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CHARACTERIZATION OF SMALL RNA CONTENT IN URINARY AND PLASMA EXTRACELLULAR VESICLES FROM PROSTATE CANCER PATIENTS

BACHELOR THESIS

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Abstract

Extracellular vesicles (EVs) mediate intercellular communication in physiological processes and various diseases, including cancer. Studying EV RNA content would allow finding new prostate cancer (PCa) biomarkers.

This work focuses on characterization of small RNA content of EVs isolated from plasma and urine of PCa patients, and tumor and non-tumor prostate tissues using RNA sequencing. Results showed that both, plasma and urinary EVs contain messenger RNA fragments, micro RNAs, long non-coding RNAs, piwi-interacting RNAs and other non-coding RNAs, however their proportions vary immensely between individuals. A fraction of these RNAs are overexpressed in tumor tissue and may represent novel PCa biomarkers. Two of these micro RNAs were chosen for validation using RT-qPCR that confirmed their suitability as PCa biomarkers.

Key words: prostate cancer, extracellular vesicles, small RNA, biomarkers

Kopsavilkums

MAZO RNS RAKSTUROŠANA ĀRPUSŠŪNAS VEZIKULĀS, KAS IEGŪTAS NO PROSTATAS VĒŽA PACIENTU URĪNA UN PLAZMAS PARAUGIEM

Ārpusšūnas vezikulas (EVs) nodrošina starpšūnu komunikāciju fizioloģiskos procesos un dažādās saslimšanās, ieskaitot ļaundabīgos audzējus. EV RNS satura izpēte dotu iespēju atklāt jaunus prostatas vēža (PCa) biomarķierus.

Šis darbs tiek fokusēts uz mazo RNS raksturošanu, kas iegūtas no PCa pacientu plazmas un urīna EVs, kā arī no PCa un normāliem prostatas audiem, izmantojot RNS sekvencēšanu. Rezultāti uzrādīja, ka gan plazmas, gan urīna EVs satur matrices RNS fragmentus, mikro RNS, garās-nekodējošās RNS, piwi-saistošās RNS un citas nekodējošās RNS, taču to proporcijas ievērojami variē starp indivīdiem. Daļa šo RNS ir paaugstināti ekspresētas vēža audos un varētu būt piemērotas kā PCa biomarķieri. Divas mikro RNS tika izvēlētas validācijai ar RT-qPCR, kas apstiprināja to piemērotību kalpot kā PCa biomarķieriem.

Atslēgas vārdi: prostatas vēzis, ārpusšūnas vezikulas, mazās RNS, biomarķieri

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LIST OF ABBREVIATIONS

°C – degree Celsius

µg – microgram

µl – microliter

µm – micrometer

A – adenine

Ago2 – Argonaute 2 protein

APS – ammonium persulfate

BCA – bicinchoninic acid assay

bp – base pair

BPH – benign prostatic hyperplasia

BRAF – B-Raf proto-oncogene

C – cytosine

C. elegans – Caenorhabditis elegans

cDNA – complimentary deoxyribonucleic acid

cfRNA – cell-free ribonucleic acid

circRNA – circular ribonucleic acid

Cu – copper

D. melanogaster – Drosophila melanogaster

DEPC – diethyl dicarbonate

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

DRE – digital rectal examination

EVs – extracellular vesicles

fmol – femtomolar

g – gram

G – guanine

GAS5 – growth arrest specific transcript 5

GS – Gleason score

H – hydrogen

HCl – hydrochloric acid

hg38 – human reference genome build 38

HOTAIR – HOX transcript antisense RNA

kDa – kilodalton

KRAS – KRAS proto-oncogene

LDL -low-density lipoproteins

lincRNA – long non-coding intergenic ribonucleic acid

lncRNA – long non-coding ribonucleic acid

logFC – logarithmic fold change

MALAT1 – metastasis associated lung adenocarcinoma transcript 1

mg – milligrams

miRNA – micro ribonucleic acid

ml – milliliters

MP – mapped reads

mRNA - messenger ribonucleic acid

mtRNA – mitochondrial ribonucleic acid

MVs – microvesicles

MYC – MYC proto-oncogene

Na – sodium

NaCl – sodium chloride

ncRNA – non-coding ribonucleic acid

ng – nanograms

nm – nanometers

nt – nucleotide

PBS – phosphate buffered saline

PCa – prostate cancer

PCA3/DD3 – prostate cancer associated transcript 3

PCAT1 – prostate cancer associated transcript 1

PCGEM1 – prostate-specific transcript 1

PCR – polymerase chain reaction

pg – picogram

pH – potential of hydrogen

PIN – prostatic intraepithelial neoplasia

piRNA – piwi-interacting ribonucleic acid

PIWI – P-element induced wimpy testis

PIWIL1/HIWI – piwi like RNA-mediated gene silencing 1 protein

PIWIL2 – piwi like RNA-mediated gene silencing 2

PIWIL2/HILI – piwi like RNA-mediated gene silencing 2 protein

PIWIL3 – piwi like RNA-mediated gene silencing 3 protein

PIWIL4/HIWI2 – piwi like RNA-mediated gene silencing 4 protein

pre-miRNA – precursor micro ribonucleic acid

pri-miRNA – primary micro ribonucleic acid

PSA – prostate specific antigen

RCC – renal cell carcinoma

RIPA – radioimmunoprecipitation assay

RISC – ribonucleic acid-induced silencing complex

RNA – ribonucleic acid

RNase – ribonuclease

RNA-seq - ribonucleic acid sequencing

RNP – ribonucleoprotein

rpm – revolutions per minute

RQ – relative quantification

rRNA – ribosomal ribonucleic acid

RT – room temperature

RT-qPCR – quantitative reverse transcription polymerase chain reaction

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC – size exclusion chromatography

snoRNA – small nucleolar ribonucleic acid

snRNA – small nuclear ribonucleic acid

sRNA – small ribonucleic acid

TBS – tris buffered saline

TEM – transmission electron microscopy

TEMED - N,N,N’,N’,-tetra-methylethylenediamine

TF – transcription factor

TNM – tumor, node, metastasis system

Tris - tris(hydroxymethyl)aminomethane

tRNA – transfer ribonucleic acid

Tsg101 – tumor susceptibility 101 protein

TUC339 – transcript uc.339

U – uracil

UV – ultraviolet light

V – volts

vRNA – vault ribonucleic acid

WB – western blot

INTRODUCTION

PCa is one of the most commonly diagnosed cancer types among men across the globe and its treatment methods involve radiation therapy, hormone therapy, chemotherapy and prostatectomy (National Cancer Institute 2015a). Prognostic markers like prostate specific antigen (PSA) blood level and diagnostic PCa markers, such as tumor stage and Gleason score (GS) have been described, although they are not sufficient to guide the choice of treatment due to PCa's high heterogeneity (Shoag and Barbieri 2016). Furthermore, PSA-based tests have low specificity, which leads to many unnecessary prostate biopsies, whilst GS is affected by implicit degree of subjectivity (Polascik *et al.* 1999; Humphrey 2004). As a result, many patients face problems with overtreatment (reviewed in Loeb *et. al.* 2014). Therefore, it is important to discover new PCa biomarkers that could serve as an early diagnostic tool and as guidance to correct treatment choice.

EVs are produced by many cell types and contain various molecules that regulate many cellular processes (Zhang 2009; reviewed in Yáñez-Mó *et al.* 2015). Cancer-derived EVs have been found in different biofluids and suggested to carry cancer-derived RNAs (reviewed in Yáñez-Mó *et al.* 2015; reviewed in Fujita *et al.* 2016). Thus, these EV-enclosed RNAs might serve as cancer biomarkers for diagnosis, prognosis and monitoring. However, the RNA cargo of plasma and urinary EVs in PCa patients has not been systematically characterized and compared to the matching tumor tissue before.

The aim of the study was to characterize the small RNA (sRNA) cargo in EVs isolated from PCa patient plasma and urine before and after radical prostatectomy and to compare it with the sRNA content in matching tumor and non-tumor prostate tissue.

The following tasks were established:

- EVs isolation from PCa patients' urine and plasma samples using size exclusion chromatography (SEC);
- Characterization of the isolated EVs by transmission electron microscopy (TEM), western blot (WB) and nanoparticle tracking analysis;
- RNA extraction from urinary and plasma EVs, and matching tumor and non-tumor prostate tissues;
- sRNA library construction and sequencing;
- Characterization of RNA species found in EVs and tissues;
- Identification of biomarker candidates;
- Validation of selected biomarker candidates.

Study was conducted in Latvian Biomedical Research and Study Centre (Riga, Latvia) and Norwegian Radium Hospital (Oslo, Norway).

1. LITERATURE REVIEW

1.1 Cancer

Cancer is a group of diseases involving rapid proliferation of abnormal cells with potential to invade surrounding tissues (National Cancer Institute 2015b). Nowadays, cancer is among the leading causes of morbidity and mortality worldwide with more than 100 different types identified (Stewart and Wild 2014; National Cancer Institute 2015b). The most widespread cancer types in Europe are breast, large bowel, lung and prostate. Among men the most prevalent types are PCa, which in 2012 composed 22,07% of all cases and lung cancer, which composed 16,04% of all cases. The most common type of cancer among women is breast cancer, contributing to 28,56% of the total number of cancer cases among females in 2012 (EUCAN 2012).

Cancer is a multi-step molecular process that arises from a single abnormal cell with an altered DNA sequence and that in the majority of cases culminates in the formation of a tumor mass (Hejmadi 2010). Ten different biological capabilities that cells acquire during the development of cancer have been described (Figure 1) (Hanahan and Weinberg 2011).

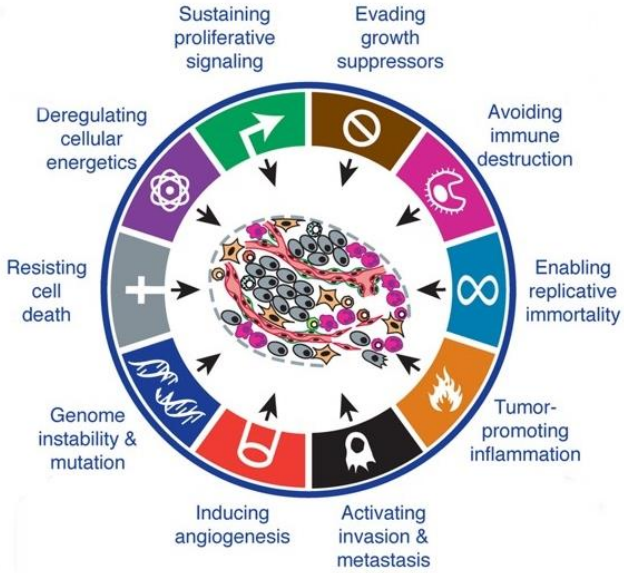

Figure 1. The hallmarks and enabling characteristics of cancer (Hanahan and Weinberg 2011). Sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, contributing to tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, causing genomic instability and mutations, resisting cell death and deregulating cellular energetics are the 10 hallmarks and enabling characteristics of human cancers.

1. attēls. Vēža raksturīgākās iezīmes un īpašības (Hanahan and Weinberg 2011). Proliferatīvas signalizēšanas saglabāšana, izvairīšanās no augšanas supresoriem un šūnas imūnās atbildes, replikatīvās nemirstības iespējošana, audzēju veicinoša iekaisuma veidošana, invāzijas un metastāzes aktivācija, angioģenēzes inducēšana, genomiskās nestabilitātes un mutāciju radīšana, pretošanās šūnu nāvei un šūnas enerģētikas regulējumu mazināšana ir 10 cilvēka vēžiem raksturīgās iezīmes un īpašības.

