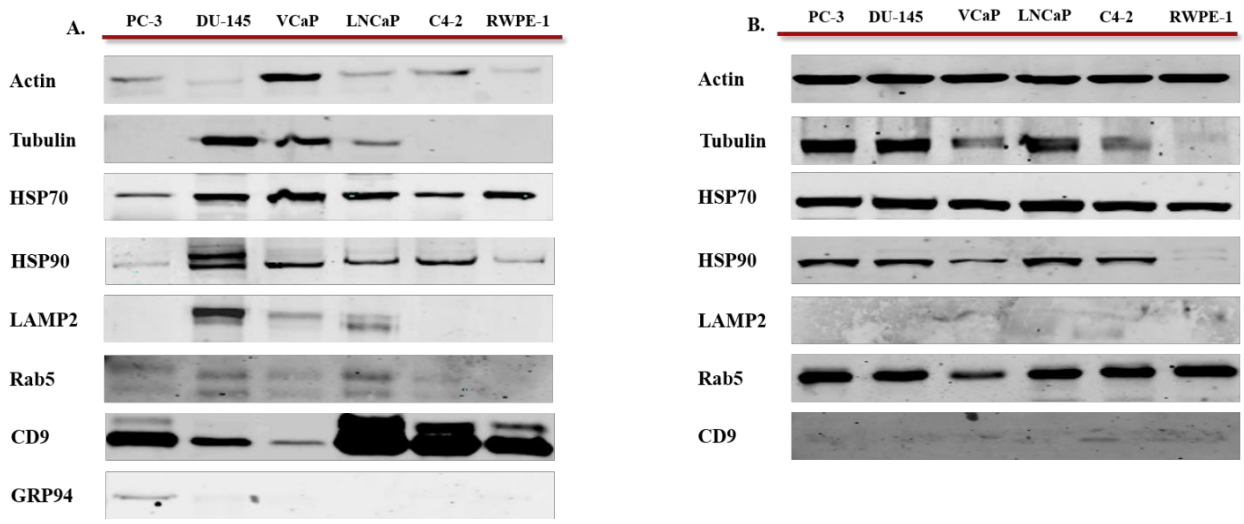


Figure 2.3 Western Blot analysis for exosomes marker.

Exosomes samples **A.** and cell lysate samples **B.** Twenty-five micrograms of total protein associated with purified exosomes derived from six different prostate cell lines **A.** and cell lysates of six different prostate cell lines **B.** were analyzed by Western Blotting using different exosome markers.

2.3.2 Uptake of Exosomes by Different Prostate Cells

We also investigated the uptake of exosomes derived from DU145 cells by five other prostate cell lines, to assess if released exosomes are taken up by surrounding cells after release. We isolated and purified exosomes from DU145 cells as this cell line is known to be one of the most aggressive PCa cell lines and also produced the highest yield of exosomes (**Appendix**).

Purified DU145 exosomes were first stained with Cell Tracker^{TM} Orange CMTMR tetramethylrhodamine. Once stained, the exosomes were used to demonstrate their transfer to surrounding cells. Stained DU145 exosomes were incubated with PC3, VCaP, LNCaP, C4-2 and RWPE-1 for 12 hours (overnight). Cells were then fixed and stained with DAPI to mark nuclei prior to imaging of the cells using confocal microscopy.

Figure 2.4 A. clearly shows the uptake of exosomes by all five prostate cell lines as demonstrated by the presence of orange/red flecks in and around the cells. Furthermore, the z-stack confocal fluorescence imaging in **Figure 2.4 B.** clearly shows cellular uptake of DU145 exosomes by other prostate cells. This image demonstrates clearly that transferred exosomes are not only attached to the cell membrane of host cells but have actually been taken up by these cells and are present in their cytoplasm. Interestingly, exosomes appear to be concentrated around the nuclei of benign RWPE-1 cells as compared with cancer cells in which they are scattered within the cytoplasm.

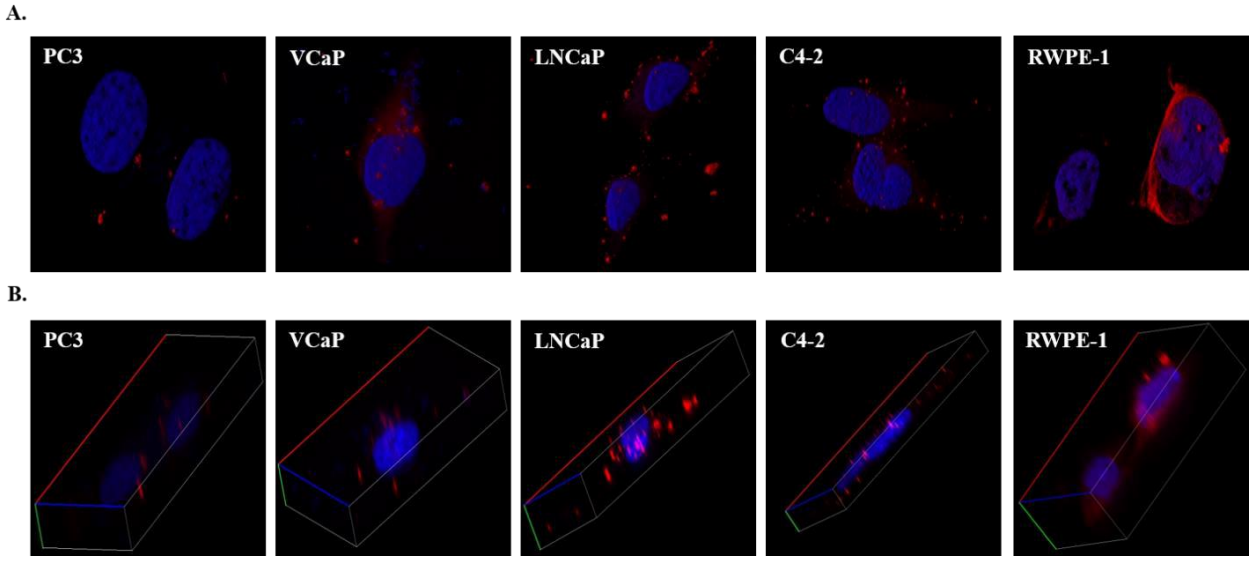


Figure 2.4 Confocal microscopy.

Confocal microscopy was used to visualize purified DU145 derived exosomes, which were stained with Cell Tracker^{TM}Orange CMTMR teramethylrhodamine. PC3, VCaP, LNCaP, C4-2 and RWPE-1 cells (10^4) were cultured on each chamber slide and incubated for 12 hours with purified- stained exosomes. Confocal micrograph clearly demonstrates that transferred DU145 derived exosomes are not only attached to the cell membrane of host cells but have actually been taken up by these cells and are present in their cytoplasm.

In a separate experiment we isolated exosomes from CLU_{GFP} stably over-

expressing LNCaP cell line. Isolated exosomes which contained CLU_{GFP} were incubated with PC3 (AR –ve) and LNCaP (AR +ve) for 12 hours (overnight) cells were further fixed and stained with DAPI and E-Cadherin prior to imaging of the cells with confocal microscopy (**Figure 2.4 C.**).

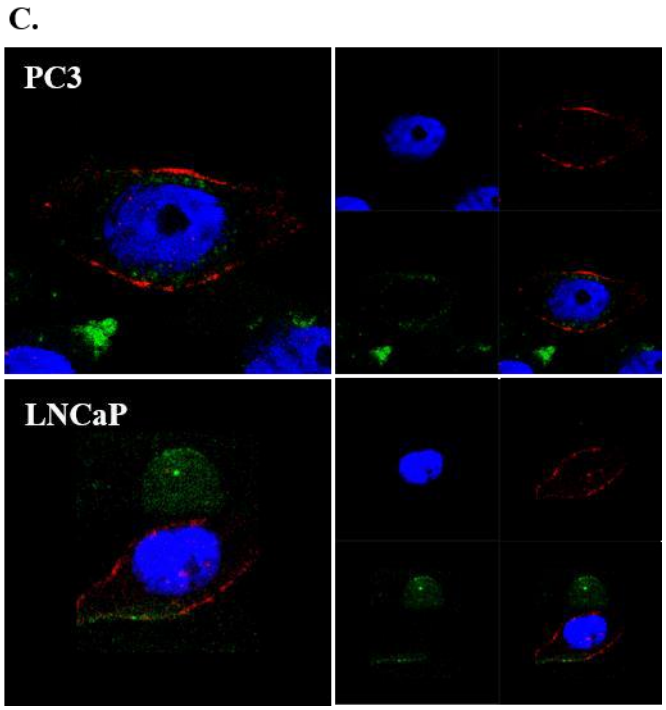


Figure 2.4 C. Confocal microscopy.

Confocal microscopy was also used to visualize freshly isolated exosomes derived from a CLU_{GFP} stably over-expressing LNCaP cell line, which contains CLU_{GFP} , being taken up by PC3 (AR-ve) and LNCaP (AR+ve) PCa cell lines after overnight incubation. Both cell lines were further fixed and stained with DAPI and E-Cadherin prior to imaging of the cells by confocal microscopy.

2.3.3 Proteomic Analysis of Exosomes

The main goal of our proteomic analysis was to understand the potential role of exosomes in the tumour microenvironment as mediators of cell-cell communication during PCa progression. We therefore established a comprehensive list of proteins present

in the different prostate cell lines. Two biological replicates of each cell line were obtained to ensure consistent results. Exosomes were sonicated and trypsin digested to release exosomal peptides, which were analyzed by LC-MS as described. Proteins were identified using ProteinLynx Global Server software (PLGS) and Mascot software. Proteomic profiles of two AR null PCa cell lines (DU145 and PC3) were compared to AR +ve cells including; VCaP, LNCaP and C4-2 (PCa Cells) and RWPE-1 (benign epithelial prostate cell line) cells.

This process resulted in the identification of 220 proteins with more than 2 matching peptides and a Mascot score higher than 40 (**Table 2.1**) representing a broad range of functional proteins. A comparison of our proteomic data with other published articles (>25 articles reviewed on exosomes derived from a variety of sources ranging from human cell lines including human keratinocytes, human breast carcinoma cells, human mesothelioma cells, cortical neurons, dendritic cells, intestinal epithelial cells to biological fluids such as plasma and urine) confirmed the presence of more than 58 common and 159 unique proteins in exosomes derived from the different prostate cells we analyzed. These include heat shock proteins, cytoskeletal proteins, tetraspanins, multivescular bodies and endosomal markers. A list of all the identified protein with their subcellular localization, type, Mascot score and number of peptide matches is provided in **Table 2.1**. This Proteomic data is also available for download at ExoCarta, a free database containing information on exosomal proteins, RNA and Lipids (Mathivanan *et al*., 2012) (Simpson *et al*., 2012).

Table 2.1 has been organized based on the subcellular localization of proteins where known. As shown in **Table 2.1**, 85% of nuclear proteins belong to the Histone family. The other 15% include nucleophosmins-involved in the transport of small proteins to the nucleus- and RBP2, an enzyme implicated in vitamin A uptake and intracellular metabolism.

The largest fraction of proteins identified in exosomes were enzymes which were classified as peptidases, kinases and phosphatases as well as other regulatory proteins such as *14-3-3* which plays a key role in ERK5 signaling, IGF-1 signalling, Myc mediated apoptosis signaling, PI3K/AKT signaling and protein kinase A signaling or *ATP citrate lysate,* important in citrate cycle; insulin receptor signaling, and *ACTC* involved in calcium and caveolar-mediated endocytosis signaling.

Table 2.1 Identification of exosome-associated proteins from six different prostate cell lines.

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
H2A2B_HUMAN	Histone cluster 2, H2ab	Nucleus	other			+	+	+		79	3	
H2AW_HUMAN	H2A histone family, member Y2	Nucleus	other					+		42	2	
H2AX_HUMAN	H2A histone family, member X	Nucleus	other				+	+		119	4	
H2AY_HUMAN	H2A histone family, member Y	Nucleus	other					+		54	2	
H2B1B_HUMAN	Histone cluster 1, H2bb	Nucleus	other			+	+	+	+	111	2	
H2B1D_HUMAN	Histone cluster 1, H2bd	Nucleus	other		+	+				148	3	
H2B1H_HUMAN	Histone cluster 1, H2bh	Nucleus	other				+			226	4	
H2B1M_HUMAN	Histone cluster 1, H2bm	Nucleus	other		+					106	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
H2B3B_HUMAN	Histone cluster 3, H2bb	Nucleus	other				+			145	5	
H12_HUMAN	Histone cluster 1, H1c	Nucleus	other					+		41	3	
H15_HUMAN	Histone cluster 1, H1b	Nucleus	other					+		48	2	
NPM_HUMAN	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	Nucleus	transcription regulator		+	+	+	+		103	2	
RBP2_HUMAN	RAN binding protein 2	Nucleus	enzyme					+		48	3	
1433E_HUMAN	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	Cytoplasm	other	+	+	+		+	+	129	2	1
1433T_HUMAN	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	Cytoplasm, nucleus	Other		+	+				112	2	
1433Z_HUMAN	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Cytoplasm	enzyme		+					119	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
ACLY_HUMAN	ATP citrate lyase	Cytoplasm	enzyme		+	+	+			133	2	
ACPH_HUMAN	N-acylaminoacyl-peptide hydrolase	Cytoplasm	peptidase					+		116	3	
ACTA_HUMAN	Actin, alpha 2, smooth muscle, aorta	Cytoplasm	other	+	+	+	+	+	+	173	3	
ACTB_HUMAN	Actin, beta	Cytoplasm	other	+	+	+	+	+	+	132	2	1, 3, 4,5, 6, 7, 8, 9, 10, 11
ACTC_HUMAN	Actin, alpha, cardiac muscle 1	Cytoplasm	enzyme		+	+				393	9	
ACTG_HUMAN	Actin, gamma 1	Cytoplasm	other		+					409	6	1, 3, 4, 5, 6, 7, 8, 10, 11, 12
ACTN1_HUMAN	Actinin, alpha 1	Cytoplasm	other	+	+	+			+	267	6	1
ACTN2_HUMAN	Actinin, alpha 2	Cytoplasm, nucleus	transcription regulator		+	+				54	2	
ACTN3_HUMAN	Actinin, alpha 3	Cytoplasm	other		+	+				107	2	
ACTN4_HUMAN	Actinin, alpha 4	Cytoplasm	other		+	+				514	8	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
ALDOA_HUMAN	Aldolase A, fructose-bisphosphate	Cytoplasm	enzyme		+	+	+		+	181	5	1, 2, 3, 13
ALDOC_HUMAN	Aldolase C, fructose-bisphosphate	Cytoplasm	enzyme		+					101	2	
CALR_HUMAN	Calreticulin	Cytoplasm	transcription regulator	+						90	2	
CAND1_HUMAN	Cullin-associated and neddylation-dissociated 1	Cytoplasm	transcription regulator		+					56	2	
CATD_HUMAN	Cathepsin D	Cytoplasm	peptidase	+						4	1, 13 (preprotein)	
CH60_HUMAN	Heat shock 60kDa protein 1 (chaperonin)	Cytoplasm	enzyme				+	+		295	5	
COF2_HUMAN	Cofilin 2 (muscle)	Cytoplasm, nucleus	other		+					74	2	
DPOG1_HUMAN	Polymerase (DNA directed), gamma	Cytoplasm	enzyme			+				40	4	
DPYL2_HUMAN	dihydropyrimidinase-like 2	Cytoplasm	enzyme		+					204	4	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
EF1A1_HUMAN	Eukaryotic translation elongation factor 1 alpha 1	Cytoplasm	translation regulator		+	+	+			75	3	
EF1A2_HUMAN	Eukaryotic translation elongation factor 1 alpha 2	Cytoplasm	translation regulator		+					158	4	
EF1G_HUMAN	Eukaryotic translation elongation factor 1 gamma	Cytoplasm	translation regulator		+	+	+			108	2	
EF2_HUMAN	Eukaryotic translation elongation factor 2	Cytoplasm	translation regulator		+	+	+	+		244	7	1
EIF3A_HUMAN	Eukaryotic translation initiation factor 3, subunit A	Cytoplasm	translation regulator		+					46	2	
ENOA_HUMAN	Enolase 1, (alpha)	Cytoplasm	transcription regulator		+	+			+	379	7	2, 15, 16
ENOB_HUMAN	Enolase 3 (beta, muscle)	Cytoplasm	enzyme		+					245	4	
ENOG_HUMAN	Enolase 2 (gamma, neuronal)	Cytoplasm	enzyme		+					176	2	
ENPL_HUMAN	Heat shock protein 90kDa beta (Grp94), member 1	Cytoplasm	other	+	+					172	3	1

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
FAS_HUMAN	Fatty acid synthase	Cytoplasm	enzyme		+	+	+	+		112	3	
FLNA_HUMAN	Flamin A, alpha	Cytoplasm	other		+	+				102	2	1
FLNB_HUMAN	Filamin B, beta	Cytoplasm	other		+	+				212	4	1, 13
FLNC_HUMAN	Filamin C, gamma	Cytoplasm	other		+	+				63	2	
FSCN1_HUMAN	Fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	Cytoplasm	other		+					50	2	
GLU2B_HUMAN	Protein kinase C substrate 80K-H	Cytoplasm	enzyme	+						129	3	
G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasm	enzyme	+	+	+	+	+	+	403	6	1, 2, 3, 4 , 5, 6, 7, 8, 9, 10, 17
GANAB_HUMAN	Glucosidase, alpha; neutral AB	Cytoplasm	enzyme	+		+				207	2	
GRP78_HUMAN	Heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	Cytoplasm	other	+	+					121	3	3, 11

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
HS71L_HUMAN	Heat shock 70kDa protein 1-like	Cytoplasm	other				+			126	4	
HS90A_HUMAN	Heat shock protein 90kDa alpha (cytosolic), class A member 1	Cytoplasm	other		+	+		+		316	7	1, 15, 16, 18 (15, 16, 18 listed as HSP90)
HS90B_HUMAN	Heat shock protein 90kDa alpha (cytosolic), class B member 1	Cytoplasm	other	+	+	+	+	+	+	472	11	1, 15, 16, 18 (15, 16, 18 listed as HSP90)
HS905_HUMAN	Putative heat shock protein HSP 90-alpha A5 OS=Homo sapiens GN=HSP90AA5P PE=1 SV=1	Cytoplasm	Other		+					106	4	
HSP7C_HUMAN	Heat shock 70kDa protein 8	Cytoplasm	enzyme		+	+				198	5	3, 4, 5, 6, 7, 8, 9, 10, 12, 17, 19, 20

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
HSP71_HUMAN	Heat shock 70 kDa protein 1 OS=Homo sapiens GN=HSPA1A PE=1 SV=5	Cytoplasm	Other				+			107	4	
HSPB1_HUMAN	Heat shock 27kDa protein 1	Cytoplasm	other						+	124	2	1, 13
IMB1_HUMAN	Karyopherin (importin) beta 1	Cytoplasm, nucleus	transporter			+				147	4	
IPO5_HUMAN	Importin 5	Cytoplasm, nucleus	transporter		+	+				57	2	
K1C10_HUMAN	Keratin 10	Cytoplasm	other					+		200	7	1
K1C14_HUMAN	Keratin 14	Cytoplasm	other						+	203	4	1
K1C9_HUMAN	Keratin 9	Cytoplasm	other					+	+	87	2	1
K2C1_HUMAN	Keratin 1	Cytoplasm	other	+						69	2	
K22E_HUMAN	Keratin 2	Cytoplasm	other					+		143	3	
K2C5_HUMAN	Keratin 5	Cytoplasm	other						+	200	5	1
K2C8_HUMAN	Keratin 8	Cytoplasm	other		+	+		+		113	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
KLHL4_HUMAN	Kelch-like 4 (Drosophila)	Cytoplasm	other		+					41	3	
KPYM_HUMAN	Pyruvate kinase, muscle	Cytoplasm	kinase	+	+	+	+	+	+	834	15	1
KPYR_HUMAN	Pyruvate kinase, liver and RBC	Cytoplasm	kinase		+	+				45	2	
LDHA_HUMAN	Lactate dehydrogenase A	Cytoplasm	enzyme	+	+	+	+	+		228	5	1, 2
LDH6B_HUMAN	L-lactate dehydrogenase A-like 6B OS=Homo sapiens GN=LDHAL6B PE=1 SV=3	Cytoplasm	Enzyme/ oxidoreductase		+					53	2	
LDHB_HUMAN	Lactate dehydrogenase B	Cytoplasm	enzyme		+	+		+	+	256	3	2
MDHC_HUMAN	malate dehydrogenase 1, NAD (soluble)	Cytoplasm	enzyme		+					49	2	
MX1_HUMAN	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	Cytoplasm, nucleus	enzyme			+				119	3	
MYH9_HUMAN	myosin, heavy chain 9, non-muscle	Cytoplasm	enzyme		+					88	2	1, 13, 21
NDKA_HUMAN	non-metastatic cells 1, protein (NM23A) expressed in	Cytoplasm, nucleus	kinase		+					113	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
NQO1_HUMAN	NAD(P)H dehydrogenase, quinone 1	Cytoplasm	enzyme			+				78	2	
NUCL_HUMAN	nucleolin	Cytoplasm, nucleus	other				+			54	3	
PGAM1_HUMAN	phosphoglycerate mutase 1 (brain)	Cytoplasm	phosphatase		+					119	2	1, 3
PGAM2_HUMAN	phosphoglycerate mutase 2 (muscle)	Cytoplasm	phosphatase		+					60	2	
PGK1_HUMAN	phosphoglycerate kinase 1	Cytoplasm	kinase		+					138	3	1
PLOD1_HUMAN	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	Cytoplasm	enzyme	+						274	5	
PRDX1_HUMAN	peroxiredoxin 1	Cytoplasm	enzyme		+	+				98	4	3
PRDX2_HUMAN	peroxiredoxin 2	Cytoplasm	enzyme			+				49	2	
PRGR_HUMAN	progesterone receptor	Cytoplasm, nucleus	ligand-dependent nuclear receptor				+	+		40	2	
PROF1_HUMAN	profilin 1	Cytoplasm	other		+	+				59	3	
PSA1_HUMAN	proteasome subunit, alpha type, 1	Cytoplasm	peptidase		+	+	+	+	+	73	3	1, 2

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
PSA2_HUMAN	proteasome subunit, alpha type, 2	Cytoplasm	peptidase		+	+				239	5	1, 2
PSA3_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 3	Cytoplasm	peptidase		+	+		+		130	2	
PSA4_HUMAN	proteasomesubunit, alpha type, 4	Cytoplasm	peptidase		+	+	+	+	+	203	3	1, 2
PSA5_HUMAN	proteasome subunit, alpha type, 5	Cytoplasm	peptidase		+	+	+	+	+	110	3	1
PSA6_HUMAN	proteasome subunit, alpha type, 6	Cytoplasm	peptidase		+	+	+	+	+	218	5	2
PSA7_HUMAN	proteasome subunit, alpha type, 7	Cytoplasm	peptidase		+	+		+		206	3	1, 2
PSA7L_HUMAN	proteasome (prosome, macropain) subunit, alpha type, 8	Cytoplasm	peptidase					+		109	2	
PSB1_HUMAN	proteasome subunit, beta type, 1	Cytoplasm	peptidase		+	+			+	191	4	2
PSB2_HUMAN	proteasome subunit, beta type, 2	Cytoplasm	peptidase		+	+		+	+	81	3	2
PSB4_HUMAN	proteasome (prosome, macropain) subunit, beta type, 4	Cytoplasm	peptidase		+	+				74	2	
PSB5_HUMAN	proteasome subunit, beta type, 5	Cytoplasm	peptidase		+		+		+	165	3	1, 2

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
PSB6_HUMAN	proteasome subunit, beta type, 6	Cytoplasm	peptidase		+	+	+	+		92	2	1, 2
PSB7_HUMAN	proteasome (prosome, macropain) subunit, beta type, 7	Cytoplasm	peptidase		+					68	3	
PSB9_HUMAN	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	Cytoplasm	peptidase			+				123	3	
QSOX1_HUMAN	quiescin Q6 sulfhydryl oxidase 1	Cytoplasm	enzyme					+		155	3	1
RADI_HUMAN	radixin	Cytoplasm	other		+					69	2	
RAN_HUMAN	RAN, member RAS oncogene family	Cytoplasm, nucleus	enzyme		+	+				71	2	
RLA1_HUMAN	ribosomal protein, large, P1	Cytoplasm	other			+				43	2	
RSSA_HUMAN	ribosomal protein SA	Cytoplasm	translation regulator		+					118	2	
RLA2_HUMAN	ribosomal protein, large, P2	Cytoplasm	other		+	+				73	2	
S10A6_HUMAN	S100 calcium binding protein A6	Cytoplasm	transporter		+					72	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
S10AB_HUMAN	S100 calcium binding protein A11	Cytoplasm	other		+					95	2	
SAHH_HUMAN	Adenosylhomocysteinase	Cytoplasm	enzyme		+	+				85	2	
SYTC_HUMAN	threonyl-tRNA synthetase	Cytoplasm, nucleus	enzyme		+					44	2	
TAGL2_HUMAN	transgelin 2	Cytoplasm	other		+					105	3	
TBA1A_HUMAN	tubulin, alpha 1a	Cytoplasm	other					+		294	5	
TBA1B_HUMAN	tubulin, alpha 1b	Cytoplasm	other	+	+	+	+	+	+	263	5	
TBA1C_HUMAN	tubulin, alpha 1c	Cytoplasm	other		+		+	+		249	5	
TBA3C_HUMAN	Tubulin alpha-3C/D chain OS=Homo sapiens GN=TUBA3C PE=1 SV=3	Cytoplasm	Other		+	+	+	+		149	3	
TBA4B_HUMAN	tubulin, alpha 4b (pseudogene)	Cytoplasm	other		+	+				95	2	
TBA8_HUMAN	tubulin, alpha 8	Cytoplasm	other		+	+	+	+		80	3	
TBB1_HUMAN	tubulin, beta 1	Cytoplasm	other					+		92	3	
TBB2A_HUMAN	tubulin, beta 2A	Cytoplasm	other		+					401	6	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
TBB2C_HUMAN	tubulin, beta 2C	Cytoplasm	other		+	+	+	+		192	5	
TBB3_HUMAN	tubulin, beta 3	Cytoplasm	other		+	+	+	+		141	3	
TBB5_HUMAN	tubulin, beta	Cytoplasm	other		+	+	+	+		220	6	3, 4, 6, 7, 8, 9, 10, 11, 12, 17, 20
TBB6_HUMAN	tubulin, beta 6	Cytoplasm	other				+			60	3	
TCPB_HUMAN	chaperonin containing TCP1, subunit 2 (beta)	Cytoplasm	kinase					+		123	4	
TCPE_HUMAN	chaperonin containing TCP1, subunit 5 (epsilon)	Cytoplasm	other		+	+		+		57	2	
TCPG_HUMAN	chaperonin containing TCP1, subunit 3 (gamma)	Cytoplasm	other			+		+		58	2	
TCPH_HUMAN	chaperonin containing TCP1, subunit 7 (eta)	Cytoplasm	other			+	+	+		40	2	
TCPQ_HUMAN	chaperonin containing TCP1, subunit 8 (theta)	Cytoplasm	enzyme			+		+		103	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
TCPZ_HUMAN	chaperonin containing TCP1, subunit 6A (zeta 1)	Cytoplasm	other			+		+		64	2	
TERA_HUMAN	valosin-containing protein	Cytoplasm	enzyme		+	+	+			756	17	13
TITIN_HUMAN	titin	Cytoplasm	kinase	+	+	+				42	10	
TKT_HUMAN	transketolase	Cytoplasm	enzyme		+					331	6	1
TPM3_HUMAN	tropomyosin 3	Cytoplasm	other		+					48	2	
TRAP1_HUMAN	TNF receptor-associated protein 1	Cytoplasm	enzyme					+		117	2	
UBA1_HUMAN	ubiquitin-like modifier activating enzyme 1	Cytoplasm	enzyme			+				96	2	
UGPA_HUMAN	UDP-glucose pyrophosphorylase 2	Cytoplasm	enzyme		+					67	2	
1B54_HUMAN	major histocompatibility complex, class I, B	Plasma Membrane	Trans-membrane receptor			+				212	3	
1A25_HUMAN	major histocompatibility complex, class I, A	Plasma Membrane	Trans-membrane receptor			+				182	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
1B07_HUMAN	major histocompatibility complex, class I, B	Plasma Membrane	transmembrane receptor			+				147	2	
1B73_HUMAN	major histocompatibility complex, class I, B	Plasma Membrane	Trans-membrane receptor			+				219	3	
4F2_HUMAN	solute carrier family 3 , member 2	Plasma Membrane	transporter	+					+	189	2	13
A4_HUMAN	amyloid beta (A4) precursor protein	Plasma Membrane	other	+						239	4	
AGRIN_HUMAN	agrin	Plasma Membrane	other	+			+	+	+	116	2	1 (precursor)
ANK3_HUMAN	ankyrin 3, node of Ranvier (ankyrin G)	Plasma Membrane	other			+				41	6	
ANXA1_HUMAN	annexin A1	Plasma Membrane	other		+					110	2	
ANXA2_HUMAN	annexin A2	Plasma Membrane	other	+	+	+			+	98	2	1, 3, 4, 6, 9, 12, 19, 20
CD81_HUMAN	CD81 molecule	Plasma Membrane	other	+			+	+		124	2	18, 24, 25
CD9_HUMAN	CD9 molecule	Plasma Membrane	other	+			+	+		128	2	24

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
CHLE_HUMAN	butyrylcholinesterase	Plasma Membrane	enzyme				+			147	3	
CLH1_HUMAN	clathrin, heavy chain (Hc)	Plasma Membrane	other		+	+				111	4	
CLH2_HUMAN	clathrin, heavy chain-like 1	Plasma Membrane	other			+				49	2	
CSTN1_HUMAN	calsyntenin 1	Plasma Membrane	other	+			+	+		57	2	
DMD_HUMAN	dystrophin	Plasma Membrane	other					+		46	7	
EPCAM_HUMAN	epithelial cell adhesion molecule	Plasma Membrane	other				+			160	2	
FAT1_HUMAN	FAT tumor suppressor homolog 1	Plasma Membrane	other				+			71	3	
FAT2_HUMAN	FAT tumor suppressor homolog 2	Plasma Membrane	other						+	63	2	
FINC_HUMAN	fibronectin 1	Plasma Membrane	enzyme	+				+	+	588	15	1, 21 (isoform 3 preprotein)
FOLH1_HUMAN	folate hydrolase (prostate-specific membrane antigen) 1	Plasma Membrane	peptidase					+		54	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
FPRP_HUMAN	prostaglandin F2 receptor negative regulator	Plasma Membrane	other				+	+		131	3	1
K2C6A_HUMAN	keratin 6A	Plasma Membrane	other						+	80	3	1
LG3BP_HUMAN	lectin, galactoside-binding, soluble, 3 binding protein	Plasma Membrane	transmembrane receptor	+		+	+		+	270	5	
MPRI_HUMAN	insulin-like growth factor 2 receptor	Plasma Membrane	transmembrane receptor					+		89	2	
PTPRF_HUMAN	protein tyrosine phosphatase, receptor type, F	Plasma Membrane	phosphatase				+			81	3	
SDC4_HUMAN	syndecan 4	Plasma Membrane	other				+	+	+	44	2	
TFR1_HUMAN	transferrin receptor	Plasma Membrane	transporter				+	+		120	2	24
A2MG_HUMAN	alpha-2-macroglobulin	Extracellular Space	transporter	+	+	+	+	+		76	2	
ALBU_HUMAN	albumin	Extracellular Space	transporter	+	+	+	+	+		100	2	13 (precursor), 21 (precursor)

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
APOA1_HUMAN	apolipoprotein A-I	Extracellular Space	transporter	+			+			50	2	
C1R_HUMAN	complement component 1, r subcomponent	Extracellular Space	peptidase			+				99	2	21
CLUS_HUMAN	clusterin	Extracellular Space	other	+		+				244	4	1, 21 (isoform)
CO1A1_HUMAN	collagen, type I, alpha 1	Extracellular Space	other		+					171	5	
CO6A1_HUMAN	collagen, type VI, alpha 1	Extracellular Space	other		+					592	11	
CO3_HUMAN	complement component 3	Extracellular Space	peptidase			+				619	14	
COCA1_HUMAN	collagen, type XII, alpha 1	Extracellular Space	other		+	+	+	+		108	2	
FETUA_HUMAN	alpha-2-HS-glycoprotein	Extracellular Space	other		+					122	3	
G6PI_HUMAN	glucose-6-phosphate isomerase	Extracellular Space	enzyme	+						147	3	1, 13
GDF15_HUMAN	growth differentiation factor 15	Extracellular Space	growth factor				+			148	3	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
GRN_HUMAN	granulin	Extracellular Space	growth factor		+					219	6	
HEMO_HUMAN	hemopexin	Extracellular Space	transporter						+	680	18	
ITIH2_HUMAN	inter-alpha (globulin) inhibitor H2	Extracellular Space	other		+					125	3	
ITIH4_HUMAN	inter-alpha (globulin) inhibitor H4	Extracellular Space	other						+	266	6	
LAMA5_HUMAN	laminin, alpha 5	Extracellular Space	other				+	+	+	245	9	1
LAMB1_HUMAN	laminin, beta 1	Extracellular Space	other				+		+	119	3	
LAMB2_HUMAN	laminin, beta 2 (laminin S)	Extracellular Space	enzyme					+		105	4	
LAMC1_HUMAN	laminin, gamma 1 (formerly LAMB2)	Extracellular Space	other				+	+	+	173	2	
LAMC2_HUMAN	laminin, gamma 2	Extracellular Space	other						+	361	7	
MFGM_HUMAN	milk fat globule-EGF factor 8 protein	Extracellular Space	other		+		+			69	2	
MK_HUMAN	midkine (neurite growth-promoting factor 2)	Extracellular Space	growth factor				+			102	3	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
MYH14_HUMAN	myosin, heavy chain 14, non-muscle	Extracellular Space	other	+						72	3	
PTX3_HUMAN	pentraxin 3, long	Extracellular Space	other	+	+	+				255	5	
SAP_HUMAN	prosaposin	Extracellular Space	other		+					234	8	13 (isoform preprotein)
SPON2_HUMAN	spondin 2, extracellular matrix protein	Extracellular Space	other				+	+		114	4	
TENA_HUMAN	tenascin C	Extracellular Space	other	+						125	4	1
TFPI1_HUMAN	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	Extracellular Space	other					+		311	5	
TRFE_HUMAN	transferrin	Extracellular Space	transporter						+	804	23	1, 13
TSP1_HUMAN	thrombospondin 1	Extracellular Space	other					+	+	200	5	
ABCAD_HUMAN	ATP-binding cassette, sub-family A (ABC1), member 13	unknown	transporter		+					42	5	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
ACTBL_HUMAN	actin, beta-like 2	unknown	other	+	+	+	+	+	+	99	2	
AHNK2_HUMAN	AHNAK nucleoprotein 2	unknown	other		+	+		+		49	3	
AN18B_HUMAN	ankyrin repeat domain 18B	unknown	other					+		41	3	
ARP3B_HUMAN	ARP3 actin-related protein 3 homolog B (yeast)	unknown	other		+					40	2	
AXA2L_HUMAN	annexin A2 pseudogene 2	unknown	other	+	+					126	2	
DYH2_HUMAN	dynein, axonemal, heavy chain 2	unknown	other	+						42	3	
EF1A3_HUMAN	eukaryotic translation elongation factor 1 alpha 1 pseudogene 5	unknown	other			+				470	7	
H2AV_HUMAN	H2A histone family, member V	unknown	other					+		178	3	
H90B2_HUMAN	heat shock protein 90kDa alpha (cytosolic), class B member 2 (pseudogene)	unknown	other		+	+		+		40	2	
HS904_HUMAN	heat shock protein 90kDa alpha (cytosolic), class A member 4 (pseudogene)	unknown	other			+				69	2	

ID	Protein name	Subcellular Location	Type(s)	Cell Lines						Mascot Score	Peptide matches	Reference[1]
				PC3	DU145	VCaP	LNCaP	C4-2	RWPE-1			
HSP76_HUMAN	heat shock 70kDa protein 6 (HSP70B')	unknown	other		+		+			98	2	
HSP77_HUMAN	heat shock 70kDa protein 7 (HSP70B)	unknown	other					+		71	2	
KATL2_HUMAN	katanin p60 subunit A-like 2	unknown	other		+					55	2	
MSMP_HUMAN	microseminoprotein, prostate associated	unknown	other	+						151	2	
POTEE_HUMAN	POTE ankyrin domain family, member F	unknown	other		+			+		361	6	
PUR6_HUMAN	phosphoribosylamino imidazole carboxylase,	unknown	enzyme		+	+	+	+		120	2	
PXDN_HUMAN	peroxidasin homolog	unknown	other			+		+		111	3	
TBB8B_HUMAN	tubulin, beta polypeptide 4, member Q	unknown	other					+		48	2	

Proteomic profiles were compared for prostate cancer cells that are AR -ve: PC3, DU145 versus AR +ve: VCaP, LNCaP and C4-2 in addition to the benign epithelial prostate cell line, RWPE-1. Proteins with Mascot scores ≥40 and ≥2 peptide matches were considered statistically significant ($P<0.05$) and listed in this table (n=2).

[1]Indicate the references that have been referred in Table 1, which have been listed below.

1) Chavez-Munoz, C., R. T. Kilani, et al. (2009). "Profile of exosomes related proteins released by differentiated and undifferentiated human keratinocytes." Journal of cellular physiology 221(1): 221-231.

2) Jansen, F. H., J. Krijgsveld, et al. (2009). "Exosomal secretion of cytoplasmic prostate cancer xenograft-derived proteins." Molecular & cellular proteomics : MCP 8(6): 1192-1205.

3) Staubach, S., H. Razawi, et al. (2009). "Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7." Proteomics 9(10): 2820-2835.

4) Hegmans, J. P., M. P. Bard, et al. (2004). "Proteomic analysis of exosomes secreted by human mesothelioma cells." The American journal of pathology 164(5): 1807-1815.

5) Mears, R., R. A. Craven, et al. (2004). "Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry." Proteomics 4(12): 4019-4031.

6) Fevrier, B. and G. Raposo (2004). "Exosomes: endosomal-derived vesicles shipping extracellular messages." Current opinion in cell biology 16(4): 415- 421.

7) Potolicchio, I., G. J. Carven, et al. (2005). "Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism." Journal of immunology 175(4): 2237-2243.

8) Faure, J., G. Lachenal, et al. (2006). "Exosomes are released by cultured cortical neurones."Molecular and cellular neurosciences 31(4): 642-648.

9) Aoki, N., S. Jin-no, et al. (2007). "Identification and characterization of microvesicles secreted by 3T3-L1 adipocytes: redox- and hormone-dependent induction of milk fat globule-epidermal growth factor 8-associated microvesicles." Endocrinology 148(8): 3850-3862.

10) Rodrigues, M. L., E. S. Nakayasu, et al. (2008). "Extracellular vesicles produced by Cryptococcus neoformans contain protein components associated with virulence." Eukaryotic cell 7(1): 58-67.

11) Pisitkun, T., R. F. Shen, et al. (2004). "Identification and proteomic profiling of exosomes in human urine." Proceedings of the National Academy of Sciences of the United States of America 101(36): 13368-13373.

12) Lamparski, H. G., A. Metha-Damani, et al. (2002). "Production and characterization of clinical grade exosomes derived from dendritic cells." Journal of immunological methods 270(2): 211-226.

13) Gonzales, P. A., T. Pisitkun, et al. (2009). "Large-scale proteomics and phosphoproteomics of urinary exosomes." Journal of the American Society of Nephrology : JASN 20(2): 363-379.

14) Gambim, M. H., O. do Carmo Ade, et al. (2007). "Platelet-derived exosomes induce endothelial cell apoptosis through peroxynitrite generation: experimental evidence for a novel mechanism of septic vascular dysfunction." Critical care 11(5): R107.

15) Thery, C., L. Zitvogel, et al. (2002). "Exosomes: composition, biogenesis and function." Naturereviews. Immunology 2(8): 569-579.

16) Van Niel, G., G. Raposo, et al. (2001). "Intestinal epithelial cells secrete exosome-like vesicles." Gastroenterology 121(2): 337-349.

17) Wubbolts, R., R. S. Leckie, et al. (2003). "Proteomic and biochemical analyses of human B cell derived exosomes. Potential implications for their function and multivesicular body formation." The Journal of biological chemistry 278(13): 10963-10972.

18) Thery, C., A. Regnault, et al. (1999). "Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73." The Journal of cell biology 147(3): 599-610.

19) Van Niel, G., J. Mallegol, et al. (2003). "Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice." Gut 52(12): 1690-1697.

20) Segura, E., C. Nicco, et al. (2005). "ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming." Blood 106(1): 216-223.

21) Looze, C., D. Yui, et al. (2009). "Proteomic profiling of human plasma exosomes identifies PPAR gamma as an exosome-associated protein." Biochemical and biophysical research communications 378(3): 433-438.

22) Giri, P. K., N. A. Kruh, et al. (2010). "Proteomic analysis identifies highly antigenic proteins in exosomes from M. tuberculosis-infected and culture filtrate protein-treated macrophages." Proteomics 10(17): 3190-3202.

23) Kinlough, C. L., P. A. Poland, et al. (2004). "MUC1 membrane trafficking is modulated by multiple interactions." The Journal of biological chemistry 279(51): 53071-53077.

24) Caby, M. P., D. Lankar, et al. (2005). "Exosomal-like vesicles are present in human blood plasma." International immunology 17(7): 879-887.

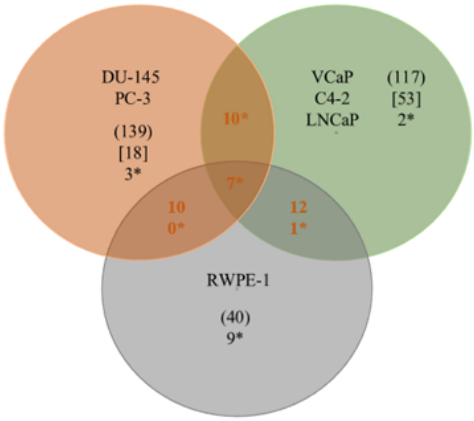
25) Escola, J. M., M. J. Kleijmeer, et al. (1998). "Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human Blymphocytes." The Journal of biological chemistry 273(32): 20121-20127.

26) Skokos, D., S. Le Panse, et al. (2001). "Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes." Journal of immunology 166(2):868-876.

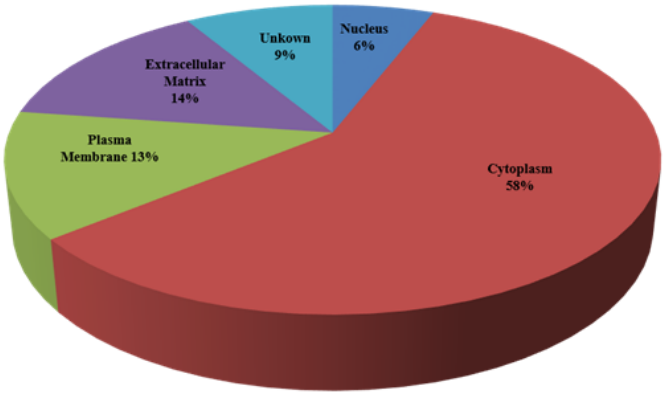
27) Clayton, A., J. Court, et al. (2001). "Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry." Journal of immunological methods 247(1-2): 163-174.

As demonstrated in **Figure 2.5 (A)**, 139 proteins identified from our isolated exosome samples were presented in one or the other of the AR –ve DU145 or PC3 cells. Of them, 18 proteins were present in both DU145 and PC3 cells with only 3 proteins (ENPL, GRP78 and AXA2L) being mutual only to the AR -ve DU145 or PC3 cells and not present in any of the AR +ve cell lines (VCaP, C4-2, LNCaP and RWPE-1).

2.5 A. Mutuality of proteins in exosomes derived from respective cell lines.



2.5 B. Subcellular localization



In the case of VCaP, C4-2 and LNCaP PCa cells, which are AR +ve, 117 proteins were present in exosomes derived from at least one of the AR +ve PCa cell lines, however, 53 of these proteins were present in exosomes from all of the AR +ve PCa cell lines and at least one of the AR -ve or benign RWPE-1 cells. Interestingly, there were only two proteins

identified as present in exosomes derived from all three AR +ve PCa cell lines (e.g. TCPH and H2A2B) that were not present in the AR -ve cell lines (DU145 and PC3) or RWPE-1 cells.

Comparison of the proteomic profiles of isolated exosomes from the prostate cell lines analyzed indicates the presence of 40 proteins, which were present in the benign AR+ve cell line (and also present in at least one of the other PCa cell lines). The nine unique proteins, which were determined to be contained in RWPE-1 exosomes only include ITIH4, LAMC2, TRFE, HEMO, K2C6A, FAT2, K2C5, K1C14 and HSPB1, none of which have been previously identified in exosomes derived from PCa cells. It would be interesting to determine the relevance of the specificity of these proteins being in exosomes derived from non-malignant cells, however comparison of a larger panel of non-malignant cells would be required. Other major groups of proteins present in exosomes are cytoskeletal proteins such as actin and tubulin, heat shock proteins (HSP90, HSP70 and CLU), elongation factors involved in protein synthesis and proteosomal proteins, and lipid-related proteins.

Using the Ingenuity and Mascot software all proteins were further assigned a subcellular localization and biological process. **Figure 2.5** displays the distribution of proteins in various prostate cell lines (**2.5 A.**), their subcellular localization (**2.5 B.**) their functions (**2.5 C.**) as well as the main canonical pathways they are involved in (**2.5 D.**).

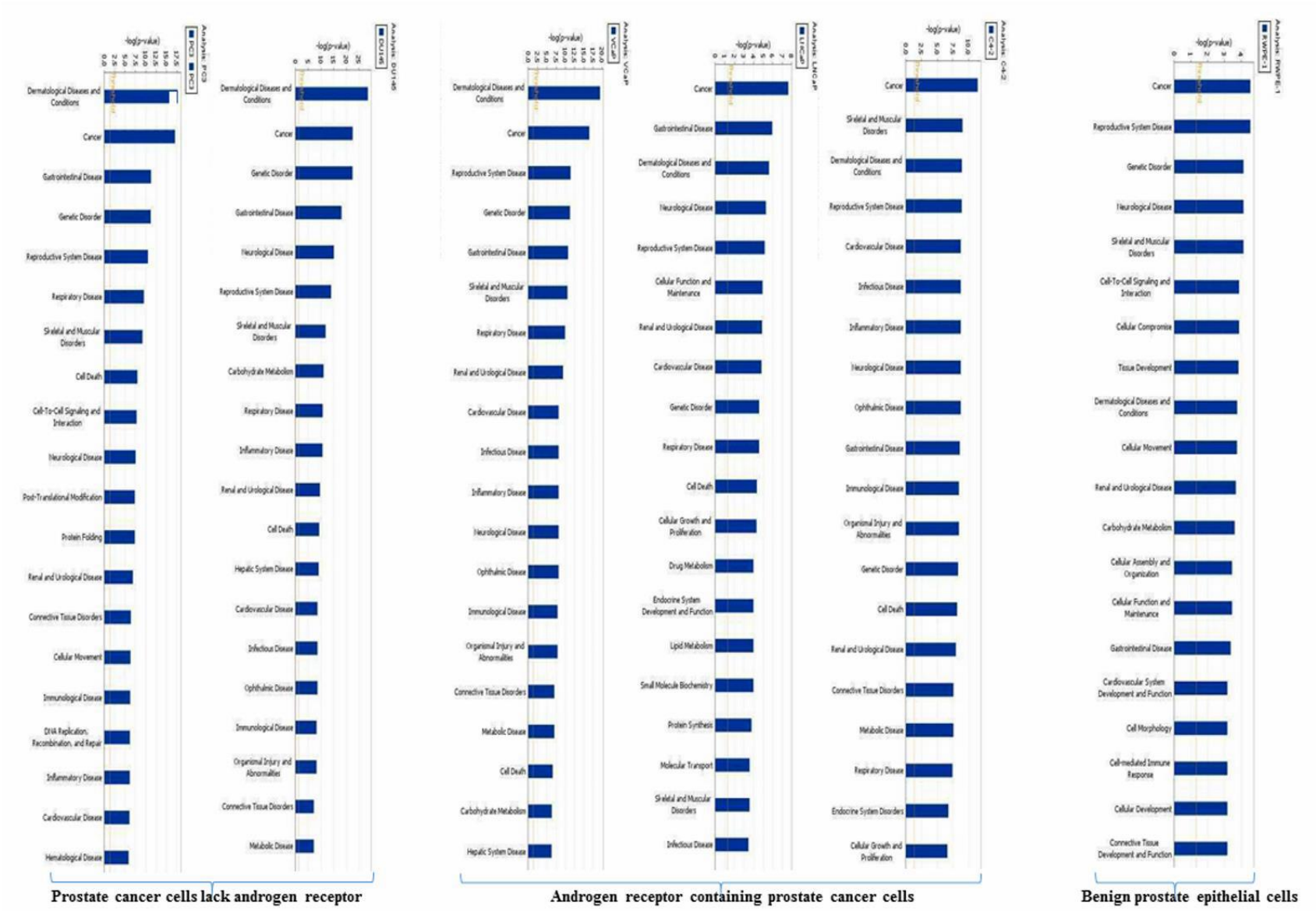
As shown in **Figure 2.5 B.**, 58% of prostate exosomal proteins (127 proteins) are localized in the cytoplasm, 14% in the extracellular matrix, 13% in plasma membrane and 6% in the nucleus. Nineteen proteins have been identified with unknown subcellular localization (9%) in this analysis.

All identified proteins could be categorized as enzymes (32%), (peptidase (9%), kinase (3%) and phosphatase (1%)), transporters (5%), transmembrane receptors (3%) translational regulators (3%), transcriptional regulators (2%), and growth factors (1%). 53% of these proteins have been categorized as protein with "other" function.

Data was also analyzed using Ingenuity Pathway Software (IPS) to gain an understanding of the function of these proteins. Our results indicate that exosomal proteins derived from prostate cells are mainly involved in cancer, dermatological disease, gastrointestinal disease, genetic disorders and neurological disease. More specifically our IPS analysis indicates the role of proteins such as ACTC1, ALDOC, ANXA2, CCT5,8, CLTC ENO1-3, GAPDH, HPX, MX1, MYH9,14, TNC, TUBA8, TUBA1B, 1C, 4B, TUBB, TUBB3, TUBB2A, 2C in cancer, reproductive system disease and cardiac inflammation, while exosomal proteins such as FAT2, GANAB, HIST1, H2BD, KATNAL2, MX1, NPM1 and TAGLN2 tend to play a pivotal role in cell death and cell to cell signaling.

Using the IPS knowledge base tool, we also identified the canonical pathways which suggest the main possible functions of proteins identified in exosomes. All 220 proteins derived from the six prostate cell lines were included in this analysis. As indicated in **Figure 2.5 D.** the protein ubiquitination pathway and germ cell-sertoli cell junction signaling are the major canonical pathways present in exosomal proteins from PCa cells.

2.5 C. Exosomal protein function in exosomes derived from different prostate cell lines



2.5 D. Main canonical pathway function in exosomes derived from different prostate cell lines

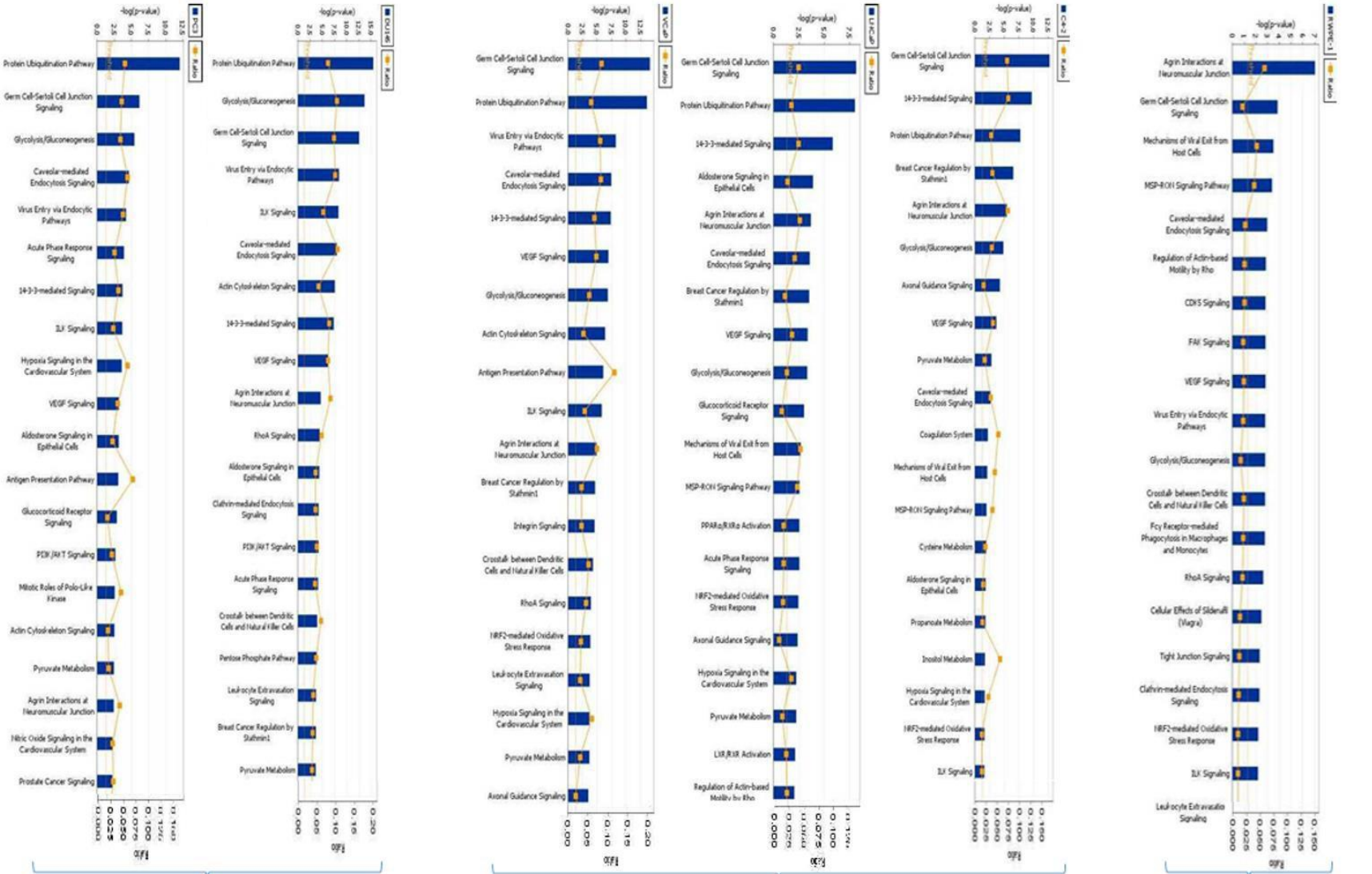


Figure 2.5 Proteomic analysis of different prostate cancer cell lines.

Venn diagram describing the mutuality of proteins in exosomes derived from the benign epithelial prostate cell line (RWPE-1) versus five different PCa cell lines categorized based on androgen sensitivity (PC3, DU145 and VCaP, LNCaP, C4-2). Numbers in ( ) are representative of the total number of proteins present in each cell lines, Numbers in [ ] are representative of proteins present in either designated category and not present in any other undesignated category. Numbers denoted with * are the mutual proteins present in all cell lines in each category. **B.** Pie chart showing the subcellular localization of proteins found in exosomes derived from six different prostate cell lines.
C. Bar chart indicating the cellular function of proteins found within exosomes determined using Ingenuity software. **D.** Predicted top canonical pathways are represented by the identified exosomal proteins.

Furthermore, IPS also was used to establish a list of novel biomarker signatures as well as their application in various pathologic conditions. Subcellular localization of the proteins in this biomarker list is summarized in **Fig. 2.6** As reported in **Table 2.2**, PCa specific biomarkers, including ANXA2 (Annexin A2), CLSTN1 (Calsyntenin 1), FASN (Fatty Acid Synthesis), FLNC (Filamin C, gamma), FOLH1 (Folate Hydrolase (prostate specific membrane antigen)-1), GDF15 (Growth Differentiation Factor 15), are present in PCa cells derived exosomes. To strengthen conclusions drawn from the IPA data, that exosomes are a rich source of biomarkers, we performed a statistical test to determine the probability of finding biomarkers by chance in a random set of proteins (expressed as a p-value). Our results indicated that among 220 proteins randomly selected from the UniProt human protein database, 5 were identified as biomarkers, of which zero were associated with PCa diagnosis. A p-value less than 0.0001 was determined for the chance of finding 50 biomarkers in the enriched exosome sample compared to the 5 found in the random protein group. Similarly a p-value of 0.0302 was subsequently determined for the chance of finding 6 PCa biomarkers when there were zero found in the random selection of proteins.

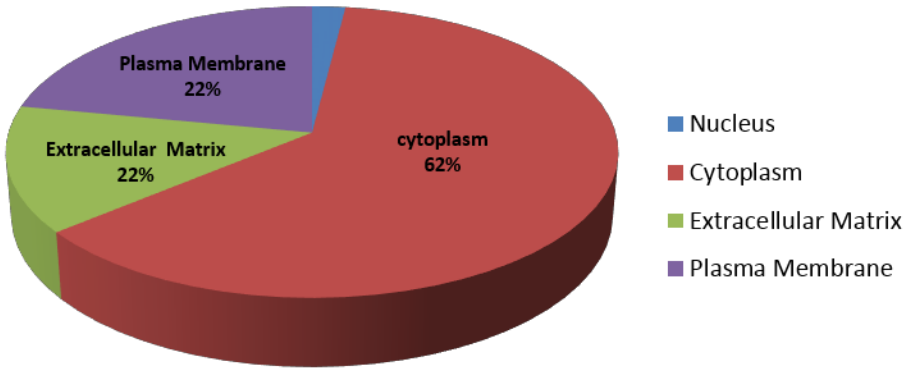


Figure 2.6 Biomarker proteins subcellular localization.

Pie chart showing the subcellular localization of biomarkers found in exosomes derived from PCa cell lines; PC3, DU145, VCaP, LNCaP and C4-2.

Table 2.2 Proteomic analysis of different biomarkers in exosomes derived from PCa cell lines.

Symbol	PC3	DU145	VCaP	LNCaP	C4-2	Biomarker Application(s)	Type of Cancer(s)
ACLY	+	+	+			Unspecified Application	Cancer, Connective Tissue Disorders, Gastrointestinal Disease
ACTG1		+				Unspecified Application	Auditory Disease, Cancer, Gastrointestinal Disease
AHCY		+				Unspecified Application	Cancer, Dermatological Diseases and Conditions, Gastrointestinal DiseaseDisorders
AHCY		+	+			Unspecified Application	Cancer, Dermatological Diseases and Conditions, Gastrointestinal DiseaseDisorders
ANXA1		+				Diagnosis, Prognosis, Unspecified Application	Cancer, Cardiovascular Disease, Connective Tissue Disorders
ANXA2	+	+	+		+	Diagnosis, Unspecified Application	Cancer, Cardiovascular Disease, Dermatological Diseases and Conditions, Gastrointestinal Disease
CALR	+					Unspecified Application	Cancer, Cardiovascular Disease, Connective Tissue Disorders
CCT6A			+		+	Diagnosis	Cancer, Renal and Urological Disease
CD9	+	+	+			Efficacy	Cancer, Gastrointestinal Disease, Genetic Disorder
CLSTN1	+			+	+	Diagnosis	Cancer, Gastrointestinal Disease, Genetic Disorder

Symbol	PC3	DU145	VCaP	LNCaP	C4-2	Biomarker Application(s)	Type of Cancer(s)
CLU	+	+				Diagnosis, Efficacy, Unspecified Application	Cancer, Cardiovascular Disease, Connective Tissue Disorders, Endocrine System Disorders,
CTSD		+				Diagnosis, Unspecified Application	Cancer, Cardiovascular Disease, Developmental Disorder
EEF1A2		+				Prognosis	Cancer, Cardiovascular Disease, Genetic Disorder
ENO1	+	+	+			Diagnosis	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
ENO2		+				Diagnosis, Efficacy, Prognosis	Cancer, Cardiovascular Disease, Gastrointestinal Disease
EPCAM				+		Unspecified Application	Cancer, Endocrine System Disorders, Gastrointestinal Disease
FASN		+	+	+	+	Diagnosis, Efficacy, Prognosis	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
FLNC		+	+			Diagnosis	Cancer, Genetic Disorder, Organismal Injury and Abnormalities
FN1	+	+	+			Diagnosis, Disease Progression, Efficacy, Prognosis, Unspecified	Cancer, Cardiovascular Disease, Connective Tissue Disorders
FOLH1					+	Diagnosis, Prognosis	Cancer, Cardiovascular Disease, Gastrointestinal Disease

Symbol	PC3	DU145	VCaP	LNCaP	C4-2	Biomarker Application(s)	Type of Cancer(s)
FSCN1		+				Unspecified Application	Cancer, Dermatological Diseases and Conditions, Gastrointestinal Disease
GDF15		+				Diagnosis	Cancer, Cardiovascular Disease, Dermatological Diseases and Conditions
GPI		+				Unspecified Application	Cancer, Cardiovascular Disease, Connective Tissue Disorders
GRN	+					Diagnosis, Disease Progression	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
HLA-A			+			Efficacy, Response to Therapy	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
HLA-B		+	+			Safety	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
HSP90AA1	+	+	+			Safety, Unspecified Application	Cancer, Dermatological Diseases and Conditions, Gastrointestinal Disease
HSPA5	+	+				Unspecified Application	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
HSPA8	+	+				Diagnosis	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
HSPB1		+				Diagnosis	Cancer, Endocrine System Disorders, Gastrointestinal Disease

Symbol	PC3	DU145	VCaP	LNCaP	C4-2	Biomarker Application(s)	Type of Cancer(s)
IGF2R					+	Diagnosis	Cancer, Cardiovascular Disease, Gastrointestinal Disease
KRT14		+				Unspecified Application	Cancer, Dermatological Diseases and Conditions, Gastrointestinal Disease
KRT5		+				Diagnosis, Efficacy, Unspecified Application	Cancer, Dermatological Diseases and Conditions, Genetic Disorder
KRT8	+	+	+			Prognosis	Cancer, Dermatological Diseases and Conditions, Developmental Disorder
LAMA5	+	+	+			Diagnosis	Cancer, Cardiovascular Disease, Dermatological Diseases and Conditions
LDHA	+	+	+	+	+	Diagnosis, Disease Progression, Efficacy, Prognosis, Unspecified	Cancer, Gastrointestinal Disease, Genetic Disorder
MDK					+	Diagnosis	Cancer, Cardiovascular Disease, Connective Tissue Disorders
NPM1	+	+	+		+	Disease Progression, Unspecified Application	Cancer, Genetic Disorder, Hematological Disease
NQO1			+			Diagnosis, Unspecified Application	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
PFN1		+	+			Unspecified Application	Cancer, Cardiovascular Disease, Dermatological Diseases and Conditions
POLG			+			Diagnosis	Cancer, Dermatological Diseases and Conditions, Gastrointestinal Disease

Symbol	PC3	DU145	VCaP	LNCaP	C4-2	Biomarker Application(s)	Type of Cancer(s)
PRDX1	+	+				Diagnosis	Cancer, Connective Tissue Disorders, Genetic Disorder
PSMA2	+	+				Unspecified Application	Cancer, Dermatological Diseases and Conditions, Developmental Disorder
TFRC	+	+				Diagnosis	Cancer, Connective Tissue Disorders, Dermatological Diseases and Conditions
TNC		+				Diagnosis, Efficacy, Response to Therapy	Cancer, Dermatological Diseases and Conditions, Gastrointestinal Disease
TPM3		+				Diagnosis, Unspecified Application	Cancer, Dermatological Diseases and Conditions, Genetic Disorder
TUBA1A					+	Unspecified Application	Cancer, Cardiovascular Disease, Connective Tissue Disorders
TUBB3		+	+	+	+	Efficacy, Response to Therapy, Unspecified Application	Cancer, Cardiovascular Disease, Connective Tissue Disorders
YWHAE	+	+	+	+	+	Diagnosis	Cancer, Cardiovascular Disease, Dermatological Diseases and Conditions
YWHAZ		+				Diagnosis	Cancer, Genetic Disorder, Neurological Disease

Table indicating the presence of different biomarkers in exosomes derived from five different PCa cell lines including PC3, DU145, VCaP, LNCaP and C4-2. This table also describes the type of cancer associated with the biomarker and the application of that biomarker in the specific disease.

2.3.4 Cholesterol Content

Cholesterol content of exosomes and corresponding cells was determined using LC-MS. As shown in **Figure 2.7 A.**, cholesterol content of RWPE-1 cells was significantly higher than that of the PCa cell lines. The average cholesterol concentration of PCa cell lysates was 13.1 µg cholesterol/ µg protein which was approximately half that determined for RWPE-1 cells (24.4 µg cholesterol/ µg proteins) (**Figure 2.7 B.**). Interestingly, the average cholesterol content of exosomes derived from PCa cells was three times higher than exosomes derived from RWPE-1, benign prostate cells.

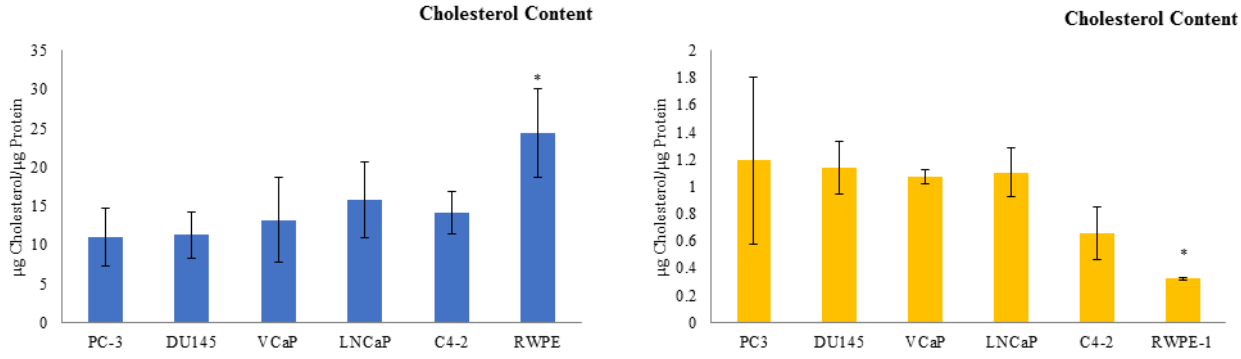


Figure 2.7 Cholesterol concentration.

The bar diagrams show the cholesterol concentration of **A.** lysates of PC3, DU145 and VCaP, LNCaP, C4-2 and RWPE-1 cells **B.** exosomes derived from the six different prostate cell lines. Cholesterol results were normalized to protein concentration of each sample and expressed as µg Cholesterol/µg Protein.

* indicate significantly difference ($p<0.05$), ($n\geq2$).

2.3.5 Lipidomic Analysis

We next investigated the lipid profile (glycerolipid, glycerophospholipid, sphingolipid, glycosphingolipid) of exosomes for comparison with their parent cells. Our data indicated that glycerophospholipid was the most abundant class of lipids with an average of 86.3% and 65.1% determined in both cells and exosomes respectively for all six different prostate cell lines. Sphingolipid (9.6% in cells, 30.2% in exosomes) was the second most abundant lipid class in all different groups, followed by glycerolipid (3.6%, 3.4%) and glycosphingolipid (0.5%, 1.3%).

Data shown in **Figure 2.8 A.** establishes that there is no significant difference between the glycerolipid content between different exosomes and their corresponding cells.

The glycerophospholipid distribution determined in the various prostate cells and their exosomes was more balanced compared to other groups of lipids. PC3, DU145, VCaP and LNCaP and RWPE-1 cells were shown to have a significantly higher glycerolipid content compared to their derived exosomes (**Figure 2.8 B.**).

The most striking difference between the lipid profiles determined for exosomes and their corresponding cells were their sphingolipid contents. In agreement with many other published reports we show enrichment in the sphingolipid content in all of our exosomes samples (Laulagnier *et al*., 2004). As demonstrated in **Figure 2.8 C.**, PC3, VCaP, C4-2, LNCaP and RWPE-1 have a significantly higher sphingolipid content compared with their corresponding parent cells.

Finally, our lipid analysis showed that only DU145, VCaP and LNCaP derived exosomes have a higher glycosphingolipid content compared to their parent cells.

No significant differences were seen between the cell lysates and exosomes of respective cell lines in any of lipid class.

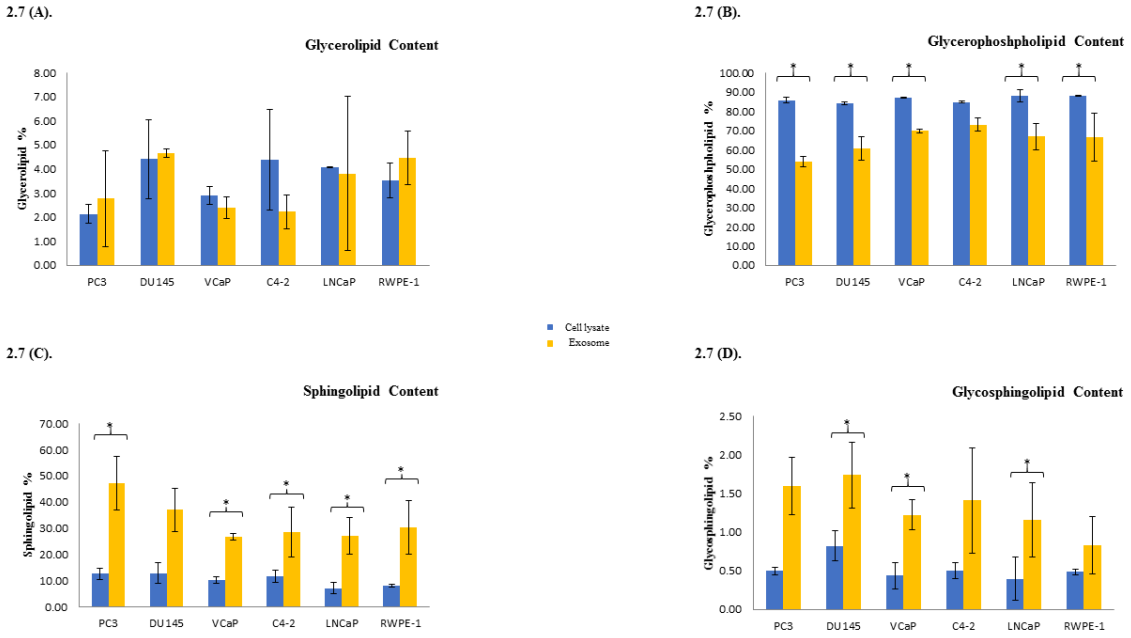


Figure 2.8. Exosome lipidomic data.

The lipid content of four major lipid classes was measured in PC3, DU145 and VCaP, LNCaP, C4-2 and RWPE-1 cells and compared with their derived exosomes, using LC-MS. The bar diagrams are representative of **A.** Glycerolipid, **B.** Glycerophospholipid, **C.** Sphingolipid, **D.** Glycosphingolipid in cell lysates and exosomes. Relative amounts of each lipid group were calculated as (sum of all AUC's for a particular lipid group) ÷ (sum of AUC's for all lipid groups) x 100%.

* denotes a significant difference ($p<0.05$) between exosomes and their corresponding cells. No significant differences were seen between the cell lysates and exosomes of respective cell lines in any of lipid class, ($n\geq2$).

2.4 Discussion

Exosomes are complex nanovesicles secreted from a wide range of cells including neoplastic cells (Trams *et al.*, 1981) dendritic cells (Zitvogel *et al.*, 1998), reticulocytes (Pan *et al.*, 1985), T cells (Zhang *et al.*, 2011), mastocytes (Raposo *et al.*, 1997), differentiated and un-differentiated keratinocytes (Chavez-Munoz *et al.*, 2009),

platelets (Heijnen *et al*., 1999), and neurons (Lachenal *et al*., 2011). They have recently been studied in different pathological conditions including cancer, atherosclerosis, vascular disease, pulmonary hypertension and thrombosis as well as bacterial infections (Amderson *et al*., 2010). Exosomes have been isolated and characterized from many different biological fluids such as blood components, urine, amniotic fluids, malignant effusions, breast milk and brochoalveolar lavage fluid and contain an array of proteins and lipids as well as genetic material such as mRNA and miRNA (Schorey *et al*., 2008). Collectively all these studies highlight membrane and cytoplasmic proteins/mRNA as exosome cargo, many of which have known functional importance in cellular function.

In this study we have characterized exosomes derived from different AR +/- ve PCa cell lines. The goal was not only to understand the difference between exosomes secreted from different cells as a basis for evaluating transfer of identified composite exosome proteins between cells as part of a recognized cell communication phenomenon, but also to determine potential biomarkers for different stages of PCa as well as therapeutic targets(Andre *et al*., 2002)(Brad *et al*., 2004).

The initial challenge of this work was to experimentally isolate homogenous samples of exosomes for study. In order to maintain reproducible data sets we used a protocol developed according to Lamparski et al's (2002) which removes cell debris and protein aggregates using filtration and we were thus able to isolate a homogenous exosome mixture based on the unique density of exosomes in sucrose using ultracentrifugation(Lamparski *et al*., 2002).

While some controversy exists regarding the exact nature of exosome biomarkers several classes of proteins have been characteristically assigned as such. These include cytoskeletal proteins, HSPs and tetraspanins (Thery *et al*., 2002)(Schorey *et al*., 2008). Similarly the absence of Endoplasmic Reticulum (ER), mitochondrial and nuclear proteins are considered to vouch for 'lack of significant contamination' of an exosome preparation (Fevrier *et al*., 2004)(Welton *et al*., 2010). This being said, our understanding of exosome biology is in its infancy, and further work is required to validate protocol in this regard. We were surprised in fact to find proteins identified by our LCMS proteomic analyses which are normally considered to be located in the nucleus and therefore not likely candidates for exosome cargo. Rather than assume that these proteins are contaminants of our exosome preparation we would rather tend to the idea that they might play a role in cell-cell communication via exosomal transfer (Papp *et al*., 2008). In keeping with this notion, it is thought that dysregulation of key biological mechanisms in cancer cells, such as those regulating protein synthesis and transportation, may result in altered distribution of proteins within and outside of defined intracellular compartments (Barboro *et al*., 2009). One example, which our data supports is the presence of a protein called nucleophosmin in exosomes. This protein is involved in a multitude of cellular functions including ribosome biogenesis, cell proliferation, regulation of tumor suppressor p53, and protein chaperoning which could imply 'shuttling' between the cytoplasm and the nucleus. Although this protein is predominantly nuclear, and might be considered as a contaminant in our exosome sample, its minor role as a chaperone in the cytoplasm would allow for detection in exosomes.

We were able to generate exosomes from all six prostate cell lines. All isolated vesicles contained exosomal markers and lacked ER marker (except PC3), as validated using Western Blotting, and were observed to be cup-shaped under TEM (Thery *et al*., 2002). While the mechanism involved in exosome internalization by other prostate cells was not under investigation as part of this study, this has been studied by others previously using ovarian cell lines (Escrevente *et al*., 2011) human saliva, plasma and breast milk derived exosomes (Lasser *et al*., 2011). For the purpose of this study we have simply confirmed the uptake of exosomes derived from DU145 PCa cells into other PCa and benign cells and furthermore, transfer of exosomal CLU^{GFP} derived from LNCaP cell line to PC3 and LNCaP. While more research is needed to investigate the mechanism involved in the uptake of exosomes by neighboring cells in the PCa microenvironment, our finding support the widely accepted hypothesis for the role of exosomes as cell-cell communication vesicles in tumor growth (Liu *et al*., 2006)(Iero *et al*., 2008) cell migration (Esser *et al*., 2010)(McCready *et al*., 2010)(Vrijsen *et al*., 2010), metastasis (Janowska-Wieczorek *et al*., 2005)(Hood *et al*., 2011) and angiogenesis (Skog *et al*., 2008).

Cancer is a complex disease, which involves disruption of numerous cell cycle regulatory pathways (Hanahan and Weinberg, 2000)(Hanahan and Weinberg, 2011) and several lines of evidence indicate that, for each unique cancer cell subtype, the path to malignancy is different. Over time, depending on the type of cancer, tumor cells break away from the primary site and invade surrounding tissues before migrating to distant tissue (Chaffer *et al*., 2011). While metastasis is responsible for more than 90% of cancer related deaths, the mechanisms involved in metastasis are not fully understood. Recently there has been an increasing interest in the potential link between microvesicles and local

invasion of primary cancer cells within the microenvironment, as well as metastasis to foreign microenvironments. Further, our present study attempts to understand this process by comparing the protein profiles of exosomes derived from different PCa cell lines as well as a non-malignant epithelial prostate cell line. We used mass spectrometry to successfully identify 220 proteins in exosomes derived from six prostate cell lines. By reporting proteins with more than two peptides and a Mascot score higher than 40 we have a high level of confidence that our proteomic data accurately represents proteins found in exosome samples. To better understand the differences between exosomes derived from a variety of PCa cells as well as RWPE-1 cells we further categorized them as AR +ve and AR –ve cells.

Reinforcing the conclusions of a recent published proteomic analysis of exosomes derived from the AR null PC3 cell line (Sandvig *et al*., 2012), this proteomic study also supports the premise that exosomes provide a pool of proteins which are enriched in biomarkers for potential use in PCa diagnosis via a relatively non-invasive diagnostic test. Our list consists of 50 candidate protein biomarkers, which have been previously reported to have potential diagnosis, prognosis, disease progression, efficacy and response to therapy for a variety of pathological diseases. We also identified novel potential biomarker proteins in exosomes derived from PCa cells, which may be more specific to PCa diagnosis.

Proteins, which were identified mutually within exosomes derived from PCa cell lines and benign RWPE-1 cells include GAPDH, PKM2, TUBA1B and THBS1. These were removed from the biomarker list however we recognize that comparison of a larger panel of non-malignant cells would be required to definitively determine the relevance of the specificity of these proteins being in exosomes derived from non-malignant cells. Several biomarker candidates in our list include ANXA2, CLSTN1, FASN, FLNC, FOLH1 and GDF15 which

have all been previously reported as candidates for PCa diagnosis (http://clinicaltrials.gov/show/NCT00340717, NCT00897234 and NCT00438906). While ANXA2 and FOLH1 have been reported in exosomes derived from hepatocyte, bladder, tracheobronchial epithelial and PCa cells, we are the first group to report the presence of CLSTN1, FASN, FLNC, and GDF15 in different PCa cell exosomes. The presence of these biomarkers, in addition to the CYP17 protein which we previously characterized in serum exosomes, and is known to play a significant role in CRPC (Leon *et al*., 2010)(Locke *et al*., 2009), could be further explored for potential indicators of disease progression. The presence of CLU (Clusterin) in exosomes is also of significant relevance since this molecule has already been characterized as a therapeutic target for CRPC (Lamoureux *et al*., 2011)(Chi *et al*., 2011)(Zoubeidi *et al*., 2010) and antisense oligonucleotide OGX-011, which was designed to target CLU, is currently in phase III clinical trial. In our study we went on to more fully characterize exosomes by analyzing their cholesterol and lipid profiles compared with respective cells of origin. As our results demonstrate, although there is a significant difference in lipid content for some lipid classes in some of the cell lines, no significant differences have been seen between AR +ve and AR -ve cells and their exosomes or between PCa cells/exosomes and RWPE-1 cells or derived exosomes. We hypothesize that the significant differences in lipid content of exosomes and their cells of origin could imply that there is a mechanism in existence, which stabilizes exosomes in the circulation for subsequent fusion with the plasma membranes of recipient cells to which they are delivered. Interestingly our quantitative cholesterol data suggests that exosomes derived from PCa cell lines contain significantly more cholesterol than their benign counterpart cell line RWPE-1. This is in line with our previous work, which indicates that cholesterol is

likely to play a role in PCa progression (Locke *et al*., 2008)(Leon *et al*., 2010).

While exosome biogenesis is still under investigation it is very well known that exosome membranes have the same topology as the cell membranes from which they are derived and are correspondingly rich in lipid rafts (de Gassart *et al.*, 2003). In agreement with previously published studies our lipidomic data shows a significant enrichment of sphingolipid and glycosphingolipid in exosomes, both very well-known markers of lipid rafts which are contained within almost all exosomes derived from prostate cells (Wubbolts *et al*., 2003)(Izquierdo-Useros *et al*., 2009).

Lastly, it has been previously proposed that endocytic sorting behavior of lipids depends exclusively on the chemistry of their hydrophobic tails (Mukherjee *et al*., 1999). In the long term, further study to generate a more thorough understanding of the differences in lipid content of cells and their derived exosomes would be useful to define the role of each lipid class in exosome formation as well as mechanisms involved in exosome-mediated communication within the tissue microenvironment.

Chapter 3: Exosomes as Biomarker Enriched Microvesicles: Characterization of Exosomal Protein Derived from the Biological Fluids Obtained from Prostate Cancer Patients.

3.1 Introduction

EVs could be a major potential source of biomarkers for many different pathological disorders including cancer and PCa. The accessibility of these vesicles in biological fluids such as blood (Caby *et al*., 2005)(Taylor *et al*., 2006), urine (Pisitkun *et a*l., 2004), milk (Andersen *et al*. 1997)(Admyre *et al*., 2007)(Andersen *et al*., 1997), saliva (Marzesco *et al*., 2009)(Xiao *et al*., 2012) malignant ascites (Andre *et al*., 2002)(Bard *et al*., 2004), amniotic fluid (Asea *et al*., 2008) and bronchoalveolar lavage fluid (Admyre *et al*., 2003)(Hawari *et al*., 2004) have resulted in intensive investigation in their use for many recent biomarker discovery studies. During the last several years numerous and distinct EVs have been identified and studied for their role in biological and pathological processes such as communication vesicles, or messengers/transporters (Zocco *et al*., 2014)(Braicu *et al*., 2015). Their accessibility through non- invasive procedures as well as the presence of different classes of proteins (Thery *et al*., 2001) and genetic material (DNA and RNA) (Lotvall and Valadi, 2007)(Huang *et al*., 2013)(Kahlert *et al*., 2014)(Thakur *et al*., 2014) in these vesicles make them an interesting avenue for biomarker researchers to explore.

Therefore the main purpose of this study was to purify EVs from the blood and urine of normal and cancer patients and attempt to determine differences between the two sources (blood vs urine) and the two sample groups (normal vs cancer). In follow up to our first paper (Hosseini-Beheshti *et al*., 2012) we also performed a comprehensive MS-based proteomic analysis on these samples to understand the major differences between EV derived from

blood vs. urine in control and cancer groups. As well as possible underlying differences in protein profiles, an additional part of this study was to investigate the cholesterol levels in exosome isolates as a potential diagnostic tool.

Taken together this study examines the potential of direct MS based proteomic and/or cholesterol profiling of EV derived from different biological fluids and their use for biomarker analysis.

3.2 Material and Methods

3.2.1 Sample Collection

Fifteen CRPC patients' samples, in addition to four healthy volunteers (3 male, 1 female (female sample was only used as a comparison in exosome isolation methodology development)) were selected for this study. All patients were confirmed positive for PCa by biopsy. Patient information including serum PSA, age and treatment history of all patients was collected as part of a clinical trial designed to study Abiraterone (clinical trials.gov number NCT01857908) and is summarised in **Table 3.1** Control arm patients were used for the purpose of this study. Human samples were used in accordance with Ethics Board Approval Cert. H09-01010 obtained from the University of British Columbia, Canada.

The average age for CRPC patients (9 urine donors and 12 blood donors) was 71 (57-81) while the average age for healthy control donors was 29 (26-33).

We categorized the patient samples into two different groups assigned as PSA lower and higher than 50 ng/ml.

Blood: 5ml whole blood samples were collected in red top no additive tubes by venipuncture. Blood was allowed to coagulate for 30min and serum fractions were separated

by centrifugation at 1,000g for 20 minutes and stored at -80°C until further analysis.

Urine: PCa patients provided a pooled 24-hour urine collection of which 50ml per patient was retained in sterile containers. All samples were frozen in -80°C until further analysis.

Table 3.1 Patient information.

Patient Number	Subject ID	Age	PSA result	Treatment History
1	100	79	39.12	Casodex
2	101	65	60.21	Flutamide, Docetaxel
3	102	73	17.5	Casodex, Corticosteroid, Flutamide
4	103	60	76.85	Casodex, Corticosteroid
5	105	69	151.36	Casodex
6	106	70	253.14	Casodex, Docetaxel
7	107	74	39.65	Casodex, Corticosteroid, ketoconazole, Carbo-taxol, Taxotere
8	109	80	227.36	Casodex, Docetaxel
9	110	57	8.56	Casodex, Corticosteroid, Flutamide
10	111	73	12.16	Casodex, Docetaxel
11	113	81	32.16	Decetaxel
12	115	73	20.23	Casodex, Docetaxel
13	118	65	281.76	Casodex
14	119	72	29.4	Casodex
15	120	78	33.9	Casodex, Corticosteroid, ketoconazole, Docetaxel

15 PCa patients were used in this study. Of these 15 patient 12 were blood donor and 9 were urine donor.

3.2.2 Exosome Isolation

Blood: Frozen serum or plasma samples (n=15) were thawed at room temperature and centrifuged at 500g for 5 minutes at 10°C to remove any cell debris. The supernatants of the cell-free serum/plasma were transferred to fresh centrifuge tubes for the second centrifugation cycle at 3,000g for 20minutes at 10°C. The supernatant fraction was again transferred to fresh centrifuge tubes for the next centrifuge at 12,000g for 20 minutes at 10°C followed by an ultracentrifugation step in a 30% sucrose cushion at 100,000g for 70 minutes at 10°C (using a fixed angel 70.1 Ti rotor (Beckman Coulter)). The 300 µl exosome pellets were then washed with PBS followed by a final ultracentrifugation at 100,000g for 70 minutes at 10°C. Washed exosome pellets (300 µl) were then stored in -80°C freezer until further analysis.

Urine: Urine samples (n=12) were thawed at room temperature and 2 tablets of protease inhibitor (commercial protease inhibitor mixture Complete (Roche Applied Science) were added to each 25ml sample immediately after thawing prior to exosome isolation.

Samples were then vortexed for 1 minute to create a homogenous sample before centrifugation at 500g for 5 minutes at 4°C to remove the cell-debris. The cell-free urine supernatant was then transferred to fresh centrifuge tubes for the next centrifugation at 17,000g for 20 minutes at 4°C. The supernatant was ultracentrifuged at 200,000g for 70 minutes at 4°C (using a fixed angel 70.1 Ti rotor (Beckman Coulter)). Urinary exosome pellets (300 µl) were then washed with PBS followed by a final ultracentrifugation at 200,000g for 70 minutes at 4°C (**Figure 3.1)**.

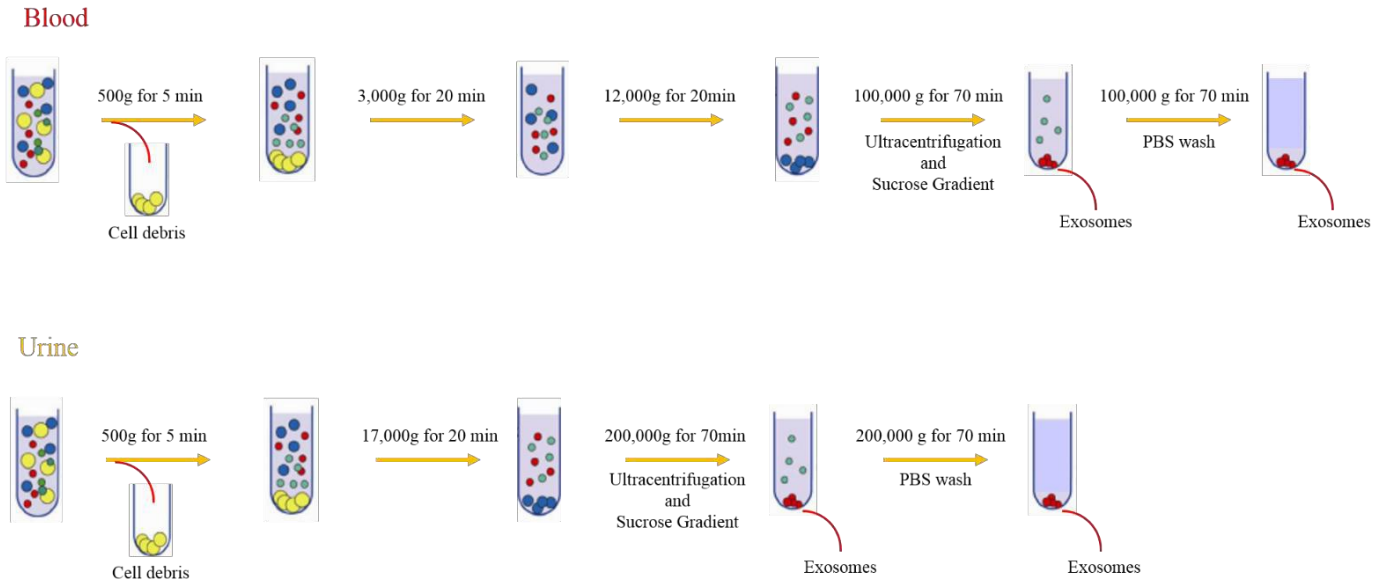


Figure 3.1 EV enrichment workflow in A. Serum/Plasma **B.** Urine.

3.2.3 Transmission Electron Microscopy

Isolated EVs (2.5µl) were dried onto freshly glow discharged 300 mesh formvar/carbon- coated TEM grids (Ted Pella, Redding, CA), negatively stained with 2% aqueous uracyl acetate and observed with a Hitachi H7600 TEM (Hitachi High-Technologies Corp., Tokyo, Japan) operated at 80kV. Images were captured with a side mounted 1K AMT Advantage digital camera (Advanced Microscopy Techniques, Corp. Woburn, MA).

3.2.4 Western Blot Analysis

Blood, urine and their EVs were analyzed for total protein concentration using the BCA protein determination kit (Sigma, Oakville, Ontario, Canada). Thirty micrograms of total protein associated with purified serum/plasma, urine and EV derived from blood and/or urine were loaded on 12% acrylamide gel. Relative enzyme levels were quantified

using antibodies specific for exosome markers; mouse monoclonal Alix, mouse monoclonal CD63, mouse monoclonal HSP70 and goat polyclonal HSP90 α/β (1:1000 Santa Cruz Biotechnology, Inc., Santa Cruz, CA).Mouse monoclonal TSG101 (1:1000 Abnova Corporation) and Rabbit polyclonal LAMP2 (Lysosomal-associated membrane protein)(1:1000 Abcam).

3.2.5 NanoSight^{TM} Tracking Analysis

Size distribution and the estimated concentration of nanoparticles in purified EV isolated were analysed using a light scattering technology via measurement of the rate of Brownian motion with the NanoSight^{TM} LM10 system (NanoSight^{TM} Ltd, Amesbury, UK) configured with a (488 nm) laser and a high sensitivity digital camera (OrcaFlash2.8, Hamamatsu C11440, NanoSight^{TM} Ltd).

All samples were diluted with nanoparticle-free water so the concentration was within the range of 5×10^{7} to 5×10^{9} (nanoparticle number/mL). Samples were infused and recorded under controlled flow (infusion rate of 100) using a NanoSight^{TM} syringe pump and script control system. The ambient temperature was set at 25ºC, with the camera sensitivity and detection threshold set between 9 to 12 for maximum particle detection. Five different videos of 60 seconds from 3 different replicates were collected and analysed using NTA-software (version 2.3) for each sample.

3.2.6 Coomassie Blue Staining of EV Derived Proteins

Thirty micrograms of protein (serum, plasma, urine and their EVs) was loaded per lane onto the 12% polyacrylamide gel. Gels were fixed for 1hr with (50% (v/v) ethanol in

water with 10% Acetic Acid). The fixed gels were then washed with 50% (v/v) methanol in water containing 10% Acetic Acid at room temperature (RT). Gels were fixed overnight after which they were submerged into of Coomassie Blue staining solution (0.1% (w/v) coomassie blue, 10% (v/v) methanol and 10% (v/v) acetic acid) at room temperature for 4-6 hrs. The stained gels were de- stained with 50% methanol in water with 10% (v/v) acetic acid gently agitated until clear protein bands could be observed without any background staining. Finally gels were equilibrated in storage solution (5% (v/v) acetic acid solution) for at least an hour.

Gel lanes containing serum and plasma EVs were removed and cut into 25-35 sequential bands for subsequent proteomic analysis by mass spectrometry to obtain representative protein profiles for these isolates.

3.2.7 Proteomic Analysis

3.2.7.1 In Gel Digestion

Coomassie Blue stained gels bands were cut into 1mm pieces, neutralized with 50mM ammonium bicarbonate (100µl) twice for 10min and dehydrated twice using 50% acetonitrile (100µl) for 10min before fully drying under vacuum using a Centrivap for 10mins. Reduction and alkylation was carried out with 10mM DTT (50µl) for 35min at 65ºC followed by addition of 55mM iodoacetamide (30µl) for 30min at RT in the dark after removal of excess DTT. Gel pieces were then washed twice with 50mM ammonium bicarbonate for 10 minutes and dried as above with 50% acetonitrile and Centrivap before addition of 10µl trypsin solution (10ng/µl, Roche cat# 418025). The trypsin solution was allowed to absorb into the dried gel pieces on ice, excess was removed and 50mM ammonium

carbonate added to cover gel pieces after which they were incubated overnight at 37ºC. Supernatant peptide solutions were transferred to eppendorfs and gel pieces extracted for 15min twice with 50% acetonitrile/5% FA (50µl). Extracts were pooled, dried down to 15µl and transferred to LC vials for analysis. LC-MS analysis of the resulting peptide was similar to that used for the in solution trypsin digest generated samples described below.

3.2.7.2 In Solution Digestion

An in solution trypsin digestion protocol was used to generate peptides for LC-MS analysis. Serum and urine isolated EVs were initially sonicated for 5 min and 40µg protein equivalent sample then precipitated with 5-10x volumes of acetone (-20°C, 1 hour). Precipitate was sedimented by centrifugation (20,000g, 5min) and the pellet re-dissolved in 36µl of 25mM ammonium bicarbonate. DTT was added (1µl of 100mM solution) and samples incubated for 35 minutes at 65°C, followed by the addition of 100mM iodoacetamide (2µl) and incubation for an additional 30 minutes at RT in the dark. Upon addition of 1µl of 100ng/µl trypsin the sample was incubated overnight at 37°C. The resulting peptides were separated using a 75µm x 100mm 1.7µm BEH130 C18 column using a 3-40% linear acetonitrile gradient with 0.1% FA present throughout, at 0.3µl/minute over 40minutes using a NanoAcquity™ LC (Waters). The column was re-equilibrated for 20min between runs. Column eluate was directed into a Synapt™ mass spectrometer through a 20µm capillary held at 3.2kV. Instrument calibration was carried out using Glu-Fibrinogen fragments and Glu-Fibrinogen was also used as a lock mass to compensate for any calibration drift. The instrument was run in V-mode with a mass resolution of approximately 10,000. A data dependent method was used with a 1 second scan followed by up to 3 fragment scans,

using ion intensity and charge state as the main selection criteria. The accumulated data was analysed using ProteinLynx Global Server software (PLGS) using peptide and fragment mass accuracies of 25ppm and 0.1Da respectively. Uniform carbamido methyl C and variable N-terminal acetyl, M oxidation, N deamidation and C propionamide were selected as permitted modifications with a maximum protein MW of 250K. This search engine was applied to the full Uniprot database, human species. A search with similar parameters was also carried out using Mascot using the pkl peak list files generated in PLGS.

3.2.8 Pathway Analysis

Pathway analysis was performed on data generated from proteomic mass spectrometric analysis using Ingenuity Pathway Analysis (IPA; Ingenuity System, Redwood city, CA, www.ingenuity.com).

IPA generated data were used for identification of biological pathways, cellular function, top canonical pathways and potential biomarker signatures and their application in different pathological conditions, in particular cancer and specifically PCa.

3.2.9 Cholesterol Analysis

Serum, urine and derived EVs were extracted similar to the methodology of Matyash (Matyash *et al.*, 2008) and derivatized prior to analysis by the method of Liebisch et al. (Liebisch, Binder *et al*. 2006). Briefly, samples (5µl serum, 20µl serum EVs, or 100µl urine/urine EV) were spiked with 200ng d6 cholesterol and vortexed with 1ml 20:80 MeOH:MTBE (methanol:methyl- t-butyl ether) in glass tubes for 30 minutes; ddH_2O (500µl) was added followed by another 10 minutes vortex. Samples were centrifuged for

5 minutes (Centrivap) and the upper layer collected. A second extraction using 1ml MeOH:MTBE was carried out, pooled with the first and the extracts dried in the Centrivap under vacuum. Dried extracts were dissolved in 200µl of acetyl chloride/chloroform 1/5 and incubated at room temperature for 1hr. Samples were then left uncapped in a fume hood for 15 minutes before a final drying in the Centrivap. Residue was re-dissolved in 60µl of 70/30 methanol/chloroform, further diluted with 140µl methanol and centrifuged at 15,000g for 5 minutes before transferring to LC vials.