In non-cancerous cells the release of growth-promoting signals is carefully controlled thereby regulating cell growth, division, aging and dying. However, cancer cells are able to sustain proliferative signaling by deregulating these signals (Does *et al.* 2003; Hanahan and Weinberg 2011). Studies have shown that many of these processes are affected by mutations in DNA repair genes or by mutations in oncogenes, such as *KRAS, BRAF* and *MYC* (Gurel *et al*. 2008; Morkel *et al.* 2015). Therefore, specific genomic mutations and genome instability only contributes to cancer progression (Hanahan and Weinberg 2011). Moreover, cancer cells are capable of inactivating tumor suppressor genes (Hanahan and Weinberg 2011). Normal cells often undergo apoptosis during high physiological stress conditions or when their DNA is damaged. On the contrary, cancer cells do not lose their ability to divide and can resist cell death even when there are mutations present in their genome (Does *et al.* 2003; Hanahan and Weinberg 2011). Furthermore, cancer cells can enable replicative immortality and continue proliferating without ever reaching senescence state or apoptosis (Hanahan and Weinberg 2011). Additionally, cancer cells can avoid immune destruction and inflammation caused by immune cells may be tumor-promoting (Hanahan and Weinberg 2011). Alike non-cancerous cells, cancer cells also require nutrients and oxygen to grow and ability to dispose of metabolic waste products and carbon dioxide. In order to take care of these needs, they can induce angiogenesis. Moreover, cancer cells are capable of reprogramming energy metabolism thus promoting cancer growth (Hanahan and Weinberg 2011). Last but not least, cancers progress by activating multistep process of invasion and metastasis (Hanahan and Weinberg 2011). Metastasis involves cancer cell passage from primary tumor to distant tissues through lymphatic and hematogenous systems and the formation of cancer cell colonies in other parts of the body (Talmadge and Fidler 2010). Furthermore, recent studies show that high-fat diet has a clear impact on promoting metastasis (Pascual *et al.* 2017; reviewed in Peinado *et al.* 2017).

1.2 Prostate cancer

Prostate is a walnut size male exocrine gland located between the bladder and the penis, which secretes a specific fluid that, together with spermatozoa and seminal vesicle fluid, compose semen (Cramer and Alcamo 2007). Prostate is comprised of multiple secretory acini that are made of epithelial basal and glandular cells surrounded by fibromuscular stromal cells. Basal cells, which have been shown to exhibit stem cell-like and neurogenic properties, synthesize the components of the basal lamina of the acini, acting as a physical barrier between the glandular cells and stroma; while glandular cells secrete prostate fluid (Cramer and Alcamo 2007; Zhang *et al*. 2016). Within the basal layer rare neuroendocrine cells responsible for sex hormones secretion can also be found (Cramer and Alcamo 2007).

PCa is the most frequent malignant and heterogeneous neoplasia reported in males (Torre *et al.* 2015). However, even though the incidence rates for PCa in Europe in 2012 were the highest, contributing to 22,07% of all cancer cases, the estimated mortality was 9,45%, setting PCa behind lung (26,07%) and large bowel cancer (11,59%) (EUCAN 2012).

There are several types of PCa, but the predominant type is acinar adenocarcinoma, which originates from glandular epithelial tissue (Stewart and Wild 2014). In most cases, PCa arises from prostate gland cells located in the peripheral zone of the prostate. During such development, cancer cells remain confined by normal prostate glandular cells causing development of prostatic intraepithelial neoplasia (PIN), which is considered to be the earliest stage of carcinogenesis (Bostwick and Qian 2004). Further cancer progression implicates formation of invasive carcinoma and metastasis (Figure 2) (Abate-Shen and Shen 2000).

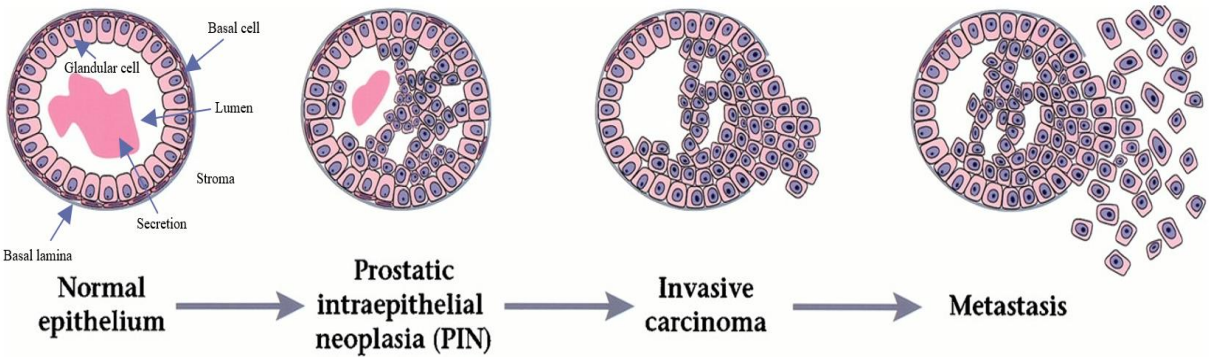

Figure 2. PCa progression pathway (Abate-Shen and Shen 2000). At first cells in the peripheral zone of the prostate mutate and formation of PIN occurs. Further progression of cancer involves formation of invasive carcinoma and metastasis. Note: picture has been taken from Abate-Shen and Shen 2000 and modified.

2. attēls. PCa progresēšanas ceļš (Abate-Shen and Shen 2000). Vēža attīstības sākumā, šūnas, kas atrodas prostatas perifērajā zonā, mutē un veidojas prostatas intraepiteliālā neoplāzija. Turpmākā vēža attīstība ietver invazīvas karcinomas un metastāzes veidošanos. Piezīme: attēls aizgūts no Abate-Shen and Shen 2000 un ir ticis pārveidots.

To determine cancer stage, usually number staging system and/or Tumor Node Metastasis (TNM) staging system is used. In TNM system, T category describes the size and/or the extent of the primary tumor, N category indicates whether cancer has spread to the lymph nodes and M category specifies whether it has metastasized and advanced to other parts of the body or not. Each TNM system category is codified by numbers ranging from zero (non-existent) to four (high) or with letter X, in case the category cannot be assessed (Greene *et al.* 2002). There are four (I – IV) stages in the number system as well - stage I referring to relatively small cancer, contained within the prostate and stage IV meaning that cancer has become metastatic (Greene *et al.* 2002).

Early PCa is usually asymptomatic and only in later stages, once cancer has metastasized, symptoms can be noticed (Miller *et al*. 2003). Ultrasonography, digital rectal examination (DRE) and magnetic resonance imaging are the most commonly used techniques for PCa detection. These methods are used to find prostate abnormalities, in order to identify targets to biopsy (Bonekamp *et al.* 2011). Unfortunately, all the previously mentioned methods are invasive and unpleasant. Due to this, special interest has arisen regarding new approaches that could allow identification of PCa in a non-invasive way (Nilsson *et al.* 2009). Among the hitherto markers identified for PCa, the most commonly used serum marker is PSA. PSA is a glycoprotein enzyme, member of kallikrein family, which is secreted by prostate epithelial cells (Balk *et al.* 2003). In normal prostate tissue, PSA is first secreted into the lumen, whilst in PCa the basal cell layer and basal lamina is disrupted allowing PSA to pass directly into bloodstream contributing to higher level of serum PSA (Balk *et al.* 2003). However, taking that into account that PSA level varies from one individual to another and also increases with age (Table 1), as consensus, the threshold for performing prostate biopsies has been set at four nanograms per milliliter of serum total PSA and amounts below that are considered to be normal (Balk *et al.* 2003; Connolly *et al*. 2007). Nevertheless, caution should be taken into consideration as it has been proven that PSA level can also be affected by other non-cancer related genitourinary diseases, such as prostatitis and benign prostatic hyperplasia (BPH) (Balk *et al.* 2003).

Table 1

PSA average and median values depending on male age and PCa presence (Connolly *et al*. 2007).

1. tabula

PSA vidējā vērtība un mediāna atkarībā no vīrieša vecuma un PCa klātbūtnes (Connolly *et al*. 2007).

Age	**<50**		**50-59**		**60-69**		**≥70**	
PCa	Yes	No	Yes	No	Yes	No	Yes	No
PSA average value (ng/ml)	30.1	1.2	91.9	1.8	105.3	3.1	182.0	8.9
PSA median value (ng/ml)	4.2	0.7	8.2	1.0	10.8	1.5	24.7	2.4

For instance, study by Nadler *et al.* in 1995 showed that prostatitis and BPH contribute to serum PSA elevation (Nadler *et al.* 1995). In addition, study carried out by Thompson *et. al.* in 2004 proved that PCa patients can present PSA levels of four nanograms per milliliter or lower, which are considered normal PSA levels found in serum in non-cancer patients (Thompson *et al.* 2004). These controversial findings make PSA a not completely reliable marker when diagnosing PCa. As a result, PSA-based test low specificity gives high amount PCa false positive rates leading to a large number of unnecessary prostate biopsies (Polascik *et al.* 1999). Alternative approaches, such as PSA density, PSA velocity, age-specific PSA levels

and free to total PSA ratio, have been suggested, to improve PSA diagnostic performance (Polascik *et al.* 1999). All of them have their own pros and cons, but as they rely on the same initial target, they are still subjected to false positivity, thus additional biomarkers for PCa diagnostics are needed.

Prostate biopsy is performed to analyze prostate tissue and its morphological changes (National Cancer Institute 2015c). In cases of cancer being detected, in order to determine the grade of morphological cellular changes in the tissue, GS is used. GS system is a PCa specific histological grading method that serves to assess the prognosis of men with PCa (Humphrey 2004). It uses five histological Gleason patterns, which are graded from one to five, respectively. Each pattern is associated with different features, most of them related to prostate gland dedifferentiation (Gleason patterns are explained in detail in Figure 3) (Humphrey 2004). Primary pattern grade is assigned to the dominant archetype seen in the tumor specimen, secondary grade to the next most frequent archetype and sometimes tertiary grade to the third most frequent archetype. In cases when only two archetypes are seen, sum of primary and secondary pattern grade establish the final GS. In case a third archetype is found, the sum of the two highest pattern grades prevails. GS can range from two to ten; score below six is considered to be relatively low whilst score above eight refers to aggressive PCa (Zelefsky *et al.* 2011). Unfortunately, GS is affected by implicit degree of subjectivity and underrating of tumor tissue is a common problem (Humphrey 2004).

As there are still unmet clinical needs regarding PCa diagnostics and prognostics it is important to develop better non-invasive PCa detection methods to enable early detection of PCa and guide choice of treatment as to avoid overtreatment as well as unnecessary prostate biopsies and prostatectomy.