LC-MS analysis was carried out with a Waters Acquity UPLC coupled to a Quattro Premier XE using a 2.1x50mm, BEH 1.7µM C18 column. Mobile phase consisted of acetonitrile/0.1M ammonium acetate 9/1 (A) and isopropanol (B) with the following gradient: 0.2minute, 25%B; 5-8minutes, 70%B, 8.1minutes, 25% B with a 10min run length. Instrument parameters were optimized for the m/z's of ammonium adducts of acetate derivatized cholesterol and the m/z369 fragment was used for MRM quantitation. AUC's for cholesterol acetate and d6 cholesterol acetate were obtained using Quanlynx and a linear calibration curve from 0.2- 10µg/ml, R^2>0.99, was generated using AUC ratios.

3.2.10 Statistical Analysis

Unless indicated, analyses were performed on data generated from triplicate exosome preparations. Results were expressed as mean ± standard deviation. Statistical significance for differences between serum and urine derived exosomes in different groups were evaluated by Student's t-test ($p<0.05$).

3.3 Results

3.3.1 Urine and Blood EVs Purification and Characterization

The presence of EVs in both blood (serum and plasma) and urine samples were confirmed using TEM, WB and Nanoparticle Tracking Analysis (NTA).

Isolated EVs from healthy and PCa patients' serum and urine were fixed onto formvar- coated carbon EM grids for visualization under TEM (**Figure 3.2 A. and 3.3 A.**). TEM data revealed the presence of cup-shaped (artifact of sample preparation for TEM) nanovesicles with a size range of 30-200 nm in both samples types.

Protein quantitation assays were performed using the BCA assay followed by WB analysis to confirm the presence of exosome markers in our exosome isolates derived from serum, plasma and urine. As shown in **Figure 3.2 B.** both serum and plasma EVs contain LAMP2 (Lysosome-associated membrane protein 2), TSG101 (Tumor susceptibility gene) as well as CD63. Similarly urine samples (urine and urinary EVs) were examined for the presence of exosomal markers. As shown in **Figure 3.3 B.** all the tested exosomal markers (Alix, HSP70, HSP 90, TSG101, LAMP2) were present and enriched in the urinary EVs when compared to urine samples.

NTA was used to measure the EV isolation protocol efficacy in both serum and urine samples. The NTA data (**Figure 3.2 C, D.** and **3.3 C.**) demonstrates that between 83 to 87% of isolated nanoparticles in our isolate has the size of 30-200nm (exosomes size), while 13 to 16% are within the size of large microvesicles (200-1,000nm). While serum EVs didn't show increase in nanovesicle concentrations of 30-200 nm when compared to whole serum (**Data not shown**), the average concentration of urinary EVs was at least two times higher when compared to urine samples (**Fig 3.3 D.**).

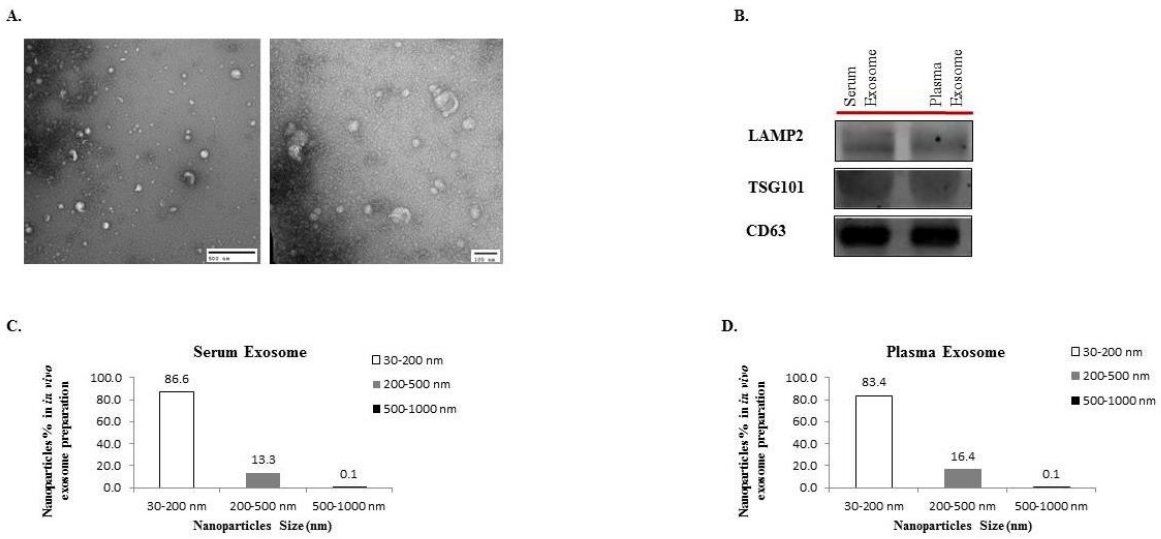


Figure 3.2 Serum EV characterization.

A.Transmission Electron Microscopy. TEM images of EVs derived from Human Plasma and Serum. EVs were negatively stained with 2% uracyl acetate after removing the extra moisture. Cup-shaped structures, with 30-200 nm size were identified as being exosomes. **B. Western Blot analysis for exosomes marker in Plasma and serum derived EVs.** EVs have been purified based on their unique size and density by ultracentrifugation with 30% sucrose-deuterium. Thirty micrograms of total protein associated with purified EVs were analyzed by Western Blot using different exosome markers. **C, D. Nanoparticle Tracking Analysis.** Bar chart showing the average percentage of nanoparticles within 30-200nm, 200-500nm, and 500-1000nm size in EVs derived from **C.** Serum and **D.** Plasma.

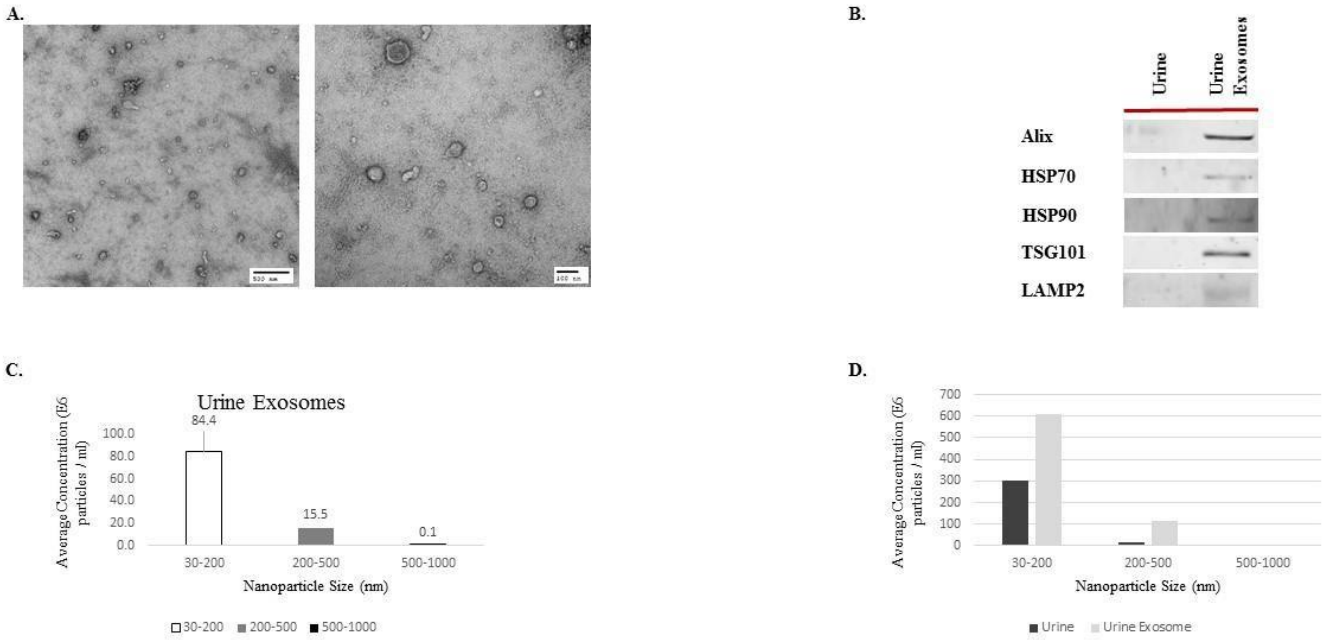


Figure 3.3 Urine EV characterization.

A. Transmission Electron Microscopy. TEM images of EV derived from Human Urine. EVs were negatively stained with 2% uracyl acetate after removing the extra moisture. Cup-shaped structures, with 30-200 nm size were identified as being exosomes. **B. Western Blot analysis for exosomes marker in Urinary exosomes.** EVs have been purified based on their unique size and density by ultracentrifugation. Thirty micrograms of total protein associated with purified EVs were analyzed by WB using different exosome markers. **C, D. Nanoparticle Tracking Analysis.** Bar chart showing the **C.** average percentage of nanoparticles within 30-200nm, 200- 500nm, and 500-1000nm size in urinary EVs, **D.** average particle number/mL for Urine and urinary EVs.

Finally the number and size of the nanoparticles were measured for both serum and urine samples using NanoSight^{TM} LM10 system. All data were determined for comparisons between control (n=3 healthy donors) and PCa patients with PSA lower (<50, n=3) or higher (>50, n=3) than fifty.

As shown in the **Figure 3.4 A.** the total serum derived nanoparticle numbers (particle/ml) were significantly higher in both PCa patients groups when compared with the control group (similar to that reported in our previous paper, (Hosseini-Beheshti *et al*., 2012)). Interestingly the size of nanoparticles derived from PCa patient serum were slightly smaller (104-159nm) when compared to those derived from the healthy control group (159-187nm) (**Figure 3.4 B.**).

When compared to urine derived EVs, while neither the total nanoparticle number nor the average nanoparticle size were significantly different between controls and PCa patients, there seemed to be a trend towards lower number and larger size of nanoparticles derived from PCa patient urine particularly when compared to those found in serum.

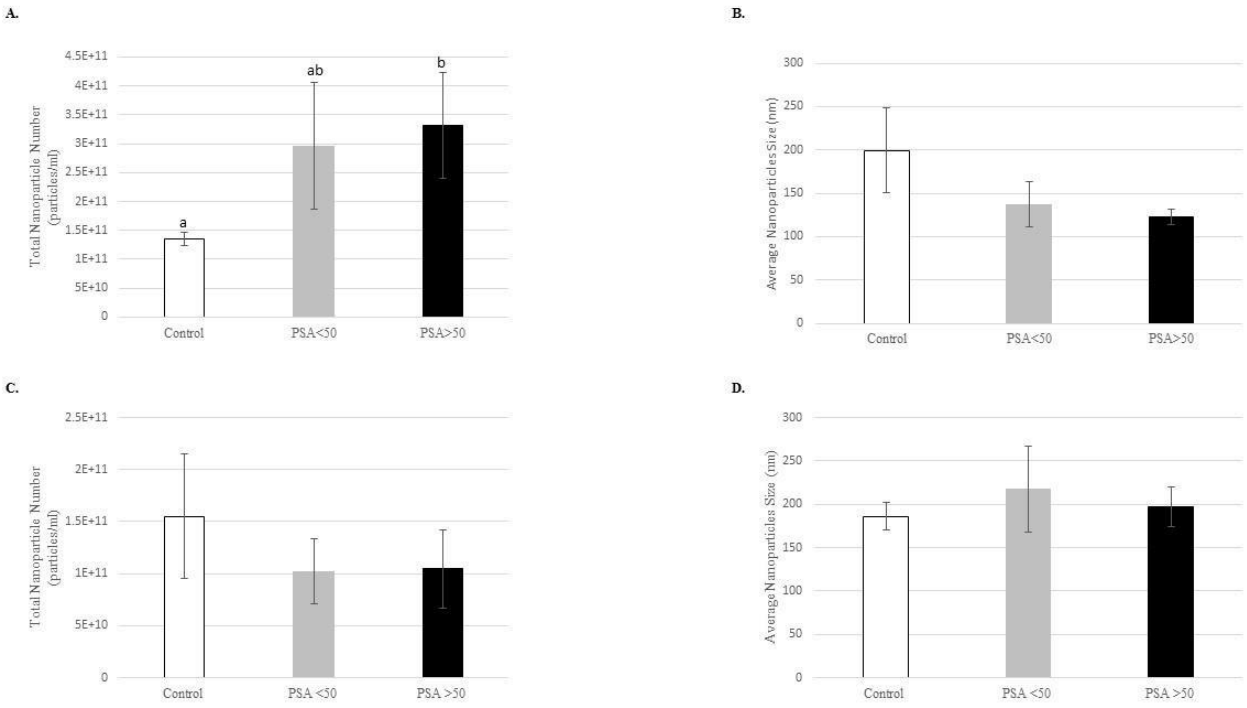


Figure 3.4 Nanoparticle Tracking Analysis.

Bar chart showing the Nanoparticles: **A, C.** number/ml for each step of exosome purification in serum EVs (n(control)=3, n (PSA<50)=7, n(PSA>50)=5) and urine derived EVs. (n (control)=3, n (PSA<50)=4, n(PSA>50)=5) **B, D.** Average nanoparticle size (nm) in Serum and urine derived EVs.

3.3.2 Proteomic Analysis of EVs Derived from Serum and Urine

3.3.2.1 In Gel Digestion

The protein molecular weight distribution of serum and plasma and their EVs from healthy male and female donors were compared using Coomassie Blue staining. This was also used with patient samples for both with and without AlbuminOUT^{TM} (G-Biosciences A Geno Technology, Inc. (USA)).

The main goal of this study was first to study and compare the protein distribution and profile of different samples and also to examine the clean-up of albumin which persisted in our exosome samples.

As shown in **Figure 3.5 A.** the albumin level decreased significantly in all EVs samples. We also used the AlbuminOUT^{TM} column (albumin removal with Cibacron Blue) to minimize albumin interference from patient serum EVs, prior to analysis. As shown in **Figure 3.5 B.** the albumin level has been significantly decreased in both serum EVs from all patients samples.

The Coomassie stained gels were cut into 25-35 pieces before being processed and analyzed by LC-MS. In our proteomic analysis carried out using one sample per group they yielded more than 160 proteins including 50 proteins with biomarker potential (**Data not shown**).

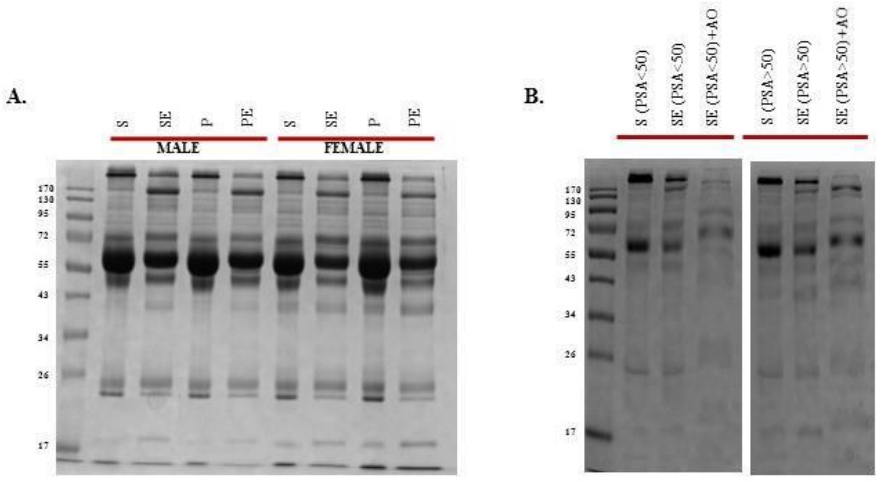


Figure 3.5 Coomassie blue staining showing the protein profile of healthy male and female serum vs serum derived EVs and plasma vs plasma derived EVs.

25 µl (**A**) or 15 µl (**B**) of each samples were resolved through 12% SDS-PAGE, followed by Coomassie blue staining. **A.** serum, serum exosomes, plasma and plasma exosomes of healthy male and female Samples loaded on SDS-PAGE. **B.** Comparison between patients' serum and serum EVs before and after using albumin out.

S:Serum; **SE:** Serum Derived EVs; **P:**Plasma; **PE:** Plasma Derived EVs; **AO:** AlbuminOut.

3.3.2.2 In Solution Digestion

In order to investigate the PCa patients' serum/urine protein profile and also to follow up with our previous proteomic analysis in PCa cell line derived exosomes (Hosseini-Beheshti *et al*. 2012), serum/urine EVs of 7 (serum)/4 (urine) PCa patients with PSA<50 and 5 (serum)/ 5 (urine) patients with PSA>50 were analyzed using LC-MS. Two biological replicates of samples obtained from each donor to ensure consistent results.

IPA software were also used to analyze the data, this software is able to analyze and interpret the data via 4 different assessments. Core analysis, Metabolomics, Toxicity and Biomarker Analysis. IPA Core and Biomarker Analysis were used for a rapid assessment of the signaling and metabolic pathways and identification of the most promising and relevant biomarker candidates.Our core and biomarker proteomic analysis resulted in identification of more than 77 serum exosome proteins and 74 urine exosome protein (**Table 3.2., 3.3.**).

Core analysis data were compared to the biomarker analysis list and as both lists were very similar we decided to only include the biomarker list in this paper. We then compared our biomarker proteomic data to that described in our previous published paper (Hosseini-Beheshti *et al*., 2012) and confirmed the presence of six mutual proteins including Alpha-2 macroglobulin, Albumin, Apolipoprotein A-I, Complement component 3, Hemopexin, and Transferin in both set. A list of all identified proteins with their protein ID, Subcellular location, Type, Drug, Biomarker application, Mascot peptide score and peptide matches is provided in **Table 3.2.** and **3.3.** for serum derived and urine derived EVs respectively.

Table 3.2 Identification of serum exosome-associated proteins from PCa Patients with PSA lower and higher than 50 as well as a control group.

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P04217	alpha-1-B glycoprotein	Extracellular Space	other		+	+	+		173	18	
P01023	alpha-2-macroglobulin	Extracellular Space	transporter		+	+	+		227	21	*
P01019	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Extracellular Space	growth factor		+	+	+	efficacy	89	11	
P02768	albumin	Extracellular Space	transporter		+	+	+	diagnosis,efficacy,prognosis,safety, unspecified application	168	21	*
P02647	apolipoprotein A-I	Extracellular Space	transporter		+	+	+	diagnosis,efficacy,unspecified application	129	17	*
P02652	apolipoprotein A-II	Extracellular Space	transporter		+	+	+		165	23	
P04114	apolipoprotein B	Extracellular Space	transporter	mipomersen	+	+	+	diagnosis,efficacy	137	15	
P01024	complement component 3	Extracellular Space	peptidase	IgG	+	+	+	efficacy	136	16	*
P0C0L4	complement component 4B (Chido blood group)	Extracellular Space	other	IgG	+	+	+		171	19	
P04003	complement component 4 binding protein, alpha	Extracellular Space	other		+	+	+		105	12	
O75155-2	cullin-associated and neddylation-dissociated 2 (putative)	Nucleus	transcription regulator		+				14	1	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P00450	ceruloplasmin (ferroxidase)	Extracellular Space	enzyme		+	+	+	efficacy	84	9	
A8K855-2	EF-hand calcium binding domain 7	Other	other		+				35	1	
Q9NY74	Ewing tumor-associated antigen 1	Other	other		+				16	2	
P02671-2	fibrinogen alpha chain	Extracellular Space	other	F2	+			diagnosis	124	7	
P68871	hemoglobin, beta	Cytoplasm	transporter	iron dextran	+	+	+		114	12	
P02042	hemoglobin, delta	Other	transporter		+		+		32	4	
P00738	haptoglobin	Extracellular Space	peptidase		+	+	+	diagnosis,efficacy, unspecified application	166	20	
P00739	haptoglobin-related protein	Extracellular Space	peptidase		+	+	+		106	12	
P02790	hemopexin	Extracellular Space	transporter		+	+	+		151	17	*
P04196	histidine-rich glycoprotein	Extracellular Space	other		+	+	+		105	11	
P01876	immunoglobulin heavy constant alpha 1	Extracellular Space	other		+	+	+		136	17	
P01877	immunoglobulin heavy constant alpha 2 (A2m marker)	Extracellular Space	other		+	+	+		129	29	
P01857	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other		+	+	+		155	17	
P01859	immunoglobulin heavy constant gamma 2 (G2m marker)	Plasma Membrane	other		+	+	+		122	15	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P01860	immunoglobulin heavy constant gamma 3 (G3m marker)	Extracellular Space	other		+	+	+		146	16	
P01861	immunoglobulin heavy constant gamma 4 (G4m marker)	Extracellular Space	other		+	+	+		121	14	
P01871-2	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor		+	+	+		164	18	
P01834	immunoglobulin kappa constant	Extracellular Space	other		+	+	+	unspecified application	144	14	
P0CG05	immunoglobulin lambda constant 2 (Kern-Oz-marker)	Extracellular Space	other		+	+	+		121	14	
P02763	orosomucoid 1	Extracellular Space	other		+	+	+	unspecified application	132	12	
P19652	orosomucoid 2	Extracellular Space	other		+	+	+		122	13	
P00747	plasminogen	Extracellular Space	peptidase	tenecteplase, PLAT, tranexamic acid, aprotinin, 6-aminocaproic acid, reteplase	+				17	1	
P01009-2	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Extracellular Space	other		+	+	+	diagnosis,unspecified application	108	13	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P01008	serpin peptidase inhibitor, clade C (antithrombin), member 1	Extracellular Space	enzyme	heparin, enoxaparin, ardeparin, SR-123781A, glucuronyl glucosamine glycan sulfate, fondaparinux, nadroparin	+				18	1	
Q149N8-4	SNF2 histone linker PHD RING helicase, E3 ubiquitin protein ligase	Nucleus	transcription regulator		+				23	1	
Q5JUK2-2	spermatogenesis and oogenesis specific basic helix-loop-helix 1	Cytoplasm	transcription regulator		+				14	2	
P02549-2	spectrin, alpha, erythrocytic 1 (elliptocytosis 2)	Cytoplasm	other		+				29	1	
Q8NHU6-2	tudor domain containing 7	Cytoplasm	other		+	+	+		21	1	
P02787	transferrin	Extracellular Space	transporter		+	+	+	efficacy,prognosis	110	12	*
Q15361	transcription termination factor, RNA polymerase I	Nucleus	transcription regulator		+				19	1	
P04004	vitronectin	Extracellular Space	other		+	+	+		112	12	
Q96GC6-4	zinc finger protein 274	Nucleus	transcription regulator		+				26	2	
Q68DY1-2	zinc finger protein 626	Other	other		+				18	8	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P02747	complement component 1, q subcomponent, C chain	Extracellular Space	other			+	+		75	10	
P01031	complement component 5	Extracellular Space	cytokine	eculizumab, IgG		+	+		94	11	
O75808	calpain 15	Other	peptidase			+	+		15	1	
O43866	CD5 molecule-like	Plasma Membrane	transmembrane receptor			+	+		165	16	
P08603	complement factor H	Extracellular Space	other			+	+	unspecified application	45	14	
P50461	cysteine and glycine-rich protein 3 (cardiac LIM protein)	Nucleus	other			+			17	1	
A7MBM2	dispatched homolog 2 (Drosophila)	Other	other			+			14	1	
O14490-5	discs, large (Drosophila) homolog-associated protein 1	Plasma Membrane	other			+			24	2	
Q96MC2	dynein regulatory complex subunit 1 homolog (Chlamydomonas)	Extracellular Space	other			+			14	1	
A4FU69-2	EF-hand calcium binding domain 5	Other	other			+			14	2	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P00734	coagulation factor II (thrombin)	Extracellular Space	peptidase	enoxaparin, desirudin, dabigatran etexilate, Fibrinogen, ximelagatran, antithrombin alfa, aspirin/dabigatran etexilate, dabigatran, argatroban, bivalirudin, lepirudin		+		diagnosis,unspecified application	93	11	
O95837	guanine nucleotide binding protein (G protein), alpha 14	Plasma Membrane	enzyme			+			14	2	
Q02846	guanylate cyclase 2D, membrane (retina-specific)	Plasma Membrane	kinase			+			14	1	
P0CG04	immunoglobulin lambda constant 1 (Mcg marker)	Cytoplasm	other			+	+		298	4	
Q8IWB1	inositol 1,4,5-trisphosphate receptor interacting protein	Extracellular Space	other			+	+		19	1	
O43272-1	proline dehydrogenase (oxidase) 1	Cytoplasm	enzyme			+		diagnosis	14	1	
P08519	lipoprotein, Lp(a)	Extracellular Space	other			+		efficacy	45	14	
P47972	neuronal pentraxin II	Extracellular Space	other			+		diagnosis	14	2	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
Q8NG80	olfactory receptor, family 2, subfamily L, member 5	Plasma Membrane	other			+			31	1	
O95837	guanine nucleotide binding protein (G protein), alpha 14	Plasma Membrane	enzyme			+			14	2	
Q02846	guanylate cyclase 2D, membrane (retina-specific)	Plasma Membrane	kinase			+			14	1	
P0CG04	immunoglobulin lambda constant 1 (Mcg marker)	Cytoplasm	other			+	+		298	4	
Q8IWB1	inositol 1,4,5-trisphosphate receptor interacting protein	Extracellular Space	other			+	+		19	1	
O43272-1	proline dehydrogenase (oxidase) 1	Cytoplasm	enzyme			+		diagnosis	14	1	
P08519	lipoprotein, Lp(a)	Extracellular Space	other			+		efficacy	45	14	
P47972	neuronal pentraxin II	Extracellular Space	other			+		diagnosis	14	2	
Q8NG80	olfactory receptor, family 2, subfamily L, member 5	Plasma Membrane	other			+			31	1	
P20742	pregnancy-zone protein	Extracellular Space	other			+			125	4	
Q9NWS8-2	required for meiotic nuclear division 1 homolog (S. cerevisiae)	Cytoplasm	other			+			25	1	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Serum EVs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P0DJI8	serum amyloid A1	Extracellular Space	transporter			+	+	diagnosis,unspecified application	85	13	
O14907	Tax1 (human T-cell leukemia virus type I) binding protein 3	Cytoplasm	transcription regulator			+		unspecified application	23	1	
Q96PF1	transglutaminase 7	Other	enzyme			+	+		31	2	
O75157-2	TSC22 domain family, member 2	Extracellular Space	other			+			15	1	
Q99856	AT rich interactive domain 3A (BRIGHT-like)	Nucleus	transcription regulator				+		19	1	
P02750	leucine-rich alpha-2-glycoprotein 1	Extracellular Space	other				+		87	13	
Q0VAA2	leucine rich repeat containing 74	Other	other				+		17	1	
P46199	mitochondrial translational initiation factor 2	Cytoplasm	translation regulator				+		21	1	
Q6IFG1	olfactory receptor, family 52, subfamily E, member 8	Plasma Membrane	G-protein coupled receptor				+		21	2	
P06702	S100 calcium binding protein A9	Cytoplasm	other				+	diagnosis,unspecified application	61	10	
P0DJI9	serum amyloid A2	Extracellular Space	other				+		84	11	
O75533	splicing factor 3b, subunit 1, 155kDa	Nucleus	other				+		27	2	

Proteins with Mascotscores lower than 40 are listed in highlighted column this table, (n (control)=3, n (PSA<50)=7, n(PSA>50)=5). Proteins denoted with * are the mutual proteins present in our *in vitro* EVs proteomic analysis.

Table 3.3 Identification of urine exosome-associated proteins from PCa Patients with PSA lower and higher than 50 as well as a control group.

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P02768	Albumin	Extracellular Space	transporter		+	+	+	diagnosis,efficacy,prognosis,safety, unspecified application	150	18	*
P02760	alpha-1-microglobulin/ bikunin precursor	Extracellular Space	transporter		+	+	+	safety,unspecified application	97	17	
H0Y4T9	ankyrin repeat domain 26	Nucleus	transcription regulator		+						
C9JF17	apolipoprotein D	Extracellular Space	transporter		+		+	safety	56	7	
C9JEV0	alpha-2-glycoprotein 1, zinc-binding	Extracellular Space	transporter		+				81	14	
P53634	cathepsin C	Cytoplasm	peptidase		+			unspecified application	13	1	
P01859	immunoglobulin heavy constant gamma 2 (G2m marker)	Plasma Membrane	other		+	+			21	1	
P01834	immunoglobulin kappa constant	Extracellular Space	other		+	+	+	unspecified application	132	13	
P04264	keratin 1	Cytoplasm	other		+		+	diagnosis	81	9	
P13645	keratin 10	Cytoplasm	other		+				80	8	
P35908	keratin 2	Other	other		+				67	9	
P02538	keratin 6A	Other	other		+			diagnosis	77	9	
P04259	keratin 6B	Cytoplasm	other		+			diagnosis	66	9	
Q86Y46	keratin 73	Extracellular Space	other		+						
P35527	keratin 9	Other	other		+			diagnosis	47	5	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
O00187-2	mannan-binding lectin serine peptidase 2	Extracellular Space	peptidase		+			prognosis	22	1	
P02763	orosomucoid 1	Extracellular Space	other		+	+	+	unspecified application	98	10	
P19652	orosomucoid 2	Extracellular Space	other		+	+	+		66	9	
H0Y5A1	prostaglandin D2 synthase 21kDa (brain)	Cytoplasm	enzyme		+	+	+	efficacy	59	8	
D6RCM9	RAN binding protein 3-like	Other	other		+				19	1	
P07998	ribonuclease, RNase A family, 1 (pancreatic)	Extracellular Space	enzyme		+	+			43	7	
Q9HAT2	sialic acid acetylesterase	Cytoplasm	enzyme		+				28	1	
P10451-2	secreted phosphoprotein 1	Extracellular Space	cytokine		+			diagnosis,efficacy,unspecified application	38	2	
P01023	alpha-2-macroglobulin	Extracellular Space	transporter			+			82	9	*
P02647	apolipoprotein A-I	Extracellular Space	transporter			+		diagnosis,efficacy,unspecified application	102	11	*
P02652	apolipoprotein A-II	Extracellular Space	transporter			+			37	8	
P04114	apolipoprotein B	Extracellular Space	transporter	mipomersen		+		diagnosis,efficacy	109	15	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P25311	alpha-2-glycoprotein 1, zinc-binding	Extracellular Space	transporter			+	+		79	10	
P02747	complement component 1, q subcomponent, C chain	Extracellular Space	other			+			42	8	
P01024	complement component 3	Extracellular Space	peptidase	IgG		+		efficacy	60	8	*
E9PNW4	CD59 molecule, complement regulatory protein	Plasma Membrane	other			+			77	1	
O43866	CD5 molecule-like	Plasma Membrane	transmembrane receptor			+			107	14	
C9J979	cleavage and polyadenylation specific factor 3-like	Nucleus	other			+			19	1	
D6RAK8	group-specific component (vitamin D binding protein)	Extracellular Space	transporter			+			53	110	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
C9JV37	coagulation factor II (thrombin)	Extracellular Space	peptidase	enoxaparin, desirudin, dabigatran etexilate, Fibrinogen, ximelagatran, antithrombin alfa, aspirin/dabigatran etexilate, dabigatran, argatroban, bivalirudin, lepirudin		+		diagnosis, unspecified application	33	1	
P00738	haptoglobin	Extracellular Space	peptidase			+		diagnosis,efficacy,unspecified application	58	8	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P02790	hemopexin	Extracellular Space	transporter			+			85	12	*
P01876	immunoglobulin heavy constant alpha 1	Extracellular Space	other			+			81	22	
P01857	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other			+			153	17	
P0CG05	immunoglobulin lambda constant 2 (Kern-Oz-marker)	Extracellular Space	other			+			102	11	
P0DJD7	pepsinogen 5, group I (pepsinogen A)	Extracellular Space	peptidase	sucralfate		+			32	1	
C9JMK5	phosphoinositide -3-kinase interacting protein 1	Other	other			+	+		36	1	
P01009-2	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	Extracellular Space	other			+		diagnosis,unspecified application	58	9	
H7C4W7	stabilin 1	Plasma Membrane	transporter			+			18	2	
P02787	transferrin	Extracellular Space	transporter			+		efficacy,prognosis	92	9	*

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P01871-2	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor			+			81	11	
Q7L0Y3	tRNA methyltransferase 10 homolog C (S. cerevisiae)	Cytoplasm	other			+			16	2	
P04004	vitronectin	Extracellular Space	other			+			57	9	
Q16853	amine oxidase, copper containing 3	Plasma Membrane	enzyme	hydralazine, hydralazine/ hydrochlorothiazide/reserpine, hydralazine/ hydrochlorothiazide, hydralazine/isosorbide dinitrate			+		30	1	
P08571	CD14 molecule	Plasma Membrane	transmembrane receptor				+	efficacy	78	8	
E9PNW4	CD59 molecule, complement regulatory protein	Plasma Membrane	other				+		77	10	
J3KRR7	cylindromatosis (turban tumor syndrome)	Nucleus	transcription regulator				+		28	1	
Q8IV36-2	HID1 domain containing	Plasma Membrane	other				+		18	1	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P52272-2	heterogeneous nuclear ribonucleoprotein M	Nucleus	other				+		41	6	
Q8N3X6-2	ligand dependent nuclear receptor corepressor-like	Nucleus	transcription regulator				+		17	1	
P02750	leucine-rich alpha-2-glycoprotein 1	Extracellular Space	other				+		74	8	
O60487	myelin protein zero-like 2	Plasma Membrane	other				+		24	1	
Q8NGX3	olfactory receptor, family 10, subfamily T, member 2	Plasma Membrane	other				+		20	1	
B1AVU8	prosaposin	Extracellular Space	other				+		29	2	
Q5VY30	retinol binding protein 4, plasma	Extracellular Space	transporter				+	unspecified application	53	4	
G3V3A0	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	Extracellular Space	other				+	unspecified application	62	2	
H7C5E6	solute carrier family 26 (anion exchanger), member 8	Plasma Membrane	transporter				+		23	1	
D6R9C5	secreted phosphoprotein 1	Extracellular Space	cytokine				+	diagnosis,efficacy,unspecified application	23	1	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P51809-2	vesicle-associated membrane protein 7	Cytoplasm	transporter				+		18	1	
Q8N3X6-2	ligand dependent nuclear receptor corepressor-like	Nucleus	transcription regulator				+		17	1	
P02750	leucine-rich alpha-2-glycoprotein 1	Extracellular Space	other				+		74	8	
O60487	myelin protein zero-like 2	Plasma Membrane	other				+		24	1	
Q8NGX3	olfactory receptor, family 10, subfamily T, member 2	Plasma Membrane	other				+		20	1	
B1AVU8	prosaposin	Extracellular Space	other				+		29	2	
Q5VY30	retinol binding protein 4, plasma	Extracellular Space	transporter				+	unspecified application	53	4	
G3V3A0	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	Extracellular Space	other				+	unspecified application	62	2	
H7C5E6	solute carrier family 26 (anion exchanger), member 8	Plasma Membrane	transporter				+		23	1	
D6R9C5	secreted phosphoprotein 1	Extracellular Space	cytokine				+	diagnosis,efficacy,unspecified application	23	1	

ID	Protein Name	Subcellular location	Type(s)	Drug(s)	Urine Evs			Biomarker Application(s)	Mascot score	Peptide matches	Reference
					Control	PSA <50	PSA >50				
P51809-2	vesicle-associated membrane protein 7	Cytoplasm	transporter				+		18	1	

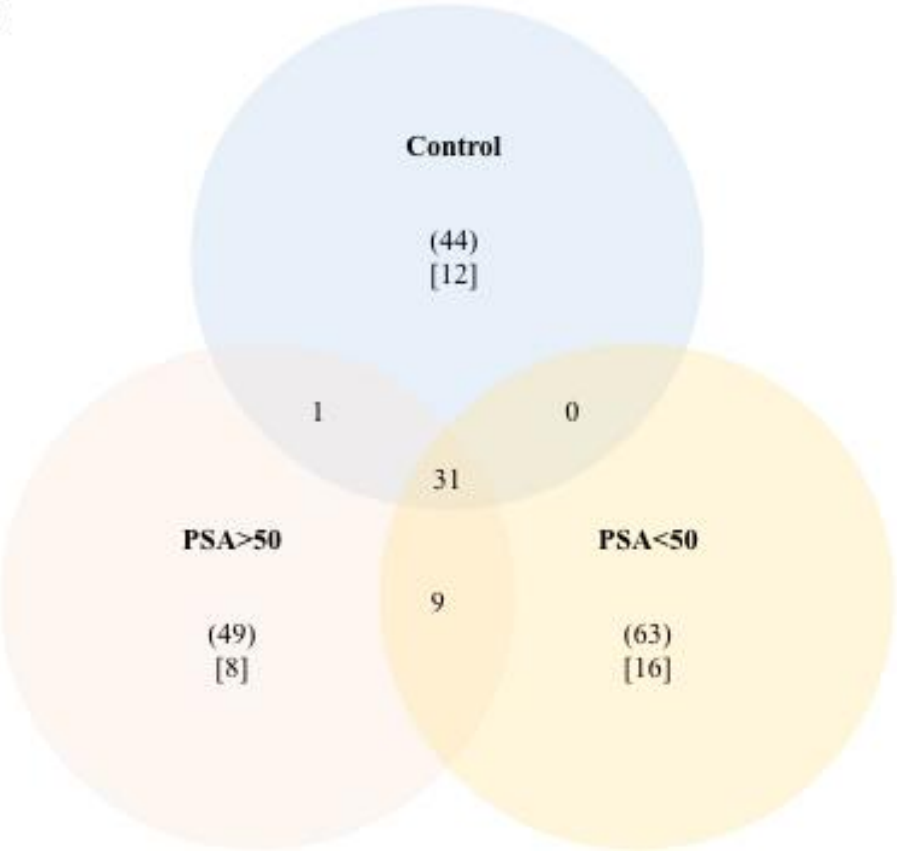
Proteins with Mascotscores lower than 40 are listed in highlighted column this table, (n (control)=3, n (PSA<50)=4, n(PSA>50)=5). Proteins denoted with * are the mutual proteins present in our *in vitro* EVs proteomic analysis.

3.3.2.2.1 Serum EVs

As demonstrated in **Figure 3.6 A.** analysis of serum EVs identified proteins in all three groups, 12 proteins were [only] belong to control groups (including: cullin-associated and neddylation-dissociated 2 (putative), EF-hand calcium binding domain 7, Ewing tumor-associated antigen 1, fibrinogen alpha chain, plasminogen, serpin peptidase inhibitor-clade C (antithrombin)- member 1 SNF2 histone linker PHD RING helicase, E3 ubiquitin protein ligase, spermatogenesis and oogenesis specific basic helix-loop-helix 1, spectrin, alpha, erythrocytic 1 (elliptocytosis 2), transcription termination factor- RNA polymerase I, zinc finger protein 274, zinc finger protein 626.), 16 proteins were unique in PCa patients with PSA lower than 50 (including: cysteine and glycine-rich protein 3 (cardiac LIM protein), dispatched homolog 2 (Drosophila), discs- large (Drosophila) homolog-associated protein 1, dynein regulatory complex subunit 1 homolog (Chlamydomonas), EF-hand calcium binding domain 5, coagulation factor II (thrombin), guanine nucleotide binding protein (G protein)-alpha 14, guanylate cyclase 2D- membrane (retina-specific), proline dehydrogenase (oxidase) 1, lipoprotein, Lp(a), neuronal pentraxin II, olfactory receptor- family 2- subfamily L-member 5, pregnancy-zone protein, required for meiotic nuclear division 1 homolog (S. cerevisiae), Tax1 (human T-cell leukemia virus type I) binding protein 3, TSC22 domain family, member 2) and only 8 proteins were identified in PCa patients with PSA higher than 50 (including: AT rich interactive domain 3A (BRIGHT-like), leucine-rich alpha-2-glycoprotein 1, leucine rich repeat containing 74, mitochondrial translational initiation factor 2, olfactory receptor, family 52, subfamily E-member 8, S100 calcium binding protein A9, serum amyloid A2, splicing factor 3b- subunit 1- 155kDa) and not any other groups. 31 identified proteins were mutual in all three different groups (control, PSA< 50, PSA>50) 9 proteins were mutual between the

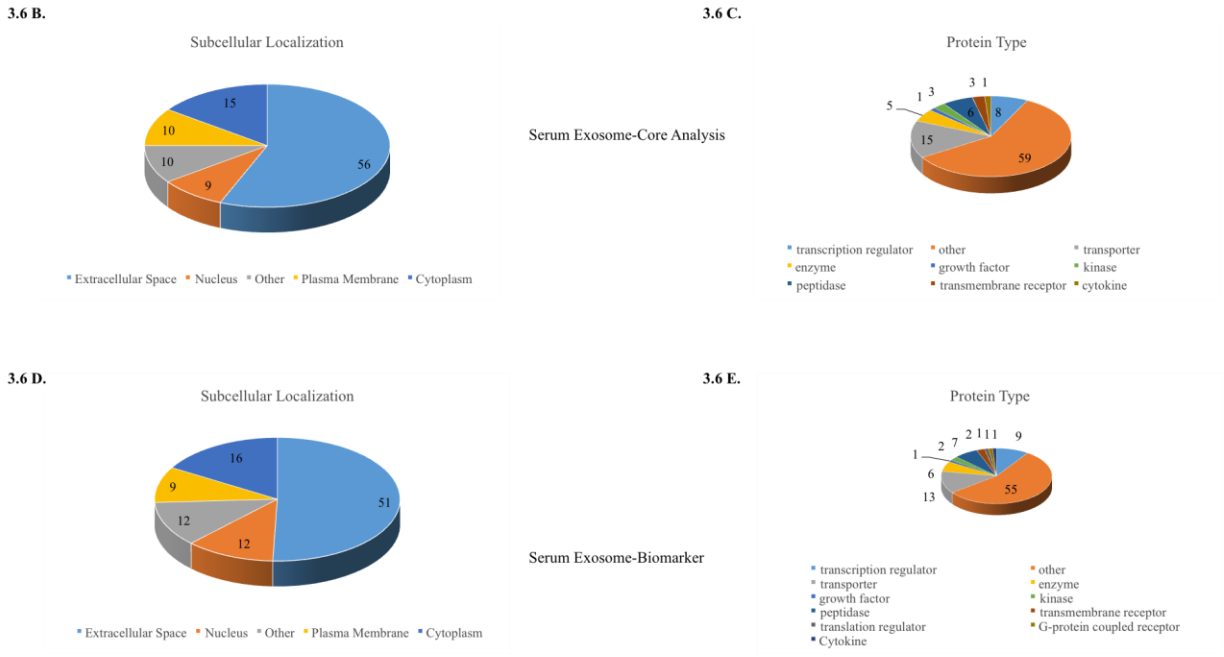
two PCa patients group (PSA<50, PSA>50) (including: complement component 1- q subcomponent- C chain, complement component 5, calpain 15, CD5 molecule-like, complement factor H, immunoglobulin lambda constant 1 (Mcg marker), inositol 1,4,5-trisphosphate receptor interacting protein, serum amyloid A1, Tax1 (human T-cell leukemia virus type I) binding protein 3, transglutaminase 7), 1 proteins were mutual between control and PSA>50 group (hemoglobin, delta) and no mutual proteins were found between control and PSA<50 group.

3.6A.



The protein subcellular localization and type of both core and biomarker analysis determined using Ingenuity pathway analysis have been reported and compared in **Figure 3.6. B-E.** The majority of these proteins were localized in the extracellular space (>50%), followed by 15-16% from cytoplasm, 9-10% plasma membrane and 9-12% from nucleus.

Between 10-12% of these proteins were identified with unknown subcellular localization in this analysis (**Figure 3.6. B, D)**.



All these proteins were identified as Transporters (13-15%), Transcription regulators (8-9%), Peptidases (6-7%), Other enzymes (5-6%), Kinases (2-3%), Transmembrane receptor (2-3%), Growth factors (1%), Cytokine (1%) Translation regulators 1% and G-protein coupled receptor 1%. The remaining 55-59% of these proteins have been categorized as protein with "other" function.

Our Ingenuity pathway analysis indicates that the main top five cellular function of serum EVs derived from healthy donors were involved in Embryonic Development, Organismal Development, Cardiovascular Disease, Endocrine System Disorders, Organismal Injury and Abnormalities and Reproductive System Disease, while serum EVs derived from PCa patients (with PSA lower and higher than 50) were mainly for Ophthalmic Disease, Organismal Injury and Abnormalities, Cardiovascular Disease,

Embryonic Development, Organismal Development Cell-To-Cell Signalling and Interaction and Endocrine System. Top predicted canonical pathways were very similar in all three groups of serum derived EVs (**Figure 3.6 F.,G.**).

3.6 F. Exosomal protein function in serum derived EVs

3.6 F.

3.6 G. Main canonical pathway function in urine derived EVs

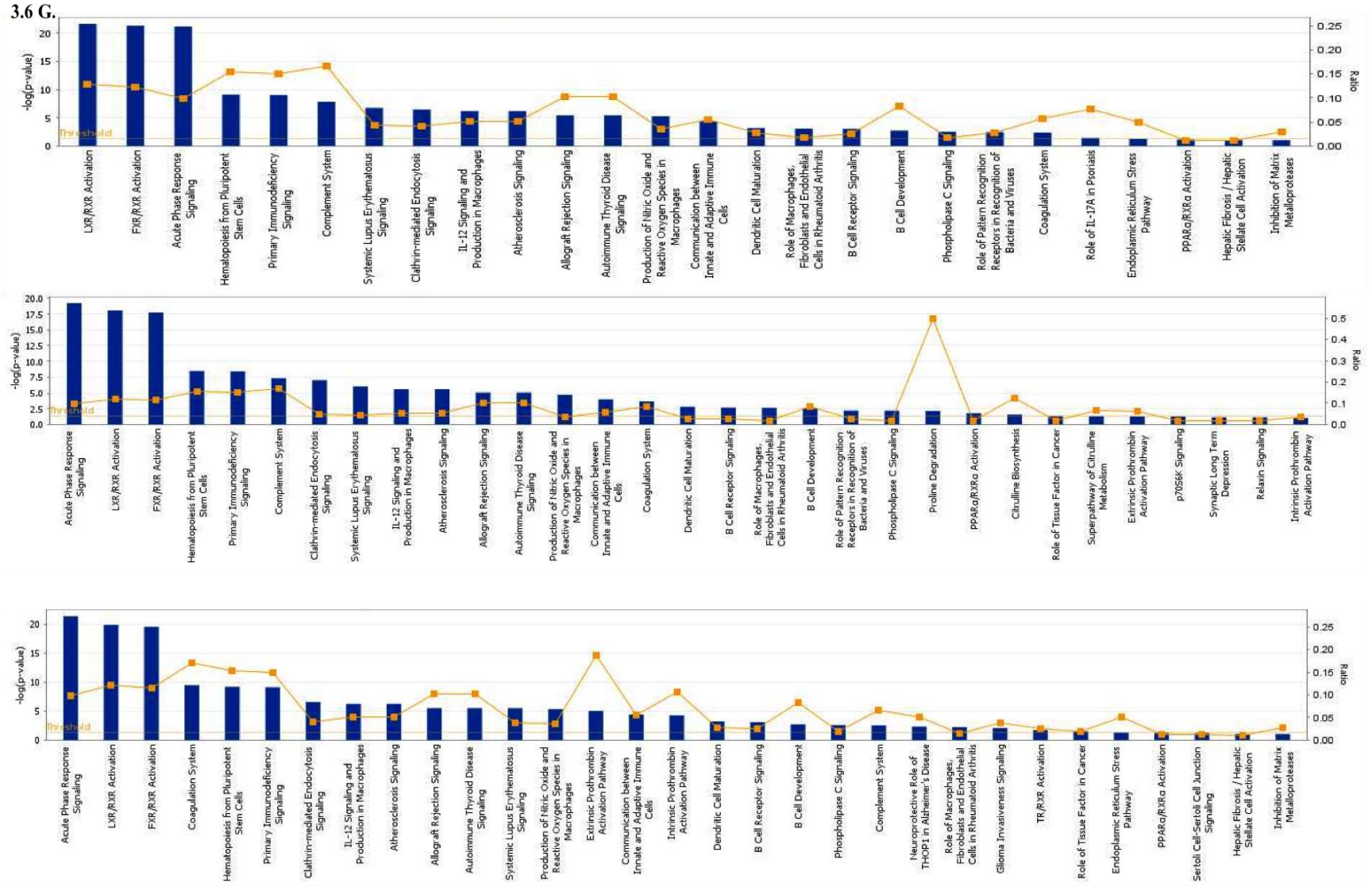


Figure 3.6. Proteomic analysis of different patient groups.

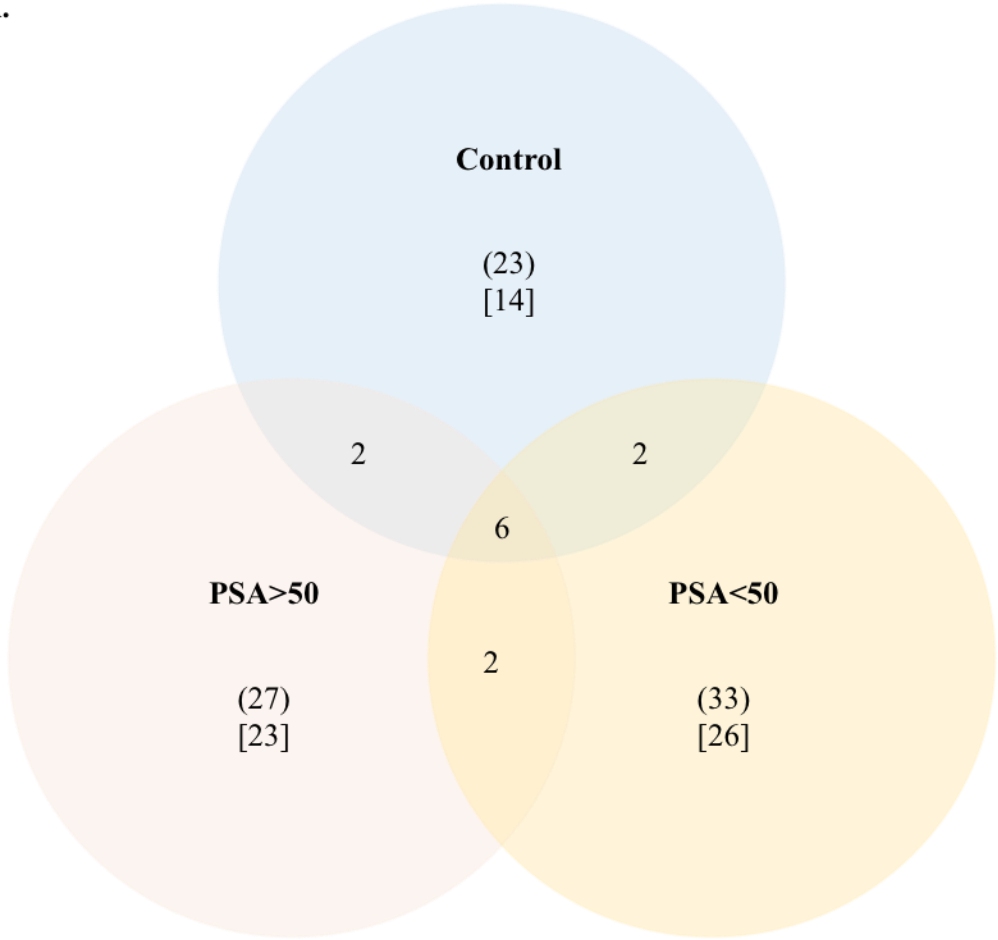
Venn diagram describing the mutuality of proteins in serum EVs derived from the control group versus serum EVs from patients with PSA lower or higher than 50. Numbers in ( ) are representative of the total number of proteins present in each group, Numbers in [ ] are representative of proteins present in either designated category and not present in any other undesignated category **B-E.** Pie chart showing the subcellular localization and Type of proteins found in serum derived EVs by core analysis and biomarker analysis using Ingenuity software. **F.** Bar chart indicating the disease and cellular function cellular function of proteins found within EVs determined using Ingenuity software. **G.** Predicted top canonical pathways are represented by the identified exosomal proteins. (n(control)=3, n (PSA<50)=7, n(PSA>50)=5)

3.3.2.2.2 Urinary EVs

As shown in **Figure 3.7 A.**, 14 unique proteins were identified in our urinary exosome proteomic analysis in the control (including: ankyrin repeat domain 26, alpha-2-glycoprotein 1- zinc-binding, cathepsin C, keratin 1, keratin 10, keratin 2, keratin 6A, keratin 6B, keratin 73, keratin 9, mannan-binding lectin serine peptidase 2, RAN binding protein 3-like, sialic acid acetylesterase, secreted phosphoprotein 1), 26 proteins were unique in PCa patients with PSA lower than 50 (including: alpha-2-macroglobulin, apolipoprotein A-I, apolipoprotein A-II, apolipoprotein B, complement component 1-q subcomponent-C chain, complement component 3, CD59 molecule- complement regulatory protein, CD5 molecule-like, cleavage and polyadenylation specific factor 3-like, group-specific component (vitamin D binding protein), coagulation factor II (thrombin), haptoglobin, hemopexin, immunoglobulin heavy constant alpha 1, immunoglobulin heavy constant gamma 1 (G1m marker), immunoglobulin lambda constant 2 (Kern-Oz- marker), pepsinogen 5- group I (pepsinogen A), serpin peptidase inhibitor- clade A (alpha-1 antiproteinase, antitrypsin)-member 1, stabilin 1, transferrin, immunoglobulin heavy constant mu, tRNA methyltransferase 10 homolog C (S. cerevisiae), vitronectin) and 23 proteins were unique in PCa patients with PSA>50 (including: amine oxidase, copper containing 3, CD14 molecule, CD59 molecule-complement regulatory protein, cylindromatosis (turban tumor syndrome), HID1 domain containing, heterogeneous nuclear ribonucleoprotein M, ligand dependent nuclear receptor corepressor-like, leucine-rich alpha-2-glycoprotein 1, myelin protein zero-like 2, olfactory receptor, family 10- subfamily T- member 2, prosaposin, retinol binding protein 4- plasma, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin)-member 3, solute carrier family 26 (anion exchanger)-member 8, secreted

phosphoprotein 1, vesicle-associated membrane protein 7, ligand dependent nuclear receptor corepressor-like, leucine-rich alpha-2-glycoprotein 1, myelin protein zero-like 2, olfactory receptor, family 10-subfamily T- member 2, prosaposin, retinol binding protein 4-plasma, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin)-member 3, solute carrier family 26 (anion exchanger)- member 8, secreted phosphoprotein 1, vesicle-associated membrane protein 7) groups respectively. Our data also shows that there were 6 proteins mutual to all three groups (Albumin, Alpha-1-microglubin/bikunin precursor, immunoglobulin kappa constant, orosomucoid 1, orosomucoid 2 and prostaglandin D2 synthase), 2 proteins mutual to control and PSA<50 groups (immunoglobulin heavy constant gamma 2 and ribonuclease RNase A family, 1), 2 protein mutual tothe control and PSA>50 (apolipoprotein D And keratin 1) groups and lastly 2 proteins mutual to PSA<50 and PSA>50 groups (Alpha-2-glycoprotein 1, zinc-binding and phosphoinositide-3-kinase interacting protein 1).

3.7 A.



Similar to what we saw in serum EVs the majority of identified proteins in urine derived EVs were from extracellular space (53-55%), followed by 19-20% from plasma membrane, 11% from cytoplasm, 8% from nucleus and 8% from unknown location (**Figure 3.7 B., D.**).

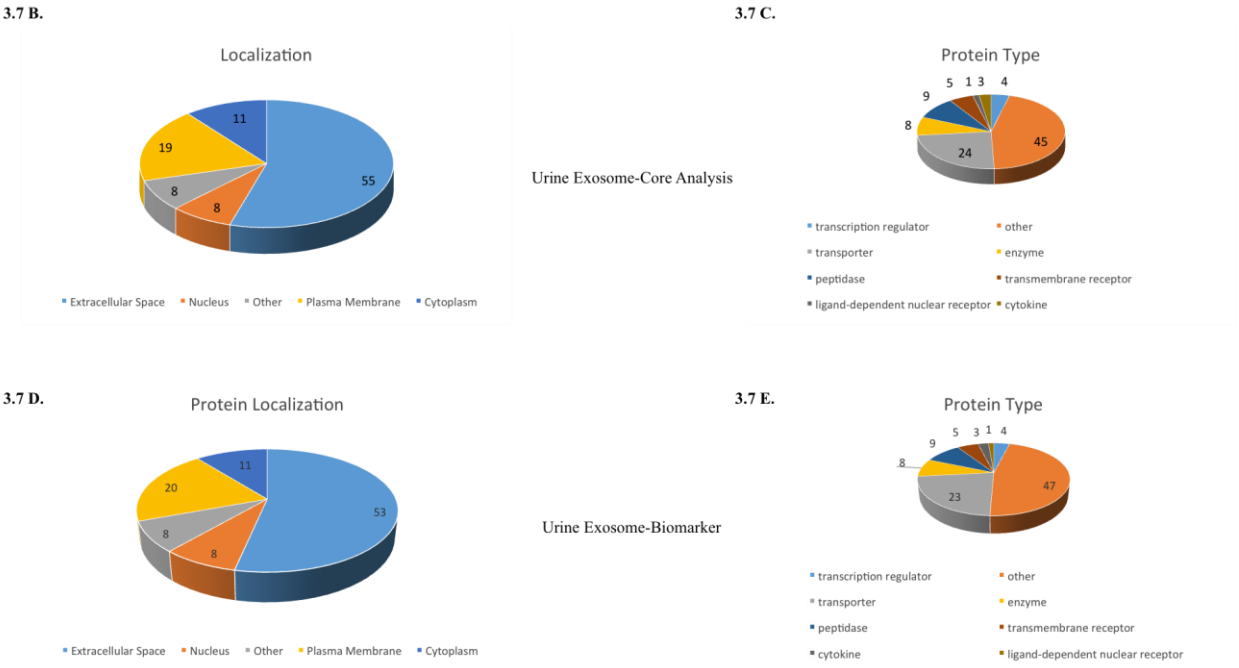
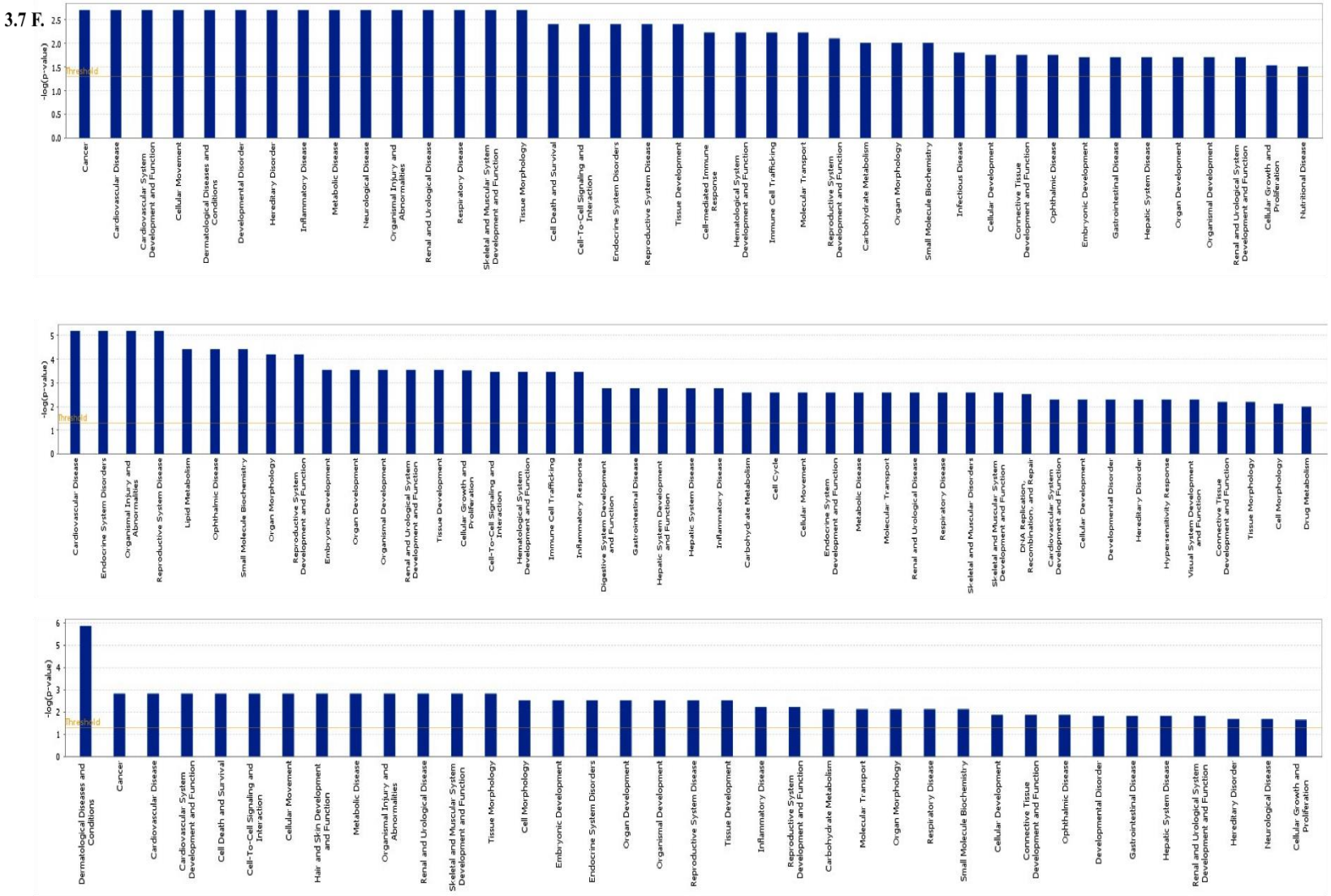


Figure 3.7 C, E. indicates that all the proteins have been categorized to 8 protein types. With the exception of proteins that had unidentified protein type (45-47%) most of the proteins were Transporters (23-24%) followed by Peptidases (9%), Other enzymes (8%), Transmembrane receptors (5%), Transcription regulators (4%), Cytokines (3%) and Ligand-dependent nuclear receptors (1%).

Our IPA data for urinary EVs demonstrated that while Dermatological Disease and Conditions, Cancer, Cardiovascular Disease, Cardiovascular System Development and Function and Cell Death and Survival were the top five disease and cellular functions identified in the control group, Cancer, Cardiovascular Disease, Endocrine System Disorders, Organismal Injury and Abnormalities, Reproductive System Disease, Lipid Metabolism, Cellular Movement and Dermatological Disease and Function were the major ones determined for the PCa patient groups (both PSA<50 and PSA>50). When compared, the top canonical pathways in these three groups: Acute Phase Response Signalling, LXR/PXR

Activation and FXR/PXR Activation, were higher than the control group.

3.7 F. Exosomal protein function in urine derived EVs



3.7 G. Main canonical pathway function in urine derived EVs

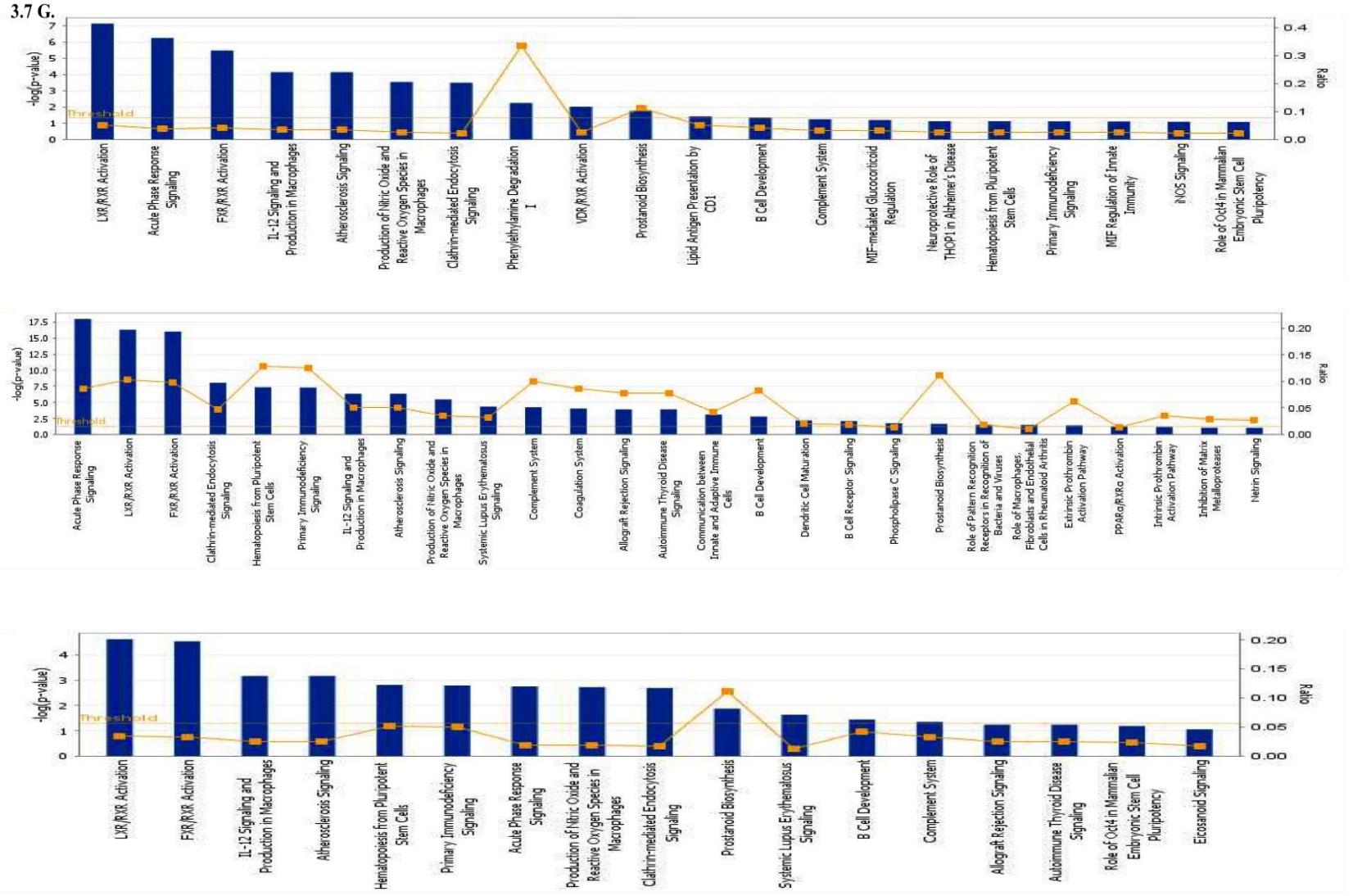


Figure 3.7 Proteomic analysis of different patient groups

A. Venn diagram describing the mutuality of proteins in urine EVs derived from the control group versus urine EVs from patients with PSA lower or higher than 50. Numbers in ( ) are representative of the total number of proteins present in each group, Numbers in [ ] are representative of proteins present in either designated category and not present in any other undesignated category **B-E.** Pie chart showing the subcellular localization and Type of proteins found in urine derived EVs by core analysis and biomarker analysis using Ingenuity software. **F.** Bar chart indicating the disease and cellular function of proteins found within EVs determined using Ingenuity software. **G.** Predicted top canonical pathways are represented by the identified exosomal proteins. (n(control)=3, n (PSA<50)=4, n(PSA>50)=5)

.

3.3.3 Cholesterol Content

The cholesterol content of all samples (serum, urine, serum derived EVs, urine derived EVs) was determined using LC-MS. As shown in **Figure 3.8 A.** while the average cholesterol level of PCa patient serum EVs (n=12) seems to be slightly higher than the control group (n=3) (10.75 µg/ml vs 14.50 µg/ml respectively) there was no significant difference between the cholesterol content of serum and serum derived EVs in all three samples.

Interestingly, as shown in **Figure 3.8 B.** the urinary EVs were in general enriched in cholesterol when compared to the urine cholesterol content of each group. The cholesterol level of the PCa patients urinary EVs in both PSA<50 and PSA>50 is significantly lower than the control group (2.95 µg/ml vs 0.96 µg/ml respectively).

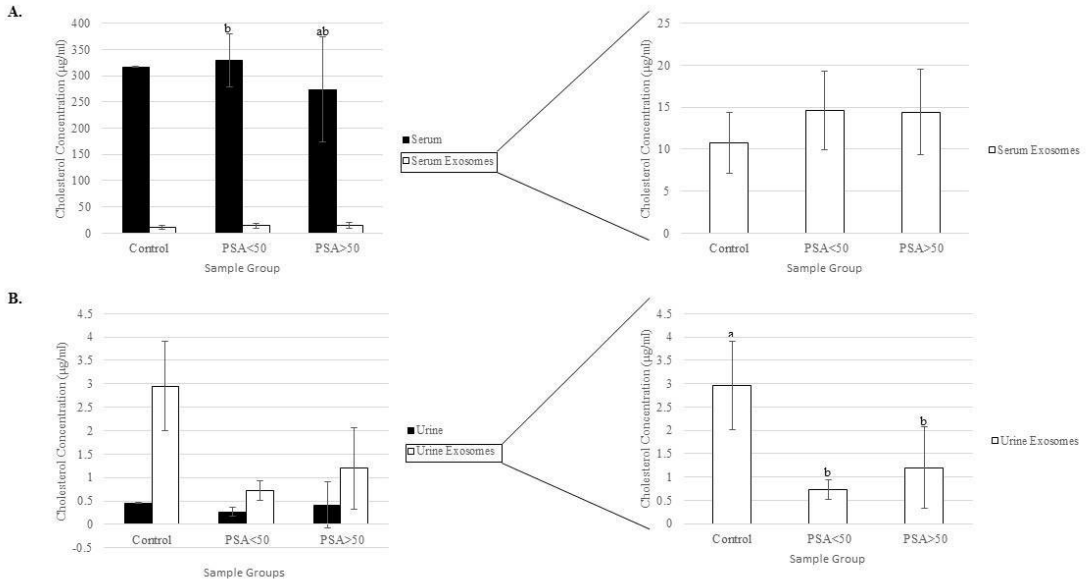


Figure 3.8 Cholesterol concentration.

The bar diagrams show the cholesterol concentration of **A.** Serum and Serum EVs , (n(control)=3, n (PSA<50)=7, n(PSA>50)=5) **B.** Urine and Urine EVs (n (control)=3, n (PSA<50)=4, n(PSA>50)=5)

3.4 Discussion

The presence of EVs in accessible biological fluids (e.g. blood and urine) combined with the presence of preserved and bioactive differential exosomal protein, lipid and genetic markers involved in cancer development and progression is an avid area of research interest in areas of both cancer biomarker discovery and therapeutic targeting. In this study we characterized EVs from two biological fluids and performed proteomic analysis to identify the most promising and relevant exosomal biomarker candidates in PCa patient samples.

To date exosome isolation especially from biological fluids has remained one of the major and initial challenges in EVs research. Many different protocols and commercial kits have been developed in order to improve the efficiency and purity of exosome isolation. Even though, the lack of specific exosomal markers makes it extremely challenging to validate the efficiency and specificity of these protocols, EVs researchers have mainly used differential centrifugation-based protocols to purify different classes of microvesicles from cell debris, protein aggregate and other EVs. While differential centrifugation could eliminate the presence of some contamination, it can not eliminate the co-sedimentation of other vesicles, platelet derived microparticles (in plasma) (Siljander *et al.*, 2011) or protein aggregates of similar size to specific classes of microvesicles. Therefore in the lack of standardized isolation protocol, some EVs researchers have added different steps of filtration or sucrose gradients to their protocols in the hope of eliminating the presence of some of these co-sediments. However all these additional steps for homemade EV isolation have improve the quality of isolated vesicles to some degree, the current isolation protocols (especially for biological samples) are very labour intensive, time consuming and expensive. Even though one of the main challenges of this research was to develop a

protocol which suits our further proteomic analysis, we need to recognize that the co-presence of other EVs from different sources and secretory proteins within our EV isolate was unavoidable.

Access to the limited volumes of biological fluids available compared to conditioned media (µl vs ml) used in *in vitro* studies, as well as the presence of abundant housekeeping proteins (e.g. albumin, uromodulin) in clinical samples adds an extra layer of complexity to the development of an isolation protocol which provides an acceptable degree of purity for further content analysis of specific microvesicles.

In the present study EVs were isolated from two biological fluids; blood and urine, using differential centrifugation with a final ultracentrifugation step. To confirm the identity of isolated vesicles with our protocol their morphological and biochemical characteristics were determined using TEM, NTA and WB analysis. The presence and enrichment of some exosomal markers in addition to the classical exosome morphology and size in purified vesicles revealed the isolation of homogenous EVs in both biological fluids.

While blood derived EVs could potentially originate from any tissue, organ or cell type in the body, urine derived EVs are mainly derived from kidney, bladder, seminal vesicle, prostate, urethra and immune cell infiltrate (Drake and Kislinger 2014). To better understand the major differences of these two sources for different applications both blood and urine were used and compared in this study.

In agreement with previous *in vitro* and *in vivo* reports, the number of PCa patient serum derived EVs was significantly higher than healthy controls. Taylor et al (2008) have also reported that the level of circulating EVs increased as the ovarian cancer stage progressed, suggesting that cancer cells not only secrete higher level of EVs into the blood, but that this

number also elevates as the disease progresses (Taylor *et al*., 2008). Lazaro-Ibanez et al. (2014) have also demonstrated that levels of plasma-derived EVs are significantly higher in PCa patients when compared to healthy donors (Lazaro-Ibanez *et al*., 2014). In a similar study, Tavoosidana et al. (2011) also reported an elevated amount of plasma derived EVs in PCa patients compared to controls, their data also suggested that the increased level of plasma derived EVs in PCa patients is positively correlated to their GS (Tavoosidana *et al*., 2011). Yu et al. (2006) reported that in response to stress, the tumour suppressor protein p53 induces the function of the endosome compartment thereby regulating the transcription of tumor-suppressor activated pathway-6 (TSAP-6) to enhance exosome production (Yu *et al*., 2006). Another explanation for these observations in cancer cells could be due to the mutation and upregulation of Rab GTPase family (Rab Family are very well-known to promote exosome secretion) in cancer cells (Ponnambalam and Baldwin, 2003)(Ostrowski *et al*., 2010). In this study while a significant difference was observed both in number and size of cancer serum derived EVs ,statistically significant differences were not observed in number or size of PCa patient urine derived EVs when compared to urine EVs from healthy controls, although a trend towards lower and higher respectively was apparent. Therefore based on NTA data alone, monitoring the number of EVs released into blood is superior to urine and could lead to an earlier diagnosis for determining prognosis of pathological disorders including PCa.

During the last several years mass spectrometry-based EVs analyses (proteomic and lipidomic) have greatly facilitated the understanding of EVs molecular biology and their membrane structure. While these technologies have tremendously improved our understanding of the molecular mechanisms and proteins involved in EVs cargo sorting

and biogenesis, the major aim of clinical EV proteomic analysis is to discover and identify disease biomarkers in biological fluids which could facilitate the inexpensive, easy and early diagnosis of the disease (Choi *et al*., 2014)(Pisitkun *et al*., 2004). The main challenge in this process is to overcome the proteome complexity of biological fluids and improve the detectability of low abundance proteins with diagnostic and/or prognostic biomarker potential.

Blood samples in particular typically contain more than 10,000 different proteins, the concentration of the high serum abundance protein, albumin, is 35-50 mg/ml which is 10^{10} higher than the concentration of Interleukin 6 with the 0.5 pg/ml concentration in plasma (Honda *et al*., 2013). Glauser et al (2000) have also determined that the concentration of THP/Creatinine varies between 1.38 to 2.08 mg/mmol in healthy male and female donors (Glauser *et al*., 2000). Therefore, improving both EV isolation protocols to remove abundant proteins as well as proteomic technologies to provide more in depth information about low abundance proteins is urgently needed. To this date conventional proteomic analysis falls short in biomarker discovery with respect to EVs due to the limited separation peak capacity as well as the dynamic range of detection (Zocco *et al*., 2014)

Adding several steps of washing with different buffers, using buoyant density gradient ultracentrifugation as well as incubation of urine derived EVs with DTT are some of the procedures that have been used to eliminate or denature the abundant proteins in biological fluid derived EVs (Pisitkun *et al*., 2004)(Choi *et al*., 2014). While all these preparative steps may improve the removal of these proteins partially, the final proteomic analysis demonstrates the presence of these highly abundant circulating proteins remaining predominant in the analysis. This is almost certainly due to association of these proteins with EV proteins or EV membrane (Hiemstra *et al*., 2011) as their removal leads to

extensive loss of EVs as observed in serum processed using an 'Albumin Out' column which resulted in quite effective and parallel removal of both albumin and EV's when compared in proteomic analysis (**Data not shown**). Zhou et al (2006) has studied the collection, storage and preservation of human urinary exosomes. Their result revealed several critical parameters for future urinary exosomes research. They have demonstrated that protease inhibitors are necessary for preservation of exosome-associated proteins during the collection process. Their results also confirmed that -80°C is the best temperature for urine storage to preserve their urinary exosome-associated proteins as well as demonstrating that extensive vortexing after thawing improves the recovery of the exosome fraction (Zhou *et al*., 2006).

For the purpose of our study even though urine samples were stored at -80°C and were vortexed extensively after thawing the absence of protease inhibitor (which is essential to inhibit the degradation of exosome-associated proteins) at the time of urine sample collection could explain the lower number of identified proteins in our proteomic analysis when compared to similar proteomic research carried out on urinary EVs.

In addition, Zhou et al (2006) has indicated that only 3% of the total urinary protein excreted from normal human adult subjects was in exosomes (48% was in sediment and 49% was soluble)(Zhou *et al*., 2006). Therefore isolation and purification of urinary EVs can lead to a very large enrichment of urinary proteins which could therefore be very valuable in biomarker studies.

When populations of biological fluid derived EVs are being studied, it is essential to aim for longitudinal sampling (especially for urine) and to eliminate any co-contamination to ensure authentic and reliable results. In this study, in addition to PBS washing steps that

were added to the exosome isolation protocol, as referred to previously, we also used an "Albumin Out" column and DTT incubation for blood and urine sample respectively. While the Coomassie staining profile of our serum/urine derived EVs reveals a significant reduction in the level of both proteins after these treatments, no significant differences were observed regarding the quality of our proteomic analyses.

In addition to searching for PCa biomarkers, many researchers have investigated prostate specific exosome protein markers to purify the prostate derived EVs subpopulation from those derived from other organs. FOLH-1, CD13, survivin and PTEN are a few examples of claimed prostate or PCa specific protein markers that have been used for prostate derived exosome purification (Tavoosidana *et al*., 2011)(Khan *et al*., 2012)(Gabriel *et al*., 2013).

In our previous publication we successfully identified 220 proteins in six different prostate cell lines (Mascot score>40 and Peptide number 2), out of these 50 protein biomarkers were identified. Our list also consisted of protein biomarkers that were present in cancer derived exosomes (AR+/- ve) and not in our benign cell line (Hosseini-Beheshti *et al*., 2012).

While we were expecting to see more overlap in proteins identified from exosomes derived from our clinical and PCa cells, an overlap of only 6 proteins were observed when the two sets were compared. This is in agreement with the observation of Duijvesz et al. (2013) where only 42 mutual proteins were determined upon comparison of their PCa derived exosomes proteomic analysis to ours (Hosseini-Beheshti et al., 2012)(Duijvesz *et al*., 2013). In addition to the described complexities of biological samples this difference could be partially due to different isolation protocols used for clinical samples

versus PCa cell line derived exosomes.

Building upon our previous findings we successfully identified 85 proteins in serum derived EVs from 12 patients and 3 control serum samples. To better understand the differences between the patient derived and healthy control derived groups and considering the lack of detailed information about the tumor stage or GS, we further categorized them as PSA<50 and PSA>50.

In comparison to other published blood or urine derived EVs proteomic data, we found that more than 20 proteins (from mutual data set of all three groups) have already been reported in other blood or urinary exosome proteomics research (e.g. Hemopaxin, lysosomal-associated membrane protein 2, regulator of G-protein signaling 20, complement component 3, alpha-2- macroglobulin, CD14, CD59, ceruloplasmin, vitamin D binding protein, haptoglobin, heterogeneous nuclear ribonucleoprotein M, inter-alpha-trypsin inhibitor heavy chain 2, leucine- rich alpha-2-glycoprotein 1, mannan-binding lectin serine peptidase 2, phosphoinositide-3-kinase interacting protein 1, transferrin (Prunotto *et al*., 2013)(Zubiri *et al*., 2013)(Saraswat *et al*., 2014) (Benito-Martin *et al*., 2013). Prunotto et al. (2013) have published a very comprehensive proteomic analysis of the podocyte exosome-enriched fraction from normal human urine and identified more than 1,000 proteins with at least two unique peptides using one-dimensional gel electrophoresis and LC-MS/MS in normal urinary EVs (Prunotto *et al.*, 2013). Conversely, there are several reports describing the detection of prostate tumor exosomes and their protein content. In an attempt to discover urine markers for the specific detection of PCa, Chen et al. (2011) recently published a list of blood and urinary markers (Chen *et al*., 2011). Immunoglobulin superfamily member 8 (blood and urine), Apolipoprotein D (urine), complement factor H (blood) and retinol binding protein

4 (urine) were mutually identified in our proteomic data set when compared to the list provided by Chen et al. (2011).

Among the proteins identified with biomarker potential such as diagnosis, prognosis, disease progression, efficacy and response to therapy, 11 serum exosome (Albumin, apolipoprotein A-I, apolipoprotein B, fibrinogen alpha chain, Haptoglobin, serpin peptidase inhibitor- clade A (alpha-1 antiproteinase, antitrypsin)-member 1 coagulation factor II (thrombin), proline dehydrogenase (oxidase) 1, neuronal pentraxin II, serum amyloid A1, S100 calcium binding protein A9) and 12 urine exosome proteins were identified with known diagnostic application (Albumin, keratin 1, keratin 6A, keratin 6B, keratin 9, secreted phosphoprotein 1, apolipoprotein A-I, apolipoprotein B, coagulation factor II (thrombin), haptoglobin, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1). All of these diagnostic proteins were investigated to obtain further information pretaining specifically to PCa and Apoliprotein, a high density lipoprotein which is mainly produced in the brain and testes and is a biomarker for androgen insensitivity syndrome, was the only protein that we identified that has already been reported as a potential PCa biomarker.

Using WB analysis we also attempted to validate our previously published *in vitro* PCa biomarker list using the data obtained for the serum and/or urine derived exosomes (Hosseini- Beheshti *et al*., 2012). While it is not possible to draw any conclusions from the data obtained (**data not shown**) considering the lack of descriptive information regarding the stage of PCa in our samples, FOLH1, FASN, FLMN and ANXA2 data suggest some significant differences when compared to control samples and could be interesting targets for further PCa biomarker studies.

Similar to our previous publication we went on to fully characterize the biological fluid derived EVs and to investigate whether EVs cholesterol content could be a diagnostic indicator of PCa. Our group has previously shown evidence of *de novo* androgen synthesis from cholesterol pre-cursors within local tumor microenvironment and shown that this mechanism contributes to CRPC (Locke *et al*., 2008). Furthermore, our group was the first group who demonstrated the presence of CYP17, a member of the cytochrome P450 enzyme family and a key enzyme in *de novo* steroidogenesis, in human serum exosomes, which confirmed the role of exosomes in PCa progression (Locke *et al*., 2009). In line with these observations we also reported that exosomes derived from PCa cell lines contain significantly more cholesterol than their benign counterpart cell line RWPE-1(Hosseini-Beheshti *et al*., 2012). Therefore, in this study the cholesterol content of both serum and urine derived exosomes were measured and compared to their source of origin.

While no enrichment or significant differences were observed between different groups of serum derived exosomes (Control, PSA<50, PSA>50) when compared to serum, our research revealed a significant enrichment in the cholesterol content of urinary exosomes when compared to urine. This may be a reflection of higher overall enrichment during urine processing and/or lower lipid/lipoproteins available in urine for association and co-extraction with EV's. Our quantitative cholesterol content of urinary EVs also shows that the cholesterol level of PCa derived EVs are significantly lower than the control group. These findings are in line with our previous statement about the role of cholesterol in *de novo* synthesis of androgen and CRPC. While more studies are needed to draw definitive conclusions, any correlative decrease in cholesterol content of urinary exosomes may be an interesting PCa diagnostic or prognostic indicator.

In conclusion, the cumulative biomarker discovery path contextual to the characterization of PCa EVs holds an encouraging future. In this study we attempt to form a solid platform for PCa derived exosome biomarker study. There are clearly substantial issues that require improvement in EV isolation from clinical specimens to further drive a discovery based biomarker search using EVs. Future blood and urinary EVs proteomic and lipidomic analysis will most certainly extend and build on what we have described and ultimately progress to allow development of more highly multiplexed targeted proteomic and lipidomic assessment of EV enriched specimens. Thus, future proteomic and lipidomic analysis of EVs can extend and build on what is have reported herein.

Chapter 4: Exosomes Confer Pro-Survival Signals to Alter the Phenotype of Prostate Cells in Their Surrounding Environment

4.1 Introduction

Since the initial description by Trams in 1981 (Trams, 1981) followed by Pan and Johnston (Pan and Johnstone, 1983) EV research has grown exponentially. Cancer cell derived exosomes in particular have been one of the main areas of interest for EV scientists not only because of their biomarker potential but also because of their detrimental effects on the immune system. These effects occur via blocking or inducing specific pathways and is possible as a result of their extensive range of bioactive molecules (Taylor & Gercel-Taylor, 2005)(Liu *et al*., 2006)(Abusamra *et al*., 2005)(Koga *et al*., 2005)(Qu *et al*., 2009).

It is very well known that cancer cells produce many of their own growth factors in order to sustain independent proliferative growth signalling. MAPK and PI3K/Akt pathways are recognized as the main cytoplasmic signalling pathways that play a central role in growth signalling (Arcaro *et al*., 2007). Due to their containment of a large array of proteins as well as their role in disease progression, numerous studies have already looked into the effects of cancer cell derived exosomes on different signalling pathways within the neighbouring cells in their microenvironment (Umezu *et al*., 2014)(Ostenfeld *et al*., 2014).

Therefore, in the present study we investigate the role of AR +/- ve, PCa cell derived exosomes on PCa tumour growth and progression. We have also reported the effects of exosomes derived from PCa cells on the PSA level and tumor growth of mice bearing human PCa tumour xenografts when they have been systemically introduced via IV injection.

While the primary emphasis of this research was to understand the effects of different PCa cell derived exosomes, with distinct AR phenotypes, on cell-cell communication as they confer changes in cellular properties of neighboring cells in a tumour population, further studies are required to achieve a deeper and more precise understanding of the role of exosomes at the molecular level as it pertains to cancer progression and metastasis.

4.2 Materials and Methods

4.2.1 Cell Culture

PC3 and DU145 human PCa cells (ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM), LNCaP cells (ATCC) were cultured in RPMI 1640 supplemented with 5% FBS (Invitrogen) and antibiotic, at 37 °C in 5% CO_2. RWPE-1 (ATCC) cells were maintained in keratinocyte-SFM (KSFM) with growth supplement (GIBCO) and 1% penicillin streptomycin (Invitrogen, Carlsbad, CA). CLUGFP stably over-expressing LNCaP cells were maintained in 200 mg/ml G418 (Invitrogen) containing RPMI supplemented with 10% FBS and 1% antibiotics at 37 °C in 5% CO_2.

All cells were grown to 60–70% confluency, washed with sterile PBS buffer and removed from serum and incubated in culture media for 72 hours for exosome collection and purification.

4.2.2 Exosome Isolation

Exosomes were purified from the serum free media of AR +ve and –ve PCa cell lines following exposure to LNCaP and DU145 cells for 72 hours. For exosome purification,

200 ml of each cell line's conditioned medium was cleared by centrifugation at 6,000 g at 4°C for 10 minutes to remove cell debris and protein aggregates. The precleared medium was concentrated to 2 ml using a 100 kDa MWCO Centricon Plus-20 filter capsule (Millipore, Billerica, MA). Samples were transferred to ultracentrifuge tubes containing 300µl of 30% sucrose-deuterium oxide (D2O). Sample tubes were then ultracentrifuged at 100,000g for 70 minutes at 4°C (using a fixed angel 70.1 Ti rotor (Beckman Coulter)). Purified exosomes (350 µl) were collected from the cushion of sucrose and washed with PBS prior to any exosome treatment (350 µl exosomes were isolated from 10,000,000 originally seeded cells).

4.2.3 Transmission Electron Microscopy of Exosomes

Isolated exosomes (2.5µl) were dried onto freshly 'glow discharged' 300 mesh formvar/carbon- coated TEM grids (Ted Pella, Redding, CA), negatively stained with 2% aqueous uracyl acetate and observed with a Hitachi H7600 TEM (Hitachi High-Technologies Corp., Tokyo, Japan) operated at 80kV. Images were captured with a side mounted 1K AMT Advantage digital camera (Advanced Microscopy Techniques, Corp. Woburn, MA).

4.2.4 Western Blot Analysis

Exosomes and cell lysates were analyzed for total protein concentration using the BCA protein determination kit (Sigma, Oakville, Ontario, Canada). Thirty micrograms of total protein associated with purified exosomes as well as their cell lysate were loaded on 12% acrylamaide gel. Relative enzyme levels were quantified using antibodies specific for exosome markers, specficially: mouse monoclonal Actin (1:1000 Sigma) mouse

monoclonal Alix and mouse monoclonal HSP70 (1:1000 Santa Cruz Biotechnology, Inc., Santa Cruz, CA). In order to evaluate the purity of the exosome preparations, all exosomes samples were also blotted against GRP94 (1:1000 Cell Signaling) to demonstrate the absence of cellular contaminants from cell lysate in our exosome preparation. The activation of MEK/ERK pathway was demonstrated using rabbit polyclonal p-MEK1/2, t-MEK1/2, p-ERK1/2, and t-ERK1/2 (1:1000 Cell signalling) antibodies.

4.2.5 Nanoparticle Tracking Analysis of Exosomes

Size distribution and the estimated concentration of nanoparticles in purified exosomes isolate were analysed using a light scattering technology via measurement of the rate of Brownian motion with the NanoSight^{TM} LM10 system (NanoSight^{TM} Ltd, Amesbury, UK) configured with a (488 nm) laser and a high sensitivity digital camera (OrcaFlash2.8, Hamamatsu C11440, NanoSight^{TM} Ltd).

All samples were diluted with nanoparticle-free water so the concentration was within the range of 5×10^{7} to 5×10^{9}. Samples were administered and recorded under controlled flow (infusion rate of 100) using a NanoSight^{TM} syringe pump and script control system. The ambient temperature was set at 25ºC, with the camera sensitivity and detection threshold set between 9 to 12 for maximum particle detection. Five different videos of 60 seconds from 3 different replicates were collected and analysed using NTA-software (version 2.3) for each sample.

4.2.6 Confocal Microscopy

In order to study the uptake of exosomes by different cancerous or non-cancerous

prostate cell lines (with distinct AR expression phenotypes) equal numbers of cells were seeded in four- well chamber slides (Lab-Tek II chamber slide with cover, Thermo Fisher scientific). In the next step as previously reported (Hosseini-Beheshti *et al.*, 2012) fresh CLUGFP labelled exosomes were incubated with PC3 (AR-ve) and LNCaP (AR+ve) PCa cell lines as well as RWPE-1 representing a benign epithelial prostate cell line, for 12 h at 37 °C and 5% CO_2. CLU_{GFP} tagged exosomes were isolated from a CLU_{GFP} stably overexpressing LNCaP cell line. After removal of media, cells were fixed with ice-cold MeOH/Acetone (3:1) for 10 minutes, and then washed in TBS buffer and permeabilized in 0.1% Triton X-100 in TBS for 15 minutes at room temperature (RT). Non-specific binding was avoided by blocking in odyssey solution for 30 minutes at RT. Primary purified mouse anti E-Cadherin (1:250 BD Transduction Laboratories™) or rabbit anti Caveolin-1(1:250 Santa Cruz, CA) were diluted in blocking agent and incubated with cells for 1 hour at RT. Secondary antibody, Alexa Fluor® 568 goat antimouse IgG or Alexa Fluor® 555 Donkey Anti-Rabbit IgG (1:500, Invitrogen), was incubated with cells for 30 minutes at RT. Finally, as described above, all slide chambers were mounted and monitored using a x63 objective on a confocal microscopy (LSM 780 Ziess, Heidelberg, Germany).

4.2.7 Apoptosis Assay

Caspase- 3/7 assay was carried out by mixing 10 μg of total protein extracts prepared from cells as above with Caspase-Glo 3/7 substrates (Promega) (Chan *et al.*, 2010). The relative luminescence units (RLU) were measured using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc.). The percentage of apoptosis based on caspase 3/7 activity was calculated relative to that of control samples.

4.2.8 Real Time Cell Analysis (xCELLigence)

4.2.8.1 Proliferation

A blank reading was taken with 30µl of RPMI + 5% FBS in each well. DU145 cells were seeded at a density of 7,000 cells per well and LNCaP and RWPE-1 cells were seeded at a density of 20,000 cells per well with a final volume of 100µl. Treatments of either DU145 or LNCaP exosomes (0-400µg/ml) were added after 24 hours to a final volume of 200µl. Data was recorded once every 5 minutes for the first 25 sweeps, followed by once every 10 minutes till completion of the experiment 72 hours post treatment. Data was normalized to a time point shortly prior to treatment delivery.

4.2.8.2 Migration

Media with 10% FBS was added to the bottom chamber and 30µl of serum free media was added to the wells of the top chamber of the CIM migration plates (Post equilibration of the two chambers). DU145 and RWPE-1 cells were seeded at a density of 20,000 cells per well with a final volume of 100µl. Treatments of either DU145 or LNCaP exosomes (100µg/ml) was added after 24 hours to a final volume of 200µl. Data was recorded once every 5 minutes for the first 25 sweeps, followed by once every 10 minutes till completion of the experiment 48 hours post treatment. Data was normalized to a time point shortly prior to treatment delivery.

4.2.8.3 Cell Motility

In vitro cell migration assays were performed in a 24-well Transwell plate with 8-µm polycarbonate membrane filters (Corning) separating the lower and upper culture

chambers.RWPE-1 cells were grown to subconfluence (~75%–80%) and were incubated with LNCaP or DU145-derived exosomes (100 µg/ml) or serum-free medium for 48 hours. After detachment with trypsin, cells were washed with PBS and resuspended in serum-free medium, after which the cell suspension (1×105 cells), supplemented with exosomes (100 µg/ml) or serum-free medium, was added to the upper chamber. Medium containing 10% FBS and exosomes (100 µg/ml) or serum-free medium was added to the bottom wells of the chamber. The cells that had not migrated were removed from the upper face of the filters using cotton swabs, and the cells that had migrated to the lower face of the filters were fixed with methanol and stained with 0.5% crystal violet solution. Images of at least 10 random fields were captured from each membrane using a x10 objective, and the number of migratory cells was counted. All values are representative of at least two independent experiments with similar results determined in each case.

4.2.9 3D Migration

Three-dimensional multicellular spheroids were prepared by the liquid overlay technique (Weiswald *et al*., 2010). In brief, tissue culture microplates were coated with 75µl of 1% agarose in water. RWPE-1 cells grown as a monolayer were resuspended with trypsin, and 2×10^3 cells were seeded in microwells so as to obtain, after 3 days, a single spheroid per well.

Serum-free medium or serum-free medium supplemented with LNCaP or DU145 exosomes (final concentration, 0.1 µg/µl) was added to the microwells containing spheroids.

48 hours later, we performed the spheroid-based assay (Vinci *et al.*, 2013). Briefly, RWPE-1 spheroids were transferred on a 50 µg/ml collagen I-coated surface (single

spheroid/96- well; 6 spheroids/treatment) in 300 µl respective media in the presence or absence of LNCaP or DU145 exosomes (final concentration, 0.1 µg/µl). The spheroids were imaged (Canon EOS Digital) and the migration was quantified recording the total area covered by RWPE-1 cells at the start of the experiment and at 12 and 24 hours. The covered areas were manually measured using ImageJ and the data have been normalized to the original size of each spheroid recorded at t = 0 (formula: (migrated area at t = x /migrated area at t = 0) × 100). *In vivo* Study in Mice bearing LNCaP Human Tumor Xenografts Six to eight week-old nude mice (Harlan Sprague Dawley, Inc.) weighing 25-31 grams were subcutaneously inoculated with LNCaP cells (10^6 cells in BD matrigel, BD Biosciences, New Jersey, USA) at the right posterior dorsal site. Body weight, tumor volume and serum PSA levels were measured weekly. When the tumor volume reached 100 mm^3, mice were randomized into 3 treatment groups; vehicle, low dose (10 µg protein of LNCaP or DU145 derived exosomes) and high dose (100 µg LNCaP or DU145 derived Exosomes) treatment. All mice were treated intravenously via the tail vein twice a week for 4 weeks. Calipers were used to measure the three perpendicular axes of each tumor.

$$V = (L \times W \times H)\frac{\pi}{6}$$

The above formula where L is the length, W the width, and H the height, was used to calculate the tumor volume. Mice were also weighed weekly and monitored daily for signs of toxicity including death, lethargy, blindness and disorientation.