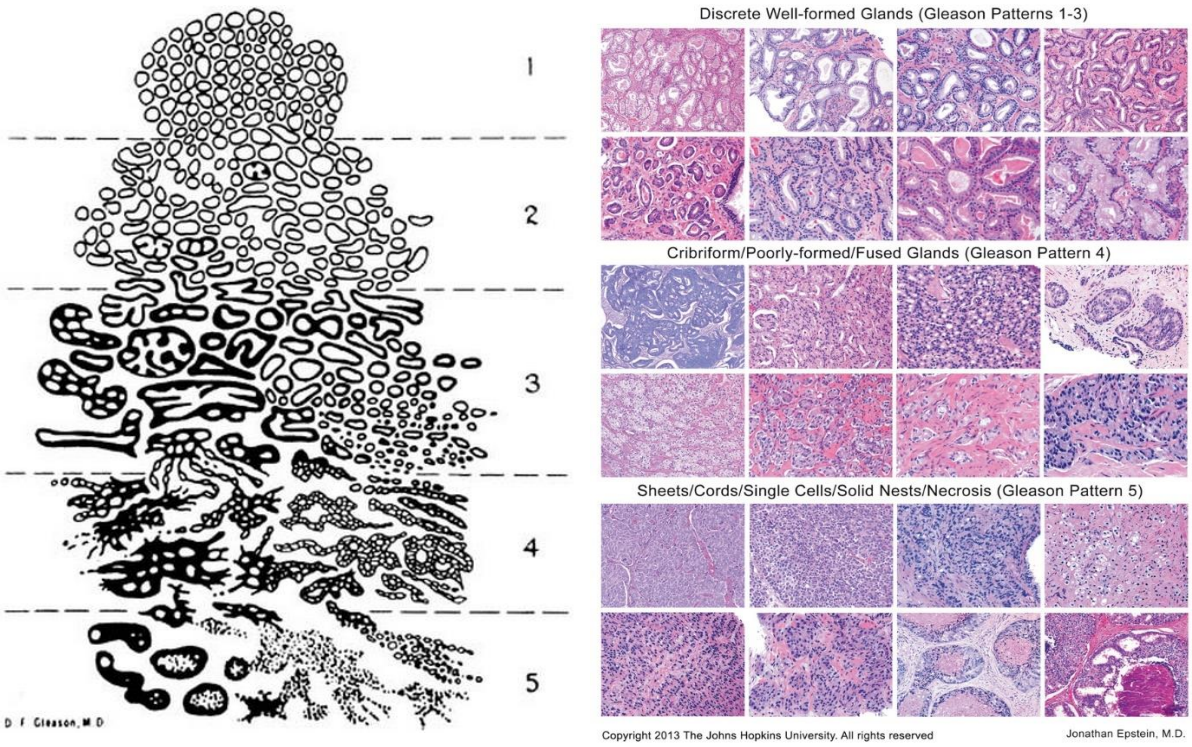

Figure 3. Five histologic Gleason patterns (Gleason 1977; Johns Hopkins Medicine Pathology 2013). Pattern 1 – cancerous tissue resembles normal prostate tissue. Discrete, well-formed glands that are closely packed. Well differentiated carcinoma. Pattern 2 – glands are still well-formed but larger in size and have bigger stroma between them. Moderately differentiated carcinoma. Pattern 3 – variations in glandular size, distinctly infiltrative margins. Moderately differentiated carcinoma. Pattern 4 - irregular masses of atypical cells, poorly formed and fused glands, extensive infiltration into surrounding tissues. Poorly differentiated carcinoma. Pattern 5 – very few or no gland formations at all, sheets of cells. Anaplastic carcinoma.

3. attēls. Piecas histoloģiskās Glīsona pakāpes (Gleason 1977; Johns Hopkins Medicine Pathology 2013). 1. pakāpe – vēža audi līdzinās normāliem prostatas audiem. Diskrēti, labi formēti dziedzeri, kas atrodas tuvu viens otram. Labi diferencēta karcinoma. 2. pakāpe – dziedzeri arvien ir labi formēti, bet ir palielinājies to izmērs un stromas lielums starp tiem ir pieaudzis. Vidēji diferencēta karcinoma. 3. pakāpe – atšķirīga izmēra dziedzeri, attāli infiltratīvas robežas. Vidēji diferencēta karcinoma. 4. pakāpe – neregulāras atipisku šūnu masas, vāji formēti un saplūduši dziedzeri, ekstensīva apkārtesošo audu infiltrācija. Vāji diferencēta karcinoma. 5. pakāpe – dažas vai pilnīgi neviena dziedzeru formācija, šūnu pārpalikumi. Anaplastiska karcinoma.

1.3 Extracellular vesicles

EVs are membrane bound vesicles secreted into the extracellular space by a variety of cells from different organisms, such as prokaryotes, higher eukaryotes and plants (reviewed in Yáñez-Mó *et al.* 2015). EVs have attracted large attention since 2006 – 2007, when it was discovered that they contain RNA and that EVs engage in cell-to-cell communication by transferring different molecules from one to another, affecting recipient cell's functions (Ratajczak *et al*. 2006; Valadi *et al.* 2007; Choi *et al*. 2013). Furthermore, the amount of studies

related to EVs and their functions have significantly increased over the past decade. Nevertheless, it is still unknown in how many physiological processes EVs are involved.

Two theories have been proposed regarding RNA sorting into EVs. First theory suggests that EV cargo largely reflects the content of the parental cell, while the second one proposes that molecules are sorted into EVs selectively not randomly (Nolte-'t Hoen *et al*. 2012; Li *et al.* 2015). Independently of the mechanisms that sorts molecules into EVs, transfer of these enclosed molecules makes EVs important players in cellular communication (Yáñez-Mó *et al.* 2015). EV cargo includes proteins, lipids, ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA), mitochondrial RNA (mtRNA), long non-coding RNA (lncRNA), circular RNA (circRNA) and different sRNA species, such as microRNA (miRNA), Y RNA, vault RNA (vRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and piwi-interacting RNA (piRNA), among others (Nolte-'t Hoen *et al.* 2012; Choi *et al*. 2013; Huang *et al.* 2013; Li *et al*. 2015). In order to compile all this information, Vesiclepedia, database that stores records of molecules detected in EVs, was created and nowadays stores entries for 92897 proteins, 27642 mRNAs, 4934 miRNAs and 584 lipids from 538 studies in 33 different species (Vesiclepedia v3.1 2015).

EVs include a large and heterogeneous population of vesicles, which present different functions, biophysical properties and have been originated by different biogenesis routes. That is why it is difficult to establish a unified vesicle nomenclature (Gould and Raposo 2013). Based on EV approximate size and biogenesis, they have been classified in three different main types – exosomes, microvesicles (MVs) and apoptotic bodies (György *et al*. 2011) (Figure 4; Table 2). Although EVs are often classified based on their size, it is not possible to completely separate different subpopulations and accurately measure their size, as EVs tend to co-precipitate during their isolation process (Soboļevska 2016). Additionally, no consensus has been reached regarding exact size of different EV subpopulations as it tends to overlap. As a result, different size ranges are often being used for the same subpopulations (György *et al.* 2011; Crescitelli *et al*. 2013; Keerthikumar *et al*. 2015). Furthermore, protein markers can only be used to characterize EVs in general instead of uniquely identifying each of the EV types since there is no single EV subpopulation-specific marker (Gould and Raposo 2013; Witwer *et al*. 2013; Yoshioka *et al*. 2013).

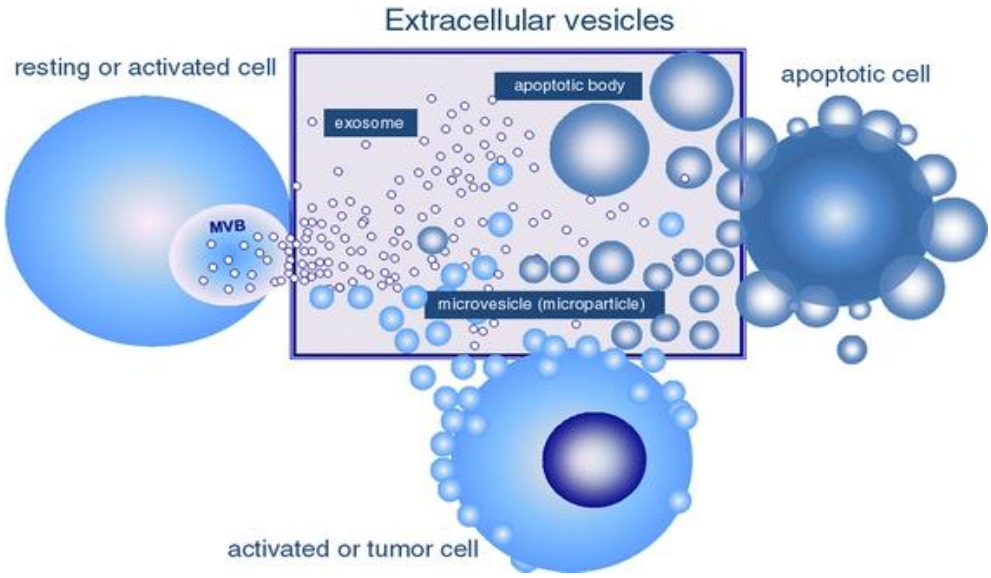

Figure 4. Schematic representation of EV populations (György *et al.* 2011). Main EV populations include exosomes, MVs and apoptotic bodies. *Abbreviations*: MVB - multi-vesicular body.

4. attēls. Shematisks EV populāciju attēlojums (György *et al.* 2011). Lielākās populācijas iekļauj eksosomas, mikrovezikulas un apoptotiskos ķermenīšus. *Apzīmējumi*: MVB - multivezikulārais ķermenītis.

Table 2

Summary of different EV type size, protein markers and biogenesis routes.

2. tabula

Dažādu EV tipu lielums, atbilstošie proteīnu marķieri un bioģenēzes ceļi.

EV type	Approximate size, nm	Protein markers	Biogenesis route	References
Exosomes	40 – 100	CD9 Alix Tsg101	Originated in the multi-vesicular body, which is later released into the extracellular environment upon fusion of multivesicular body with the plasma membrane	Mathivanan and Simpson 2009; Lee *et al.* 2011; Crescitelli *et al.* 2013
MVs (microparticles, ectosomes)	100 – 1000	CD9 Alix Tsg101 Vimentin	Produced by outward budding of plasma membrane followed by fission of their own membrane stalk	Mathivanan and Simpson 2009; Lee *et al.* 2011; Crescitelli *et al.* 2013; Keerthikumar *et al.* 2015
Apoptotic bodies	1000 – 5000	CD9 Vimentin	Released as blebs from apoptotic cells	Hristov *et al.* 2004; Crescitelli *et al. 2013;* Keerthikumar *et al.* 2015

Characterization of EVs is commonly conducted and proteins found in EV subpopulations, such as CD9, Tsg101 and Alix are often used as markers (Witwer *et al.* 2013). Tetraspanin CD9 is a membrane glycoprotein, which has been observed in exosomes, MVs and apoptotic bodies (Crescitelli *et al*. 2013). It is involved in cell differentiation, adhesion and signal transduction (NCBI 2017a). Alix and Tsg101, both cytosolic proteins, are considered to be exosome and MV markers (Lee *et al.* 2011). Alix is involved in intraluminal endosomal vesicle formation and its overexpression can block apoptosis but Tsg101 plays a role in cell growth and acts as a negative growth regulator (NCBI 2017b; NCBI 2017d). Recently it has been shown that vesicles secreted from cell membrane (MVs & apoptotic bodies) are enriched in Vimentin that is a cytoskeletal protein involved in maintenance of cell shape, integrity of cytoplasm and stabilization of cytoskeletal interactions (Keerthikumar *et al.* 2015; NCBI 2017c). However, all of the described protein presence differs in certain EV subgroups. Additionally, study by Tauro *et al*. in 2013 showed that one type of cells could release distinct variants of the same EV subpopulation, each variant expressing a mixture of various different markers (Tauro *et al*. 2013). Therefore, additional studies are required to determine if there could be any protein markers that might be associated with specific EV subpopulations.

EV functions differ based on their enclosed cargo and physiological functions. However, their impact on cell-to-cell communication is still being studied (reviewed in Yáñez-Mó *et al.* 2015). It has been shown that cancer cell-derived EVs enable communication between cancer cells and distant organs thus promoting cancer pre-metastatic niche formation. This process is mediated by EVs in multiple types of cancer, such as lung, breast, skin and prostate (reviewed in Peinado *et al.* 2017). Different reports have shown the role of EVs in stimulating angiogenesis, determining differentiation of bone marrow progenitor cells, transferring oncogenic molecules to lymph nodes and accelerating tumor cell proliferation (reviewed in Fujita *et al.* 2016). EVs can also contribute to DNA and histone modification in cancer microenvironment (Qian *et al*. 2015). Enclosed oncogenic components such as miRNA and proteins can also modulate endothelial cells, fibroblasts and immune cells, which surround the primary tumor (reviewed in Fujita *et al.* 2016).

EVs have been successfully isolated from various biological fluids, such as urine, saliva, plasma, serum, bile, breast milk, nasal and bronchial lavage, amniotic and seminal fluid (reviewed in Yáñez-Mó *et al.* 2015). Urine is considered to be a rich reservoir of EVs, which were first discovered in 2004 (Pisitkun *et al*. 2004). They originate from epithelial cells of the urogenital system, kidney and prostate cells and also infiltrating inflammatory cells (Salih *et al*. 2014; Øverbye *et al*. 2015). Noteworthy, it is believed that there should be no plasma EVs present in the urine as they are unable to pass through the glomerular filtration machinery

(Ranghino *et al*. 2015). Previous studies have shown that in prostate secretions two types of EVs can be found: exosomes, small vesicles with a cup-shaped morphology, actively secreted by normal and tumors cells (Nilsson *et al.* 2009); and MVs, in diameter 150 - 500nm, produced by prostatic ductal epithelial cells; considered to be a normal component of seminal fluid and play a role in male fertility (Burden *et al.* 2006).

EVs in blood were first discovered in 1946 by Chargaff and West and called platelet "dust" (Chargaff and West 1946). Since then, the amount of studies related to blood-derived EVs has only grown as they have been shown to transfer various kinds of molecules from cell to cell thus affecting recipient cell's functions (reviewed in Fujita *et al.* 2016). EVs can be found in both plasma and serum, however multiple studies favor plasma as it has been described as "the physiological fluid of EVs in the blood" (Witwer *et al.* 2013). Usage of serum is not advised due to platelet-derived EV release during blood clotting after blood collection. The composition of plasma-derived EV content and the amount of EVs present is affected by various pathological states (Yáñez-Mó *et al.* 2015). Tumor derived exosomes have been identified in PCa, ovarian, colorectal and other cancer patient plasma, suggesting that they could be used as a non-invasive tool for new biomarker discovery (reviewed in Fujita *et al.* 2016).

1.4 Small RNA

Genomic analyses have shown that only 2% of human genome are protein coding genes, whilst the other 98% are transcribed into non-coding RNA (ncRNA), which can regulate many biological processes, including cell development, differentiation and metabolism (International Human Genome Sequencing Consortium 2004). A large group of ncRNAs are non-coding sRNAs, ranging in size from approximately 18 to 200 nucleotides (nt) (reviewed in Fritz *et al*. 2016). sRNAs regulate gene expression in plants, animals, fungi and also bacteria (Vogel and Wagner 2007; Farazi *et al.* 2008). sRNAs are usually characterized based on their size, precursor structure, biogenesis route, mechanism of action and biological function. Multiple classes of sRNAs, such as miRNAs, piRNAs, snRNAs and snoRNAs have been identified (see Appendix 1) (reviewed in Fritz *et al*. 2016). sRNAs play an important role in the regulation of many cellular processes, including cell differentiation, proliferation, migration, apoptosis as well as metabolism and defense (Zhang 2009). Their mechanisms of action encompass mRNA cleavage, regulation of chromatin structure and translational repression (Farazi *et al.* 2008). Although sRNAs have been intensively studied for almost two decades, new classes of sRNAs as well as new functions and mechanisms of actions of already known sRNAs are still being discovered. In this work, author will focus on studying miRNAs, piRNAs and lncRNAs as more

studies suggest their involvement in PCa progression (Prensner *et al*. 2011; Watahiki *et al*. 2013; Haj-Ahmad *et al*. 2014; Öner *et al*. 2016).

1.4.1 MicroRNA

miRNAs are considered to be the most abundant class of sRNAs in animals. They are a type of endogenous 19 - 22nt long, single-stranded, ncRNA molecules that play an important role in regulation of cellular processes in both animals and plants (Bartel 2004). As of now, miRBase contains information about 1881 precursors and 2588 mature human miRNA sequences (miRbase release 21 2014). Expression profiles of miRNAs are specific to different types of tissues and have been found to be imbalanced in various cancers (Lu *et al.* 2005).

miRNA precursors genes are mostly found within intergenic or intronic regions of the genome and are transcribed by either RNA polymerase II or III (reviewed in MacFarlane and Murphy 2010). miRNAs gene primary transcripts are originated from hairpin-like primary miRNAs (pri-miRNAs). In nucleus, they are cleaved by ribonuclease Drosha and processed into 60 - 90nt long hairpin shaped precursor miRNAs (pre-miRNAs). After processing, pre-miRNAs are exported from the nucleus by Exportin-5. Dicer, cytoplasmic ribonuclease class III enzyme, then cleaves both strands of the pre-miRNA hairpin to form double stranded miRNA duplex. Afterwards one strand is bound by Argonaute 2 protein (Ago2) and miRNA-induced silencing complex (RISC) is formed. RISC can either cleave specific mRNA, repress translation or deadenylate mRNA (see Figure 5) (Krol *et al.* 2004). As a result, miRNAs post-transcriptionally regulate gene expression by targeting mRNAs for translational repression or cleavage resulting in inhibition of translation initiation or degradation of mRNAs (Krol *et al.* 2004). Furthermore, miRNA have also been shown to take part in epigenetic control of gene expression (Carthew and Sontheimer 2009)

Cell-free miRNAs have been recovered from various human body fluids including plasma, urine, breast milk, saliva, colostrum, tears, seminal, cerebrospinal, pleural, amniotic and peritoneal fluids (Weber *et al.* 2010). Saliva, seminal fluid and breast milk are the biofluids where the highest concentration of miRNA has been detected whilst urine, pleural fluid and cerebrospinal fluid present the lowest concentration of miRNAs (Weber *et al.* 2010). Some miRNAs can be found in most of the biofluids, while other miRNAs are biofluid specific and can also be related to particular medical conditions (Weber *et al.* 2010).

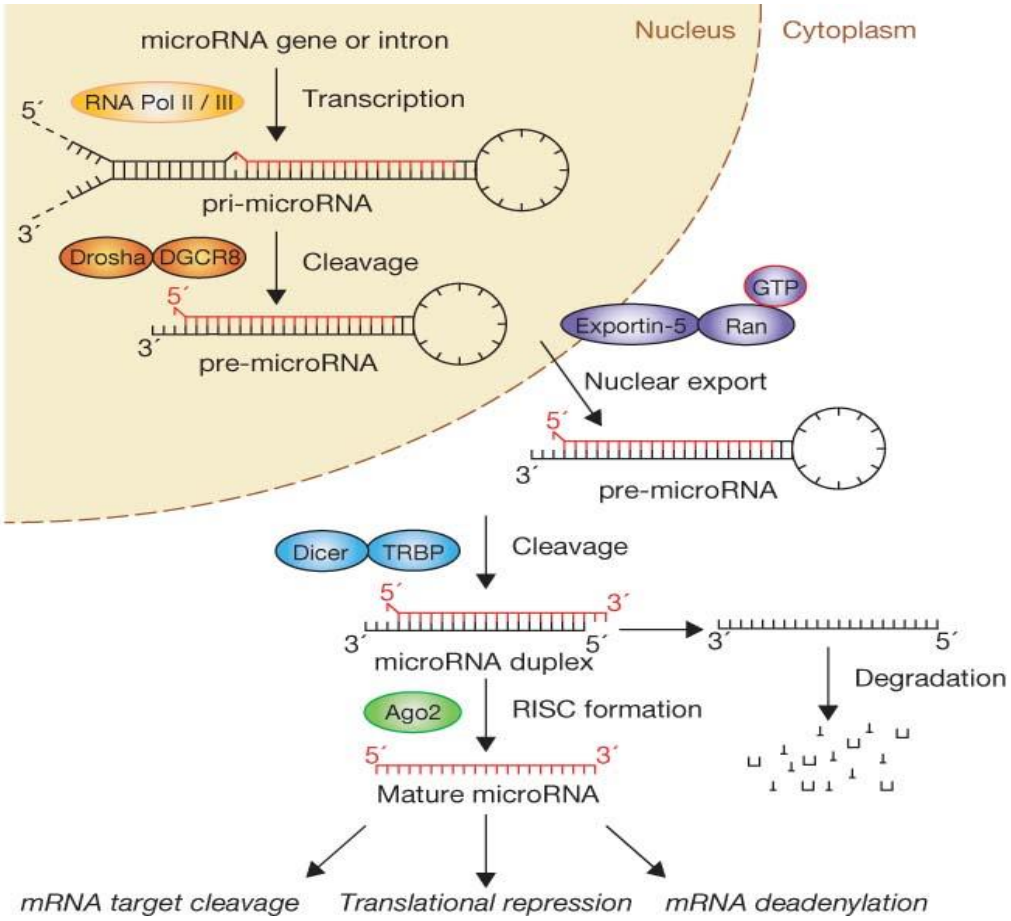

Figure 5. Biogenesis and functions of miRNA in humans (Winter *et al*. 2009). In nucleus microRNA gene or intron is transcribed by either RNA polymerase II or III and pri-microRNA is formed. It is then recognized by nuclear protein DGCR8, which is paired with Drosha enzyme, which cleaves the pri-microRNA, forming hairpin-shaped pre-microRNA. Afterwards, pre-microRNA is exported out of the nucleus into the cytoplasm by Exportin-5 protein, which is energy-dependent and uses GTP bound to a Ran protein. In cytoplasm, the pre-microRNA is cleaved by endoribonuclease Dicer and RNA-binding protein TRBP complex, forming ~ 22nt long microRNA duplex. One strand is bound by Ago2 and incorporated into the RISC. RISC can cleave mRNA, repress translation or deadenylate mRNA. Other microRNA strand undergoes degradation. *Abbreviations:* RNA Pol II/II – RNA polymerase II/III; DGCR8 - DGCR8, microprocessor complex subunit protein; RAN – ras-related nuclear protein; GTP - guanosine-5'-triphosphate; TRBP - Tar RNA binding protein; Ago2 - Argonaute protein 2; RISC – RNA-induced silencing complex.

5. attēls. miRNS bioģenēze un funkcijas cilvēka ķermenī (Winter *et al*. 2009). Kodolā miRNS gēnu vai intronu transkribē RNS polimerāze II vai III un rodas pri-mikroRNS. To vēlāk atpazīst nukleārais proteīns DGCR8, kas ir kopā ar enzīmu Drosha, kurš šķeļ pri-mikroRNS, radot matadatas formas pre-mikroRNS. Pēc tam, ar Exportin-5 proteīna palīdzību, pre-mikroRNS tiek eksportēta ārā no kodola, iekšā citoplazmā. Exportin-5 proteīns ir enerģijas atkarīgs un izmanto GTP, kas saistīts ar Ran proteīnu. Citoplazmā pre-mikroRNS šķeļ endoribonukleāzes Dicer un proteīna TRBP komplekss, radot ~22nt garu mikroRNS dubultķēdi. Vienu no ķēdēm saista Ago2 proteīns un tā tiek iekļauta RISC, kas spēj šķelt mRNS, represēt translāciju un deadenilēt mRNS. Otra mikroRNS ķēde tiek degradēta. *Apzīmējumi*: RNA Pol II/II – RNS polimerāze II/III; DGCR8 -DGCR8, mikroprocesora kompleksa subvienības proteīns; RAN – ras saistīts nukleārais proteīns; GTP – guanozīn-5'-trifosfāts;TRBP – Tar RNS saistošs proteīns; Ago2 - Argonauta proteīns 2; RISC – RNS-inducēts noklusēšanas komplekss.

miRNAs can either exist in a vesicle-free form associated with Ago2, nucleoplasmin, or high-density lipoproteins, but they can also be found packed inside EVs derived from cell culture media and biofluids (Wang *et al*. 2010; Arroyo *et al*. 2011; Vickers *et al*. 2011). The proportion of vesicle-free versus vesicle-enclosed miRNAs in biofluids is still a very controversial topic. A study conducted by Arroyo *et al*. in 2011 showed that only a minority of miRNAs is enclosed in EVs, whilst the rest seem to be associated with Ago2 containing complexes (Arroyo *et al*. 2011). This correlates with the stoichiometric analysis made by Chevillet *et al*. in 2014 where they demonstrated that either there are few miRNA copies per exosome or several exosomes full of miRNAs (Chevillet *et al*. 2014). On the contrary, a study carried by Gallo *et al*. in 2012 demonstrated that miRNA concentration in exosomes was higher than in exosomes-depleted serum and saliva (Gallo *et al*. 2012). Similarly, two different studies, one conducted by Huang *et al*. in 2013 and the other one by Cheng *et al*. in 2014, showed that exosome fraction is enriched in miRNAs (Huang *et al*. 2013; Cheng *et al*. 2014).