4.2.10 Immunohistochemistry

This study was done on the total of 19 xenograft tumors from LNCaP cells. The H&E slides were reviewed and the desired areas were marked on them and their correspondent

paraffin blocks. TMA was manually constructed (Beecher Instruments, MD, USA) by punching multiple cores of 1 mm for each sample. All the specimen were from xenograft tumors.

Immunohistochemical staining was conducted by Ventana autostainer model Discover XT^{TM} (Ventana Medical System, Tuscan, Arizona) with enzyme labeled biotin streptavidin system and solvent resistant Red Map kit by using 1:500 of ki67 rabbit polyclonal antibody (Thermoscientific) and 1:2,000 concentrations of Filamin mouse monoclonal antibody (abcam). Scoring System: Values on a four-point scale were assigned to each immunostain of Filamin C, Ki67. Descriptively, 0 represents no staining by any tumor cells, 1 represents a faint or focal, questionably present stain, 2 represents a stain of convincing intensity in a minority of cells and 3 a stain of convincing intensity in a majority of cells.

Proliferation factor was scored by calculating of the average of cell counts of 3 HPF in each core.

4.2.11 Statistical Analysis

Unless indicated, analyses were performed on data generated from triplicate experiments. Results were expressed as mean ± standard deviation. For most experiments unless indicated, statistical significance for differences were evaluated by student t-test ($P<0.05$).

In Real Time Cell Analysis (xCELLigence) experiments student t-test Level of significance was set at *$P<0.05$, **$P<0.01$, ***$P<0.001$ and the slope was calculated by using the RTCA 2.0 software (ACEA).

4.3 Results

4.3.1 Purification and Characterization of Exosomes

Exosomes were isolated and purified from two different PCa cell lines including LNCaP (AR +ve) and DU145 (AR –ve) based on their size and density. TEM, WB analysis and NTA were used to characterize their integrity and morphology, purity and size distribution.Transmission Electron Microscopy

To evaluate their integrity and morphology, 2.5 µl of a diluted exosomes sample were loaded and fixed onto formvar-coated carbon EM grids and visualized by TEM after staining with 2% uracyl acetate as previously described (Hosseini-Beheshti *et al*., 2012). Our TEM observation revealed that our protocol isolated a very homogenous exosome mixture with a typical cup- shaped and round morphology with a diameter range of 30-200 nm (**Figure 4.1 A.**).

4.3.1.1 Western Blot Analysis

WB analysis was used to identify the presence or absence of a selection of exosomal and ER markers to confirm the efficiency of our exosome isolation protocol as well as purity of the exosome isolate. The presence of at least two or all the exosomes markers from three different categories including Alix (Regulating endosomal trafficking/Anti-Apoptosis), Actin (cytoskeleton) and HSP70 (Heat-Shock Protein) and the absence of GRP94 (ER marker) in our WB data confirm the purity of the exosomes isolate from both PCa cell lines (**Figure 4.1 B.**).

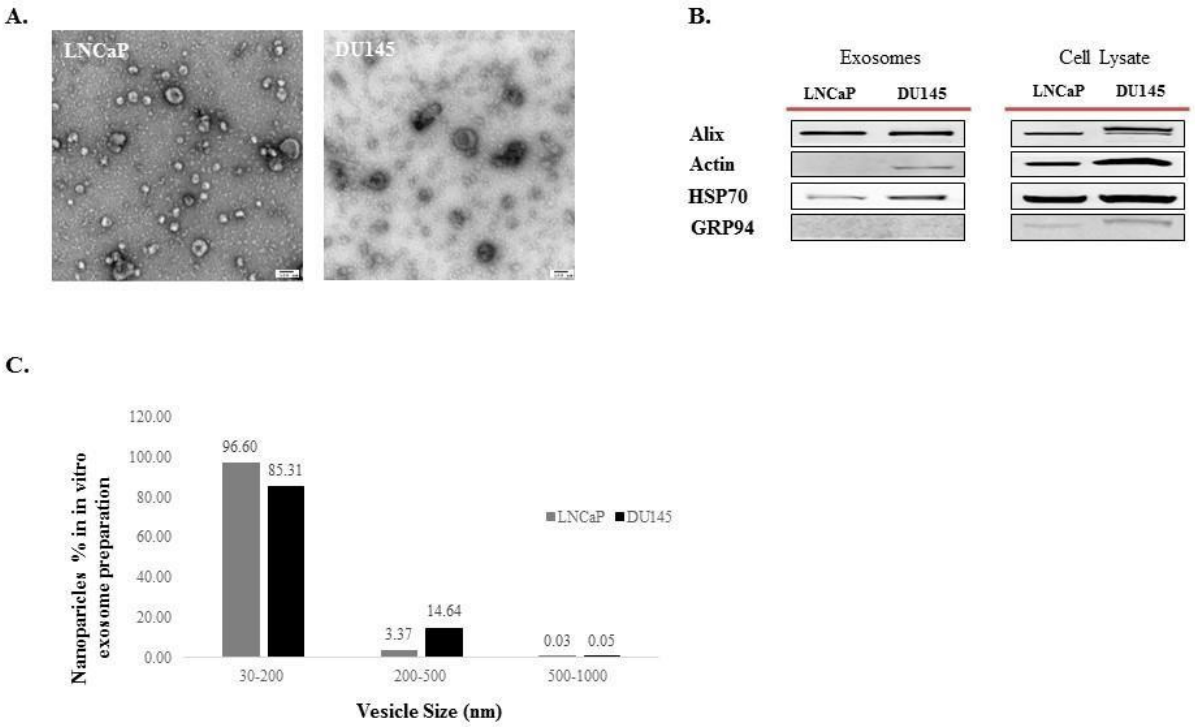


Figure 4.1 Exosome characterization.

A. Transmission Electron Microscopy. TEM images of exosomes derived from androgen sensitive and independent PCa cell lines; LNCaP and DU145. Exosomes were negatively stained with 2% uracyl acetate after removing the extra moisture. Cup-shaped structures, with 30-200 nm size were identified as being exosomes. **B. Western Blot analysis for exosomes marker in exosomes and cell lysate samples.** Exosomes have been purified based on their unique size and density by ultracentrifugation with 30% sucrose-deuterium. Thirty micrograms of total protein associated with purified exosomes or cell lysate were analyzed by WB using different exosome markers in both cell lines. **C. Nanoparticle Tracking Analysis.** Bar chart showing the average percentage of nanoparticles within 30-200nm, 200-500nm, and 500-1,000nm size in *in vitro* exosome preparation.

4.3.1.2 Sight^{TM} Tracking Analysis

NTA was used to characterize the size and estimated number/ml of isolated nanoparticles for both cell lines. To better measure the purity of our exosomes isolate, the percentage of larger nanoparticles with diameters between 200-500nm and 500-1000 nm, contained within our exosomes samples (nanoparticle with size varied between 30-200nm)

were calculated. As it has been demonstrated in Figure 4.1 C. our exosome isolation protocol which is based on size filtration and ultracentrifugation (100,000g sedimentation force) on a 30% sucrose cushion (density), purified 85-97% nanoparticles with size of 30-200 nm, 3-15% of nanoparticles with diameters of 200-500nm, and maximum of 0.05% of nanoparticles larger than 500nm (500- 1000nm).

Figure 4.2 A. and **4.2 B.** show the average size distribution of nanoparticles isolated using our exosomes isolation technique. In agreement with others peaks at 117 nm and 164 nm for nanoparticles isolated from LNCaP and DU145 respectively were observed, which are within the 30-200nm size range characteristic of these EVs (Grecel-Taylor *et al*., 2012)(Sarker *et al.*, 2014). The average number of nanoparticles were also measured using the NTA system. The average nanoparticles number/ml was 1.7×10^{11} for LNCaP and 1.5×10^{11} for DU145 (Figure 4.2 C.) (Data were compiled from five measurements per biological replicates (n=3)).

Protein concentration of exosomes was measured using a BCA assay (**Figure 4.2 D.**). While the protein concentration of LNCaP cell derived exosomes appeared to be lower than DU145 cell derived exosomes, no significant differences were seen between either the number/ml of nanoparticles or protein concentration of exosome isolates from AR +ve or –ve cell lines.

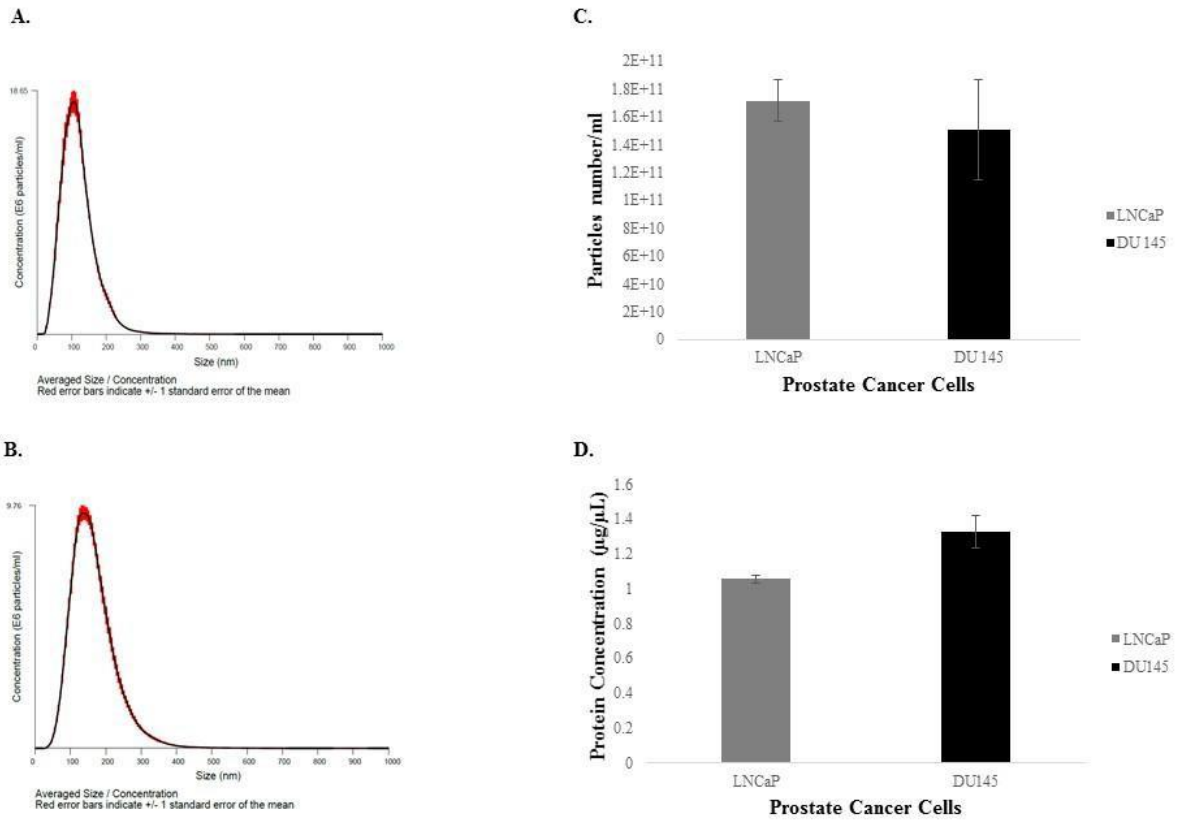


Figure 4.2 Nanoparticle Tracking Analysis of exosomes.

Size distribution of exosomes derived form **A.** DU145 and **B.** LNCaP were measured by nanoparticle tracking analysis (NTA) showed a peak at 117 +/- 0.3 nm (LNCaP) and 164 +/- 1.0 nm (DU145).

Bar Chart showing the **C.** particle number/ml for both PCa Cell lines. **D.** Protein Concentration of exosomes derived from DU145 and LNCaP Cell lines. Values are mean ± standard deviation, all values are representative of at least three independent experiments with four replicates.

4.3.2 Exosome Uptake

The uptake of exosomes into AR –ve (PC3) and AR +ve (LNCaP) PCa cell lines were compared to a benign prostate epithelial cell line RWPE-1. CLU^{GFP} tagged exosomes were isolated from a CLU^{GFP} stably overexpressing LNCaP cell line. After cells were fixed using MeOH/Acetone in order to distinguish the cellular structure all three cells were stained with DAPI (Blue, Nucleus) as well as Caveolin-1 and/or E-Cadherin (Red, Cell membrane) prior to imaging cells using confocal microscopy (**Figure 4.3 A.**, **4.3 B.** and **4.3 C.**).As shown in our results PC3 and RWPE-1 were positive for the Caveolin marker, in fact, secretion of a huge PMV or oncosome rich in Caveolin was observed as captured in the PC3 cell image (**Figure 4.3 A.**), while in contrast LNCaP were only positive for E-cadherin.

To investigate the uptake and intercellular localization of exosomes cells were incubated with 100µl of CLU^{GFP} tagged exosomes for 12 hours (overnight) at 37 ºC (**Figure 4.3 D.**, **4.3 E.** and **4.3 F.**). As can be seen in the left panel of **Figure 4.3** and in agreement with what we observed previously (Hosseini-Beheshti *et al*., 2012) exosomes have been taken up by both PCa cell lines regardless of their AR phenotype (very even distribution of CLU^{GFP} tagged exosomes in LNCaP cells cytoplasm could be observed in **Figure 4.3 E**), as well as the benign RWPE-1 cells. Upon uptake of exosomes, the invagination of the cell membrane can be clearly seen in images of the PC3 and RWPE-1 cell lines (**Figure 4.3 D**, **4.3 F**).

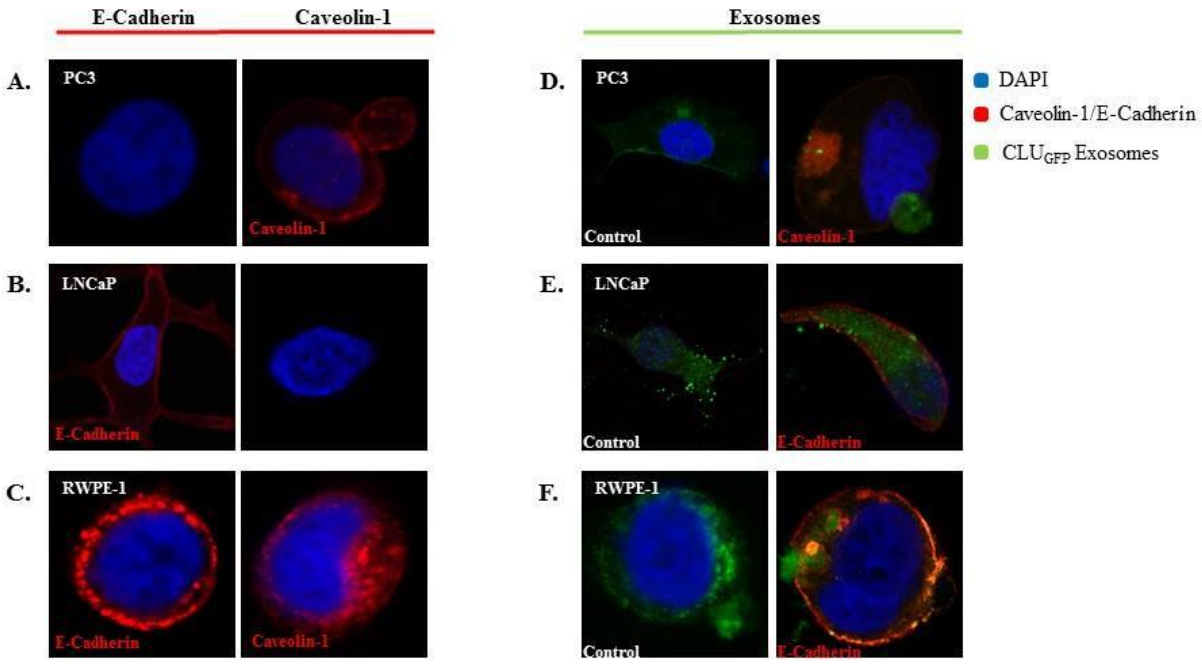


Figure 4.3 Confocal microscopy.

Confocal microscopy was used to visualize freshly isolated exosomes derived from a CLU_{GFP} stably over-expressing LNCaP cell line, which contains CLU_{GFP}, being taken up by **A.** and **D.** PC3 (AR-ve) and **B.** and **E.** LNCaP (AR+ve) PCa cell lines versus **C.** and **F.** benign epithelial prostate cell line RWPE-1, after overnight incubation. Both cell lines were further fixed and stained with DAPI and E- Cadherin/Caveolin-1 prior to imaging of the cells by confocal microscopy.

4.3.3 *In vitro* Functions of Prostate Cancer Derived Exosomes

4.3.3.1 Apoptosis Assay

The apoptotic activity of cells was assessed by measuring the activities of caspase 3 and caspase 7 as two of the key effectors in the apoptosis pathway. Caspase 3/7 activity was detected after LNCaP, DU145 and RWPE-1 cells were treated with exosomes derived from LNCaP or DU145 cells for 5, 10, 15, 30 minutes, 1, 6 and 24 hours. Our results show that while LNCaP and DU145 derived exosomes significantly reduce the caspase activity in LNCaP treated cells at almost all the time points and regardless of the exosomes source

(**Figure 4.4 A.**), none of the exosome treatments (LNCaP or DU145) influence DU145 cell apoptotic activity significantly(**Figure 4.4 B.**).

A similar phenomenon was seen with RWPE-1 cells treated with LNCaP-derived exosomes. Specifically, DU145-derived exosomes seem to be more effective in reducing the caspase 3/7 activity in the benign epithelial prostate cell line compared to LNCaP-derived exosomes (**Figure 4.4 C.**).

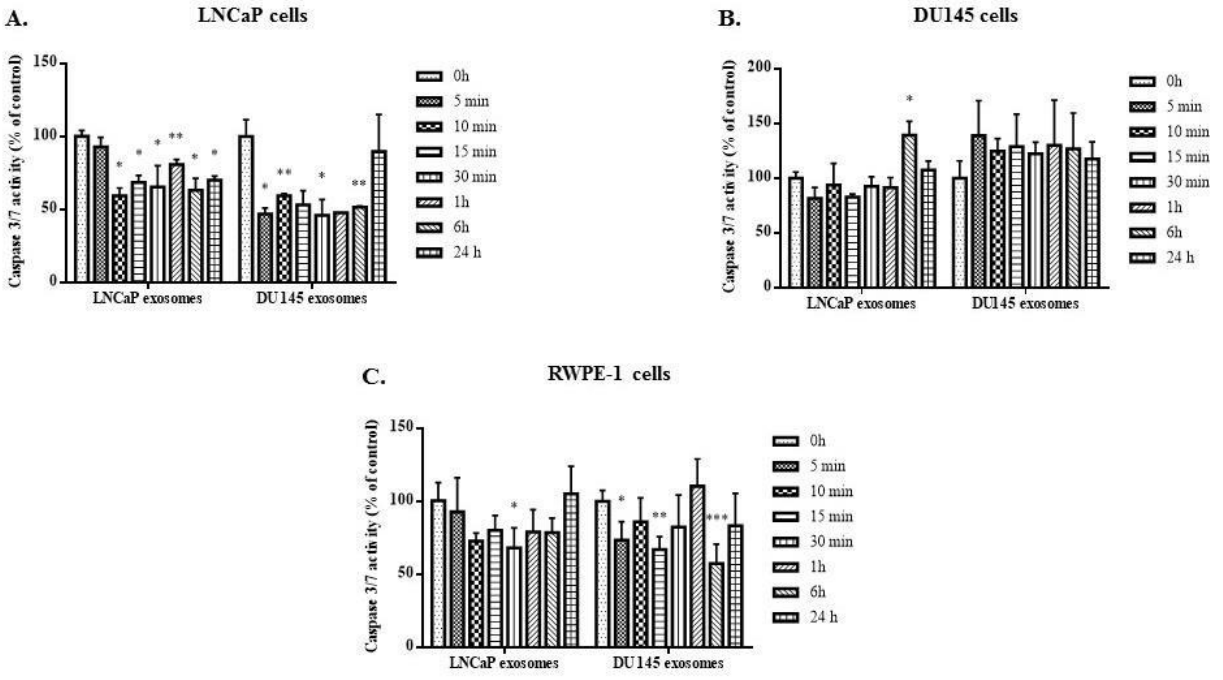


Figure 4.4 Apoptosis assay.

Analysis of apoptosis in PCa Cell lines (LNCaP and DU145) and benign epithelial prostate cell (RWPE-1) after treatment with 100 μg/mL of exosome derived from LNCaP or DU145 cells after 0, 5, 10, 15, 30 min, 1, 6, 24 hours. **A.** Both DU145 and LNCaP-derived exosomes significantly reduced the caspase 3/7 activity in LNCaP cell line in most of the time points. **B.** Exosomes derived from DU145 or LNCaP cells did not significantly influence the caspase 3/7 activity in DU145 cells. **C.** Treatment with DU145-derived exosomes led to a significant reduction of apoptosis in RWPE-1 cells whereas the RWPE-1 cells did not display significant decrease of apoptosis after treatment with exosomes derived from LNCaP cells. All values are representative of at least two independent experiments with similar results, and are presented as the percentage of caspase 3/7 activity, where non-treated cells were regarded as 100% ($P<0.05$).Real Time Cell Analysis (xCELLigence)

xCELLigence is an electrical impedance based system in which cellular events such as proliferation, migration, and invasion can be monitored in real time without the incorporation of labels. In this system impedance is measured across interdigitated micro-electrodes on tissue culture E-plates (ACEA, USA) (Roshan Moniri *et al*., 2015).

To determine the seeding concentration for all three cell lines, LNCaP, DU145 and RWPE-1 cells were seeded at numbers ranging from 2,500 to 40,000 cells/well of the E-plates. Cell adherence and time required for maximum density were then automatically monitored every 10 minutes for 72 hours to obtain the optimal cell seeding density.

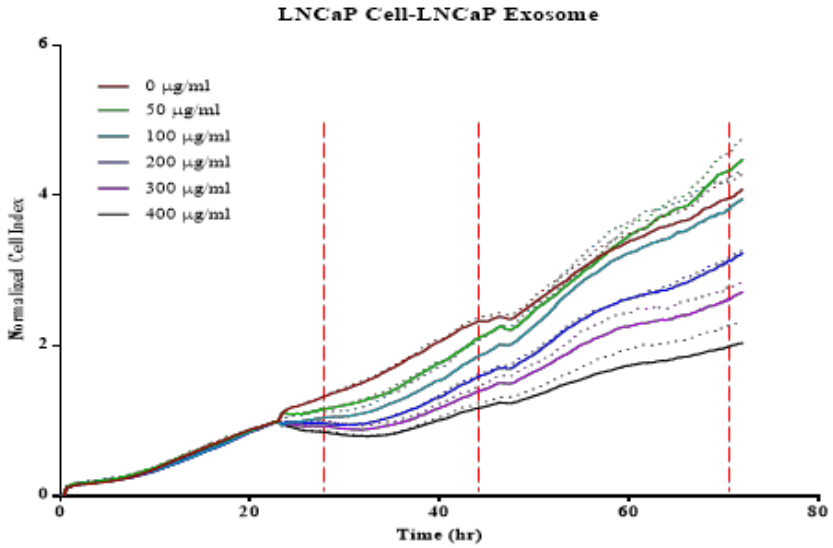
4.3.3.1.1 Proliferation Assay

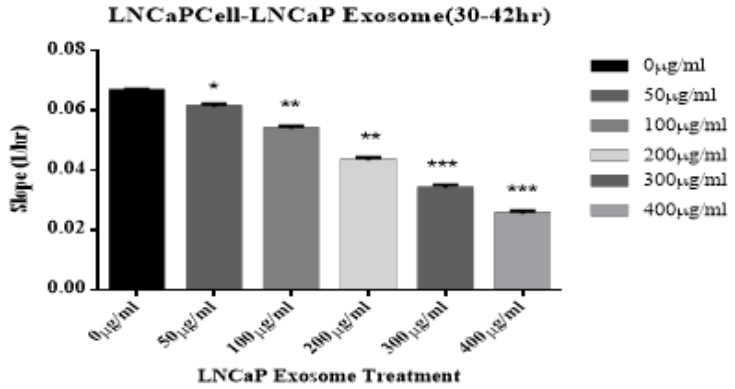
To investigate the effects of PCa derived exosomes on the proliferation or migration of different prostate cell lines (LNCaP, DU145 and RWPE-1), all three cell lines were treated with 0-400 µg/ml of exosomes derived from LNCaP or DU145 cells for up to 72 hours.

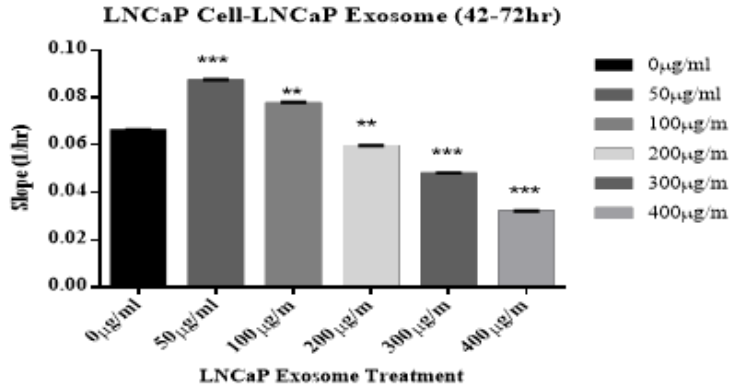
Assessment of proliferation by xCELLigence revealed a biphasic response that was concentration dependent. The concentration of LNCaP derived exosomes had a positive influence on the enhancement of proliferation in both LNCaP and DU145 cells when compared to control. Specifically, our real time cell analysis behavior determined by xCELLigence demonstrated that 50 and 100 µg/ml of LNCaP exosomes could significantly increase the proliferation rate of LNCaP cells (42-72 hour), while no significant effects were seen on DU145 cells when treated with LNCaP cell derived exosomes at different concentrations. (All slopes were compared with the control, *P<0.05, **P<0.01, ***P<0.001, n=2) (**Figure 4.5 A., 4.5 B.**).Importantly, when RWPE-1 cells were grown in the presence of 0-400 µg/ml LNCaP exosomes there was a significant reduction in the

proliferation rate of this benign epithelial prostate cell line in almost all the LNCaP cell derived exosome concentrations (except 50 µg/ml in 42-72hr) (**Figure 4.5 C.**).

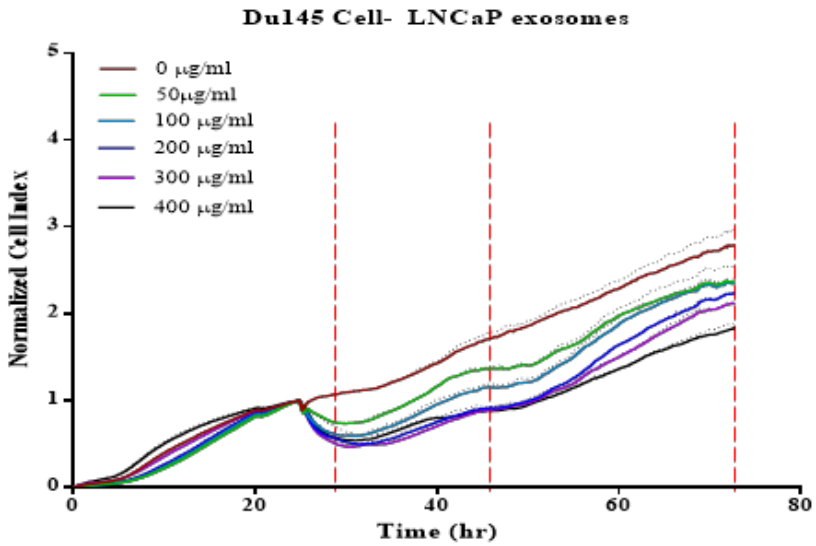
A.

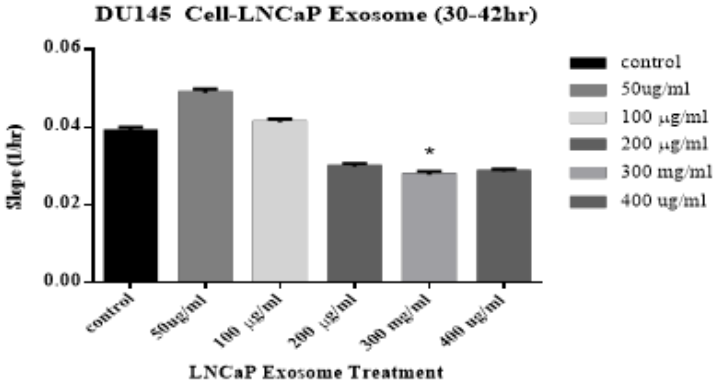

LNCaP Cell-LNCaP Exosome
0 μg/ml
50 μg/ml
100 μg/ml
200 μg/ml
300 μg/ml
400 μg/ml
Normalized Cell Index
Time (hr)

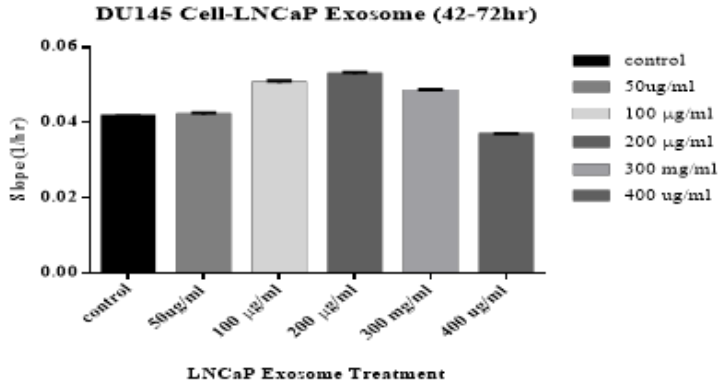

LNCaPCell-LNCaP Exosome(30-42hr)
Slope (1/hr)
LNCaP Exosome Treatment
0μg/ml
50μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml


LNCaP Cell-LNCaP Exosome (42-72hr)
Slope (1/hr)
LNCaP Exosome Treatment
0μg/ml
50μg/ml
100μg/m
200μg/m
300μg/m
400μg/m

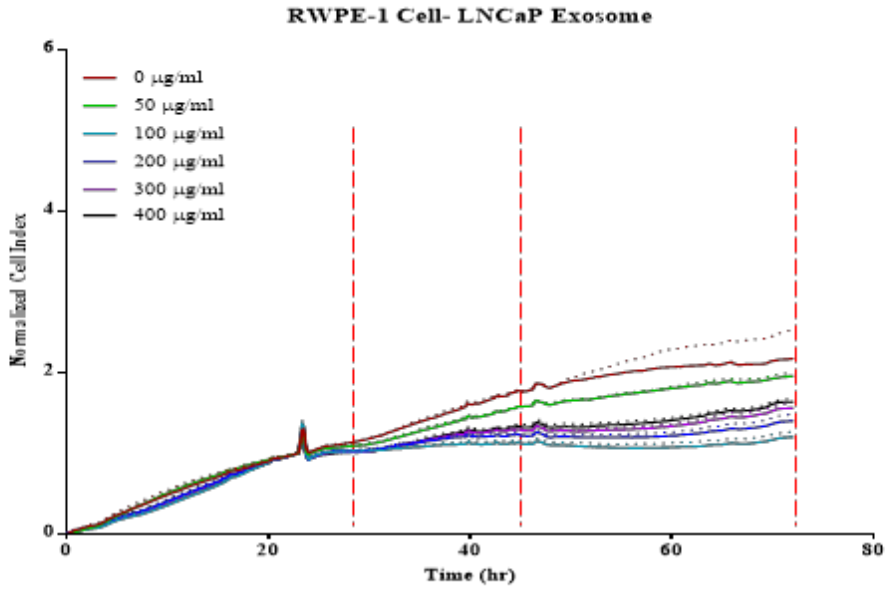
B.

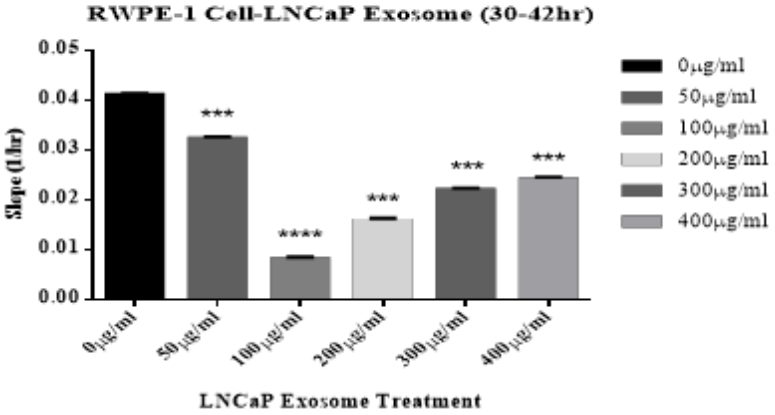

Du145 Cell- LNCaP exosomes
0 μg/ml
50μg/ml
100 μg/ml
200 μg/ml
300 μg/ml
400 μg/ml
Normalized Cell Index
Time (hr)


DU145 Cell-LNCaP Exosome (30-42hr)
Slope (1/hr)
control
50ug/ml
100 μg/ml
200 μg/ml
300 mg/ml
400 ug/ml
LNCaP Exosome Treatment


DU145 Cell-LNCaP Exosome (42-72hr)
Slope (1/hr)
control
50ug/ml
100 μg/ml
200 μg/ml
300 mg/ml
400 ug/ml
LNCaP Exosome Treatment

C.


RWPE-1 Cell- LNCaP Exosome
0 μg/ml
50 μg/ml
100 μg/ml
200 μg/ml
300 μg/ml
400 μg/ml
Normalized Cell Index
Time (hr)


RWPE-1 Cell-LNCaP Exosome (30-42hr)
Slope (1/hr)
LNCaP Exosome Treatment
0μg/ml
50μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml

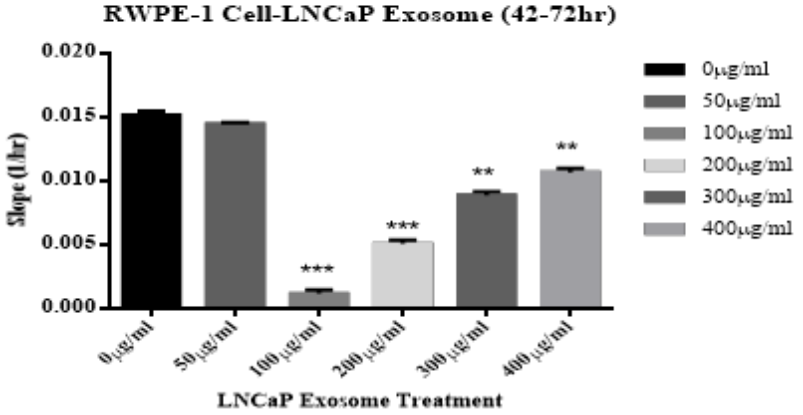

RWPE-1 Cell-LNCaP Exosome (42-72hr)
Slope (1/hr)
LNCaP Exosome Treatment
0μg/ml
50μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml

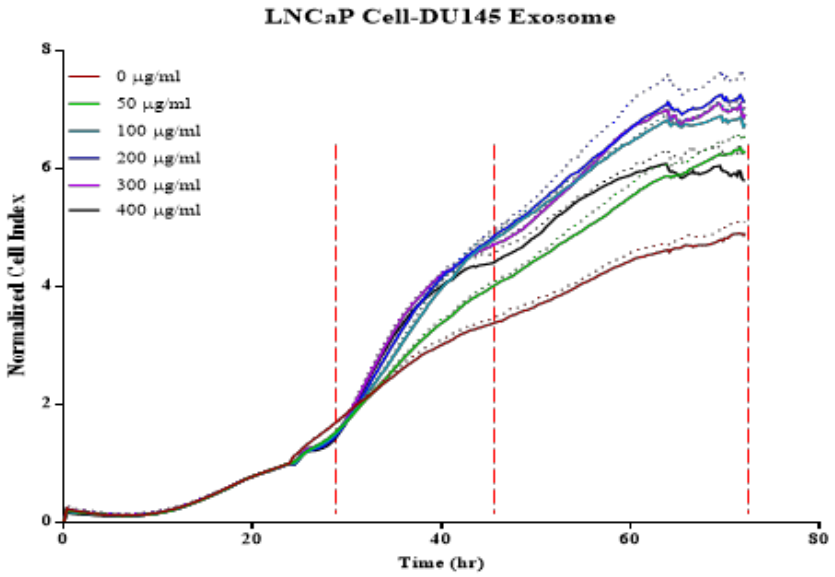
Figure 4.5 Real time proliferation cell analysis.

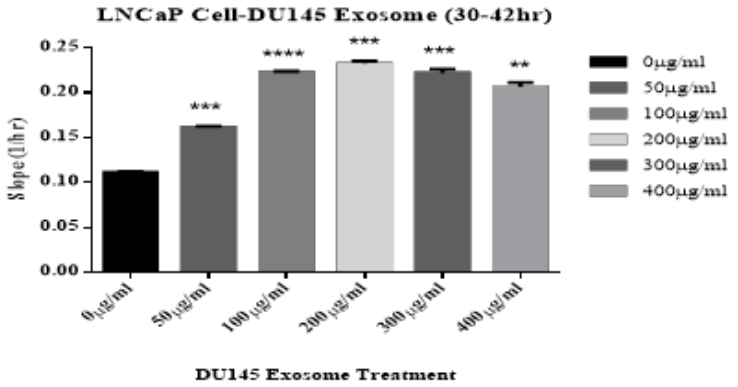
Cell growth of **A.** LNCaP, **B.** DU145 and **C.** RWPE-1 cells were analysed using the xCELLigence system which relies on the generation of electrical impedance as cell growth by 16-well plates were used in the impedance based system, cells were seeded at specific densities (LNCaP, RWPE 20,000/well, and DU145 7,000/ well) after 24 hours, cells were treated with different final concentrations of the LNCaP exoxomes. All slopes were compared with the control (black bar, and red lines in the graphs at *P<0.05, **P<0.01, ***P<0.001 n=2. Slope was calculated by using the RTCA 2.0 software (ACEA))

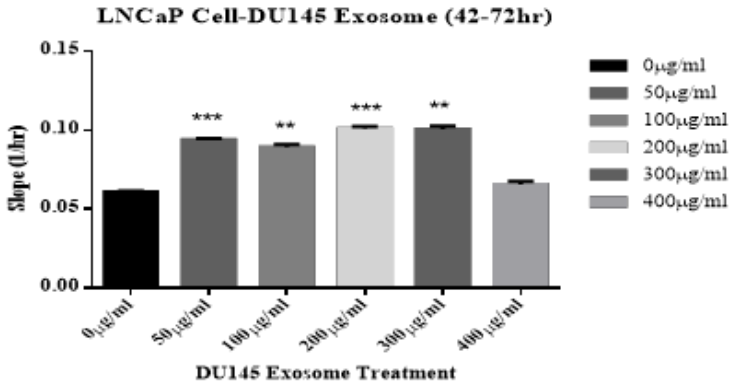
Next, we investigate the effect of DU145 exosomes on all the three cell lines (LNCaP, DU145 and RWPE-1). As may be seen in **Figure 4.5 D.** almost all concentrations of the DU145 derived exosomes significantly increase LNCaP cell proliferation (except 400 µg/ml in 42-72 hour). Similar to what we observed in our LNCaP exosome treatment no significant differences were seen in DU145 cell proliferation when treated with its own exosomes (DU145 exosomes) (**Figure 4.5 E.**)

Interestingly, as demonstrated in **Figure 4.5 F.** both exosomes have a very adverse effect on RWPE-1 cell proliferation as compared to with no treatment as well as to what have been seen in LNCaP cell derived exosome treatment. Apart from the 400 µg/ml DU145 exosomes, which surprisingly and significantly increase the RWPE-1 cell proliferation, all the other concentrations of DU145 exosomes have an adverse effect on RWPE-1 cell proliferation.

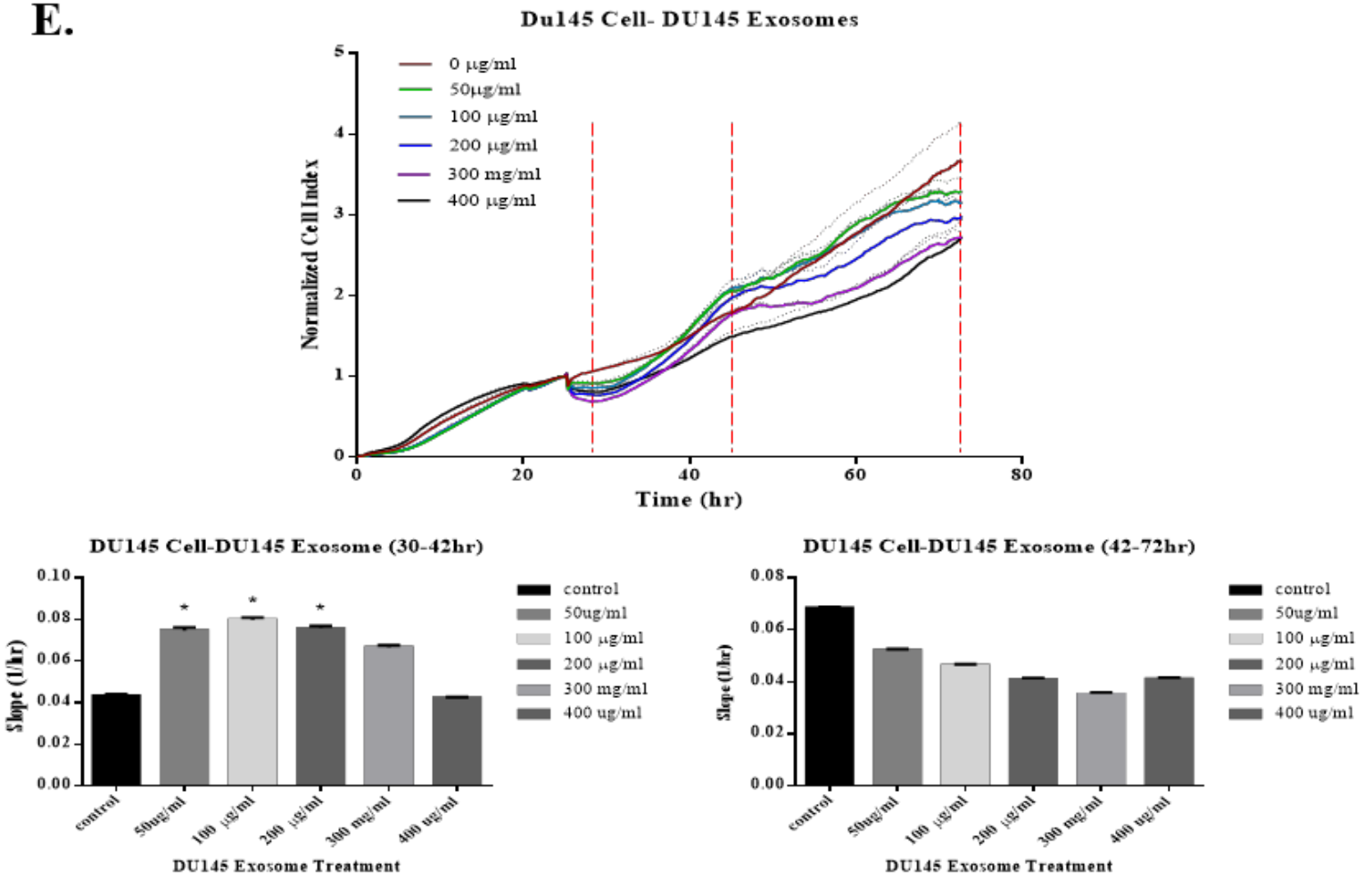
D.


LNCaP Cell-DU145 Exosome
0 μg/ml
50 μg/ml
100 μg/ml
200 μg/ml
300 μg/ml
400 μg/ml
Normalized Cell Index
Time (hr)

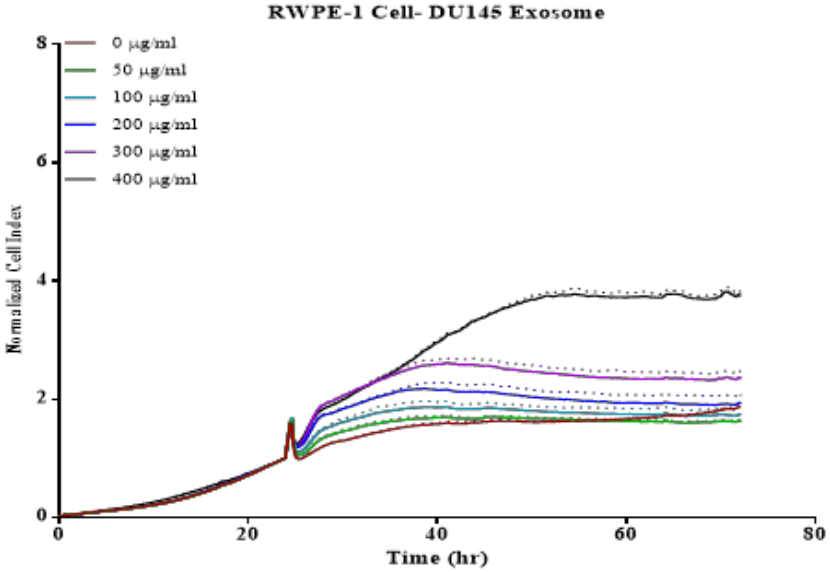

LNCaP Cell-DU145 Exosome (30-42hr)
Slope (1/hr)
0μg/ml
50μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml
DU145 Exosome Treatment

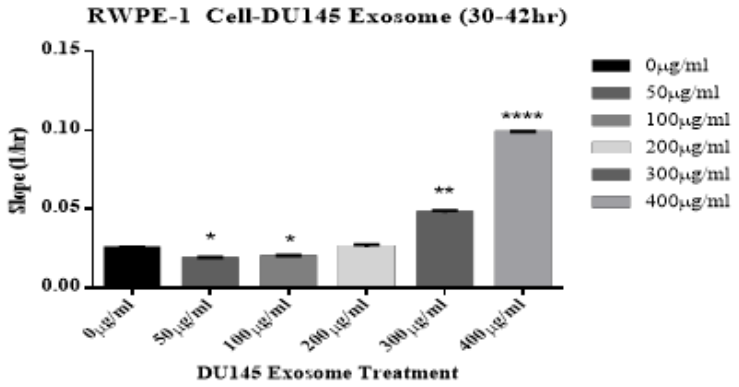

LNCaP Cell-DU145 Exosome (42-72hr)
Slope (1/hr)
0μg/ml
50μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml
DU145 Exosome Treatment

E.


Du145 Cell- DU145 Exosomes
0 μg/ml
50μg/ml
100 μg/ml
200 μg/ml
300 mg/ml
400 μg/ml
Normalized Cell Index
Time (hr)
DU145 Cell-DU145 Exosome (30-42hr)
Slope (1/hr)
control
50ug/ml
100 μg/ml
200 μg/ml
300 mg/ml
400 ug/ml
DU145 Exosome Treatment
DU145 Cell-DU145 Exosome (42-72hr)
Slope (1/hr)
DU145 Exosome Treatment

F.


RWPE-1 Cell- DU145 Exosome
0 μg/ml
50 μg/ml
100 μg/ml
200 μg/ml
300 μg/ml
400 μg/ml
Normalized Cell Index
Time (hr)


RWPE-1 Cell-DU145 Exosome (30-42hr)
Slope (1/hr)
DU145 Exosome Treatment
0μg/ml
50μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml

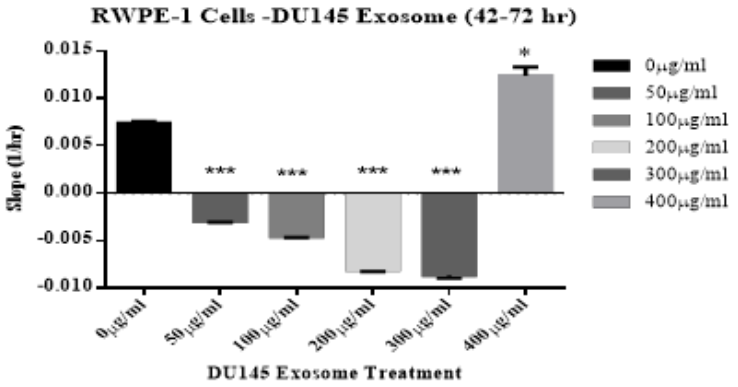

RWPE-1 Cells -DU145 Exosome (42-72 hr)
Slope (1/hr)
DU145 Exosome Treatment
0μg/ml
50μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml

Figure 4.5 Real time proliferation cell analysis.