Interestingly, miRNAs have been shown to play a major role in the development of serious illnesses, for example, cardiovascular, neurodegenerative, liver, kidney and infectious diseases (Zhang 2009). Moreover, some miRNAs have been proposed to initiate carcinogenesis and/or drive its progression (see Appendices 2-1 to 3-2) (reviewed in Di Leva *et al*. 2014). Furthermore, a large study conducted by Lu *et al*. in 2005 showed that miRNA expression profiles differ significantly among normal and tumor tissues samples in various cancers. Most miRNAs were found to be downregulated in cancer tissue samples. This led to the hypothesis that miRNA expression could be altered by cellular differentiation, which is a hallmark of cancer (Lu *et al*. 2005; Hanahan and Weinberg 2011). Moreover, study by Ozen *et al*. in 2008 found similar pattern of miRNA downregulation in PCa tissue samples when compared with BPH tissue samples (Ozen *et al*. 2008). In contrast, research conducted by Volinia *et al*. in 2006 found many miRNA upregulated in cancer tissue relatively to non-cancer tissue (Volinia *et al*. 2006). Nonetheless, studying miRNAs could help distinguish tumors from normal tissues as well as aid in classifying tumors according to aggressiveness, differentiation state and development lineage. miRNAs levels that correlate with Gleason grade and differ between localized and metastatic prostate cancer have been identified (Watahiki *et al*. 2013). Furthermore, specific miRNA expression levels differ between RNA samples isolated from PCa, BPH and healthy urine samples (Haj-Ahmad *et al*. 2014). Thus, using miRNAs as biomarkers might aid in tumor staging and determining the best treatment method for the patient.

1.4.2 Piwi-interacting RNA

An emerging and novel class of sRNAs are piRNAs, 24 to 32nt short non-coding single-stranded sRNAs, which bind to PIWI proteins, an Argonaute family subclass (Aravin *et al.* 2006; Girard *et al*. 2006; Grivna *et al*. 2006). piRNAs are transcribed from intergenic piRNA clusters and unlike during miRNA biogenesis, Dicer is not needed for their processing (Vagin *et al*. 2006). Although the full mechanism of piRNA biogenesis is not yet fully understood, it is suggested that two different pathways (primary piRNA processing pathway and ping-pong cycle) are involved in generating them. In the primary pathway piRNA precursor transcript is exported from the nucleus and then cleaved by an endonuclease and loaded onto PIWI proteins. During ping-pong cycle, the previously generated piRNA is being amplified (reviewed in Siomi *et al*. 2011). Alike other RNAs, piRNAs have been isolated from various kinds of human tissue, cell lines and also salivary, serum and urinary EVs (Cheng *et al*. 2011a; Huang *et al*. 2013; Ogawa *et al*. 2013; Li *et al*. 2014; Iliev *et al.* 2016).

piRNAs were first discovered in 2006 and since then, they have attracted a lot of attention due to theirs and PIWI protein functional roles in germline development as "selfish genetic element silencers" and their possible functions in tumorigenesis (Aravin *et al*. 2006; Girard *et al*. 2006; Grivna *et al*. 2006; Vagin *et al*. 2006; reviewed in Klattenhoff and Theurkauf 2008, Assumpção *et al*. 2015). Specifically, piRNA-PIWI complexes have been shown to function mainly as transposon repressors and mRNA regulators. Furthermore, these complexes have been proposed to mediate transgenerational epigenetic silencing in *Caenorhabditis elegans (C. elegans)* and *Drosophila melanogaster* (*D. melanogaster*). However, effects in mammals have not been reported yet (reviewed in Weick and Miska 2014). In 2003 Sasaki *et al*. identified four human PIWI proteins (PIWIL1/HIWI, PIWIL2/HILI, PIWIL3, PIWIL4/HIWI2) and it has been shown that they are mainly expressed in stem cells, germ cells and various cancers (Sharma *et al*. 2001; Qiao *et al*. 2002; Sasaki *et al.* 2003; reviewed in Suzuki *et al.* 2012).

Studies related to PIWI protein expression and roles in human cancers are only now emerging (reviewed in Suzuki *et al*. 2012). For instance, all human PIWI proteins have been found overexpressed in gastric cancer tumor tissues in comparison with non-tumor tissues (Wang *et al.* 2012). *PIWIL2* gene expression has been shown in wide range of human cancers, including PCa, where it has been proposed to act as an oncogene by inhibiting apoptosis and enhancing cell proliferation (Lee *et al*. 2005). Furthermore, in PCa *PIWIL2* expression has been shown to correlate with GS and TNM stages (Yang *et al*. 2015). Moreover, it contributes to tumor aggressiveness and its progression (Yang *et al*. 2015). Not only PIWI family proteins and their corresponding genes, but also piRNAs have been speculated to affect tumorigenesis

(reviewed in Assumpção *et al*. 2015). As of now, most studied cancer related piRNAs are piR-823 and piR-651. piR-823 has been demonstrated to be downregulated in gastric cancer and renal cell carcinoma (RCC) tissues when compared to non-cancerous tissues (Cheng *et al*. 2011b, Iliev *et al*. 2016). However, piR-823 has been found highly expressed in RCC patient biofluids in comparison with healthy donors (Iliev *et al*. 2016). Regarding piR-651, it has been shown to be upregulated in colon, lung and breast cancer tissue samples and to promote cancer cell growth (Cheng *et al*. 2011a). Interestingly, piR-651 expression seems to be affected by hormone variations as it has been shown that hormone treatment highly increases its expression in both, hormone-dependent and hormone-independent breast cancer and PCa cells (Öner *et al*. 2016). Based on all this evidence and as recent studies suggest, piRNAs could be promising cancer biomarkers. Nevertheless, functional studies related to the mechanisms through which piRNAs affect tumorigenesis are still scarce.

1.5 Long non-coding RNA

lncRNAs are ncRNA transcripts larger than 200nt that have been observed in different animal, plant, yeast and prokaryote species (Mercer *et al.* 2009; Ma *et al*. 2013). In comparison to the previously mentioned small ncRNAs, most lncRNA sequences are poorly conserved (Mercer *et al*. 2009). Similar to mRNAs, lncRNA genes are transcribed by RNA polymerase II, usually 5' capped, spliced, polyadenylated and despite of their name, some indeed do encode proteins (Schmitz *et al*. 2016).

Different lncRNA classification criteria and features have been proposed, though no consensus has yet been reached due to their complex biogenesis and length variations (reviewed in St Laurent *et al*. 2015). For example, based on their origin from multiple genomic locations, lncRNA could be classified as intergenic (lincRNA) – transcribed from intergenic DNA; intronic – transcribed from introns of protein-coding genes; sense or antisense – transcribed from the sense or antisense strand of protein-coding genes thus containing exons from the sense or antisense genes respectively (Ma *et al*. 2013).

At present, 118 777 lncRNAs have been annotated, although only 184 of them are known to be functional (lncRNAdb v2.0 2015; LNCipedia 2016). These RNAs are involved in a variety of functions and they have been shown to regulate gene expression at various stages (summarized in Figure 6) (reviewed in Schmitz *et al.* 2016). For instance, it has been proven that lncRNAs mediate transcription by acting as decoys for transcription factors (TF) thereby disabling their binding to the DNA targets (reviewed in Hu *et al*. 2012). Nonetheless, lncRNA can also bind TFs and promote their docking to target sites or recruit activating or repressing chromatin-modifying complexes to specific genomic loci by that promoting or inhibiting gene

expression (see 1&4, Figure 6) (Fatica and Bozzoni 2014; Xing *et al.* 2014). Furthermore, lncRNAs can act as miRNA sponges by mimicking miRNA target sites. In addition, lncRNAs can bind to regulatory proteins and form ribonucleoprotein (RNP) complexes (see 2&3, Figure 6) (reviewed in Hu *et al.* 2012). Additionally, few lncRNAs directly affect mRNA targets, which results in translation repression, mRNA splicing modulation or mRNA degradation (see 5-7, Figure 6) (reviewed in Hu *et al.* 2012). Moreover, lncRNAs have been also described as miRNA and piRNA precursors (Keniry *et al.* 2012; Ha *et al.* 2014). For instance, miR-675, a miRNA involved in tumor suppression, is derived from lncRNA H19 (Keniry *et al.* 2012). As lncRNAs possess ability to regulate many processes occurring in the cell, they have been hypothesized to be also capable of exerting negative impact on the cell by promoting tumorigenesis, cancer progression and metastasis (Fatima *et al.* 2015).

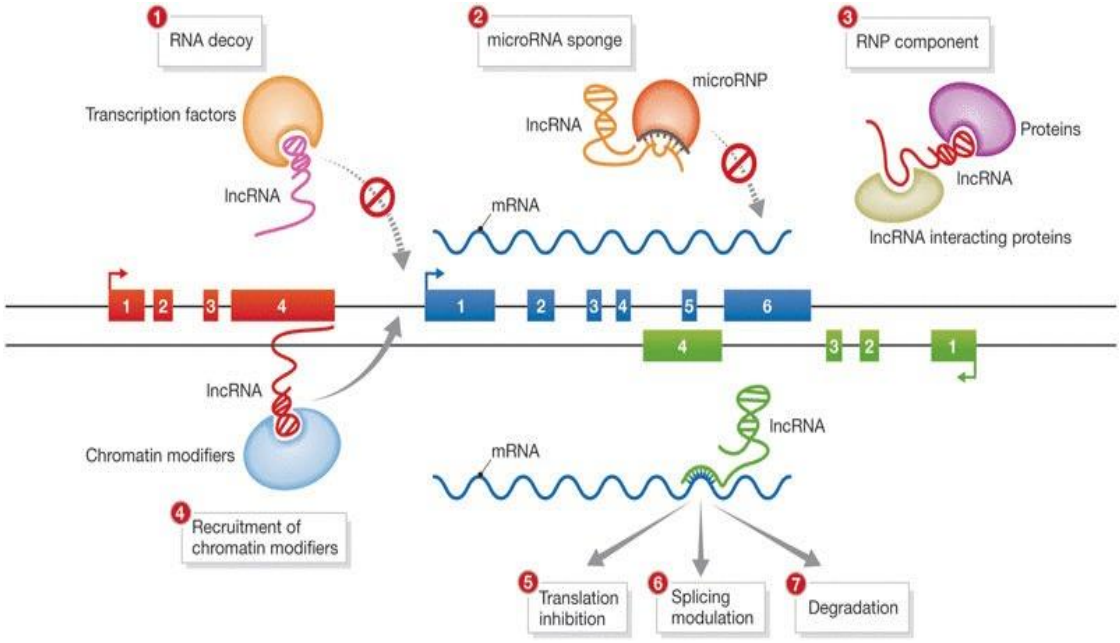

Figure 6. Schematic representation of different lncRNA functions (Hu *et al.* 2012). lncRNA binds to transcription factors and acts as decoy RNA (1). lncRNA acts as miRNA sponge by binding to microRNP and inhibiting its binding to target mRNA (2). Together with proteins, lncRNAs form RNP complexes (3). lncRNA affects chromatin modification by recruiting activating or repressing chromatin-modifying complexes (4). lncRNA interact directly with mRNA resulting in translation inhibition (5), splicing modulation (6) or mRNA degradation (7). Note: not all lncRNA functions are shown. *Abbreviations:* lncRNA – long non-coding RNA, mRNA – messenger RNA, RNP – ribonucleoprotein.

6. attēls. Shematisks dažādu garo nekodējošo RNS funkciju attēlojums (Hu *et al.* 2012). lncRNS saistās ar transkripcijas faktoriem un darbojas kā mānekļa RNS (1). lncRNS darbojas kā miRNS sūklis, saistoties ar mikroRNP un inhibējot tā saistīšanos ar mērķa mRNS (2). Kopā ar proteīniem, lncRNS veido RNP kompleksus (3). lncRNS ietekmē hromatīna modificēšanu, piesaistot aktivējošus vai inhibējošus hromatīnu modificējošus kompleksus (4). lncRNS tiešā veidā iedarbojas uz mRNS, kā rezultātā notiek translācijas inhibīcija (5), splaisinga modulēšana (6) vai mRNS degradācija (7). Piezīme: attēlā nav parādītas visas lncRNS funkcijas. *Saīsinājumi*: lncRNA – garā nekodejošā RNS, mRNS – matrices RNS, RNP – ribonukleoproteīni.

Different lncRNAs have been associated with cancer and several of them might be key players in its progression (reviewed in Fatima *et al.* 2015). Whilst various lncRNAs seems to have a potential role in oncogenesis, others might be involved in tumor-suppressive pathways and only promote cancer upon downregulation (Qi and Du 2013). In fact, it has been shown that specific lncRNAs contribute to breast, gastric, ovarian, bladder, lung, colorectal and pancreatic cancer drug resistance, making them potential targets for therapeutic applications (reviewed in Deng *et al.* 2016). Recent studies have discovered some PCa-specific lncRNAs, such as PCA3, also known as DD3 (Bussemakers *et al.* 1999; Tinzl *et al.* 2004). PCA3 has been found overexpressed in urine collected from PCa patients and also overexpressed in PCa tissue samples when compared to healthy specimens (Bussemakers *et al.* 1999; Tinzl *et al*. 2004). Furthermore, a large study by Prensner *et al*. in 2011 identified PCAT1 as the best candidate among 121 other PCa-linked lncRNAs (Prensner *et al*. 2011). PCAT1 has been proposed as a repressor of tumor suppressor and DNA repair genes and as inhibitor of homologous recombination (Prensner *et al*. 2011; Prensner *et al*. 2014). Multiple other lncRNAs, such as H19, MALAT1, PCGEM1 and GAS5, affect cellular pathways and alteration of their functions has been shown to promote PCa development and progression (reviewed in Martens-Uzunova *et al*. 2014).

It has been observed that lncRNAs, likewise the other RNAs, can be contained in EVs and transferred from one cell to another (Huang *et al*. 2013). EVs containing TUC339, a lncRNA involved in cancer cell proliferation, have been isolated from culture medium of hepatocellular carcinoma cells (Kogure *et al.* 2013). Furthermore, expression of a lncRNA HOTAIR, which is associated with increased cancer invasiveness and poor prognosis, correlates in bladder cancer patient urinary exosomes and corresponding patient tissue (Berrondo *et al*. 2016). It has also been shown that lncRNAs are enriched in cancer cell-derived exosome RNA when compared to RNA isolated from the cancer cells directly, suggesting selective lncRNA packing into exosomes (Gezer *et al.* 2014). Regarding PCa, expression levels of lncRNA-p21 have been observed to be higher in PCa-derived urinary exosomes in comparison to BPH-derived urinary exosomes, proposing that lncRNA-p21 could be used to distinguish PCa from BPH (Işın *et al*. 2015). Correspondingly, further studying lncRNA and their functions could lead to new diagnostic and prognostic PCa biomarker discovery.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Clinical samples

This study was performed according to the Declaration of Helsinki and was approved by the ethical review board of Riga East University Hospital. The enrollment criteria included that (1) patient has been diagnosed with PCa based on histopathological result after prostate biopsy and that (2) radical prostatectomy has been scheduled. Patient exclusion criteria included (1) another oncological illness, (2) chemotherapy, radiation or hormonal therapy done prior to the study, (3) urinary tract infection, (4) blood or its component transfusion in the past six months, (5) treatment involving usage of 5α-reductase inhibitor drugs and/or (6) long-term urinary catheter use prior to operation. After informed consent, urine, blood, PCa tumor and non-tumor tissue samples were collected from four individual PCa patients (Table 3). Sample collection was executed at different time points (Figure 7) by specialists in Riga East University Hospital.

Table 3
Patient data.
3. tabula
Dati par pacientiem.

Patient	P38	P56	P57	P60
Age	65	58	57	53
PSA value before operation, ng/ml	6,67	12,60	7,00	7,55
Biopsy GS	4 + 5 = 9	3 + 3 = 6	3 + 4 = 7	3 + 2 = 5
Tissue GS	4 + 5 = 9	4 + 5 = 9	4 + 4 = 8	3 + 4 = 7

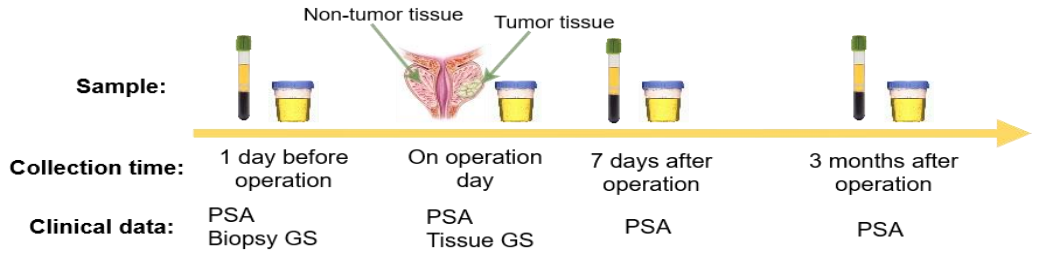

Figure 7. Summary of sample collection scheme. Urine was collected one day before operation, on operation day, seven days and three months after operation. Plasma was collected before operation, seven days and three months after operation. Non-tumor and tumor tissues were collected on operation day. Clinical data from patients included PSA levels, biopsy and tissue GS. *Abbreviations*: PSA – prostate specific antigen, GS – Gleason score.

7. attēls. Paraugu ievākšanas shēmas kopsavilkums. Urīns tika ievākts vienu dienu pirms operācijas, operācijas dienā, septiņas dienas un trīs mēnešus pēc operācijas. Plazma tika ievākta vienu dienu pirms operācijas, septiņas dienas un trīs mēnešus pēc operācijas. Pacientu klīniskajos datos tika iekļauts PSA līmenis, biopsijas un audu Glīsona skaitlis. Apzīmējumi: PSA - prostatas specifiskais antigēns, GS – Glīsona skaitlis.

Approximately 60ml of urine were collected per patient per time, centrifuged at 2000x*g* for 15 minutes at room temperature (RT) to eliminate cell debris, aliquoted and stored at -80°C until use. 10ml of blood were collected per patient per time and centrifuged at 1500x*g* for 10 minutes at RT to separate plasma fraction. After plasma fraction acquisition, it was centrifuged at 2000x*g* for 10 minutes at RT to eliminate cell debris, aliquoted and stored at -80°C. PCa tumor and non-tumor tissues were collected after radical prostatectomy by pathologist. Small section of each tissue sample was sliced, stained and assessed by pathologist in order to confirm or deny the tumor or non-tumor nature of the collected tissue. Tissues were immersed in RNAlater Stabilization Solution right after collection and stored at -20°C to preserve RNA integrity.

For RNA sequencing (RNA-seq) analysis were used: 40ml of each urine sample collected before operation and/or on operation day and seven days after operation; 3ml of each plasma sample collected before operation and seven days after operation; 20mg of PCa tumor and non-tumor tissue sample. However, for PCa miRNA biomarker validation experiments only 20ml of each urine sample, 0.5ml of each plasma samples, including ones collected 3 months after operation, and 20mg of PCa tumor and non-tumor tissues were used. Metastatic PCa cell line PC3 (ATCC® CRL-1435™, USA) was used as a positive control in WB analysis.

2.1.2 Chemical reagents

Table 4
Used chemical reagents and their producer.
4. tabula
Izmantotie ķīmiskie reaģenti un to ražotājs.

Chemical reagents	Producer
Acetic acid (99,8%)	Lach-ner, Czech Republic
Acrylamide	Roanal, Hungary
Ammonium persulfate (APS)	Bio-Rad Laboratories, USA
Bis-acrylamide (Bis-acrylamide crosslinker)	Bio-Rad, USA
Bromophenol blue	Sigma-Aldrich, USA
Carestream® Kodak® autoradiography GBX developer and replenisher	Sigma-Aldrich, USA
Chloroform	Bárta a Cihlář, spol. s r.o., Czech Republic
Diethyl dicarbonate (DEPC)	Alfa Aesar, USA
Ethanol (96%)	РЕАХИМ, Russia
Ethanol for molecular biology (100%)	Merck, Germany
ExiLENT SYBR® Green master mix	Exiqon, USA
Glycerol	Merck, Germany

Glycine	SIA “Unikems”, Latvia
Hydrochloric acid (HCl)	Merck, Germany
Kodak® READYMATIC Dental fixer and replenisher	Carestream Health, France
Marvel Original dried skimmed milk	Marvel, USA
N,N,N’,N’,-tetra-methylethylenediamine (TEMED)	Bio-Rad Laboratories, USA
PageRuler™ Prestained Protein Ladder	Thermo Fisher Scientific, USA
Phosphate buffered saline (PBS)	Amresco, USA
Ponceau-S	Reanal, Hungary
Proteinase K Solution (20 mg/ml)	Thermo Fisher Scientific, USA
RNAlater® Stabilization Solution	Thermo Fisher Scientific, USA
RNase A (10 mg/ml)	Thermo Fisher Scientific, USA
RNaseZap® RNase Decontamination Solution	Ambion®, Thermo Fisher Scientific, USA
Sepharose™ CL-6B	GE Healthcare Bio-Sciences AB, Sweden
Sodium azide (Na-azide)	Merck, Germany
Sodium chloride (NaCl)	VWR, USA
Sodium deoxycholate (Na deoxycholate)	Sigma-Aldrich, USA
Sodium dodecyl sulfate (SDS)	Merck, Germany
Tris(hydroxymethyl)aminomethane (Tris)	VWR, USA
Triton X-100	Intertechnique, France
Tween® 20	Sigma-Aldrich, USA
β-mercaptoethanol (2-mercaptoethanol)	Serva Electrophoresis GmbH, Germany

2.1.2.1 Antibodies

Table 5
Used antibodies, host species and producer.
5. tabula
Izmantotās antivielas, organisms, kurā tās producētas, un ražotājs.