Cell growth of **D.** LNCaP, **E.** DU145 and **F.** RWPE-1 cells were analysed using the xCELLigence system which relies on the generation of electrical impedance as cell growth by 16-well plates were used in the impedance based system, cells were seeded at specific densities (LNCaP, RWPE 20,000/well, and DU145 7,000/ well) after 24 hours, cells were treated with different final concentrations of the DU145 exoxomes. All slopes were compared with the control (black bar, and red lines in the graphs (*P<0.05, **P<0.01, ***P<0.001, n=2)

4.3.3.1.2 Migration Assay

We also investigated whether LNCaP or DU145 cell derived exosome treatments promote the migration of DU145 and/or RWPE-1 Cells (LNCaP cells didn't migrate (**data not shown**)). Similar to the proliferation assay the effect of exosomes on DU145 and RWPE-1 cells were monitored using the xCELLigence CIM-plates. As described in the experimental procedure (section **4.2.8.2.**) 100µg/ml of exosomes were added to each chamber and the real time migration of each cells from the upper chamber to the lower chamber were monitored over 48 hours. As indicated in **Figure 4.6 A.** both LNCaP and DU145 exosomes significantly increase the migration of DU145 cells when compared to the control (*P<0.05, **P<0.01, n=4). Importantly, DU145 cell derived exosomes have a greater effect on the migratory properties of DU145 cells in comparison to LNCaP cell derived exosomes.

To test the effect of PCa derived exosomes on benign epithelial prostate cell line, RWPE- 1, 100µg/ml of LNCaP and DU145cell derived exosomes were added to RWPE-1, seeded in CIM-plates. Both PCa cell derived exosomes significantly stimulated and increased the migration of RWPE-1 cells in culture. Similar trends were seen in the migration slope of RWPE-1 cells when compared to DU145 cells. In both cases the DU145 cell derived exosomes had a greater effect (almost twice as LNCaP cell derived exosomes) on migratory properties of both cell lines (**Figure 4.6 B.**). Specifically, the migratory effects of both exosome treatments (LNCaP and DU145 exosomes) on RWPE-1 cell were more than 2 times higher when compared to DU145 cells.

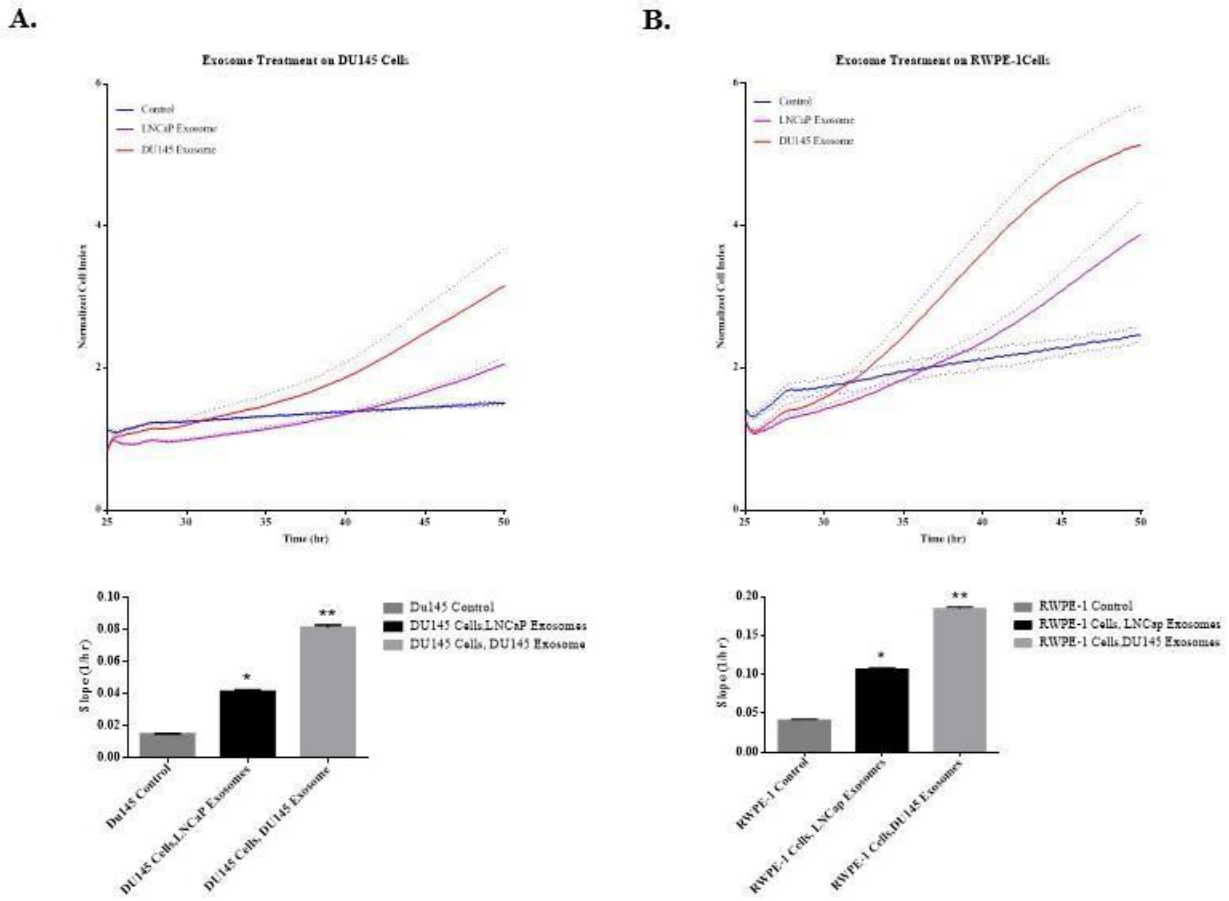


Figure 4.6 Time-dependent migratory pattern of DU145 and RWPE-1 cells after LNCaP and DU145 exosome treatment using xcelligence technology.

A. DU145 and **B.** RWPE-1 cells were treated with 100µg/mL of LNCaP or DU145 derived exosomes. Effect of exosome treatment on migratory properties of DU145 and RWPE-1 cells were determined using xCELLigence technology with CIM-16 plates. 20,000 cells were seeded per well and treated with exosomes after 24 hr. All values are representative of at least 4 independent experiments with similar results, and are presented as cell index on the top, and slope of the lines in the bar graphs $P<0.05$, $**P<0.01$ n=4.

4.3.3.2 Cell Motility

The effects of exosomes derived from both PCa cell lines on migration properties of RWPE-1 cells have been confirmed using transwell (**Figure 4.7**) and tumor spheroid-based migration assays (**Figure 4.8**). Exosome-educated RWPE-1 cells have been allowed to migrate to the underside of the chamber in the presence of 100 µg/ml of LNCaP or DU145 derived exosomes or serum free medium in both chambers using fetal bovine serum as the chemoattractant. This result in a significant increase of the cell number that migrated through the membrane pores upon treatment with exosomes derived from LNCaP (**Figure 4.7 A.**) and DU145 (**Figure 4.7 B.**).

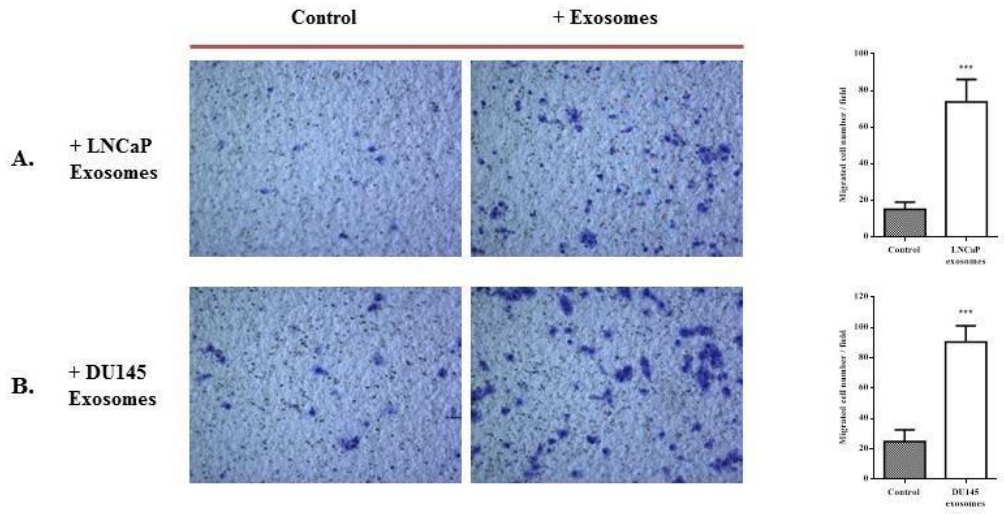


Figure 4.7 Exosomes increase RWPE-1 cell migration.

RWPE-1 cells were incubated for 48 hours with exosomes (100 µg/ml) derived from **A.** LNCaP, **B.** DU145 cells or corresponding serum-free medium and loaded into the upper chamber of a transwell. Exosomes concentrations (100 µg/ml) or serum free-medium were maintained in upper and lower chambers. After 24 h incubation, the migration activity was quantified by counting the migrated cells on the lower surface of the membrane of at least five fields per chamber using a x10 objective. Representative photographs are shown in the left panel. Quantification of migrating cells is shown in the right panel. All values are representative of at least two independent experiments with similar results, and are displayed as mean ± SD, where ***$p < 0.001$.

4.3.3.3 3D migration

The importance of studying cancer cells in three-dimensional (3D) models has been emphasized because of the greater relevance to *in vivo* tissue structures (Smalley *et al*., 2008). When cultured on agarose, the RWPE-1 cell line is able to form spheroids, a model considered to be mimicking micrometastasis or inter-capillary micro regions of solid tumors. We used the tumor spheroid-based migration assay described by Vinci et al. (2013) (Vinci *et al*., 2013). This assay attempts to mimic tumor cells spreading from a solid micro-tumor or micrometastasis. Exosome-educated spheroids were transferred onto type I collagen-coated microwells and migration was scored after 12 and 24 hours by measuring the migrated cell area in presence of 100 µg/ml of LNCaP or DU145 exosomes or serum free medium. Our data showed that in the presence of exosomes derived from LNCaP (**Figure 4.8 A.**) and DU145 (**Figure 4.8 B.**) following 12 hours of incubation, RWPE-1 cells have a significantly greater propensity to disseminate from the spheroid, validating the results obtained using 2D standard migrations assays.

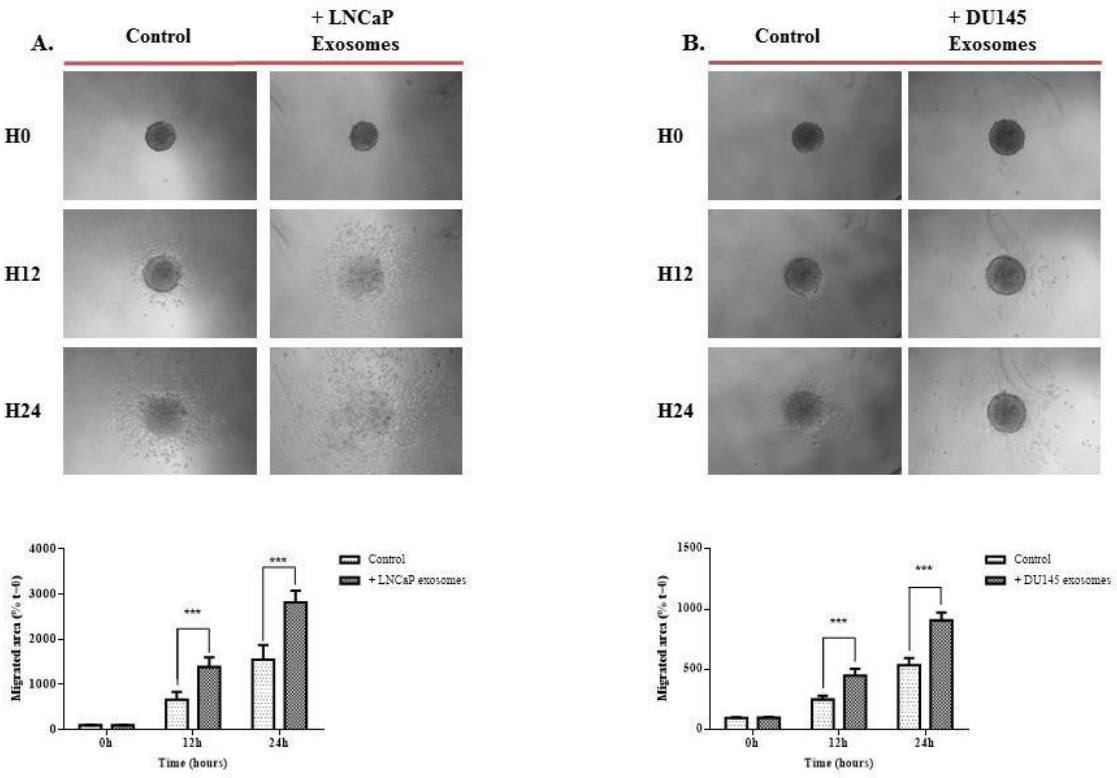


Figure 4.8 Exosomes increase RWPE-1 cell migration on collagen I.

RWPE-1 spheroids were incubated for 48 hours with exosomes (100 µg/ml) derived from **A.** LNCaP, **B.** DU145 cells or corresponding serum-free medium and transferred to microwells coated with type I collagen. The cell migration was scored at t=12 hours and t=24 hours by measuring the migrated cell area and normalizing to the migration seen at t=0. Representative photographs are shown in the upper panel. Quantification of migration area is shown in the lower panel. All values are representative of at least two independent experiments with similar results, and are displayed as mean ± SD, where ***$p < 0.001$.

4.3.4 Pathway Analysis

Overexpression of the MEK/ERK pathway has been associated with CRPC and poor prognosis (Gioeli *et al.*, 1999)(Mukherjee *et al.*, 2005)(Weber *et al.*, 2004). While the mechanism of activation of this cascade in PCa is not fully understood we attempt to assess whether the observed decrease in apoptosis, increase of proliferation and migration after treatment of cell lines with exosomes correlate with MEK/ERK activation. We examined the activity of MEK1/2 and ERK1/2 at different time points (5, 10, 15 and 30

minutes and 1, 6 and 24 hours) after PC3, DU145, LNCaP, C4-2 and RWPE-1 cells were treated with 100 µg/ml of LNCaP cell derived exosomes. As shown in **Figure 4.9**, 100 µg/ml of LNCaP exosomes increased the expression of p-MEK1/2 and/or p-ERK1/2 in PC3, DU145, LNCaP and RWPE-1 (**Figure 4.9 A., 4.9 B.** and **4.9 E.**). While both DU145 and RWPE-1 cells demonstrated an increase in the p-ERK1/2 phosphorylation followed by p-MEK1/2 activation, the levels of p-ERK were exactly equivalent to control for all time points in PC3 cells treated with LNCaP cell derived exosomes. Interestingly, while only the level of p-ERK1/2 increased in LNCaP cells after just 5 minutes treatment with LNCaP cell derived exosomes, the MEK1/2 and ERK1/2 level did not change in C4-2 cells compared with the zero-time point (control) (**Figure 4.9 C., 4.9 D.**).

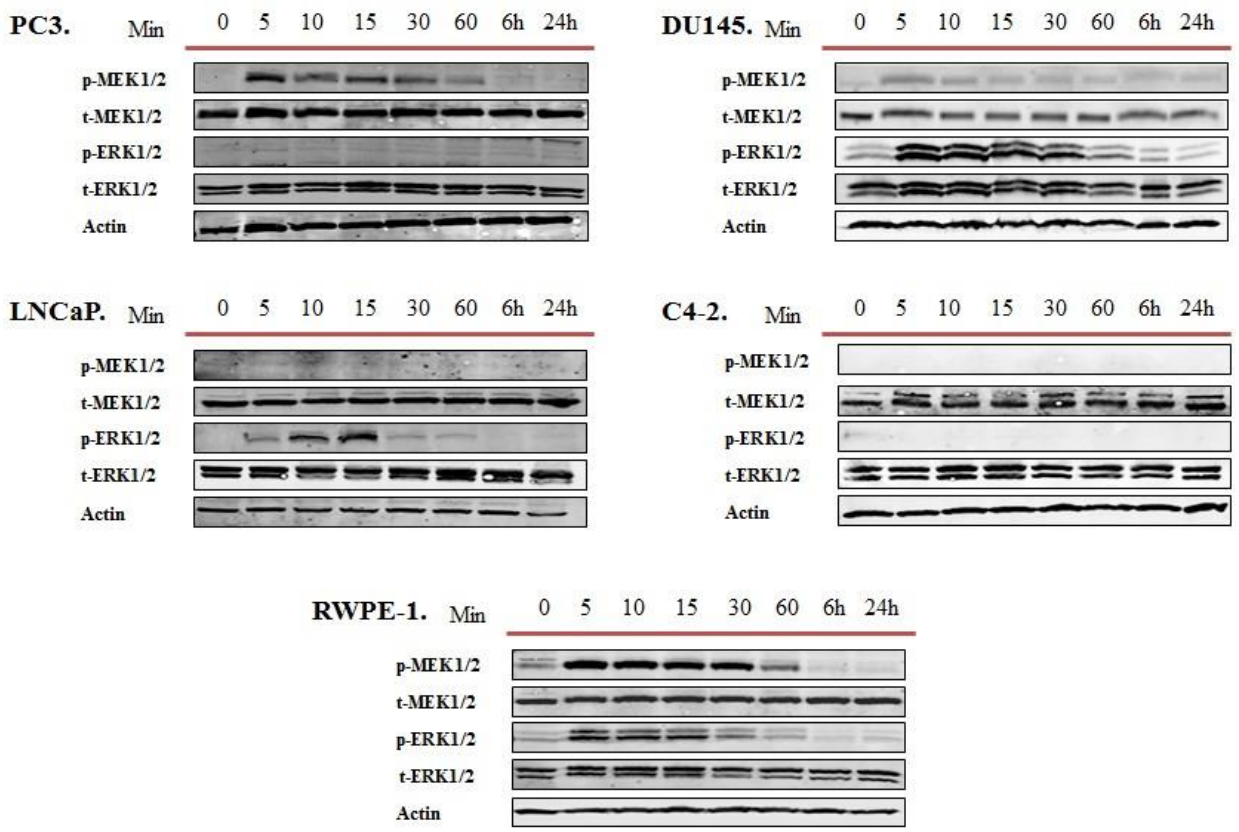


Figure 4.9 Western Blot analysis (LNCaP Exosome).

Five different prostate cell lines, including **A.** PC3, **B.** DU145 (androgen independent), **C.** LNCaP,
D. C4-2 (androgen sensitive) and **E.** RWPE-1 (benign epithelial prostate cell line) were treated with one dose of 100 µg/mL of LNCaP derived exosomes for 5, 10, 15, 30 and 60 min, 6 and 24 hr. as indicated. WB was used to analyse cell lysates with the indicated antibodies.

We then repeated the same experiment for DU145, LNCaP and RWPE-1 cells with DU145 cell derived exosomes to understand whether exosomes from AR -ve PCa cell line influence this pathway differently. As expected, and similar to what we have observed with LNCaP cell derived exosomes treatment, MEK and ERK were both phosphorylated rapidly, after 5 minutes of treatment with DU145 cell derived exosomes (**Figure 4.10 A. and 4.10 C.**).

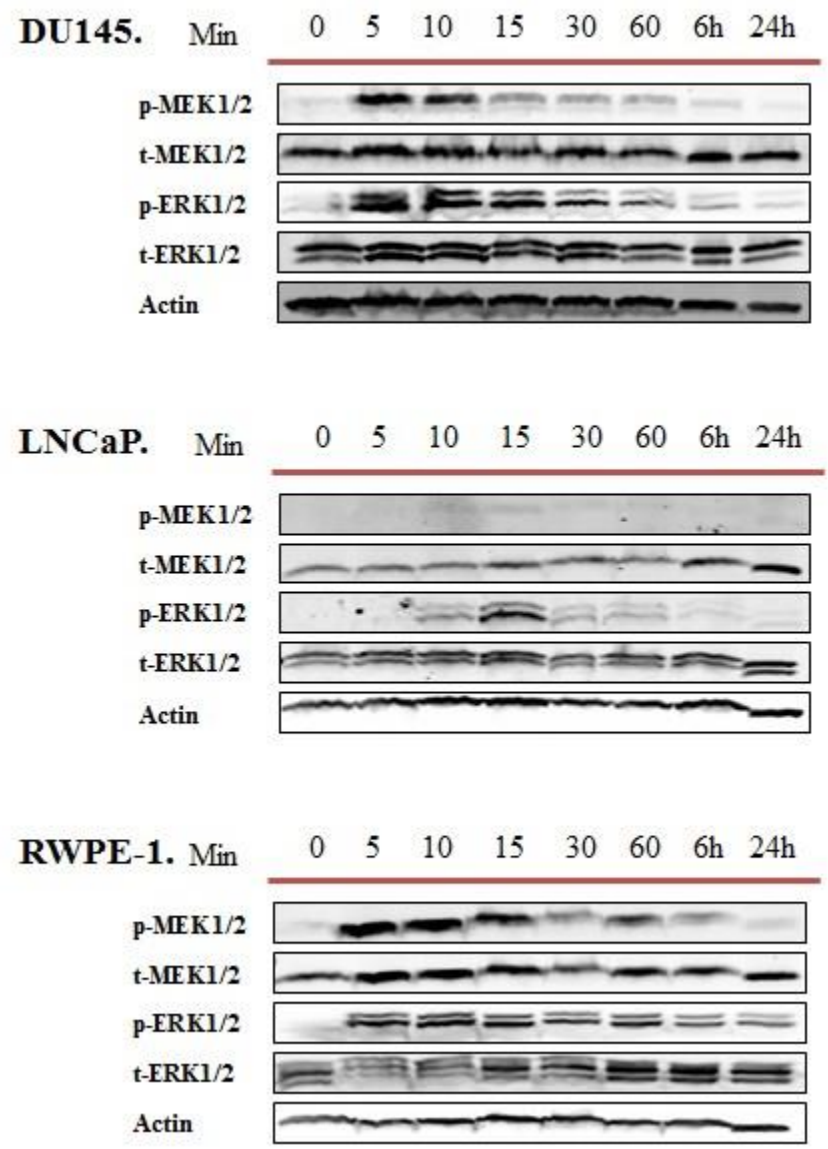


Figure 4.10 Western Blot analysis (DU145 Exosome).

Three different prostate cell lines, including **A.** DU145 (androgen independent), **B.** LNCaP (androgen sensitive) PCa cell line and **C.** RWPE-1 (benign epithelial prostate cell line) were treated with one dose of 100µg/mL of DU145 derived exosomes for 5, 10, 15, 30 and 60 min, 6 and 24 hr as indicated. WB was used to analyse cell lysates with the indicated antibodies.

4.3.5 *In vivo* Study in Mice Bearing LNCaP Human Tumor Xenografts

On the basis of the above findings we hypothesized that treatment of mice bearing human PCa tumour xenografts with PCa cell derived exosomes would increase the tumour volume hence promote PCa cancer progression in a dose dependent manner. To further examine the role of PCa derived exosomes in tumor growth *in vivo*, 10 to 15 nude mice per each group were subcutaneously inoculated with LNCaP cells (10^6 cells in BD matrigel, BD Biosciences, New Jersey, USA) at posterior dorsal site. LNCaP xenografted mice were prepared as described in section **4.2.11.** and treated (IV tail vein) with exosomes twice/week for four weeks. Our results demonstrate no significant differences in either tumor volume or PSA level of animals treated with LNCaP exosomes (low and high dose) when compared to control (**Figure 4.11 A, B**).

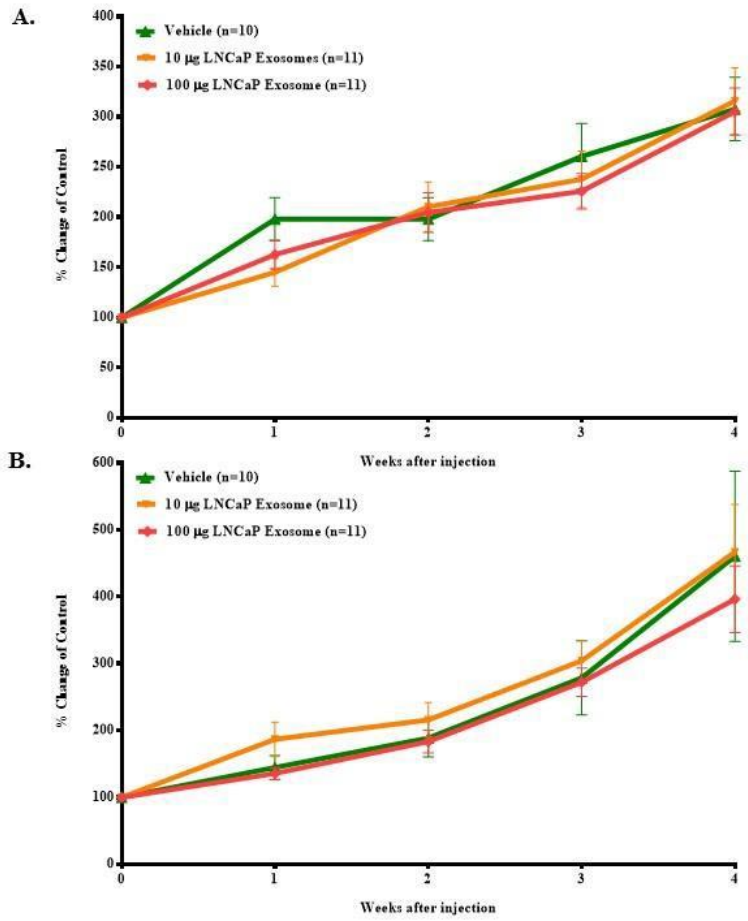


Figure 4.11 ***In vivo*** **study using LNCaP xenograft bearing mice (LNCaP Exosome).**

The *in vivo* effect of LNCaP on the **A.** tumor volume **B.** PSA level of LNCaP mice xenograft. Data are presented as Mean ± SEM. (Vehicle n=10, 10µg LNCaP Exosome group n=11 and 100µg LNCaP Exosome group n=11).

Conversely, low and high dose treatment with DU145 exosomes stimulate the tumor growth in LNCaP xenograft bearing mice. In contrast to control mice, those treated with 100µg of DU145 exosomes showed a very significant increase in tumor size starting after only one week of treatment (p value <0.001; n=11). LNCaP xenograft bearing mice treated with low dose of DU145 exosomes (10 µg) also demonstrated a very significant increase in the tumor volume after the second week of treatment with exosomes (p value <0.001;

Vehicle n=15, 10µg DU145 Exosome group n=13 and 100µg DU145 Exosome group n=11) (**Figure 4.12 A.**).

As shown in **Figure 4.12 B.**, LNCaP xenograft bearing mice treated with DU145 exosomes demonstrate a significant increase (p value <0.001, Vehicle n=15, 10µg DU145 Exosome group n=13 and 100µg DU145 Exosome group n=11) in the serum PSA level starting one and two weeks after treatment for the high and low dose group in respectively.

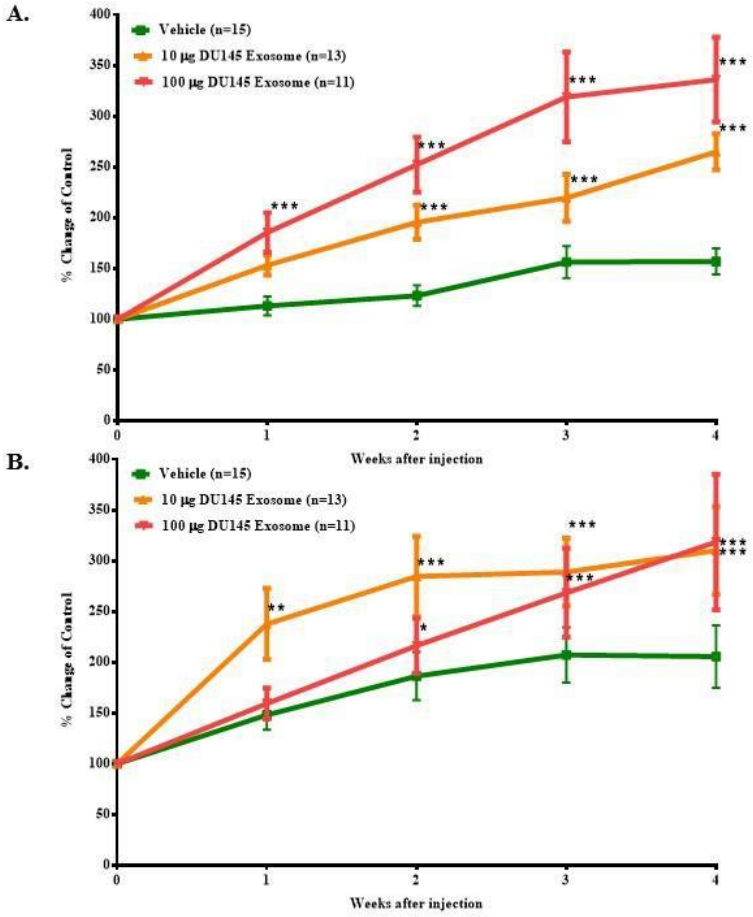


Figure 4.12 *In vivo* study using LNCaP xenograft bearing mice (DU145 Exosome).

The *in vivo* effect of DU145 on the **A.** tumor volume **B.** PSA level of LNCaP mice xenograft. Data are presented as Mean ± SEM. (***) p value < 0.001 was considered extremely significant compared vehicle treated-mice (Vehicle n=15, 10µg DU145 Exosome group n=13 and 100µg DU145 Exosome group n=11).

4.3.6 Immunohistochemistry

Upon immunohistochemical analysis of Ki67 and Filamin C expression in LNCaP tumors, obtained from LNCaP tumor-bearing nude mice treated with two different concentrations of DU145 exosomes (10 µg and 100 µg) we confirmed that the expression of Filamin C have increased upon DU145 exosome treatment. As presented in Figure 4.13 A. The level of Ki 67 has slightly increased in the LNCaP tumors treated with 100 µg DU145 exosome. This is in agreement with our *in vitro* proliferation results as well as the tumor growth. We also previously have reported the presence of ANXA2 (Annexin A2), CLSTN1 (Calsyntenin 1), FASN (Fatty acid Synthesis), FLNC (Filamin C, gamma), FOLH1 (Folate Hydrolase (prostate specific membrane antigen)-1), GDF15 (Growth Differentiation Factor 15), as PCa biomarker in PCa cells derived exosomes (Hosseini-Beheshti *et al*., 2012). Among these Filamin C was one of the proteins that were only presented in DU145 and VCaP exosomes. As revealed in Figure 4.13 B. the Filamin C level has been upregulated significantly in a dose dependent manner in LNCaP tumors upon 10 µg and 100 µg DU145 exosome treatment.

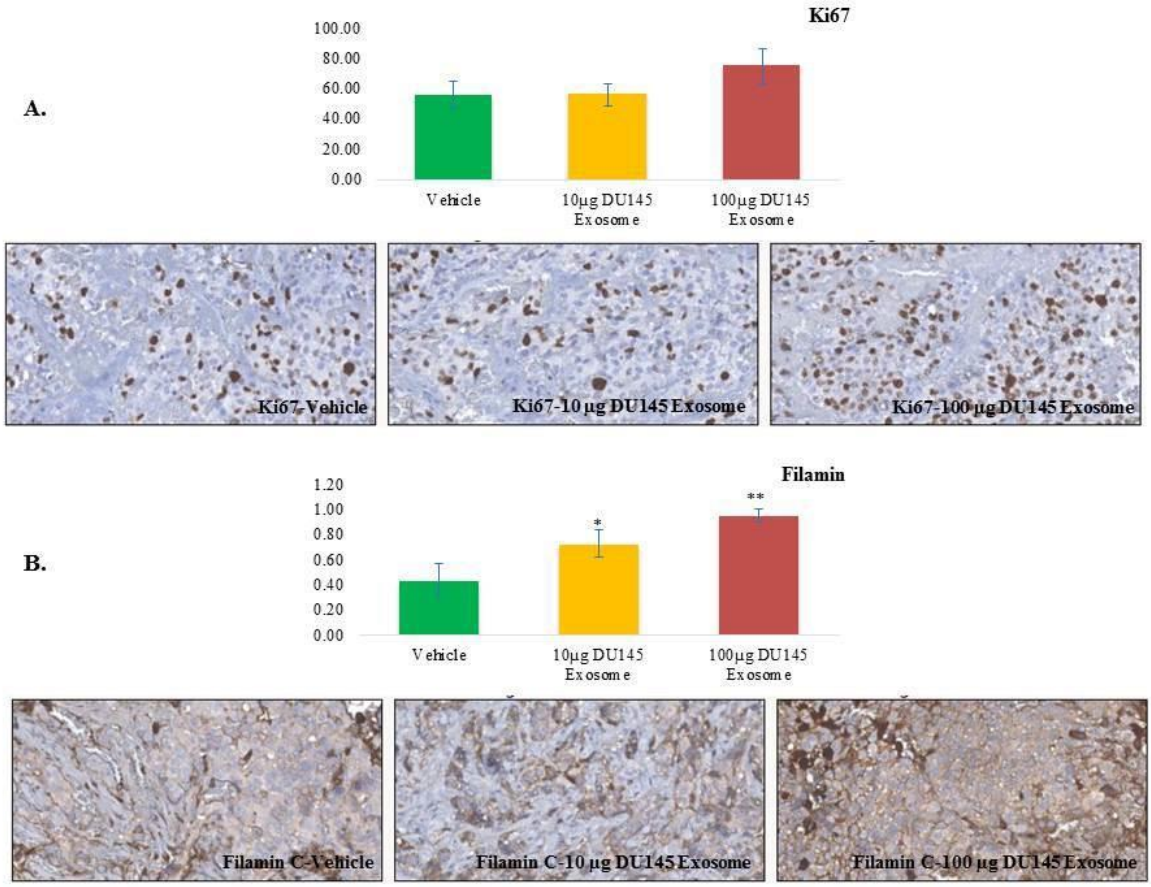


Figure 4.13 Immunohistochemical analysis.

Immunohistochemical analysis of **A.** Ki67 and **B.** Filamin C expression in LNCaP tumors upon 10 µg and 100 µg DU145 exosome treatment in comparison with vehicle treatment (* $P < 0.01$,** $P < 0.05$).

4.4 Discussion

During the last decade cancer derived EVs have been proposed to be crucial players in both anti-tumorigenic properties as well as cancer development and progression. While the molecular mechanisms regulating exosomes biogenesis and their subsequent functions, especially in cancer development and progression, have just began to be delineated, our main focus in this study was to investigate and understand the *in vitro* and *in vivo* relevance of PCa cell derived EVs using different functional assays in PCa models. The

initial step of this work was to isolate a homogenous mixture of exosomes from two different PCa cell lines with different AR phenotypes. Both isolated exosomes exhibited characteristic exosomal markers and lacked ER markers as validated by Western Blotting (Hosseini-Beheshti *et al*., 2012). TEM imaging and NTA of purified exosomes revealed the typical artificial cup-shape (due to the TEM sample preparation procedure) morphology with diameters ranging between 30-200 nm (van der Pol *et al*., 2012).

While the functional effects of EVs mainly rests on their release and uptake, mechanistic information is very limited in this area. Christianson et al (2013) have reported that several different molecules and pathways could be involved in exosome uptake. Their findings suggested that heparin sulfate proteoglycan dependent entry pathway is essential for exosome biological activity. However they proposed that exosomes may employ other functional activity through alternative internalization pathways, they found that protogelycan deficient cells may attenuate exosome mediated migration as well as ERK 1/2 activation (Christianson *et al*., 2013). Although the mechanism of exosome internalization and uptake was not the focus of this chapter, it was very important to demonstrate that all prostate cell lines in this study uptake and internalize exosomes. Similar to our previous study (Hosseini-Beheshti *et al*., 2012) the internalization of exosomal CLU_{GFP} (derived from the LNCaP cell line) to cancer and benign prostate cell lines with different AR phenotypes were clearly demonstrated.

It is very well known that the equilibrium between programmed cell death and cell survival plays a key role in ultimate outcome of cancer cell fate (Ouyang *et al*., 2012). In particular, regulation of apoptosis is known to have a central role in PCa development and its progression to CRPC partially due to the up-regulation of anti-apoptotic

genes after ADT (Li *et al*., 2004)(Zhang *et al*., 2005)(Gua *et al*., 2006). Different studies have demonstrated that increased resistance to apoptosis is the second main event associated with CRPC after therapeutic failure (Howell *et al*., 2000), due to up-regulation of anti-apoptotic genes. Tumor derived exosomes have been shown to transport apoptosis inhibitory proteins, such as survivin, which is induced under stress conditions, in order to promote survival (Khan *et al*., 2012). EVs are known to contain and carry different molecular cargo which are known to mediate the hallmarks of cancer. Yang et al. (2013) and Franzen et al (2014) have shown that bladder cancer cell derived exosomes inhibit tumor cell apoptosis through inhibition of the Akt and ERK pathways (Yang *et al*., 2013)(Franzen *et al*., 2014) . In addition, other studies have reported that cancer cell derived exosomes can create an immunosuppressive microenvironment by impairing the immune cell via induction of T-cell apoptosis. Apoptosis of T cells was found to be inducible via induction of adenosine (Liu *et al*., 2006)(Clayton *et al*., 2011) as well as FAS-FASL ligation (Huber *et al*., 2005)(Andreola *et al*., 2002) Therefore, in the next section of this chapter, the role of PCa cell derived exosomes on both cancer and benign prostate cell lines with different AR phenotypes were investigated. In agreement with what has been reported previously our results indicate that PCa derived exosomes inhibit apoptosis in both cancer and benign prostate cell lines and potentially promote tumorigenesis.

Several lines of evidence suggest that cancer derived exosomes transport paracrine signals and contribute to cancer development and progression via supporting the cancer cell or endothelial cell proliferation which results in enhanced tumor growth (Peinado *et al*., 2012) and angiogenesis (Umezu *et al*., 2014). It has been previously reported that PC3 derived EVs induced osteoclast differentiation and osteoblast proliferation (Inder *et al*., 2014) As

part of this investigation into the role of PCa derived exosomes on different functional assays, our results demonstrated that LNCaP derived exosomes only increase the proliferation of LNCaP cells at two different doses and did not influence the DU145 cell proliferation, while DU145 derived exosomes increase LNCaP cell proliferation significantly but had a minimal influence on DU145 cell proliferation. These data were in agreement with the findings of Corcoran et al (2012) and Inder et al (2014) (Corcoran *et al*., 2012)(Inder *et al*., 2014). It was very interesting to see that the effect of PCa derived exosomes on benign epithelial prostate cell line (RWPE-1) is very similar to what has been previously reported for immune system cells such as T-cells. Except for the 400µg/ml dose DU145 treatment, both LNCaP and DU145 derived exosomes attenuated the RWPE-1 cell proliferation significantly. Positive and negative influences of PCa derived exosomes observed on proliferation of PCa and benign prostate cell lines respectively support the premise that they have relevance in tumorigenesis. Extracellular trafficking of different and unique protein/genomic signatures in LNCaP and DU145 derived exosomes (as has been partially described in Chapter 2 (Hosseini-Beheshti *et al*., 2012)) as well as unique differences between recipient cells, could explain the different responses that have been observed in these functional assays.

In the next set of experiments the role of exosomes in migration and metastasis were delineated. A recent bulk of accumulating research evidence has contributed to a wealth of knowledge that exosomes are a key contributor to cell migration in both physiological and pathological conditions. Salomon et al. (2014) have established that exosomes are released into maternal blood as early as six weeks of gestation in a normal healthy pregnancy. Furthermore their results showed that the concentration of exosomes in

maternal blood increased significantly and these bioactive nanovesicles regulated endothelial cell migration (Salomon *et al*., 2014). On the other hand growing evidence has identified that cancer-derived exosomes can play a central role in various aspects of cancer progression, in particular these studies have revealed that these exosomes could promote cell motility and migration via activation of different pathways or release of different bioactive components. Activation of Wnt-planar cell polarity signaling pathway in breast cancer cells as a result of fibroblast-secreted exosomes treatment and secretion of HSP90α via breast cancer exosomes or EDIL-3 via bladder cancer are few examples which have been shown to be associated with cell migration and invasive behaviour in cancer models (Luga *et al*., 2012)(McCready *et al*., 2012)(Beckham *et al*., 2014).

In addition, Bijnsdorp et al (2013) and Morello et al. (2013) have clearly demonstrated that PCa derived exosomes or large oncosomes increase the migration and invasion of noncancerous and cancer associated fibroblasts (Bijnsdorp *et al*., 2013)(Morello *et al*., 2013). In agreement with all of the above our three independent migration assay results demonstrated that both AR+/-ve PCa derived exosomes promoted cell migration and motility significantly. Specifically, our results revealed that DU145 derived exosomes increase the cell migration in RWPE-1 and DU145 cells greater than LNCaP derived exosomes. However LNCaP cell derived exosomes appeared to have a greater effect on 3D migration when compared to DU145 derived exosomes.

As discussed earlier the difference in molecular cargo of these two different exosome sources could greatly contribute to the different responses that have been observed in these functional assays. Although more focused mechanistic studies are needed to understand the pathway involve in these functional assays one simple explanation

could be the presence of major differences in their proteins signatures. As reported in our previous study, our proteomic data revealed the presence of 65 total proteins and 31 unique proteins in our LNCaP derived exosomes while the identified proteins in our DU145 derived exosomes were 117 with 83 unique proteins and an overlap of 34 proteins in both groups (Hosseini-Beheshti *et al*., 2012). As discussed, the main aim of this study was to confirm the role and relevance of PCa derived exosomes (with two different AR phenotypes) on PCa development and progression. While our results have proved that PCa derived exosomes may in fact be one of the main players in PCa progression via reduction of apoptosis and induction of proliferation, migration and invasion, we decided to investigate if the MEK/ERK activation pathway has any role in the manifestation of these observations.

Aberrant regulation of MAPK cascade has been shown to be an essential contributor to many different cancers (Oka *et al*., 1995)(Gioeli *et al*., 1999). In particular, MAPK cascades are key signalling pathways that contribute to the regulation of cell proliferation, differentiation, survival, motility and metastasis (Pages *et al*., 1993)(Rubinfeld *et al*.,2005)(Lewis *et al*., 1998). While induction of MAPK activation has been shown to be consistent with tumor grade, stage and PCa progression (Gioeli *et al*., 1999), the inhibition of this pathway has also been the subject of intense pharmacological research scrutiny for cancer treatment (Roberts *et al*., 2007)(Deleault *et al*., 2008)(Qu *et al*., 2009)(Chalmin *et al*., 2010)(Meckes *et al*., 2010)(Sirois *et al*., 2011) .

A growing number of EV research describes the fact that exosomes attributed to cancer cell proliferation, migration and invasion via activation of MAPK (Morgan *et al*., 2011). $FASL^{+}$ exosomes have been shown to activate c-$FLIP_{L,}$ ERK and NF-κB pathways and therefore increase MMP expression in tumor cells which leads to tumor invasion (Cai *et al*.,

2012). Ye et al. (2014) have also demonstrated that nasopharyngeal carcinoma derived exosomes mediate T-cell dysfunction such as proliferation, differentiation and cytokine secretion via downregulation of MAPK1 and JAK/STAT pathways (Ye *et al*., 2014). In agreement with these observations our results support the premise that PCa derived exosomes activate MEK/ERK pathway in both cancer and benign prostate cells. Aside from the results observed with C4-2 cells, either MEK or ERK phosphorylation was enhanced in all the other cell lines upon exosomes treatment. However the molecular and cellular mechanisms involved in each of the functional activities that we have observed upon exosomes treatment has not been determined. The activation of MAPK could somewhat explain these observations.

In agreement with our *in vitro* observations we also show that DU145 derived exosomes have a role in tumour development and PSA induction in our LNCaP xenograft mouse model. The significant induction in the level of Filamin C upon the DU145 exosome treatment observed in LNCaP xenograft bearing mice seen in our immunohistochemical analysis provides further evidence supporting the role of exosomes in cancer progression and could also infer selective uptake of PCa derived exosomes by the PCa xenograft tumor.

Our study has shed light on the functionality and importance of prostate cancer derived exosomes in prostate cancer development and progression. Taken together, this study has revealed the significant potential of exosomal influence on different functional assays and suggests that prostate cancer derived exosomes are likely to play a pivotal role in prostate cancer development and progression. However, further mechanistic studies and creative experimentations are needed to understand the molecular mechanisms involved in all these processes. Our study has raised several new insights in the involvement

of PCa derived exosomes in various cancer and benign prostate cell lines. Further studies are warranted to investigate new targets for preventive and/or therapeutic interventions for PCa.

Chapter 5: Conclusion

5.1 General Conclusion

It has been more than forty years since Anderson reported the discovery of membrane- enclosed vesicles in the matrix of epiphyseal cartilage (Anderson *et al*., 1969). EVs including exosomes are bi-layers lipid membrane vesicles with a size ranging from 30 to 200 nm which carry a variety of bio-macromolecules, including nucleic acids, proteins and lipids (van der Pol *et al*., 2012).

Depending upon their cell or tissue of origin these nanovesicle entities may play different roles in physiological and pathological conditions. EVs have proven to be a key player in many different functions from embryonic development to the facilitation of immune response (Whiteside *et al*., 2013) and from their role in tumor growth (D'souza-schorey *et al*., 2012) to their tumor vaccine applications (Hsu *et al*., 2003) exosomes are proving to be unique as highly mobile, discrete packages of protein and nucleic acids that are essential for intercellular communications.

In addition to their role and relevance in attenuating or inducing different mechanisms involved in disease progression, as discussed at length in Chapters One and Four, the accessibility of these vesicles through non-invasive procedures as well as the presence of different classes of proteins, lipids and nucleic acid in these nanovesicles positions them as a promising minimally invasive and novel source of diagnostic biomarkers (liquid biopsy) (the focus of Chapters Two and Three).

In this thesis we tried to study the biomarker potential of these nanovesicles (Chapters Two and Three) as well as their role and relevance in PCa progression (Chapter Four) while I have tried to provide a very detail and precise discussion at the end of each

chapter in this final Conclusion Chapter I will mainly focus on the advantages, disadvantage/limitations, and future directions of each chapter.

5.1.1 Exosomes as Biomarker Enriched Microvesicles: Characterization of Exosomal Proteins Derived from a Panel of Prostate Cell Lines with Distinct AR Phenotypes

5.1.1.1 Advantages and Disadvantages

The first question that we asked was whether all PCa cell lines release exosomes and if yes what are the main differences between these entities released from prostate cells with different AR phenotypes?

As shown in Chapter Two, we observe the release of exosomes by six different prostate cells including PC3, DU145, VCaP, LNCaP, C4-2 and RWPE-1 and determine characteristic differences between exosomes released by parental cells of different characteristic and AR phenotypes. We then confirmed the transfer of fluorescence labeled exosomes to target cells in culture using confocal microscopy. We subsequently performed a comprehensive proteomic analysis of all six different prostate cell derived exosomes using mass spectrometry to understand differences between the protein profiles released via exosome externalization between prostate cell lines. We then investigate the difference in broad classes of lipids and cholesterol as constituents of different prostate cell lines and their exosomes.

This broad understanding of a various aspects of PCa derived exosomes form a solid platform for future PCa derived exosome research.

The main advantages of this study have been listed below: Our exosome isolation technique, which consisted of several centrifugation steps along with filtration, and a final

ultracentrifugation step at 100,000×g using a 30% sucrose cushion, decreases contamination with cell debris and produces high quality and homogenous purified exosomes, limited in contamination from other membrane vesicles or protein aggregates.

1. One of the other main advantages of this study which has recently been cited as one of the main references in PCa derived exosomes related studies (Duijvesz *et al*., 2013)(Drake and Kislinger, 2014) since it was published in 2012 was the use of six different cell lines with different AR phenotypes. While, due to the degree of the difficulty in vesicle isolation, most of the articles have focused on one or two cell lines, we decided to perform a comprehensive characterization of exosomes derived from six prostate cell lines which have distinct AR +/-ve expression phenotypes (Hosseini-Beheshti *et al*., 2012).