Antibody	Dilution factor	Host species	Producer
Alix Antibody (G-10): sc-166952	1:1000	Mouse	Santa Cruz Biotechnology, USA
Anti-Calnexin antibody (ab22595)	1:1000	Rabbit	Abcam, UK
Anti-TSG101 antibody (ab125011)	1:1000	Rabbit	
CD9 Antibody (C-4): sc-13118	1:500	Mouse	Santa Cruz Biotechnology, USA
Goat Anti-Rabbit IgG, F(ab’)$_2$-HRP: sc-3837	1:2000	Goat	
m-IgGκ BP-HRP: sc-516102	1:2000	Goat	
Vimentin Antibody (H-84): sc-5565	1:1000	Rabbit	

2.1.2.2 Reagent kits

Table 6
Reagent kits used in experiments.
6. tabula
Eksperimentos izmantotie reaģentu komplekti.

Reagent kit	Producer
3% Agarose Gel Cassettes, dye-free, 100-250bp, BluePippin	Sage Science, USA
Agilent High Sensitivity DNA Kit	Agilent Technologies, USA
Agilent Small RNA Kit	
Ion PI™ Chip Kit v3	Thermo Fisher Scientific, USA
Ion PI™ Hi-Q™ OT2 200 Kit	
Ion PI™ Hi-Q™ Sequencing 200 Kit	
Ion Sphere™ Quality Control Kit	
Ion Total RNA-Seq Kit v2	
Ion Xpress™ RNA-Seq Barcode 1-16 Kit	
miRNeasy Micro Kit	Qiagen, Germany
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, USA
RNase-Free DNase Set	Qiagen, Germany
Western Blotting Detection Reagent kit	GE HealthCare Lifesciences, Germany
UniSp6 LNATM PCR primer set V2, UniRT	Exiqon, USA
Universal cDNA Synthesis Kit II	Exiqon, USA

2.1.2.3 Solutions, buffers and gels

10% APS: 0.1g APS, 1ml distilled water.

10% milk: 1g milk powder – Marvel Original dried skimmed milk, 10ml 1xTBS + 0.05% Tween.

10% SDS: 10g SDS, fill up the rest to 100ml with distilled water.

10% separating gel for WB: 4.4ml distilled water, 3.67ml 30% acrylamide, 2.75ml lower Tris buffer (pH 8.8), 110µl 10% SDS, 110µl 10% APS, 11µl TEMED.

10x sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer: 30.3g Tris, 144g glycine, 10g SDS, fill up the rest to 1000ml with distilled water.

1x Tris buffered saline (TBS) + 0.1% Tween: 10ml 20xTBS, 200µl Tween® 20, add distilled water until 200ml.

1x TBS + 0.05% Tween: 10ml 20xTBS, 100µl Tween® 20, add distilled water until 200ml.

20x TBS: 48g Tris, 175.3g NaCl, add distilled water until 1000ml. Using concentrated HCl lower the pH to 7.5.

30% acrylamide: 29.2g acrylamide, 0.8g bis-acrylamide, fill up the rest to 100ml with distilled water. Store at +4°C.

4x Laemmli Sample buffer: 1ml glycerol, 3ml 10% SDS, 1.25ml Tris+10%SDS pH 6.8, 20% β-mercaptoethanol, 100µl bromophenol blue.

6% stacking gel for WB: 3.225ml distilled water, 1ml 30% acrylamide, 625µl upper Tris buffer (pH 6.8), 50µl 10% SDS, 50µl 10% APS, 5µl TEMED.

DEPC–treated water: dissolve 500µl of DEPC commercial reagent in 500ml distilled water. Leave overnight in fume hood, then autoclave.

Lower Tris buffer (pH 8.8): 36.3g Tris, 8ml 10% SDS, add distilled water until 180ml, using concentrated HCl lower pH to 8.8, fill up the rest to 200ml with distilled water.

PBS solution: dissolve one PBS tablet in 100ml of distilled water, autoclave.

Ponceau-S staining buffer: 0.1g Ponceau-S, 500µl acetic acid, add distilled water until 50ml.

Radioimmunoprecipitation assay buffer (RIPA buffer): 150 ml NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1 % SDS, 50 ml Tris.

Upper Tris buffer (pH 6.8): 6.06g Tris, 4ml 10% SDS, add distilled water until 90ml, using concentrated HCl lower the pH to 6.8, fill up the rest to 100ml with distilled water.

WB transfer buffer: 14.4g glycine, 3g Tris, 100ml methanol, 900ml distilled water.

2.1.3 Apparatus and equipment

Table 7
Used apparatus and equipment.
7. tabula
Izmantotā aparatūra un aprīkojums.

Apparatus	Description
10ml syringe	Jiangsu Kanghua Medical Equipment Co., China
Autoclave	Labo Autoclave MLS-3020U, SANYO, Japan
Bioanalyzer	Agilent 2100 Bioanalyzer, Agilent Technologies, USA
Centrifugal filter units with membrane (concentration tubes)	Amicon® Ultra – 15 ml, Ultracel® 100K, Merck Milipore, Ireland; Amicon® Ultra – 0,5 ml, Ultracel® 3K, Merck Milipore, Ireland;
Centrifuges	Microspin-FV-2400, Biosan, Latvia; Sigma 1-15PK, DJB Labcare, UK; Eppendorf® Microcentrifuge 5415, Sigma-Aldrich, USA
Chromatography Frits	TELOS™ 20 µm Polyethylene Frits, Kinesis, UK
DNA size selection system	BluePippin System, Sage Science, USA

Electrophoresis apparatus	Mini-PROTEAN Tetra Cell PowerPac Basic, BIO-RAD, USA
Emulsion PCR system	Ion OneTouch™ 2 System, Thermo Fisher Scientific, USA
Filtration columns	TELOS™ SPE filtration column 15 ml, Kinesis, UK
Homogenizer	FastPrep®-24 Classic Instrument, MP Biomedicals, USA
Hypercassette	Amersham Biosciences,UK
Hyperfilm (photographic paper)	Amersham Hyperfilm™ ECL, GE HeatlhCare Lifesciences, Japan
Ice block	Eppendorf, Germany
Incubator	Ecotron, Inforst HT, Switzerland
Laminar	Biowizard, KOJAIR®, Germany
Lysing matrix tubes	Lysing Matrix A, 2ml tubes, MP Biomedicals, USA
Magnetic stirrer	Labmixer Magnetic stirrer, Labotex
Microplate spectrophotometer	μQuant™, BioTek® Instruments, USA
Nanoparticle tracking systems	Zetasizer Nano ZS, NanoSight NS500, Malvern Instruments, UK
Optical adhesive film	MicroAmp® Optical Adhesive Film, Thermo Fisher Scientific, USA
Orbital shaker	Orbital Shaker OS-20, Biosan
Parafilm M®	Bemis, USA
pH meter	Ph/mV^0/C meter, 510, Oakton, Singapore
RNaseZap® RNase Decontamination Wipes	Ambion®, Thermo Fisher Scientific, USA
RT-qPCR plates	Pick-&-Mix microRNA PCR Panel, 96 well Ready-to-Use (RO), Exiqon, USA
RT-qPCR system	ViiA 7 Real-Time PCR System, Thermo Fisher Scientific, USA
Sequencing systems	Ion Proton™ System, Thermo Fisher Scientific, USA; Illumina HiSeq 2500 System, Illumina, USA
Thermocycler	GeneAmp PCR System 9700, Thermo Fisher Scientific, USA
Thermostat	Thermo Block TDB-120, Biosan
Trans-Blot® Pure Nitrocellulose Membrane	Bio-Rad Laboratories, USA
Transmission electron microscope	JEOL JEM-1230, JEOL, USA
Vortex mixers	Vortex-Genie® 2, Scientific Industries, USA; Bio Vortex V1, Biosan; IKA® MS3 Vortexer, Germany
Water bath	2219 Multitemp II, LKB Bromma, Sweden
Whatman filter paper	GE HealthCare Lifesciences, Germany

2.1.4 Safety precautions

It is advised to wear protective clothing, namely, gloves, laboratory coat and goggles to prevent chemical substances from getting on clothing, on the skin and into the eyes. Avoid exposure to UV light while working in laminar.

DEPC should be handled with care, as it is toxic and harmful if swallowed, inhaled or absorbed through skin. Upon contact with eyes, skin, mucous membranes or upper respiratory tract DEPC causes irritation.

Extreme caution should be used when working with APS, TEMED, acrylamide, β-mercaptoethanol and Na-azide.

APS dusts cause airway irritation when inhaled. Contact can irritate skin, eyes, nose and throat. It acts as a strong oxidizer and high exposure may cause a build-up fluid in the lungs. To avoid contact use gloves and work in fume hood while handling the substance.

TEMED is flammable. Contact with skin can cause burns and its vapor may irritate the airways. It is advised to work in fume hood and wear gloves while using the substance.

Acrylamide is cancerogenous and easily adsorbed by the skin. In addition, acrylamide can cause irritation and is considered to be neurotoxic. Use gloves when handling the substance.

β-mercaptoethanol is a toxic substance that causes irritation to the respiratory tract and nasal passageways when inhaled. Upon ingestion, vomiting and stomach pain occurs. It can cause lethal outcome if severe exposure happens.

Na-azide is highly toxic poison. It can cause hypotension, blindness and necrosis and may be fatal upon contact with skin or if swallowed. It is mandatory to wear gloves, laboratory coat and work in fume hood when handling the substance.

Chloroform's vapor can cause dizziness and upon oral or dermal exposure or inhalation, it is well absorbed and metabolized. High exposures can contribute to kidney and liver damage. It is advised to work in fume hood to avoid vapor inhalation.

When working with any kind of concentrated acids use extreme caution. Upon contact with skin acids cause skin burns.

2.2 Methods

2.2.1 Size exclusion chromatography

SEC was used in order to isolate EVs from urine and plasma samples as it has been previously shown that SEC effectively isolates high purity EVs (Böing *et al*. 2014). SEC was performed using heteroporous beads constructed of a neutral, cross-linked polymeric support packed into a column. Each molecule in the sample solution passes through these porous beads

based on their hydrodynamic radii resulting in different-sized molecule separation (Bhatia 2005). In the experiment, TELOS^{TM} SPE filtration columns filled with Sepharose CL-6B were used.

15ml of sepharose CL-6B were poured in a beaker, stirred and let to settle for 60 minutes. Afterwards, ethanol was removed and sepharose was washed twice using 10ml of PBS-DEPC solution. TELOS^{TM} SPE filtration column was placed on a holding rack and degassed using 10ml PBS-DEPC solution and a syringe plunger. Then column was filled with 10ml of sepharose. Frit was degassed by placing it in 10ml syringe and passing 10ml of PBS-DEPC solution through it. Afterwards, frit was removed from syringe and carefully placed on top of the sepharose. Column was capped. Additional PBS-DEPC solution was added to avoid sepharose dehydration. Column was immediately used or washed with 0.05% Na-azide PBS solution prior storage at +4°C for later use.

To isolate EVs from urine and plasma samples, filtration column was mounted in a holding rack and washed with 10ml of PBS-DEPC solution. 500µl of sample was added to the column directly after PBS-DEPC solution had drained through the frit. 16 sample fractions of 250µl were collected. Column was kept filled with PBS-DEPC solution while eluting to avoid drought. In cases where the initial sample volume exceeded 1ml, sample was concentrated until 500µl at 14000x*g* at +4°C using Amicon 15ml 100K concentration tubes. Collected sample fractions were stored at +4°C for Zetasizer Nano ZS measurement.

2.2.2 Nanoparticle tracking techniques

To measure particle size in different SEC fraction samples, Zetasizer Nano ZS (red badge) with Zetasizer Software v7.11 was used. Zetasizer is a system capable of measuring size, intensity, volume and number distributions and zeta potential of colloids and nanoparticles. Instrument has coupled a 633nm "red" laser and its size working range is from 0,6nm to 6µm (Malvern Instruments 2017a).