2. The comprehensive proteomic analysis in this study not only provides a basis for evaluating transfer of identified composite exosome proteins between different PCa cells as part of a recognised cell communication phenomenon but also forms a platform for future clinical validation research using exosomes as biomarkers for PCa diagnosis as well as potential therapeutic targets which could be important in the treatment of CRPC.

3. While the protein and nucleic acid (e.g. mRNA and miRNA) profile of nanovesicles have been the centre of attention for biomarker discovery, our cholesterol data suggests that exosomes derived from PCa cell lines contain significantly more cholesterol than their benign counterpart cell line RWPE-1. While this is in line with our previous work which indicates that cholesterol is likely to play a role in PCa progression (Locke *et al*., 2008)(Leon *et al*., 2009), this was the first time that cholesterol content of exosomes has ever been shown to be a potential biomarker for PCa diagnosis. In addition to

all listed advantages, there were also a couple of disadvantages/limitations in this study.

1. While we tried to include as many prostate cell lines as possible in this study, we strongly believe that having a couple more benign or normal prostate cell lines would have added valuable information to our proteomic/lipidomic and cholesterol analysis.

2. The lack of a standardized exosome isolation protocol as well as the absence of valid exosome marker for data normalization is a major issue in exosome research and this has been of relevance to our research also.

5.1.1.2 Future Directions for Chapter Two

As mentioned above the absence of a known exosome marker which could be used to normalize data is a major hurdle for all *in vitro* and clinical exosome studies, therefore one of the major future direction should be focused on finding a exosome marker which could be used to standardize quantitation of all different exosomes.

The work presented in this chapter provides a broad characterization of PCa derived exosomes. A comparison study between exosomes derived from Prostatic Intraepithelial Neoplasia (PIN) and PCa derived exosomes would provide interesting insight into the development of prostate tumors and androgen responsiveness.

Similar studies investigating the difference between BPH derived exosomes and PCa derived exosomes would be useful to better understand the role of prostatic inflammation in the initiation of BPH and PCa. Exosomes as Biomarker Enriched Microvesicles Characterization of Exosomal Protein Derived from the Biological Fluids Obtained from Prostate Cancer Patients

5.1.1.3 Advantages and Disadvantages

In follow up to our novel findings in the second chapter of this thesis, we purify EVs from the blood and urine of normal and PCa patients and attempt to determine differences between the two sources (blood vs urine) and the two sample groups (normal vs PCa). In follow up to our previous chapter, we also performed a comprehensive MS-based proteomic analysis on these samples to understand the major differences between exosomes derived from blood vs. urine in control and cancer groups as well as possible underlying differences in protein profiles. An additional part of this study was to investigate the cholesterol level in EVs isolates as a potential diagnostic tool. Taken together in this chapter, we examined the potential of direct MS based proteomic and/or cholesterol profiling of EVs derived from different biological fluids and their uses for biomarker analysis in biological fluids.

There were considerable advantages to using clinical samples in this study:

1. Using two biological sources (blood and urine) for exosome isolation in this chapter provide a better understanding of exosomes composition.
2. This study validates some of our proteomic findings determined using cell line derived exosomes in Chapter Two.
3. This study performs a parallel comparison in blood and urine samples obtained from the same patients allowing for a better comparative analysis to be conducted and thus more concrete conclusions be determined for the use of different biological samples for different purposes.

The major disadvantages of working with biological samples were:

1. Although our study set a platform for future diagnostic EV research, it suffers from the lack of standardized EV collection, isolation and storage protocol.

2. Access to the limited volumes of biological fluids compared to conditioned media (µl vs ml) as well as the presence of abundant housekeeping proteins (e.g. albumin, uromodulin) in these samples which adds an extra layer of complexity to the development of an isolation protocol to purify the targeted class of nanovesicles which have an acceptable degree of purification for further analysis such as MS based proteomics.

3. The presence of abundant proteins (serum albumin, uromodulin) in our exosomes samples compromise our ability to procure proteomic data to some degree.

4. While extra washing and centrifugation steps may be needed to improve the removal of the abundant proteins partially, the final proteomic analysis demonstrates the presence of these highly abundant circulating proteins remaining predominant in the analysis. This is almost certainly due to association of these proteins with EV proteins or EV membranes (Hiemstra *et al*., 2011) as their removal leads to extensive loss of EVs.

5.1.1.4 Future Directions for Chapter Three

Standardized methods for sample collection and storage for clinical exosomes studies is urgently need for clinical exosomes and EV research. A unified protocol for such studies could eliminate various handling/storing errors inherent to the process and make data analysis easier and more streamlined. It is also very important to focus on developing a protocol for better quality and cleaner exosome preparation from different biological sources, especially blood and urine. In addition to the removal of unwanted debris and abundant proteins this protocol is needed to purify exosomes of interest from the total population of

exosomes in biological samples (e.g. Purification of PCa derived exosomes from other exosomes in blood samples).

Using two-dimensional gel electrophoresis proteomics or targeted proteomic analysis could provide more information on the protein profile of these exosomes.

5.1.2 Exosomes Confer Pro-survival Signals to Alter the Phenotype of Prostate Cells in their Surrounding Environment

5.1.2.1 Advantages and Disadvantages

In the last chapter of this thesis we focused on investigation of the role of AR +/- ve, PCa cell derived exosomes on PCa tumour growth and progression. We have also reported the effects of exosomes derived from PCa cells on the PSA level and tumor growth of mice bearing human PCa tumour xenografts when they have been systemically introduced via IV injection.

The advantages of this study were:

4. Similar to the second chapter we used exosomes derived from cell lines with different AR phenotypes to investigate their role and relevance in both in PCa development and progression in both *in vitro* and in animal models.

5. We performed several different functional experiments to address the role of PCa derived exosomes in PCa development and progression.

5.1.2.2 Future Directions for Chapter Four

Similar to the second chapter in which we included exosomes derived from normal/benign prostate cell lines, this strategy could improve on and provide a clearer

conclusion for future investigation into the role and relevance of PCa derived exosomes in PCa development and progression.

5.2 Future Direction for Exosome Research

EV research is a very fast-growing and exciting field which, during last decade, has provided numerous plausible new rationales to explain various physiological and pathological phenomena. As I have attempted to highlight some potential future directions for each chapter of my thesis, there are still many unknowns in EV field.

I strongly believe that once some of the technical challenges in the EV field have been overcome, such as the limitations in isolation and purification of subpopulations of EVs, and the definition of specific markers for each EV categories, many more functional and mechanistic studies could be done to better understand the role of specific EV populations in different physiological and pathogenic functions. This understanding then could assist scientists and physicians working in the field to develop novel diagnostic and therapeutic strategies that exploit EVs.

Although pages could be written (beyond the scope of this thesis) on hundreds of different specific future directions we propose three major directions for **future novel therapeutic strategies** using EVs.

- While the major focus of this thesis was to introduce exosomes as potential biomarkers for PCa diagnosis, exosomes could be a potential chemotherapeutic drug transporter that may be able to target the metastatic sites therefore increase the drug efficacy (Pitt *et al.*, 2014)(Tran *et al.*, 2015)(Gong *et al.*, 2015) .
- Successful delivery of macromolecular therapeutic agents across the blood

brain barrier to the central nervous system is a major current challenge in the treatment of neurological diseases. Recently exosome research has shown that these nanovesicles cross the brain blood barrier and therefore provide a basis for a novel drug delivery vehicle. More studies are needed to investigate the potential of these vesicles as drug delivery system in different pathological disorders (El Andaloussi *et al*., 2013)(Yang *et al*., 2015).

- Elimination of EVs derived from cancer cells in the circulation, inhibition of EVs formation, blocking EVs release and inhibition of EVs uptake by target cells are also other exciting avenues of research which have the potential to be developed in the future (Shelke *et al*., 2014)(Roseblade *et al.*, 2015).

Bibliography

Abusamra, A. J., Zhong Z., Zheng X., Li M., Ichim T. E., Chin J. L., Min W. P. (2005). "Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis." Blood Cells Mol Dis 35(2): 169- 173.

Admyre, C., Grunewald J., Thyberg J., Gripenback S., Tornling G., Eklund A., Scheynius A., Gabrielsson S. (2003). "Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid." The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology 22(4): 578-583.

Admyre C., Johansson S. M., Qazi K. R., Filen J. J., Lahesmaa R., Norman M., Neve E. P., Scheynius A., Gabrielsson S. (2007). "Exosomes with immune modulatory features are present in human breast milk." Journal of immunology 179(3): 1969-1978.

Akduman, B. and Crawford E. D. (2006). "Treatment of localized prostate cancer." Rev Urol 8 Suppl 2: S15-21.

Al-Nedawi, K., Meehan B., Micallef J., Lhotak V., May L., Guha A. and Rak J. (2008). "Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells." Nat Cell Biol 10(5): 619-624.

Albertsen P. C., Hanley J. A., Gleason D. F., Barry M. J. (1998). "Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer." JAMA 280(11): 975-980.

Andersen M. H., Berglund L., Rasmussen J. T., Petersen T. E. (1997). "Bovine PAS-6/7 binds alpha v beta 5 integrins and anionic phospholipids through two domains." Biochemistry 36(18): 5441-5446.

Anderson H. C. (1969). "Vesicles associated with calcification in the matrix of epiphyseal cartilage." J Cell Biol 41(1): 59-72.

Anderson H. C., Mulhall D., Garimella R. (2010). "Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis." Laboratory investigation; a journal of technical methods and pathology 90(11): 1549-1557.

Andre F., Schartz N. E., Movassagh M., Flament C., Pautier P., Morice P., Pomel C., Lhomme C., Escudier B., Le Chevalier T., Tursz T., Amigorena S., Raposo G., Angevin E., Zitvogel L. (2002). "Malignant effusions and immunogenic tumour-derived exosomes." Lancet 360(9329): 295-305.

Andreola G., Rivoltini L., Castelli C., Huber V., Perego P., Deho P., Squarcina P., Accornero P., Lozupone F., Lugini L., Stringaro A., Molinari A., Arancia G., Gentile M., Parmiani G., Fais S. (2002). "Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles." J Exp Med 195(10): 1303-1316.

Andres S., Abraham K., Appel K. E., Lampen A. (2011). "Risks and benefits of dietary isoflavones for cancer." Crit Rev Toxicol 41(6): 463-506.

Antonyak M.A., Cerione R.A. (2014)."Microvesicles as mediators of intercellular communication in cancer." Methods Mol Biol. 1165:147–73.

Arcaro A. and Guerreiro A. S. (2007). "The phosphoinositide 3-kinase pathway in human cancer: genetic alterations and therapeutic implications." Curr Genomics 8(5): 271-306.

Arienti G., Carlini E., Verdacchi R., Cosmi E.V., Palmerini C.A. (1997)." Prostasome to sperm transfer of CD13/aminopeptidase N (EC 3.4.11.2)." Biochim Biophys Acta;1336:533–8.

Arscott W.T., Tandle A.T., Zhao S., Shabason J.E., Gordon I.K., Schlaff C.D., Zhang G., Tofilon P.J., Camphausen K.A. (2013). " Ionizing radiation and glioblastoma exosomes: implications in tumor biology and cell migration." Transl Oncol. 1;6(6):638-48.

Arroyo J.D., Chevillet J.R., Kroh E.M., Ruf I.K., Pritchard C.C., Gibson D.F., Mitchell P.S., Bennett C.F., Pogosova-Agadjanyan E.L., Stirewalt D.L., Tait J.F., Tewari M. (2011). "Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma." Proc Natl Acad Sci U S A. 22;108(12):5003-8

Asea A., Jean-Pierre C., Kaur P., Rao P., Linhares I. M., Skupski D., Witkin S. S. (2008). "Heat shock protein-containing exosomes in mid-trimester amniotic fluids." Journal of reproductive immunology 79(1): 12-17.

Azrad M., Zhang K., Vollmer R. T., Madden J., Polascik T. J., Snyder D. C., Ruffin M. T., Moul J. W., Brenner D., Hardy R. W., Demark-Wahnefried W. (2012). "Prostatic alpha-linolenic acid (ALA) is positively associated with aggressive prostate cancer: a relationship which may depend on genetic variation in ALA metabolism." PLoS One 7(12): e53104.

Baj-Krzyworzeka M., Szatanek R., Weglarczyk K., Baran J., Urbanowicz B., Branski P., Ratajczak M. Z., Zembala M. (2006). "Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes." Cancer Immunol Immunother 55(7): 808-818.

Balk S. P. (2002). "Androgen receptor as a target in androgen-independent prostate cancer." Urology 60(3 Suppl 1): 132-138; discussion 138-139.

Barboro P., Repaci E., Rubagotti A., Salvi S., Boccardo S., Spina B., Truini M., Introini C., Puppo P., Ferrari N., Carmignani G., Boccardo F., Balbi C. (2009). "Heterogeneous nuclear ribonucleoprotein K: altered pattern of expression associated with diagnosis and prognosis of prostate cancer." British journal of cancer 100(10): 1608-1616.

Bard M. P., Hegmans J. P., Hemmes A., Luider T. M., Willemsen R., Severijnen L. A., van Meerbeeck J. P., Burgers S. A., Hoogsteden H. C., Lambrecht B. N. (2004). "Proteomic analysis of exosomes isolated from human malignant pleural effusions." American journal of respiratory cell and molecular biology 31(1): 114-121.

Barringer B. S. (1931). "Carcinoma of the Prostate." Ann Surg 93(1): 326-335.

Beckham C. J., Olsen J., Yin P. N., Wu C. H., Ting H. J., Hagen F. K., Scosyrev E., Messing E. M., Lee Y. F. (2014). "Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression." J Urol 192(2): 583-592.

Benito-Martin A., A. Ucero C., Zubiri I., Posada-Ayala M., Fernandez-Fernandez B., Cannata- Ortiz P., Sanchez-Nino M. D., Ruiz-Ortega M., Egido J., Alvarez-Llamas G., Ortiz A. (2013). "Osteoprotegerin in exosome-like vesicles from human cultured tubular cells and urine." PLoS One 8(8): e72387.

Berry R., Schroeder J. J., French A. J., McDonnell S. K., Peterson B. J., Cunningham J. M., Thibodeau S. N., Schaid D. J. (2000). "Evidence for a prostate cancer-susceptibility locus on chromosome 20." Am J Hum Genet 67(1): 82-91.

Beyer C. and Pisetsky D. S. (2010). "The role of microparticles in the pathogenesis of rheumatic diseases." Nat Rev Rheumatol 6(1): 21-29.

Bijnsdorp I. V., Geldof A. A., Lavaei M., Piersma S. R., van Moorselaar R. J., Jimenez C. R. (2013). "Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients." J Extracell Vesicles. 23;2.

Blanpain C. and Fuchs E. (2009). "Epidermal homeostasis: a balancing act of stem cells in the skin." Nat Rev Mol Cell Biol 10(3): 207-217.

Bligh E. G. and Dyer W. J. (1959). "A rapid method of total lipid extraction and purification." Canadian journal of biochemistry and physiology 37(8): 911-917.

Bobrie A., Krumeich S.,Reyal F., Recchi C.,Moita L.F.,Seabra M. C., Ostrowski M., Théry C. (2012). "Rab27a supports exosome-dependent and- independent mechanisms that modify the tumor microenvironment and can promote tumor progression." Cancer Res. 72, 4920–4930.

Borges F. T., Melo S. A., Ozdemir B. C., Kato N., Revuelta I., Miller C. A., Gattone V. H., LeBleu V. S., Kalluri R. (2013). "TGF-beta1-containing exosomes from injured epithelial cells activate fibroblasts to initiate tissue regenerative responses and fibrosis". J Urol.;192(2):583-92.

Bostwick D. G., Myers R. P., Oesterling J. E. (1994). "Staging of prostate cancer." Semin Surg Oncol 10(1): 60-72.

Braicu C., Tomuleasa C., Monroig P., Cucuianu A., Berindan-Neagoe I., Calin G. A. (2015). "Exosomes as divine messengers: are they the Hermes of modern molecular oncology?" Cell Death Differ 22(1): 34-45.

Brase J.C., Johannes M., Schlomm T., Falth M., Haese A., Steuber T., Beissbarth T., Kuner R., Sultmann H. (2013). "Circulating miRNAs are correlated with tumor progression in prostate cancer." Int. J. Cancer 2011, 128, 608–616. Int. J. Mol. Sci., 14 14797

Bryant, R. J., T. Pawlowski, J. W. Catto, G. Marsden, R. L. Vessella, B. Rhees, C. Kuslich, T. Visakorpi and F. C. Hamdy (2012). "Changes in circulating microRNA levels associated with prostate cancer." Br J Cancer 106(4): 768-774.

Bryniarski K., Ptak W., Jayakumar A., Pullmann K., Caplan M. J., Chairoungdua A., Lu J., Adams D., Sikora E., Nazimek K., Marquez S., Kleinstein S. H., Sangwung P., Iwakiri Y., Delgato E., Redegeld F., Blokhuis B. R., Wojcikowski J., Daniel A. W., Groot Kormelink T., Askenase P. W. (2013). "Antigen-specific, antibody-coated, exosome-like nanovesicles deliver suppressor T-cell microRNA-150 to effector T cells to inhibit contact sensitivity." J Allergy Clin Immunol 132(1): 170-181.

Caby M. P., Lankar D., Vincendeau-Scherrer C., Raposo G., Bonnerot C. (2005). "Exosomal-like vesicles are present in human blood plasma." International immunology 17(7): 879-887.

Cai Z., Yang F., Yu L., Yu Z., Jiang L., Wang Q., Yang Y., Wang L., Cao X., Wang J. (2012). "Activated T cell exosomes promote tumor invasion via Fas signaling pathway." J Immunol 188(12): 5954-5961.

Castellana D., Zobairi F., Martinez M. C., Panaro M. A., Mitolo V., Freyssinet J. M., Kunzelmann (2009). "Membrane microvesicles as actors in the establishment of a favorable prostatic tumoral niche: a role for activated fibroblasts and CX3CL1-CX3CR1 axis." Cancer Res 69(3): 785-793.

Cazzoli R., Buttitta F., Di Nicola M., Malatesta S., Marchetti A., Rom W. N., Pass H. I. (2013). "microRNAs derived from circulating exosomes as noninvasive biomarkers for screening and diagnosing lung cancer." J Thorac Oncol 8(9): 1156-1162.

Ceppi P. and Peter M. E. (2014). "MicroRNAs regulate both epithelial-to-mesenchymal transition and cancer stem cells." Oncogene 33(3): 269-278.

Chaffer C. L. and Weinberg R. A. (2011). "A perspective on cancer cell metastasis." Science 331(6024): 1559-1564.

Chalmin F., Ladoire S., Mignot G., Vincent J., Bruchard M., Remy-Martin J. P., Boireau W., Rouleau A., Simon B., Lanneau D., De Thonel A., Multhoff G., Hamman A., Martin F., Chauffert B., Solary E., Zitvogel L., Garrido C., Ryffel B., Borg C., Apetoh L., Rebe C., Ghiringhelli F. (2010). "Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells." J Clin Invest 120(2): 457-471.

Chan J. M., Stampfer M. J., Ma J., Gann P. H., Gaziano J. M., Giovannucci E. L. (2001). "Dairy products, calcium, and prostate cancer risk in the Physicians' Health Study." Am J Clin Nutr 74(4): 549-554.

Chan J.M., Ho S.H., Tai I.T. (2010). "Secreted protein acidic and rich in cysteine-induced cellular senescence in colorectal cancers in response to irinotecan is mediated by P53." Carcinogenesis. 31(5):812-9.

Chang C.S., Kokontis J., Liao S. T. (1988). "Molecular cloning of human and rat complementary DNA encoding androgen receptors." Science 240(4850): 324-326.

Chavez-Munoz C., Kilani R. T., Ghahary A. (2009). "Profile of exosomes related proteins released by differentiated and undifferentiated human keratinocytes." Journal of cellular physiology 221(1): 221-231.

Chen M., Wang K., Zhang L., Li C., Yang Y. (2011). "The discovery of putative urine markers for the specific detection of prostate tumor by integrative mining of public genomic profiles." PLoS One 6(12): e28552.

Chen T. C. and Holick M. F. (2003). "Vitamin D and prostate cancer prevention and treatment." Trends Endocrinol Metab 14(9): 423-430.

Chen Z.H., Zhang G.L., Li H.R., Luo J.D., Li Z.X., Chen G.M., Yang J. (2012). "A panel of five circulating microRNAs as potential biomarkers for prostate cancer". Prostate, *72*, 1443–1452.

Chi K.N., Zoubeidi A., Gleave M. E.(2008). "Custirsen (OGX-011): a second-generation antisense inhibitor of clusterin for the treatment of cancer." Expert Opin Investig Drugs 17(12): 1955-1962. Childe C. P. (1907). "The Educational Aspect of the Cancer Question." Br Med J 2(2429): 135- 138.

Cho J. A., Yeo D. J., Son H. Y., Kim H. W., Jung D. S., Ko J. K., Koh J. S., Kim Y. N., Kim C. W. (2005). "Exosomes: a new delivery system for tumor antigens in cancer immunotherapy." Int J Cancer 114(4): 613-622.

Chodak G. W., Thisted R. A., Gerber G. S., Johansson J. E., Adolfsson J., Jones G. W., Chisholm G. D., Moskovitz B., Livne P. M., Warner J. (1994). "Results of conservative management of clinically localized prostate cancer." N Engl J Med 330(4): 242-248.

Choi D.S., Kim D.K., Kim Y.K., Gho Y.S. (2014). "Proteomics of extracellular vesicles: Exosomes and ectosomes." Mass Spectrom Rev. doi: 10.1002/mas.21420. [Epub ahead of print] Christianson H. C., Svensson K. J., van Kuppevelt T. H., Li J. P., 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(43): 17380-17385.

Clayton A., Al-Taei S., Webber J., Mason M. D., Tabi Z. (2011). "Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production." J Immunol 187(2): 676-683.

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(15): 7458- 7466.

Colombo M., Moita C., van Niel G., Kowal J., Vigneron J., Benaroch P., Manel N., Moita L. F., Thery C., Raposo G. (2013). "Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles." J Cell Sci 126(Pt 24): 5553- 5565.

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

Conde-Vancells J., Gonzalez E., Lu S. C., Mato J. M., Falcon-Perez J. M. (2010). "Overview of extracellular microvesicles in drug metabolism." Expert Opin Drug Metab Toxicol 6(5): 543-554.

Corcoran C., Rani S., O'Brien K., O'Neill A., Prencipe M., Sheikh R., Webb G., McDermott R., Watson W., Crown J., O'Driscoll L. (2012). "Docetaxel-resistance in prostate cancer: evaluating associated phenotypic changes and potential for resistance transfer via exosomes." PLoS One 7(12): e50999.

Corcoran C., Rani S., 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(13): 1320-1334.

Cox R. L. and Crawford E. D. (1995). "Estrogens in the treatment of prostate cancer." J Urol 154(6): 1991-1998.

Croswell J. M., Kramer B. S., Crawford E. D. (2011). "Screening for prostate cancer with PSA testing: current status and future directions." Oncology 25(6): 452-460, 463.

Culig Z. and Bartsch G. (2006). "Androgen axis in prostate cancer." J Cell Biochem 99(2): 373- 381.

Cunha G. R., Alarid E. T., Turner T., Donjacour A. A., Boutin E. L., Foster B. A. (1992). "Normal and abnormal development of the male urogenital tract. Role of androgens, mesenchymal- epithelial interactions, and growth factors." J Androl 13(6): 465-475.

Cunha G. R., Chung L. W., Shannon J. M., Reese B. A. (1980). "Stromal-epithelial interactions in sex differentiation." Biol Reprod 22(1): 19-42.

Cunha G. R., Hayward S. W., Wang Y. Z., Ricke W. A. (2003). "Role of the stromal microenvironment in carcinogenesis of the prostate." Int J Cancer 107(1): 1-10.

Cunha G. R., Ricke W., Thomson A., Marker P. C., Risbridger G., Hayward S. W., Wang Y. Z., Donjacour A. A., Kurita T. (2004). "Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development." J Steroid Biochem Mol Biol 92(4): 221-236.

D'Amico A. V., Whittington R., Malkowicz S. B., Schultz D., Blank K., Broderick G. A., Tomaszewski J. E., Renshaw A. A., Kaplan I., Beard C. J., Wein A. (1998). "Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer." JAMA 280(11): 969-974.

Dashevsky O., Varon D., Brill A. (2009). "Platelet-derived microparticles promote invasiveness of prostate cancer cells via upregulation of MMP-2 production". Int J Cancer.124:1773–7.

De Smaele E., Ferretti E., Gulino A. (2010). "MicroRNAs as biomarkers for CNS cancer and other disorders." Brain Res 1338: 100-111.

De Stefani E., Deneo-Pellegrini H., Boffetta P., Ronco A., Mendilaharsu M. (2000). "Alpha- linolenic acid and risk of prostate cancer: a case-control study in Uruguay." Cancer Epidemiol Biomarkers Prev 9(3): 335-338.

Decker K. F., Zheng D., He Y., Bowman T., Edwards J. R., Jia L. (2012). "Persistent androgen receptor-mediated transcription in castration-resistant prostate cancer under androgen-deprived conditions." Nucleic Acids Res 40(21): 10765-10779.

Deleault K. M., Skinner S. J., Brooks S. A. (2008). "Tristetraprolin regulates TNF TNF-alpha mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways." Mol Immunol 45(1): 13-24.

Dijkstra S., Briker I.L., Smit F.P., Leyten G.H., de Reijkr T.M., van Oort I.M., Mulders P.F., Jannink S.A., Schalken J.A. (2014). "Prostate cancer biomarker profiles in urinary sediments and exosomes." J Urol.;191(4):1132-8.

Di Vizio D., Kim J., Hager M.H., Morello M., Yang W., Lafargue C.J., True L.D., Rubin M.A., Adam R.M., Beroukhim R., Demichelis F., Freeman M.R. (2009). "Oncosome formation in prostate cancer: association with a region of frequent chromosomal deletion in metastatic disease." Cancer Res. 69:5601–9.

Drake R.R. and Kislinger T. (2014). "The proteomics of prostate cancer exosomes." Expert Rev Proteomics. 11(2):167-77.

D'Souza-Schorey C., Clancy J.W. (2012). "Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers." Genes Dev. 15;26(12):1287- 99

Duijvesz D., Burnum-Johnson K. E., Gritsenko M. A., Hoogland A. M., Vredenbregt-van den Berg M. S.,Willemsen R., Luider T., Pasa-Tolic L., Jenster G. (2013)."Proteomic Profiling of Exosomes Leads to the Identification of Novel Biomarkers for Prostate Cancer". PLoS One. 31;8(12):e82589.

Duijvesz D., Luider T., Bangma C.H., Jenster G. (2011). "Exosomes as biomarker treasure chests for prostate cancer" Eur Urol. 59(5):823-31

de Gassart A., Geminard C., Fevrier B., Raposo G. and Vidal M. (2003). "Lipid raft-associated protein sorting in exosomes." Blood 102(13): 4336-4344.

El Andaloussi S., Lakhal S., Mäger I., Wood M.J. (2013)." Exosomes for targeted siRNA delivery across biological barriers" Adv Drug Deliv Rev.;65(3):391-7.

Elgass S., Cooper A.and Chopra M. (2014). "Lycopene treatment of prostate cancer cell lines inhibits adhesion and migration properties of the cells." Int J Med Sci 11(9): 948-954.

Escrevente C., Keller S., Altevogt P. and Costa J. (2011). "Interaction and uptake of exosomes by ovarian cancer cells." BMC cancer 11: 108.

Esser J., Gehrmann U., D'Alexandri F. L., Hidalgo-Estevez A. M., Wheelock C. E., Scheynius A., Gabrielsson S., Radmark O. (2010). "Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration." The Journal of allergy and clinical immunology 126(5): 1032-1040, 1040 e1031-1034.

Fabbri M., Paone A., Calore F., Galli R., Gaudio E., Santhanam R., Lovat F., Fadda P., Mao C., Nuovo G. J., Zanesi N., Crawford M., Ozer G. H., Wernicke D., Alder H., Caligiuri M. A., Nana- Sinkam P., Perrotti D., Croce C. M. (2012). "MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response." Proc Natl Acad Sci U S A 109(31): E2110-2116.

Fair W. R. and Cordonnier J. J. (1977). "The pH of prostatic fluid: a reappraisal and therapeutic implications." Trans Am Assoc Genitourin Surg 69: 65-68.

Faure J., Lachenal G., Court M., Hirrlinger J., Chatellard-Causse C., Blot B., Grange J., Schoehn G., Goldberg Y., Boyer V., Kirchhoff F., Raposo G., Garin J., Sadoul R. (2006). "Exosomes are released by cultured cortical neurones." Mol Cell Neurosci. 31(4): 642-648.

Federici C., Petrucci F., Caimi S., Cesolini A., Logozzi M., Borghi M., D'Ilio S., Lugini L., Violante N., Azzarito T., Majorani C., Brambilla D., Fais S. (2014). "Exosome release and low pH belong to a framework of resistance of human melanoma cells to cisplatin." PLoS One 9(2): e88193.

Feldman B. J. and Feldman D. (2001). "The development of androgen-independent prostate cancer." Nat Rev Cancer 1(1): 34-45.

Ferguson R. S. (1933). "Recent Advances in the Diagnosis of Carcinoma of the Prostate." Can Med Assoc J 29(5): 497-501.

Fevrier B. and Raposo G. (2004). "Exosomes: endosomal-derived vesicles shipping extracellular messages." Current opinion in cell biology 16(4): 415-421.

Franks L. M. (1954). "Atrophy and hyperplasia in the prostate proper." J Pathol Bacteriol 68(2): 617-621.

Franzen C. A., Simms P. E., Van Huis A. F., Foreman K. E., Kuo P. C., Gupta G. N. (2014). "Characterization of uptake and internalization of exosomes by bladder cancer cells." Biomed Res Int. 619;829.

Gabriel K., Ingram A., Austin R., Kapoor A., Tang D., Majeed F., Qureshi T., Al-Nedawi K. (2013). "Regulation of the tumor suppressor PTEN through exosomes: a diagnostic potential for prostate cancer." PLoS One. 8(7):e70047.

Gann P. H., Hennekens C. H., Sacks F. M., Grodstein F., Giovannucci E. L., Stampfer M. J. (1994). "Prospective study of plasma fatty acids and risk of prostate cancer." J Natl Cancer Inst 86(4): 281- 286.

Gercel-Taylor C., Atay S., Tullis R.H., Kesimer M., Taylor D.D.(2012)."Nanoparticle analysis of circulating cell-derived vesicles in ovarian cancer patients."Anal Biochem. 1;428(1):44-53.

Gioeli D., Mandell J. W., Petroni G. R., Frierson H. F., Weber Jr. and M. J. (1999). "Activation of mitogen-activated protein kinase associated with prostate cancer progression." Cancer Res 59(2): 279-284.

Giovannucci E., Rimm E. B., Colditz G. A., Stampfer M. J., Ascherio A., Chute C. G.and Willett W. C. (1993). "A prospective study of dietary fat and risk of prostate cancer." J Natl Cancer Inst 85(19): 1571-1579.

Giovannucci E., Rimm E. B., Liu Y., Stampfer M. J., Willett W. C. (2002). "A prospective study of tomato products, lycopene, and prostate cancer risk." J Natl Cancer Inst 94(5): 391-398.

Glauser A., Hochreiter W., Jaeger P., Hess B. (2000) " Determinants of urinary excretion of Tamm-Horsfall protein in non-selected kidney stone formers and healthy subjects." Nephrol Dial Transplant. 15(10):1580-7.

Gleason D. F. (1992). "Histologic grading of prostate cancer: a perspective." Hum Pathol 23(3): 273-279.

Godley P. A., Campbell M. K., Miller C., Gallagher P., Martinson F. E., Mohler J. L. and Sandler R. S. (1996). "Correlation between biomarkers of omega-3 fatty acid consumption and questionnaire data in African American and Caucasian United States males with and without prostatic carcinoma." Cancer Epidemiol Biomarkers Prev 5(2): 115-119.

Gomella L. G., Johannes J.and Trabulsi E. J. (2009). "Current prostate cancer treatments: effect on quality of life." Urology 73(5 Suppl): S28-35.

Gong J, Jaiswal R, Dalla P, Luk F, Bebawy M. (2015). "Microparticles in cancer: A review of recent developments and the potential for clinical application." Semin Cell Dev Biol. [Epub ahead of print]

Gonzales J.C., Fink L.M., Goodman O.B., Jr., Symanowski J.T., Vogelzang N.J., Ward D.C. (2011)."Comparison of circulating MicroRNA 141 to circulating tumor cells, lactate dehydrogenase, and prostate-specific antigen for determining treatment response in patients with metastatic prostate cancer". Clin. Genitourin. Cancer *9*, 39–45.

Goyal A., Delves G.H., Chopra M., Lwaleed B.A., Cooper A.J. (2006) "Prostate cells exposed to lycopene *in vitro* liberate lycopene-enriched exosomes." BJU Int.;98(4):907-11.

Greene F. L. (2002). "The American Joint Committee on Cancer: updating the strategies in cancer staging." Bull Am Coll Surg 87(7): 13-15.

Gronberg H., Damber L., Damber J. E. (1994). "Studies of genetic factors in prostate cancer in a twin population." J Urol 152(5 Pt 1): 1484-1487; discussion 1487-1489.

Gronberg H., Xu J., Smith J. R., Carpten J. D., Isaacs S. D., Freije D., Bova G. S., Danber J. E., Bergh A., Walsh P. C., Collins F. S., Trent J. M., Meyers D. A., Isaacs W. B. (1997). "Early age at diagnosis in families providing evidence of linkage to the hereditary prostate cancer locus (HPC1) on chromosome 1." Cancer Res 57(21): 4707-4709.

Guo Z., Dai B., Jiang T., Xu K., Xie Y., Kim O., Nesheiwat I., Kong X., Melamed J., Handratta V. D., Njar V. C., Brodie A. M., Yu L. R., Veenstra T. D., Chen H., Qiu Y. (2006). "Regulation of androgen receptor activity by tyrosine phosphorylation." Cancer Cell 10(4): 309-319.

Gutman, A. B. and Gutman E. B. (1938). "An Acid Phosphatase Occurring in the Serum of Patients with Metastasizing Carcinoma of the Prostate Gland." J Clin Invest 17(4): 473-478.

Guyton. A. C. H., Hall, J.E. (2010). "Guyton and Hall Textbook of Medical Physiology." Hammad F. T. (2008). "Radical prostatectomy." Ann N Y Acad Sci 1138: 267-277.

Hammerich K. H. G., E.A.; Wheeler T.M. (2009). "Anatomy of the prostate gland and surgical pathology of prostate cancer." Cambridge University, Cambridge,: 1–10.

Hanahan D. and Weinberg R. A. (2000). "The hallmarks of cancer." Cell 100(1): 57-70. Hanahan, D. and Weinberg R. A. (2011). "Hallmarks of cancer: the next generation." Cell 144(5): 646-674.

Hao S., Ye Z., Li F., Meng Q., Qureshi M., Yang J.and Xiang J. (2006). "Epigenetic transfer of metastatic activity by uptake of highly metastatic B16 melanoma cell-released exosomes." Exp Oncol 28(2): 126-131.

Harding C., Heuser J., Stahl P. (1983). "Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes." J Cell Biol 97(2): 329-339.

Harshman L. C. and Taplin M. E. (2013). "Abiraterone acetate: targeting persistent androgen dependence in castration-resistant prostate cancer." Adv Ther 30(8): 727-747.

Harvei S., Bjerve K. S., Tretli S., Jellum E., Robsahm T. E.,Vatten L. (1997). "Prediagnostic level of fatty acids in serum phospholipids: omega-3 and omega-6 fatty acids and the risk of prostate cancer." Int J Cancer 71(4): 545-551.

Hassan M., Watari H., AbuAlmaaty A., Ohba Y., Sakuragi N. (2014). "Apoptosis and molecular targeting therapy in cancer." Biomed Res Int 2014: 150845.

Haubold K., Rink M., Spath B., Friedrich M., Chun F.K., Marx G., Amirkhosravi A., Francis J.L., Bokemeyer C., Eifrig B., Langer F. (2009). "Tissue factor procoagulant activity of plasma microparticles is increased in patients with early-stage prostate cancer." Thromb Haemost.101: 1147–55.

Hawari F. I., Rouhani F. N., Cui X., Yu Z. X., Buckley C., Kaler M., Levine S. J. (2004). "Release of full-length 55-kDa TNF receptor 1 in exosome-like vesicles: a mechanism for generation of soluble cytokine receptors." Proc Natl Acad Sci U S A 101(5): 1297-1302.

Hayes D. F., Bast R. C., Desch C. E., Fritsche H., Kemeny Jr. N. E., Jessup J. M., Locker G. Y., Macdonald J. S., Mennel R. G., Norton L., Ravdin P., Taube S., Winn R. J. (1996). "Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers." J Natl Cancer Inst 88(20): 1456-1466.

Hayward S. W. and Cunha G. R. (2000). "The prostate: development and physiology." Radiol Clin North Am 38(1): 1-14.

Hayward S. W., Haughney P. C., Rosen M. A., Greulich K. M., Weier H. U., Dahiya R., Cunha G. R. (1998). "Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model." Differentiation 63(3): 131-140.

Heidenreich A., Bastian P. J., Bellmunt J., Bolla M., Joniau S., van der Kwast T., Mason M., Matveev V., Wiegel T., Zattoni F., Mottet N. (2014). "EAU guidelines on prostate cancer. part 1: screening, diagnosis, and local treatment with curative intent-update 2013." Eur Urol 65(1): 124- 137.

Heijnen H. F., Schiel A. E., Fijnheer R., Geuze H. J.and Sixma J. J. (1999). "Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules." Blood 94(11): 3791-3799.

Heller J. E. (1987). "Prostatic acid phosphatase: its current clinical status." J Urol 137(6): 1091- 1103.

Hernandez J. and Thompson I. M. (2004). "Prostate-specific antigen: a review of the validation of the most commonly used cancer biomarker." Cancer 101(5): 894-904.

Hessvik N. P., Sandvig K., Llorente A. (2013). "Exosomal miRNAs as Biomarkers for Prostate Cancer." Front Genet 4: 36.

Hiemstra T. F., Charles P. D., Hester S. S., Karet F. E., Lilley K. S. (2011). "Uromodulin exclusion list improves urinary exosomal protein identification." J Biomol Tech 22(4): 136-145.

Hilton W.M., Thompson Jr. I.M., Parekh D.J. (2013). "Prostate cancer screening: navigating the controversy". Jones JSProstate Cancer Diagnosis. Humana Press, pp 11–26.

Hoffman R. M. (2011). "Clinical practice. Screening for prostate cancer." N Engl J Med 365(21): 2013-2019.

Honda F. I., Ono M., Shitashige M., Masuda M., Kamita M., Miura N., Yamada T. (2013). "Proteomic approaches to the discovery of cancer biomarkers for early detection and personalized medicine." Jpn J Clin Oncol.;43(2):103-9.

Hood J. L., San R. S.and Wickline S. A. (2011). "Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis." Cancer research 71(11): 3792-3801.

Hood J. L., Scott M. J., Wickline S. A. (2014). "Maximizing exosome colloidal stability following electroporation." Anal Biochem 448: 41-49.

Hosseini-Beheshti E., Pham S., Adomat H., Li N., Tomlinson Guns E. S. (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(10): 863-885.

Howell S.B. (2000) "Resistance to apoptosis in prostate cancer cells" Mol Urol. ;4(3):225-9.

Hsu D.H., Paz P., Villaflor G., Rivas A., Mehta-Damani A., Angevin E., Zitvogel L., Le Pecq J.B. (2003). "Exosomes as a tumor vaccine: enhancing potency through direct loading of antigenic peptides." J Immunother. 26(5):440-50.

Hu R., Denmeade S. R., Luo J. (2010). "Molecular processes leading to aberrant androgen receptor signaling and castration resistance in prostate cancer." Expert Rev Endocrinol Metab 5(5): 753- 764.

Huang J. G. C. and Goldenberg S.L. (2014). "PSA and beyond: Biomarkers in prostate cancer." BCMJ 56(7): 334-341.

Huang X., Liang M., Dittmar R., Wang L. (2013)" Extracellular microRNAs in urologic malignancies: chances and challenges" Int J Mol Sci. 16;14(7):14785-99.

Huang X., Yuan T., Liang M., Du M., Xia S., Dittmar R., Wang D., See W., Costello B. A., Quevedo F., Tan W., Nandy D., Bevan G. H., Longenbach S., Sun Z., Lu Y., Wang T., Thibodeau S. N., Boardman L., Kohli M.and Wang L. (2014). "Exosomal miR-1290 and miR-375 as Prognostic Markers in Castration-resistant Prostate Cancer." Eur Urol.

Huang X., Yuan T., Tschannen M., Sun Z., Jacob H., Du M., Liang M., Dittmar R. L., Liu Y., Liang M., Kohli M., Thibodeau S. N., Boardman L., Wang L. (2013). "Characterization of human plasma-derived exosomal RNAs by deep sequencing." BMC Genomics 14: 319.

Huber V., Fais S., Iero M., Lugini L., Canese P., Squarcina P., Zaccheddu A., Colone M., Arancia G., Gentile M., Seregni E., Valenti R., Ballabio G., Belli F., Leo E., Parmiani G.and Huggins L. (1967). "Endocrine-induced regression of cancers." Science 156(3778): 1050-1054.

Huggins C. and Hodges C. V. (1972). "Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate." CA Cancer J Clin 22(4): 232-240.

Huggins C. and Hodges C. V. (2002). "Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941." J Urol 167(2 Pt 2): 948-951; discussion 952.

Huggins C. and Neal W. (1942). "Coagulation and Liquefaction of Semen : Proteolytic Enzymes and Citrate in Prostatic Fluid." J Exp Med 76(6): 527-541.

Humphrey P. A. (2004). "Gleason grading and prognostic factors in carcinoma of the prostate." Mod Pathol 17(3): 292-306.

Iero M., Valenti R., Huber V., Filipazzi P., Parmiani G., Fais S., Rivoltini L. (2008). "Tumour- released exosomes and their implications in cancer immunity." Cell death and differentiation 15(1): 80-88.

Inal J.M., Kosgodage U., Azam S., Stratton D., Antwi-Baffour S., Lange S. (2013) "Blood/plasma secretome and microvesicles." Biochim Biophys Acta. 1834(11):2317-25.

Inder K. L., Ruelcke J. E., Petelin L., Moon H., Choi E., Rae J., Blumenthal A., Hutmacher D., Saunders N. A., Stow J. L., Parton R. G., Hill M. M. (2014). "Cavin-1/PTRF alters prostate cancer cell-derived extracellular vesicle content and internalization to attenuate extracellular vesicle- mediated osteoclastogenesis and osteoblast proliferation." J Extracell Vesicles 3.

Isaacs J. T. (1994). "Role of androgens in prostatic cancer." Vitam Horm 49: 433-502.

Izquierdo-Useros N., Naranjo-Gomez M., Archer J., Hatch S. C., Erkizia I., Blanco J., Borras F. E., Puertas M. C., Connor J. H., Fernandez-Figueras M. T., Moore L., Clotet B., Gummuluru S., Martinez-Picado J. (2009). "Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway." Blood 113(12): 2732-2741.

Jaiswal J. K., Andrews N. W., Simon S. M. (2002). "Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells." J Cell Biol 159(4): 625-635.

Janowska-Wieczorek A., Wysoczynski M., Kijowski J., Marquez-Curtis L., Machalinski B., Ratajczak J., Ratajczak M. Z. (2005). "Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer." Int J Cancer 113(5): 752-760.

Jansen F. H., Krijgsveld J., van Rijswijk A., van den Bemd G. J., van den Berg M. S., van Weerden W. M., Willemsen R., Dekker L. J., Luider T. M., Jenster G. (2009). "Exosomal secretion of cytoplasmic prostate cancer xenograft-derived proteins." Mol Cell Proteomics 8(6): 1192-1205. Jenster G. (1999). "The role of the androgen receptor in the development and progression of prostate cancer." Semin Oncol 26(4): 407-421.

Ju S., Mu J., Dokland T., Zhuang X., Wang Q., Jiang H., Xiang X., Deng Z. B., Wang B., Zhang L., Roth M., Welti R., Mobley J., Jun Y., Miller D., Zhang H. G. (2013). "Grape exosome-like nanoparticles induce intestinal stem cells and protect mice from DSS-induced colitis." Mol Ther 21(7): 1345-1357.

Jung T., Castellana D., Klingbeil P., Cuesta Hernandez I., Vitacolonna M., Orlicky D. J., Roffler S. R., Brodt P., Zoller M. (2009). "CD44v6 dependence of premetastatic niche preparation by exosomes." Neoplasia 11(10): 1093-1105.

Kahlert C., Melo S. A., Protopopov A., Tang J., Seth S., Koch M., Zhang J., Weitz J., Chin L., Futreal A., Kalluri R. (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(7): 3869-3875.

Katakowski M., Buller B., Zheng X., Lu Y., Rogers T., Osobamiro O., Shu W., Jiang F., Chopp M. (2013). "Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth." Cancer Lett 335(1): 201-204.

Keyes M., Crook J., Morton G., Vigneault E., Usmani N., Morris W. J. (2013). "Treatment options for localized prostate cancer." Can Fam Physician 59(12): 1269-1274.

Khan S., Jutzy J. M., Valenzuela M. M., Turay D., Aspe J. R., Ashok A., Mirshahidi S., Mercola D., Lilly M. B., Wall N. R. (2012). "Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer." PLoS One 7(10): e46737.

Kim J. W., Wieckowski E., Taylor D. D., Reichert T. E., Watkins S., Whiteside T. L. (2005). "Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes." Clin Cancer Res 11(3): 1010-1020.

King H.W., Michael M.Z., Gleadle J.M. (2012). "Hypoxic enhancement of exosome release by breast cancer cells" BMC Cancer. 24;12:421.

Klotz L. (2006). "Combined androgen blockade: an update." Urol Clin North Am 33(2): 161-166, v-vi.

Klotz L. (2008). "Maximal androgen blockade for advanced prostate cancer." Best Pract Res Clin Endocrinol Metab 22(2): 331-340.

Knudsen K. E. and Scher H. I. (2009). "Starving the addiction: new opportunities for durable suppression of AR signaling in prostate cancer." Clin Cancer Res 15(15): 4792-4798.

Kong JN, He Q, Wang G, Dasgupta S, Dinkins MB, Zhu G, Kim A, Spassieva S, Bieberich E. (2015). "Guggulsterone and bexarotene induce secretion of exosome-associated breast cancer resistance protein and reduce doxorubicin resistance in MDA-MB-231 cells." Int J Cancer. [Epub ahead of print]

Kollmeier M. A. and Zelefsky M. J. (2012). "How to select the optimal therapy for early-stage prostate cancer." Crit Rev Oncol Hematol 84 Suppl 1: e6-e15.

Kooijmans S. A., Vader P., van Dommelen S. M., van Solinge W. W., Schiffelers R. M. (2012). "Exosome mimetics: a novel class of drug delivery systems." Int J Nanomedicine 7: 1525-1541.

Kovak M.R., Sarswati S., Goddard S.D., Diekman A.B. (2013). "Proteomic identification of galectin-3 binding ligands and characterization of galectin-3 proteolytic cleavage in human prostasomes." Andrology. Sep;1(5):682-91.

Krahn, M. D., Mahoney J. E., Eckman M. H., Trachtenberg J., Pauker S. G., Detsky A. S. (1994). "Screening for prostate cancer. A decision analytic view." JAMA 272(10): 773-780.

Kramer-Albers E. M., Bretz N., Tenzer S., Winterstein C., Mobius W., Berger H., Nave K. A., Schild H., Trotter J. (2007). "Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons?" Proteomics Clin Appl 1(11): 1446-1461.

Labbe D. P., Zadra G., Ebot E. M., Mucci L. A., Kantoff P. W., Loda M.and Brown M. (2014). "Role of diet in prostate cancer: the epigenetic link." Oncogene. 2014 Dec 22. doi: 10.1038/onc.2014.422. [Epub ahead of print]

Labrie F., Cusan L., Séguin C., Bélanger A., Pelletier G., Reeves J., Kelly P.A., Lemay A., Raynaud J.P. (1980). "Antifertility effects of LHRH agonists in the male rat and inhibition of testicular steroidogenesis in man" Int J Fertil. 1980;25(3):157-70.

Labrie F. (2004). "Adrenal androgens and intracrinology." Semin Reprod Med 22(4): 299-309.

Labrie F., Cusan L., Seguin C., Belanger A., Pelletier G., Reeves J., Kelly P. A., Lemay A. and Lachenal J., Pernet-Gallay G. K., Chivet M., Hemming F. J., Belly A., Bodon G., Blot B., Haase G., Goldberg Y., Sadoul R. (2011). "Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity." Molecular and cellular neurosciences 46(2): 409- 418.

Lakkaraju A. and Rodriguez-Boulan E. (2008). "Itinerant exosomes: emerging roles in cell and tissue polarity." Trends Cell Biol 18(5): 199-209.

Lamoureux F., Thomas C., Yin M. J., Kuruma H., Beraldi E., Fazli L., Zoubeidi A., Gleave M. E. (2011). "Clusterin inhibition using OGX-011 synergistically enhances Hsp90 inhibitor activity by suppressing the heat shock response in castrate-resistant prostate cancer." Cancer Res 71(17): 5838-5849.

Lamparski H. G., Metha-Damani A., Yao J.Y., Patel S., Hsu D.H., Ruegg C., Le Pecq J.B. (2002). "Production and characterization of clinical grade exosomes derived from dendritic cells." Journal of immunological methods 270(2): 211-226.

Lan C. Y., Huang H.and Liu J. H. (2008). "Prognostic value of serum CA(125) level change during chemotherapy post-surgery in patients with advanced epithelial ovarian carcinoma." Zhonghua fu chan ke za zhi 43(10): 732-736.

Lasser C., Alikhani V. S., Ekstrom K., Eldh M., Paredes P. T., Bossios A., Sjostrand M., Gabrielsson S., Lotvall J., Valadi H. (2011). "Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages." Journal of translational medicine 9: 9.

Laulagnier K., Motta C., Hamdi S., Roy S., Fauvelle F., Pageaux J. F., Kobayashi T., Salles J. P., Perret B., Bonnerot C., Record M. (2004). "Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization." The Biochemical journal 380(Pt 1): 161-171.

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

Lehmann B. D., Paine M. S., Brooks A. M., McCubrey J. A., Renegar R. H., Wang R.,Terrian D.M. (2008). "Senescence-associated exosome release from human prostate cancer cells." Cancer Res 68(19): 7864-7871.

Leon C. G., Locke J. A., Adomat H. H., Etinger S. L., Twiddy A. L., Neumann R. D., Nelson C. C., Guns E. S., Wasan K. M. (2010). "Alterations in cholesterol regulation contribute to the production of intratumoral androgens during progression to castration-resistant prostate cancer in a mouse xenograft model." Prostate 70(4): 390-400.

Lewis T. S., Shapiro P. S., Ahn N. G. (1998). "Signal transduction through MAP kinase cascades." Adv Cancer Res 74: 49-139.

Li B., Antonyak M. A., Zhang J., Cerione R. A. (2012). "RhoA triggers a specific signaling pathway that generates transforming microvesicles in cancer cells." Oncogene 31(45): 4740-4749. Li P., Yang R.,

Gao W. Q. (2014). "Contributions of epithelial-mesenchymal transition and cancer stem cells to the development of castration resistance of prostate cancer." Mol Cancer 13: 55.

Li Y., Che M., Bhagat S., Ellis K. L., Kucuk O., Doerge D. R., Abrams J., Cher M. L.and Sarkar

F. H. (2004). "Regulation of gene expression and inhibition of experimental prostate cancer bone metastasis by dietary genistein." Neoplasia 6(4): 354-363.

Liebisch G., Binder M., Schifferer R., Langmann T., Schulz B., Schmitz G. (2006). "High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS)." Biochim Biophys Acta. 1761(1):121-8.

Lima L. G., Chammas R., Monteiro R. Q., Moreira M. E., Barcinski M. A. (2009). "Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine- dependent manner." Cancer Lett 283(2): 168-175.

Liu C., Yu S., Zinn K., Wang J., Zhang L., Jia Y., Kappes J. C., Barnes S., Kimberly R. P., Grizzle W. E., Zhang H. G. (2006). "Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function." Journal of immunology 176(3): 1375-1385.

Llorente A., van Deurs B., Sandvig K. (2007). "Cholesterol regulates prostasome release from secretory lysosomes in PC-3 human prostate cancer cells." Eur J Cell Biol 86(7): 405-415.

Locke J.A., Guns E.S, Lubik A.A., Adomat H.H., Hendy S.C., Wood C.A., Ettinger S.L., Gleave M.E., Nelson C.C..(2008) " Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res. 68(15):6407-15.

Locke J. A., Fazli L., Adomat H., Smyl J., Weins K., Lubik A. A., Hales D. B., Nelson C. C., Gleave M. E., Tomlinson Guns E. S. (2009). "A novel communication role for CYP17A1 in the progression of castration-resistant prostate cancer." Prostate 69(9): 928-937.

Locke J. A., Wasan K. M., Nelson C. C., Guns E. S., Leon C. G. (2008). "Androgen-mediated cholesterol metabolism in LNCaP and PC-3 cell lines is regulated through two different isoforms of acyl-coenzyme A:Cholesterol Acyltransferase (ACAT)." Prostate 68(1): 20-33.

Logozzi M., De Milito A., Lugini L., Borghi M., Calabro L., Spada M., Perdicchio M., Marino M. L., Federici C., Iessi E., Brambilla D., Venturi G., Lozupone F., Santinam M.i, Huber V., Maio M., Rivoltini L., Fais S. (2009). "High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients." PLoS One 4(4): e5219.

Lotvall J. and Valadi H. (2007). "Cell to cell signalling via exosomes through esRNA." Cell Adh Migr 1(3): 156-158.

Lowe F. C. and Trauzzi S. J. (1993). "Prostatic acid phosphatase in 1993. Its limited clinical utility." Urol Clin North Am 20(4): 589-595.

Lowsley O. S. (1915). "The Prostate Gland in Old Age." Ann Surg 62(6): 716-737.

Lowsley O. S. and Perez Venero A. (1954). "Persistent anterior lobe of the prostate gland." J Urol 71(4): 469-474.

Luga V., Zhang L., Viloria-Petit A. M., Ogunjimi A. A., Inanlou M. R., Chiu E., Buchanan M., Hosein A. N., Basik M., Wrana J. L. (2012). "Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration." Cell 151(7): 1542-1556.

Lundholm M., Schroder M., Nagaeva O., Baranov V., Widmark A., Mincheva-Nilsson L., Wikstrom P. (2014). "Prostate tumor-derived exosomes down-regulate NKG2D expression on natural killer cells and CD8+ T cells: mechanism of immune evasion." PLoS One 9(9): e108925.

Lytton B. (2001). "Prostate cancer: a brief history and the discovery of hormonal ablation treatment." J Urol 165(6 Pt 1): 1859-1862.

Maguire C. A., Balaj L., Sivaraman S., Crommentuijn M. H., Ericsson M.,. Mincheva-Nilsson L, Baranov V., Gianni D., Tannous B. A., Sena-Esteves M., Breakefield X. O., Skog J. (2012). "Microvesicle-associated AAV vector as a novel gene delivery system." Mol Ther 20(5): 960-971.

Mahn R., Heukamp L.C., Rogenhofer S., von Ruecker A., Muller S.C., Ellinger J. (2011). " Circulating microRNAs (miRNA) in serum of patients with prostate cancer." Urology, 77, e9–16. Mandair D., Rossi R. E., Pericleous M., Whyand T. and Caplin M. E. (2014). "Prostate cancer and the influence of dietary factors and supplements: a systematic review." Nutr Metab (Lond) 11: 30.

Marzesco A.M., Wilsch-Bräuninger M., Dubreuil V., Janich P., Langenfeld K., Thiele C., Huttner W.B, Corbeil D. “Release of extracellular membrane vesicles from microvilli of epithelial cells is enhanced by depleting membrane cholesterol.” FEBS Lett. 2009 Mar 4;583(5):897-902.

Mathivanan S., Fahner C. J., Reid G. E., Simpson R. J. (2012). "ExoCarta 2012: database of exosomal proteins, RNA and lipids." Nucleic Acids Res 40(Database issue): D1241-1244.

Mathivanan S., Ji H., Simpson R. J. (2010). "Exosomes: extracellular organelles important in intercellular communication." J Proteomics 73(10): 1907-1920.

Matyash V., Liebisch G., Kurzchalia T.V., Shevchenko A., Schwudke D. (2008). " Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics." J Lipid Res. 49(5):1137-46.

Maurice M. J., Abouassaly R., Zhu H. (2014). "American trends in expectant management utilization for prostate cancer from 2000 to 2009." Can Urol Assoc J 8(11-12): E775-782.

McCarthy J.F., Stepita C.T., Johnston M.B., Killian J.A., (1928). "Biochemical studies of prostato- vesicular secretions." J Urol 19(43).

McCready J., Sims J. D., Chan D., Jay D.G. (2010). "Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: a role for plasminogen activation." BMC cancer 10: 294. McGuire W. L., Chamness G. C., Fuqua S. A. (1991). "Estrogen receptor variants in clinical breast cancer." Mol Endocrinol 5(11): 1571-1577.

McNeal J. E. (1980). "Anatomy of the prostate: an historical survey of divergent views." Prostate 1(1): 3-13.

McNeal J. E. (1981). "Normal and pathologic anatomy of prostate." Urology 17(Suppl 3): 11-16.

McNeal J. E. (1988). "Normal histology of the prostate." Am J Surg Pathol 12(8): 619-633.

Meckes D. G., Shair Jr. K. H., Marquitz A. R., Kung C. P., Edwards R. H., Raab-Traub N. (2010). "Human tumor virus utilizes exosomes for intercellular communication." Proc Natl Acad Sci U S A 107(47): 20370-20375.

Mediwala S. N., Sun H., Szafran A. T., Hartig S. M., Sonpavde G., Hayes T. G., Thiagarajan P., Mancini M. A., Marcelli M. (2013). "The activity of the androgen receptor variant AR-V7 is regulated by FOXO1 in a PTEN-PI3K-AKT-dependent way." Prostate 73(3): 267-277.

Minciacchi V.R., Freeman M.R., Di Vizio D. (2015) " Extracellular Vesicles in Cancer: Exosomes, Microvesicles and the Emerging Role of Large Oncosomes." Semin Cell Dev Biol. pii: S1084- 9521(15)00037-3

Mitchell P.S., Parkin R.K., Kroh E.M., Fritz B.R., Wyman S.K., Pogosova-Agadjanyan E.L., Peterson A., Noteboom J., O'Briant K.C., Allen A. Lin DW Urban N, Drescher C.W., Knudsen B.S,. Stirewalt D.L., Gentleman R., Vessella R.L., Nelson P.S., Martin D.B., Tewari M. (2008)."Circulating microRNAs as stable blood-based markers for cancer detection." *Proc. Natl. Acad. Sci. USA, 105*, 10513–10518.

Mitchell P. J., Welton J., Staffurth J., Court J., Mason M. D., Tabi Z., Clayton A. (2009). "Can urinary exosomes act as treatment response markers in prostate cancer?" J Transl Med 7: 4.

Mizrak A., Bolukbasi M. F., Ozdener G. B., Brenner G. J., Madlener S., Erkan E. P., Strobel T., Breakefield X. O., Saydam O. (2013). "Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth." Mol Ther 21(1): 101-108.

Mizutani K., Terazawa R., Kameyama K., Kato T., Horie K., Tsuchiya T., Seike K., Ehara H., Fujita Y., Kawakami K., Ito M., Deguchi T. (2014). "Isolation of prostate cancer-related exosomes." Anticancer Res 34(7): 3419-3423.

Mohler J. L., Kantoff P. W., Armstrong A. J., Bahnson R. R., Cohen M., D'Amico A. V., Eastham

J. A., Enke C. A., Farrington T. A., Higano C. S., Horwitz E. M., Kane C. J., Kawachi M. H., Kuettel M., Kuzel T. M., Lee R. J., Malcolm A. W., Miller D., Plimack E. R., Pow-Sang J. M., Raben D., Richey S., Roach M., Rohren E., Rosenfeld S., Schaeffer E., Small E. J., Sonpavde G., Srinivas S., Stein C., Strope S. A., Tward J., Shead D. A., Ho M. (2014). "Prostate cancer, version 2.2014." J Natl Compr Canc Netw 12(5): 686-718.

Moltzahn F. R., Volkmer J. P., Rottke D., Ackermann R. (2008). "Cancer stem cells"-lessons from Hercules to fight the Hydra." Urol Oncol 26(6): 581-589.

Moltzahn F., Olshen A.B., Baehner L., Peek A., Fong L., Stoppler H., Simko J., Hilton J.F., Carroll P., Blelloch R. (2011) "Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients". Cancer Res., 71, 550– 560.

Monroe K. R., Yu M. C., Kolonel L. N., Coetzee G. A., Wilkens L. R., Ross R. K., Henderson B. E. (1995). "Evidence of an X-linked or recessive genetic component to prostate cancer risk." Nat Med 1(8): 827-829.

Morello M., Minciacchi V. R., de Candia P., Yang J., Posadas E., Kim H., Griffiths D., Bhowmick N., Chung L. W., Gandellini P., Freeman M. R., Demichelis F., Di Vizio D. (2013). "Large oncosomes mediate intercellular transfer of functional microRNA." Cell Cycle 12(22): 3526-3536.

Morgan K., Stavrou E., Leighton S. P., Miller N., Sellar R., Millar R. P. (2011). "Elevated GnRH receptor expression plus GnRH agonist treatment inhibits the growth of a subset of papillomavirus 18-immortalized human prostate cells." Prostate 71(9): 915-928.

Mostaghel E. A., Solomon K. R., Pelton K., Freeman M. R., Montgomery R. B. (2012). "Impact of circulating cholesterol levels on growth and intratumoral androgen concentration of prostate tumors." PLoS One 7(1): e30062.

Msaouel P., Diamanti E., Tzanela M., Koutsilieris M. (2007). "Luteinising hormone-releasing hormone antagonists in prostate cancer therapy." Expert Opin Emerg Drugs 12(2): 285-299.

Mukherjee R., Bartlett J. M., Krishna N. S., Underwood M. A., Edwards J. (2005). "Raf-1 expression may influence progression to androgen insensitive prostate cancer." Prostate 64(1): 101-107.

Mukherjee S., Soe T. T., Maxfield F. R. (1999). "Endocytic sorting of lipid analogues differing solely in the chemistry of their hydrophobic tails." J Cell Biol 144(6): 1271-1284.

Muralidharan-Chari V., Clancy J., Plou C., Romao M., Chavrier P., Raposo G., D'Souza-Schorey C. (2009). "ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles." Curr Biol 19(22): 1875-1885.

Neeb A., Hefele S., Bormann S., Parson W., Adams F., Wolf P., Miernik A., Schoenthaler M., Kroenig M., Wilhelm K., Schultze-Seemann W., Nestel S., Schaefer G., Bu H., Klocker H., Nazarenko I., Cato A. C. (2014). "Splice variant transcripts of the anterior gradient 2 gene as a marker of prostate cancer." Oncotarget 5(18): 8681-8689.

Neumann E., Schaefer-Ridder M., Wang Y., Hofschneider P. H. (1982). "Gene transfer into mouse lyoma cells by electroporation in high electric fields." EMBO J 1(7): 841-845.

Nieto C. M., Rider L. C., Cramer S. D. (2014). "Influence of stromal-epithelial interactions on androgen action." Endocr Relat Cancer 21(4): T147-160.

Nilsson J., Skog J., Nordstrand A., Baranov V., Mincheva-Nilsson L., Breakefield X.O., Widmark A. (2009)" Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer." Br J Cancer. 19;100(10):1603-7.

Nguyen H.C., Xie W., Yang M., Hsieh C.L., Drouin S., Lee G.S., Kantoff P.W (2013)."Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low- risk, localized prostate cancer". Prostate. 73, 346–354.

Ogata-Kawata H., Izumiya M., Kurioka D., Honma Y., Yamada Y., Furuta K., Gunji T., Ohta H., Okamoto H., Sonoda H., Watanabe M., Nakagama H., Yokota J., Kohno T.and Tsuchiya N. (2014). "Circulating exosomal microRNAs as biomarkers of colon cancer." PLoS One 9(4): e92921.

Oka H., Chatani Y., Hoshino R., Ogawa O., Kakehi Y., Terachi T., Okada Y., Kawaichi M., Kohno M.,Yoshida O. (1995). "Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma." Cancer Res 55(18): 4182-4187.

Ostenfeld M. S., Jeppesen D. K., Laurberg J. R., Boysen A. T., Bramsen J. B., Primdal-Bengtson B., Hendrix A., Lamy P., Dagnaes-Hansen F., Rasmussen M. H., Bui K. H., Fristrup N., Christensen E. I., Nordentoft I., Morth J. P., Jensen J. B., Pedersen J. S., Beck M., Theodorescu D., Borre M.,. Howard K. A, Dyrskjot L., Orntoft T. F. (2014). "Cellular Disposal of miR23b by RAB27-Dependent Exosome Release Is Linked to Acquisition of Metastatic Properties." Cancer Res 74(20): 5758-5771.

Ostrowski M., Carmo N. B., Krumeich S., Fanget I., Raposo G., Savina A., Moita C. F., Schauer K., Hume A. N., Freitas R. P., Goud B., Benaroch P., Hacohen N., Fukuda M., Desnos C., Seabra M. C., Darchen F., Amigorena S., Moita L. F., Thery C. (2010). "Rab27a and Rab27b control different steps of the exosome secretion pathway." Nat Cell Biol 12(1): 19-30; sup pp 11-13.

Ouyang L., Shi Z., Zhao S., Wang F. T., Zhou T. T., Liu B., Bao J. K. (2012). "Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis." Cell Prolif 45(6): 487-498.

Pages G., Lenormand P., L'Allemain G., Chambard J. C., Meloche S., Pouyssegur J. (1993). "Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation." Proc Natl Acad Sci U S A 90(18): 8319-8323.

Palmer, R. (1981). "Nicolo Massa, his family and his fortune." Med Hist 25(4): 385-410.

Pan B. T. and R. M. Johnstone (1983). "Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor." Cell 33(3): 967-978.

Pan B. T., Teng K., Wu C., Adam M., Johnstone R. M. (1985). "Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes." The Journal of cell biology 101(3): 942-948.

Pan Q., Ramakrishnaiah V., Henry S., Fouraschen S., de Ruiter P. E., Kwekkeboom J., Tilanus H. W., Janssen H. L., van der Laan L. J. (2012). "Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi)." Gut 61(9): 1330-1339.

Papp K., Vegh P., Prechl J., Kerekes K., Kovacs J., Csikos G., Bajtay Z., Erdei A. (2008). "B lymphocytes and macrophages release cell membrane deposited C3-fragments on exosomes with T cell response-enhancing capacity." Molecular immunology 45(8): 2343-2351.

Park J.E., Tan H.S., Datta A., Lai R.C., Zhang H., Meng W., Lim S.K., Sze S.K. (2010). "Hypoxic tumor cell modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes." Mol Cell Proteomics 9(6): 1085-1099.

Park J.O., Choi D.Y., Choi D.S., Kim H.J., Kang J.W., Jung J.H., Lee J.H., Kim J., Freeman M. R., Lee K. Y., Gho Y. S.and Kim K. P. (2013). "Identification and characterization of proteins isolated from microvesicles derived from human lung cancer pleural effusions." Proteomics 13(14): 2125-2134.

Peinado H., Aleckovic M., Lavotshkin S., Matei I., Costa-Silva B., Moreno-Bueno G., Hergueta- Redondo M., Williams C., Garcia-Santos G., Ghajar C., Nitadori-Hoshino A., Hoffman C., Badal K., Garcia B.A., Callahan M.K., Yuan J., Martins V.R., Skog J., Kaplan R.N., Brady M.S., Wolchok J.D., Chapman P.B., Kang Y., Bromberg J., Lyden D. (2012). "Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET." Nat Med 18(6): 883-891.

Pelser C., Mondul A. M., Hollenbeck A. R., Park Y. (2013). "Dietary fat, fatty acids, and risk of prostate cancer in the NIH-AARP diet and health study." Cancer Epidemiol Biomarkers Prev 22(4): 697-707.

Pinheiro L. C., Wheeler S. B., Chen R. C., Mayer D. K., Lyons J. C., Reeve B. B. (2014). "The effects of cancer and racial disparities in health-related quality of life among older Americans: A case-control, population-based study." Cancer, 121(8):1312-20.

Pisitkun T., Shen R. F., Knepper M. A. (2004). "Identification and proteomic profiling of exosomes in human urine." Proceedings of the National Academy of Sciences of the United States of America 101(36): 13368-13373.

Pitt J.M., Charrier M., Viaud S., André F, Besse B., Chaput N., Zitvogel L. (2014) " Dendritic cell- derived exosomes as immunotherapies in the fight against cancer. J Immunol. 1;193(3):

Ponnambalam S., and Baldwin S.A. (2003). "Constitutive protein secretion from the trans-Golgi network to the plasma membrane." Mol Membr Biol.;20(2):

Prensner J. R., Rubin M. A., Wei J. T., Chinnaiyan A. M. (2012). "Beyond PSA: the next generation of prostate cancer biomarkers." Sci Transl Med 4(127): 127rv123.

Principe S., Jones E. E., Kim Y., Sinha A., Nyalwidhe J. O., Brooks J., Semmes O. J., Troyer D. A., Lance R. S., Kislinger T., Drake R. R. (2013). "In-depth proteomic analyses of exosomes isolated from expressed prostatic secretions in urine." Proteomics 13(10-11): 1667-1671.

Prunotto M., Farina A., Lane L., Pernin A., Schifferli J., Hochstrasser D. F., Lescuyer P., Moll S. (2013). "Proteomic analysis of podocyte exosome-enriched fraction from normal human urine." J Proteomics 82: 193-229.

Qu J. L., Qu X. J., Zhao M. F., Teng Y. E., Zhang Y., Hou K. Z., Jiang Y. H.,. Yang X. H., Liu Y.P. (2009). "Gastric cancer exosomes promote tumour cell proliferation through PI3K/Akt and MAPK/ERK activation." Dig Liver Dis 41(12): 875-880.

Rahman M., Miyamoto H.and Chang C. (2004). "Androgen receptor coregulators in prostate cancer: mechanisms and clinical implications." Clin Cancer Res 10(7): 2208-2219.

Raposo G., Tenza D., Mecheri S., Peronet R., Bonnerot C., Desaymard C. (1997). "Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation." Molecular biology of the cell 8(12): 2631-2645.

Ratajczak J., Miekus K., Kucia M., Zhang J., Reca R., Dvorak P., Ratajczak M.Z. (2006). "Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery." Leukemia 20(5): 847-856.

Ratajczak J., Wysoczynski M., Hayek F., Janowska-Wieczorek A., Ratajczak M. Z. (2006). "Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication." Leukemia 20(9): 1487-1495.

RennieP. S.; Murphy L.C. (2013). "Hormones and Cancer. The Basic Science of Oncology". I. F.R. Tannock P. B. , McGraw-Hill R.G. Professional Pub / McGraw-Hill: 469-501.

Roberts P. J. and Der C. J. (2007). "Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer." Oncogene 26(22): 3291-3310.

Roddam A. W., Duffy M. J., Hamdy F. C., Ward A. M., Patnick J., Price C. P., Rimmer J., Sturgeon C., White P., Allen N. E., NHS Prostate Cancer Risk Management Programme. (2005). "Use of prostate-specific antigen (PSA) isoforms for the detection of prostate cancer in men with a PSA level of 2-10 ng/ml: systematic review and meta-analysis." Eur Urol 48(3): 386-399; discussion 398-389.

Rodriguez C., McCullough M.L., Mondul A.M, Jacobs E.J., Fakhrabadi-Shokoohi D., Giovannucci E.L., Thun M.J., Calle E.E. (2003) "Calcium, dairy products, and risk of prostate cancer in a prospective cohort of United States men." Cancer Epidemiol Biomarkers Prev. 12(7):597-603.

Ronquist G. and Frithz G.G (1986) "Prostasomes in human semen contain ADP and GDP." Acta Eur Fertil 1986 17:273–6.

Ronquist K. G., Ronquist G., Larsson A. and Carlsson L. (2010). "Proteomic analysis of prostate cancer metastasis-derived prostasomes." Anticancer Res 30(2): 285-290.

Ronquist G., Brody I. (1985)." The prostasome: its secretion and function in man." Biochim Biophys Acta. 822:203–18.

Rooney I.A., Atkinson J.P., Krul E.S. Schonfeld G, Polakoski K., Saffitz J.E., Morgan B.P. (1993)." Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on extracellular organelles (prostasomes), binds cell membranes, and inhibits complementmediated lysis". J Exp Med. 177:1409–20.

Roseblade A, Luk F, Ung A, Bebawy M. (2015). "Targeting Microparticle Biogenesis: A Novel Approach to the Circumvention of Cancer Multidrug Resistance." Curr Cancer Drug Targets. [Epub ahead of print]

Roshan Moniri M., Young A., Reinheimer K., Rayat J., Dai L.J., Warnock G.L. (2015). "Dynamic assessment of cell viability, proliferation and migration using real time cell analyzer system (RTCA) ". Cytotechnology. 67(2):379-86.

Rountree R.B., Mandl S.J., Nachtwey J.M., Dalpozzo K., Do L., Lombardo J.R., Schoonmaker P.L., Brinkmann K., Dirmeier U., Laus R., Delcayre A. (2011) " Exosome targeting of tumor antigens expressed by cancer vaccines can improve antigen immunogenicity and therapeutic efficacy." Cancer Res. 1;71(15):5235-44.

Rubinfeld H. and Seger R. (2005). "The ERK cascade: a prototype of MAPK signaling." Mol Biotechnol 31(2): 151-174.

Ruijter E., van de Kaa C., Miller G., Ruiter D., Debruyne F., Schalken J. (1999). "Molecular genetics and epidemiology of prostate carcinoma." Endocr Rev 20(1): 22-45.

Safaei R., Larson B. J., Cheng T. C., Gibson M. A., Otani S., Naerdemann W., Howell S. B. (2005). "Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells." Mol Cancer Ther 4(10): 1595-1604.

Salomon C., Torres M. J., Kobayashi M., Scholz-Romero K., Sobrevia L., Dobierzewska A., Illanes S. E., Mitchell M. D., Rice G. E. (2014). "A gestational profile of placental exosomes in maternal plasma and their effects on endothelial cell migration." PLoS One 9(6): e98667.

Sanders I., Holdenrieder S., Walgenbach-Brunagel G., von Ruecker A., Kristiansen G., Muller S.C., Ellinger J. (2012). "Evaluation of reference genes for the analysis of serum miRNA in patients with prostate cancer, bladder cancer and renal cell carcinoma". Int. J. Urol. Off. J. Jpn. Urol. Assoc., 19, 1017–1025.

Sandvig K. and Llorente A. (2012). "Proteomic analysis of microvesicles released by the human prostate cancer cell line PC-3." Mol Cell Proteomics 11(7): M111 012914.

Saraon P., Jarvi K., Diamandis E. P. (2011). "Molecular alterations during progression of prostate cancer to androgen independence." Clin Chem 57(10): 1366-1375.

Saraswat M., Joenvaara S., Musante L., Peltoniemi H., Holthofer H., Renkonen R. (2014). "N- glycoproteomics of urinary exosomes." Mol Cell Proteomics.

Saraswati S., Block A.S., Davidson M.K., Rank R.G., Mahadevan M., Diekman A.B. (2011)" Galectin-3 is a substrate for prostate specific antigen (PSA) in human seminal plasma." Prostate. 1;71(2):197-208.

Sarker S., Scholz-Romero K., Perez A., Illanes S.E., Mitchell M.D., Rice G.E., Salomon C. (2014)." Placenta-derived exosomes continuously increase in maternal circulation over the first trimester of pregnancy." J Transl Med. 8;12:204.

Savina A., Furlan M., Vidal M., Colombo M. I. (2003). "Exosome release is regulated by a calcium-dependent mechanism in K562 cells." J Biol Chem 278(22): 20083-20090.

Savina A., Vidal M., Colombo M. I. (2002). "The exosome pathway in K562 cells is regulated by Rab11." J Cell Sci 115(Pt 12): 2505-2515.

Schorey J. S. and Bhatnagar S. (2008). "Exosome function: from tumor immunology to pathogen biology." Traffic 9(6): 871-881.

Selth L.A., Townley S., Gillis J.L., Ochnik A.M., Murti K., Macfarlane R.J., Chi K.N., Marshall V.R., Tilley W.D., Butler L.M. (2012). "Discovery of circulating microRNAs associated with human prostate cancer using a mouse model of disease". Int. J. Cancer, 131, 652–661.

Shao H., Chung J., Balaj L., Charest A., Bigner D.D., Carter B.S., Hochberg F.H., Breakefield X.O., Weissleder R., Lee H. (2012)."Protein typ-ing of circulating microvesicles allows real-time monitoring of glioblastomatherapy." Nat Med;18:1835–40.

Shelke G.V., Lässer C., Gho Y.S., Lötvall J. (2014) "Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum." J Extracell Vesicles. 30;3.

Shen J., Hruby G.W., McKiernan J.M., Gurvich I., Lipsky M.J., Benson M.C., Santella R.M. (2012). "Dysregulation of circulating microRNAs and prediction of aggressive prostate cancer. *Prostate, 72*, 1469–1477.

Siljander P.R. (2011) "Platelet-derived microparticles - an updated perspective." Thromb Res.;127 Suppl 2:S30-3.

Simpson R. J., Kalra H., Mathivanan S. (2012). "ExoCarta as a resource for exosomal research." J Extracell Vesicles. 16;1.

Simpson R.J., Jensen S.S., Lim J.W. (2008)."Proteomic profiling of exosomes: current perspectives." Proteomics; 8:4083–99.

Sirois I., Raymond M. A., Brassard N., Cailhier J. F., Fedjaev M., Hamelin K., Londono I., Bendayan M., Pshezhetsky A. V., Hebert M. J. (2011). "Caspase-3-dependent export of TCTP: a novel pathway for antiapoptotic intercellular communication." Cell Death Differ 18(3): 549-562.

Skog J., Wurdinger T., van Rijn S., Meijer D. H., Gainche L., Sena-Esteves M., Curry W. T., J Carter r., B. S., Krichevsky A. M., Breakefield X. O. (2008). "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers." Nature cell biology 10(12): 1470-1476.

Smalley K.S., Lioni M., Herlyn M. (2006). "Life isn't flat: taking cancer biology to the next dimension." *In Vitro* Cell Dev Biol Anim. 42(8-9):242-7.

Sowery R. D., Hadaschik B. A., So A. I., Zoubeidi A., Fazli L., Hurtado-Coll A., Gleave M. E. (2008)."Clusterin knockdown using the antisense oligonucleotide OGX-011 re-sensitizes docetaxel-refractory prostate cancer PC-3 cells to chemotherapy." BJU Int 102(3): 389-397.

Stamey T. A., Yang N., Hay A. R., McNeal J. E., Freiha F. S., Redwine E. (1987). "Prostate- specific antigen as a serum marker for adenocarcinoma of the prostate." N Engl J Med 317(15): 909-916.

Stewart A. B., Anderson W., Delves G., Lwaleed B. A., Birch B., Cooper A. (2004). "Prostasomes: a role in prostatic disease?" BJU Int 94(7): 985-989.

Stoeck A., Keller S., Riedle S., Sanderson M. P., Runz S., Le Naour F., Gutwein P., Ludwig A., Rubinstein E., Altevogt P. (2006). "A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44." Biochem J 393(Pt 3): 609-618.

Stoorvogel W., Kleijmeer M. J., Geuze H. J., Raposo G. (2002). "The biogenesis and functions of exosomes." Traffic 3(5): 321-330.

Suarez B. K., Lin J., Burmester J. K., Broman K. W.,. Weber J. L, Banerjee T. K., Goddard K. A., Witte J. S., Elston R. C., Catalona W. J. (2000). "A genome screen of multiplex sibships with prostate cancer." Am J Hum Genet 66(3): 933-944.

Svensson M., Hult M., van der Mark M., Grotta A., Jonasson J., von Hausswolff-Juhlin Y., Rossner S., Trolle Lagerros Y. (2014). "The change in eating behaviors in a Web-based weight loss program: a longitudinal analysis of study completers." J Med Internet Res 16(11): e234.

Szajnik M., Derbis M., Lach M., Patalas P., Michalak M., Drzewiecka H., Szpurek D., Nowakowski A., Spaczynski M., Baranowski W., Whiteside T. L. (2013). "Exosomes in Plasma of Patients with Ovarian Carcinoma: Potential Biomarkers of Tumor Progression and Response to Therapy." Gynecol Obstet (Sunnyvale) Suppl 4: 3.

Tagnon H. J., Whitmore W. F., Shulman Jr., N. R. (1952). "Fibrinolysis in metastatic cancer of the prostate." Cancer 5(1): 9-12.

Taichman R. S., Loberg R. D., Mehra R., Pienta K. J. (2007). "The evolving biology and treatment of prostate cancer." J Clin Invest 117(9): 2351-2361.

Takeda H., Lasnitzki I., Mizuno T. (1986). "Analysis of prostatic bud induction by brief androgen treatment in the fetal rat urogenital sinus." J Endocrinol 110(3): 467-470.

Tavoosidana G., Ronquist G., Darmanis S., Yan J., Carlsson L., Wu D., Conze T., Ek P., Semjonow A., Eltze E., Larsson A., Landegren U., Kamali-Moghaddam M. (2011) "Multiple recognition assay reveals prostasomes as promising plasma biomarkers for prostate cancer." Proc Natl Acad Sci U S A. 108(21): 8809–8814.

Taylor D. D., Akyol S., Gercel-Taylor C. (2006). "Pregnancy-associated exosomes and their modulation of T cell signaling." Journal of immunology 176(3): 1534-1542.

Taylor D. D. and Gercel-Taylor C. (2008). "MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer." Gynecol Oncol 110(1): 13-21.

Thakur B. K., Zhang H., Becker A., Matei I., Huang Y., Costa-Silva B., Zheng Y., Hoshino A., Brazier H., Xiang J., Williams C., Rodriguez-Barrueco R., Silva J. M., Zhang W., Hearn S., Elemento O., Paknejad N., Manova-Todorova K., Welte K., Bromberg J., Peinado H., Lyden D. (2014). "Double-stranded DNA in exosomes: a novel biomarker in cancer detection." Cell Res 24(6): 766-769.

Thery C., Boussac M., Veron P., Ricciardi-Castagnoli P., Raposo G., Garin J., Amigorena S. (2001)."Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles." J Immunol 166(12): 7309-7318.

Thery C., Ostrowski M., Segura E. (2009). "Membrane vesicles as conveyors of immune responses." Nature reviews. Immunology 9(8): 581-593.

Thery C., Zitvogel L., Amigorena S. (2002). "Exosomes: composition, biogenesis and function." Nat Rev Immunol 2(8): 569-579.

Théry C., Regnault A., Garin J., Wolfers J., Zitvogel L., Ricciardi-Castagnoli P., Raposo G., Amigorena S. (1999). "Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73." J Cell Biol. 147(3):599-610.

Thery C., Amigorena S., Raposo G., Clayton A. (2006). "Isolation and characterization of exosomes from cell culture supernatants and biological fluids." Curr Protoc Cell Biol, Chapter 3:Unit 3.22.

Tian Y., Li S., Song J., Ji T., Zhu M., Anderson G. J., J. Wei G. Nie (2014). "A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy." Biomaterials 35(7): 2383-2390.

Tickner J. A., Urquhart A. J., Stephenson S. A., Richard D. J., O'Byrne K. J. (2014). "Functions and therapeutic roles of exosomes in cancer." Front Oncol 4: 127.

Tourneur L., Mistou S., Schmitt A., Chiocchia G. (2008). "Adenosine receptors control a new pathway of Fas-associated death domain protein expression regulation by secretion." J Biol Chem 283(26): 17929-17938.

Trajkovic K., Hsu C., Chiantia S., Rajendran L., Wenzel D., Wieland F., Schwille P., Brugger B., Simons M. (2008). "Ceramide triggers budding of exosome vesicles into multivesicular endosomes." Science 319(5867): 1244-1247.

Trams E. G., Lauter C. J., Salem N., Heine Jr., U. (1981). "Exfoliation of membrane ecto-enzymes in the form of micro-vesicles." Biochim Biophys Acta 645(1): 63-70.

Tran T.H., Mattheolabakis G., Aldawsari H., Amiji M.(2015) " Exosomes as Nanocarriers for Immunotherapy of Cancer and Inflammatory Diseases." Clin Immunol.S1521-6616(15)00124-2 Umar, A., B. K. Dunn and P. Greenwald (2012). "Future directions in cancer prevention." Nat Rev Cancer 12(12): 835-848.

Umezu T., Tadokoro H., Azuma K., Yoshizawa S., Ohyashiki K. and Ohyashiki J. H. (2014). "Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1." Blood. 124(25):3748-57.

van den Ouden D., Bentvelsen F. M., Boeve E. R., Schroder F. H. (1993). "Positive margins after radical prostatectomy: correlation with local recurrence and distant progression." Br J Urol 72(4): 489-494.

van der Pol E., Boing A. N., Harrison P., Sturk A., Nieuwland R. (2012). "Classification, functions, and clinical relevance of extracellular vesicles." Pharmacol Rev 64(3): 676-705.

Verderio C., Muzio L.,Turola E., Bergami A., Novellino L., Ruffini F., Riganti L., Corradini I., Francolini M., Garzetti L., Maiorino C., Servida F., Vercelli A., Rocca M., Dalla Libera D., Martinelli V., Comi G., Martino G., Matteoli M., Furlan R. (2012)."Myeloid microvesiclesareamarkerand therapeutictargetforneuroin- flammation". Ann.Neurol. 72, 610–624.

Vinci M., Box C., Zimmermann M., Eccles SA. (2013). "Tumor spheroid-based migration assays for evaluation of therapeutic agents." Methods Mol Biol. 986:253-66.

Vrijsen K. R., Sluijter J. P., Schuchardt M. W., van Balkom B. W., Noort W. A., Chamuleau S. A., Doevendans P. A. (2010)."Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells." Journal of cellular and molecular medicine 14(5): 1064-1070.

Wahlgren J., De L. K. T., Brisslert M., Vaziri Sani F., Telemo E., Sunnerhagen P., Valadi H. (2012)."Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes." Nucleic Acids Res 40(17): e130.

Wan L., Tan H. L., Thomas-Ahner J. M., Pearl D. K., Erdman J. W., Moran Jr., N. E., Clinton S. K. (2014). "Dietary Tomato and Lycopene Impact Androgen Signaling- and Carcinogenesis- Related Gene Expression during Early TRAMP Prostate Carcinogenesis." Cancer Prev Res (Phila) 7(12): 1228-1239.

Wang G., Dinkins M., He Q., Zhu G., Poirier C., Campbell A., Mayer-Proschel M., Bieberich E. (2012). "Astrocytes secrete exosomes enriched with proapoptotic ceramide and prostate apoptosis response 4 (PAR-4): potential mechanism of apoptosis induction in Alzheimer disease (AD)." J Biol Chem.15;287(25):21384-95.

Watahiki A., Macfarlane R.J., Gleave M.E., Crea F., Wang Y., Helgason C.D., Chi K.N (2013)."Plasma miRNAs as Biomarkers to Identify Patients with Castration-Resistant Metastatic Prostate Cancer". Int. J. Mol. Sci., 14, 7757–7770.

Weber M. J. and Gioeli D. (2004). "Ras signaling in prostate cancer progression." J Cell Biochem 91(1): 13-25.

Weiswald L.B., Guinebretière J.M., Richon S., Bellet D, Saubaméa B., Dangles-Marie V. (2010). "In situ protein expression in tumour spheres: development of an immunostaining protocol for confocal microscopy." BMC Cancer. 22;10:106.

Welton J. L., Khanna S., Giles P. J., Brennan P., Brewis I. A., Staffurth J., Mason M. D. Clayton A. (2010). "Proteomics analysis of bladder cancer exosomes." Mol Cell Proteomics. 9(6):1324- 38.

Whiteside A.(2013). "Immune modulation of T-cell and NK (natural killer) cell activities by TEXs (tumour-derived exosomes)." Biochem Soc Trans 41(1): 245-251.

Whitmore W. F., (1956). "Hormone therapy in prostatic cancer." Am J Med 21(5): 697-713.

Williams H. and Powell I. J. (2009). "Epidemiology, pathology, and genetics of prostate cancer among African Americans compared with other ethnicities." Methods Mol Biol 472: 439-453.

Wilson K. M., Shui I. M., Mucci L. A., Giovannucci E. (2015). "Calcium and phosphorus intake and prostate cancer risk: a 24-y follow-up study." Am J Clin Nutr 101(1): 173-183.

Wood S.L., Knowles M.A., Thompson D., Selby P. J., Banks R.E. (2013). "Proteomic studies of urinary biomarkers for prostate, bladder and kidney cancers." Nat Rev Urol 10(4): 206-218.

Wranicz J. and Szostak-Wegierek D. (2014). "Health outcomes of vitamin D. Part II. Role in prevention of diseases." Rocz Panstw Zakl Hig 65(4): 273-279.

Wubbolts R., Leckie R. S., Veenhuizen P. T., Schwarzmann G., Mobius W., Hoernschemeyer J., Slot J. W., Geuze H. J., Stoorvogel W. (2003). "Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation." J Biol Chem 278(13): 10963-10972.

Xiao H, Wong DT. (2012)."Proteomic analysis of microvesicles in human saliva by gel electrophoresis with liquid chromatography-mass spectrometry." Anal Chim Acta. 20;723:61-7.

Xu J., Zheng S. L., Hawkins G. A., Faith D. A., Kelly B., Isaacs S. D., Wiley K. E., Chang B., Ewing C.M., Bujnovszky P., Carpten J.D., Bleecker E.R., Walsh P.C., Trent J.M., Meyers D. A., Isaacs W.B. (2001). "Linkage and association studies of prostate cancer susceptibility: evidence for linkage at 8p22-23." Am J Hum Genet 69(2): 341-350.

Yang L., Wu X.H., Wang D., Luo C.L., Chen L.X. (2013). "Bladder cancer cell-derived exosomes inhibit tumor cell apoptosis and induce cell proliferation *in vitro*." Mol Med Rep 8(4): 1272-1278.

Yang X. F., Wu C. J., Chen L., Alyea E. P., Canning C., Kantoff P., Soiffer R. J., Dranoff G. and Ritz J. (2002). "CML28 is a broadly immunogenic antigen, which is overexpressed in tumor cells." Cancer Res 62(19): 5517-5522.

Yang T., Martin P., Fogarty B., Brown A., Schurman K., Phipps R., Yin V.P., Lockman P., Bai S. (2015)"Exosome Delivered Anticancer Drugs Across the Blood-Brain Barrier for Brain Cancer Therapy in Danio Rerio". Pharm Res.. [Epub ahead of print]

Ye S.B., Li Z.L., Luo D.H., Huang B.J., Chen Y.S., Zhang X.S., Cui J., Zeng Y.X., Li J. (2014). "Tumor-derived exosomes promote tumor progression and T-cell dysfunction through the regulation of enriched exosomal microRNAs in human nasopharyngeal carcinoma." Oncotarget. l 30;5(14):5439-52.

Yin W., Ouyang S., Li Y., Xiao B., Yang H. (2013). "Immature dendritic cell-derived exosomes: a promise subcellular vaccine for autoimmunity." Inflammation 36(1): 232-240.

Yu S., Liu C., Su K., Wang J., Liu Y., Zhang L., Li C., Cong Y., Kimberly R., Grizzle W. E., Falkson C., Zhang H. G. (2007). "Tumor exosomes inhibit differentiation of bone marrow dendritic cells." J Immunol 178(11): 6867-6875.

Yu X., Harris S.L., Levine A.J. (2006). " The regulation of exosome secretion: a novel function of the p53 protein." Cancer Res. 1;66(9):4795-801.

Yue S., Mu W., Erb U., Zöller M. (2015). " The tetraspanins CD151 and Tspan8 are essential exosome components for the crosstalk between cancer initiating cells and their surrounding." Oncotarget. 10;6(4):2366-84.

Zhang, H., Xie Y., Li W., Chibbar R., Xiong S., Xiang J. (2011). "CD4(+) T cell-released exosomes inhibit CD8(+) cytotoxic T-lymphocyte responses and antitumor immunity." Cellular & molecular immunology 8(1): 23-30.

Zhang, H. G. and Grizzle W. E. (2011). "Exosomes and cancer: a newly described pathway of immune suppression." Clin Cancer Res 17(5): 959-964.

Zhang M., Latham D. E., Delaney M. A., Chakravarti A. (2005). "Survivin mediates resistance to antiandrogen therapy in prostate cancer." Oncogene 24(15): 2474-2482.

Zhou H., Yuen P. S., Pisitkun T., Gonzales P. A., Yasuda H., Dear J. W., Gross P., Knepper M. A., Star R. A. (2006). "Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery." Kidney Int 69(8): 1471-1476.

Zhuang, X., Xiang X., Grizzle W., Sun D., Zhang S., Axtell R. C., Ju S., Mu J., Zhang L., Steinman L., Miller D., Zhang H. G. (2011). "Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain." Mol Ther 19(10): 1769-1779.

Zitvogel L., Regnault A., Lozier A., Wolfers J., Flament C., Tenza D., Ricciardi-Castagnoli P., Raposo G., Amigorena S. (1998). "Eradication of established murine tumors using a novel cell- free vaccine: dendritic cell-derived exosomes." Nature medicine 4(5): 594-600.

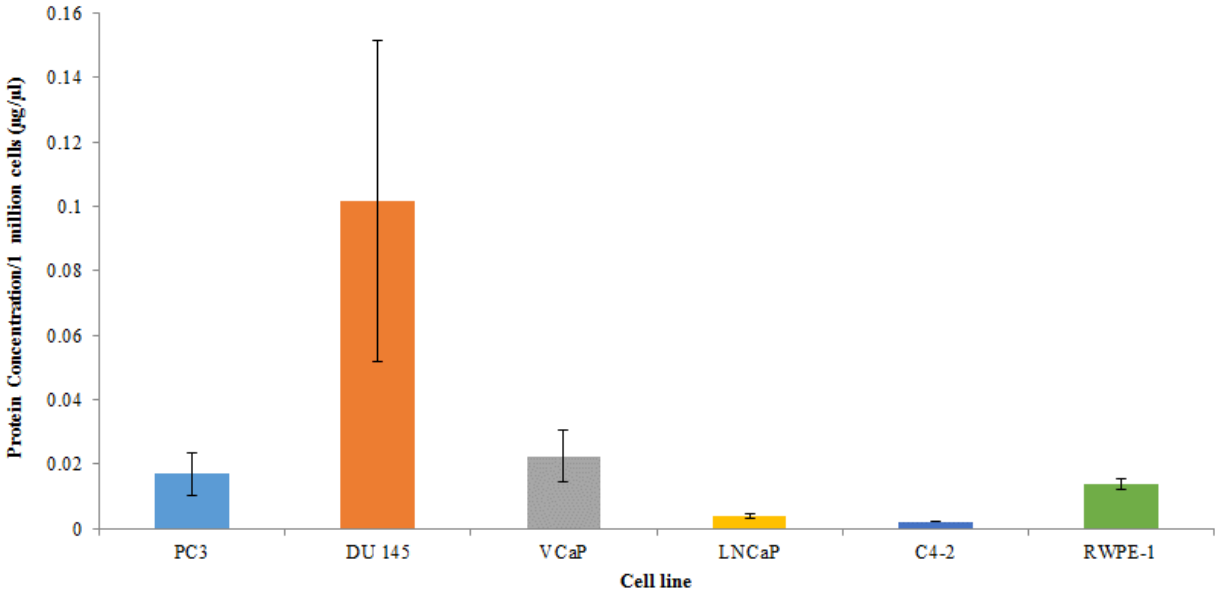
Zocco D., Ferruzzi P., Cappello F., Kuo W. P., Fais S. (2014). "Extracellular vesicles as shuttles of tumor biomarkers and anti-tumor drugs." Front Oncol 4: 267.

Zoubeidi A., Chi K., Gleave M. (2010). "Targeting the cytoprotective chaperone, clusterin, for treatment of advanced cancer." Clin Cancer Res 16(4): 1088-1093.

Zoubeidi A., Ettinger S., Beraldi E., Hadaschik B., Zardan A., Klomp L. W., Nelson C. C., Rennie P. S., Gleave M. E. (2010). "Clusterin facilitates COMMD1 and I-kappaB degradation to enhance NF-kappaB activity in prostate cancer cells." Mol Cancer Res 8(1): 119-130.

Zubiri I., Vivanco F., Alvarez-Llamas G. (2013). "Proteomic analysis of urinary exosomes in cardiovascular and associated kidney diseases by two-dimensional electrophoresis and LC-MS/MS." Methods Mol Biol 1000: 209-220.

Appendix



Prostate cell derived exosomes protein concentration

Bar chart showing the protein concentration of Prostate cell derived exosomes.