Sample was vortexed and 20µl of it was pipetted in a previously washed cuvette. Cuvette was placed inside the Zetasizer Nano ZS and measurement was made using the following settings: +25°C, measurement cycle number 10 and measurement cycle repeat number 2. After each measurement cuvette was washed. After all of the fractions were measured, those which contained more than 70% of particles bigger than 40nm, were joined together. Sample was concentrated until 100µl at 14000x*g* at +4°C using Amicon 0.5ml 3K concentration tubes.

In order to characterize EVs in concentrated samples, they were analyzed using NanoSight NS500 with NanoSight NTA Software v3.0. To define and quantify the amounts of particles present in the sample, NanoSight applies light scattering properties and Brownian

motion. Instrument is capable of measuring size and total particle concentration liquid suspensions as well as visualizing populations of nanoparticles from 10 to 2000nm (Malvern Instruments 2017b). NanoSight measurement data was obtained at Norwegian Radium Hospital (Oslo, Norway) by specialists.

2.2.3 Transmission electron microscopy

TEM is a commonly used method to study structure and morphology of different solid and soft materials, for example, proteins and vesicles. Method uses a high voltage beam of accelerated electrons and electromagnetic lenses to obtain image of the object's surface (Hayat 1970). In the experiment, TEM was used to detect EVs presence based on their visual characteristics. Experimental data was obtained by a TEM specialist. The followed protocol was based on a study carried out by Zhang *et al.* in 2013 (Zhang *et al.* 2013).

2.2.4 Bicinchoninic acid assay

In order to determine total EVs and PCa cell sample protein amounts for following WB analysis, bicinchoninic acid assay (BCA) was performed. The method relies on reduction of Cu^{+2} ions to Cu^{+1} ions, which occurs due to the presence of peptides in an alkaline medium. As a result, chelation of two molecules of bicinchoninic acid with Cu^{+1} ions form a purple colored complex that strongly absorbs light at 562nm wavelength and which absorption can be measured (Smith *et al.* 1985). BCA was done using Pierce™ BCA Protein Assay Kit following manufacturer's instructions. Samples were lysed in 1x RIPA buffer.

2.2.5 Western blot

To confirm that EVs have been successfully isolated from urine and plasma samples, WB, also known as protein immunoblot, was used. Method relies on protein separation by their charge and molecular weight using SDS-PAGE, protein transfer from gel to nitrocellulose membrane, blocking and protein detection with specific antibodies (Moore 2009).

WB was done following a modified previously established protocol (Mahmood and Yang 2012). SDS-PAGE was carried out using 10% separating gel and 6% stacking gel at +4°C at constant current of 150V until all proteins were well spread. 10µg total protein for each sample was diluted in Laemmlli buffer. After electrophoresis finished, samples were transferred from the gel onto a nitrocellulose membrane overnight at +4°C at constant current of 30V. After transfer was completed, membrane was stained using Ponceau-S to assess transfer success. Later, membrane was blocked in 10% milk for 1 hour on orbital shaker at 60 rpm to prevent unspecific antibody binding. Afterwards, membrane was incubated with primary antibody

diluted in 5% milk and secondary antibody diluted in 5% milk for 1 hour on orbital shaker at 60 rpm at RT. Antibody dilution factors are shown in Table 5 on page 28. Primary and secondary antibody excess was removed after each incubation through consecutive washes: 5 minutes with 1xTBS + 0.05% Tween, 10 minutes with 1xTBS + 0.10% Tween, 5 minutes with 1xTBS + 0.10% Tween, 10 minutes with 1xTBS + 0.05% Tween on orbital shaker at 90 rpm.

Protein expression detection was done in a dark room, in presence of only red light. Detection reagents, containing luminol solution and peroxide were mixed using 1:1 ratio. Hyperfilm™ was placed on top of membrane to capture expression. Afterwards film was developed, fixed, washed in distilled water and dried.

2.2.6 Enzymatic treatment

In the experiment proteinase K treatment was performed to reduce EV aggregation and to eliminate protein complexes, especially low-density lipoproteins (LDL), which attach to EV membranes and co-precipitate with them, as they can mimic EVs and affect experiment outcome (Sódar *et al.* 2016). Proteinase K treatment was performed at 1 mg/ml final concentration at +37°C for 1 hour. Enzyme was inactivated by heating the mixture at +70°C for 10 minutes.

To remove cell-free RNA (cfRNA) that could be attached to EV membranes or detached from protein complexes after proteinase K treatment, samples were treated with RNase A. This enables analysis of RNA exclusively enclosed inside EVs and prevents non-enclosed RNA contamination when total RNA is extracted. RNase A treatment was performed at final concentration of 100 ng/µl at +37°C for 15 minutes. Enzyme was inactivated by lysis buffer addition during sRNA extraction.

2.2.7 Total RNA extraction

To be able to quantify urinary and plasma EV and PCa tumor and non-tumor tissue sample RNA and detect its quality, sRNA extraction was performed using miRNeasy Micro Kit. Tissue RNA was isolated from 20mg of tissue sample, which was homogenized using Lysing Matrix A tubes and FastPrep®-24 homogenizer. RNA isolation was performed following miRNA-enrichment protocol specified by the manufacturer. RNA isolation from plasma and urinary EVs was performed following miRNeasy Micro Kit manufacturer's protocol. Additional on column DNase treatment step was included when isolating RNA from both EV and tissue samples, in order to remove DNA content. Treatment with DNase was done following the protocol specified in Appendix B for RNA samples lower than one microgram in miRNeasy Micro Handbook. After sRNA extraction, all samples were stored at -70°C.

2.2.8 RNA quantification

Agilent Small RNA Kit was used to assess RNA quantity and to confirm that RNA enclosed in EVs has been preserved during EV isolation and enzymatic treatment. Assay was performed on Agilent 2100 Bioanalyzer instrument using 2100 Expert Software, following Agilent Small RNA Kit manufacturer's instructions. Prior to performing assay, tissue RNA samples were diluted 1:50 in nuclease-free water.

2.2.9 Preparation of small RNA libraries

sRNA libraries were built following Ion Total RNA-Seq Kit v2 manufacturer's instructions for construction of sRNA libraries with one modification – primers and adaptors were diluted four times in order to decrease the amount of primer-dimer increase that inevitably occurs when working with low RNA concentrations. Method encompasses hybridization and ligation of RNA sample, synthesis of complimentary DNA (cDNA) from the RNA template using reverse transcription, purification and size-selection of the cDNA using magnetic beads, barcoding of libraries, polymerase chain reaction (PCR) amplification of the cDNA using Ion Xpress™ RNA-Seq Barcode 01–16 Kit and purification of the amplified cDNA using Magnetic Bead Cleanup Module.

2.2.10 Assessment of small RNA library yield and size

Assessment of sRNA library quality and quantity was done to confirm that construction of sRNA libraries has been successful and to determine the molar concentration of libraries. Assay was performed following Agilent High Sensitivity DNA Kit's manufacturers protocol on Agilent 2100 Bioanalyzer instrument using 2100 Expert Software.

2.2.11 BluePippin System

BluePippin System was used to size select the sRNA libraries' fragments of our interest and to get rid of primer-dimers that may have formed during sRNA library construction post-PCR amplification step. System uses pulse-field electrophoresis that allows fragment selection based on their size. In the experiment, size-selection was done using 3% agarose gel cassettes and 87 to 130bp size range. After performing size selection using BluePippin System, samples were repeatedly analyzed using Agilent High Sensitivity DNA chip.

2.2.12 RNA sequencing

RNA-seq was performed on Ion Proton™ System with Torrent Suite™ software. System is capable of measuring pH changes. Each time a deoxyribonucleotide triphosphate (dNTP) molecule is added to the DNA polymer, a H^+ ion is released, changing the pH. As the slide with DNA polymers is flooded with a single species of dNTP at a time, the pH change allows determining if and how many bases were added to the sequence read (Thermo Fisher Scientific 2017). Sequencing was done using Ion PI™ Chip Kit v3 and Ion PI™ Hi-Q™ Sequencing 200 Kit. Prior to sequencing, library templates were pooled together, amplified and enriched using Ion OneTouch™ 2 System and Ion PI™ Hi-Q™ OT2 200 Kit. Templated bead quality control was performed using Ion Sphere™ Quality Control Kit. All RNA-seq steps were performed by a specialist. Samples from patients P38, P56 and P60 were sequenced as described above. Patient's P57 samples were sequenced using Illumina HiSeq 2500 System by project's collaborators in Oslo, Norway.

2.2.13 RNA sequencing data analysis

In order to quantify different RNA species' amounts, compare them among samples and to identify potential sRNA biomarkers, sequencing data analysis was performed (Figure 8). Briefly, adaptor removal, trimming with 20% error rate and quality control of raw data was done using cutadapt and FastQC v0.11.5 (Martin 2011; Babraham Bioinformatics 2016). Afterwards data was mapped to miRBase release 21, piRBase v1.0 and human reference genome build 38 (hg38) annotation file using Bowtie 2 v2.3.2 (Langmead and Salzberg 2012; miRBase release 21 2014; piRBase v1.0 2014; Ensembl 2017). Selection of mapped alignments by local coverage was performed with ShortStack (Axtell 2013). Further analysis involved sRNA type catalog building from data mapped to hg38 using HTSeq, *htseq-count* script (Anders *et al.* 2015). As last, clustering and differential expression analysis was performed using R v3.2.5 *stats* and *limma* packages (R 2016). Raw sequencing data was analyzed by a specialist.

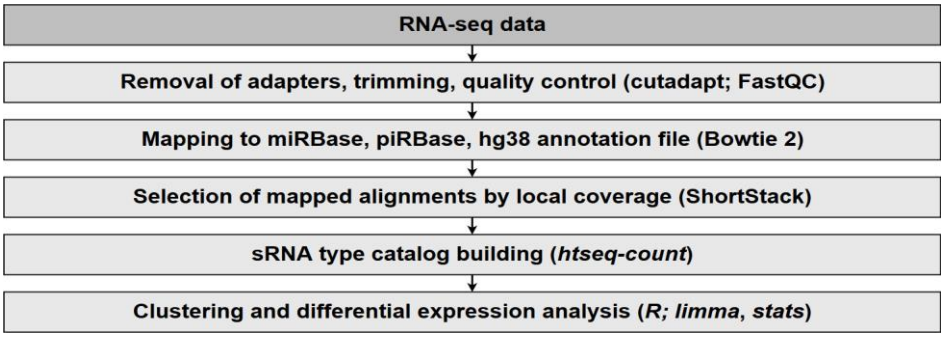

Figure 8. Sequencing data analysis pipeline.

8. attēls. Sekvencēšanas datu analīzes secība.

2.2.14 Complimentary DNA synthesis

Prior to performing quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay, cDNA was synthesized using Universal cDNA Synthesis Kit II. 2µl 5x Reaction buffer, 0.2µl (0.03fmol) UniSp6 RNA spike-in template (directed for use in normalization), 1µl Enzyme mix, containing reverse transcription polymerase, 450pg EV RNA or 30ng tissue RNA sample (volume calculated based on Agilent Small RNA chip) and appropriate volume of nuclease-free water to have 10µl total reaction volume were mixed by pipetting and spun down afterwards. Reaction mix was incubated in a thermocycler as follows: 60 minutes at +42°C, 5 minutes at +95°C and indefinite time at +4°C until sample was removed from thermocycler. Afterwards, cDNA samples were stored at -70°C for later use.

2.2.15 Quantitative reverse transcription polymerase chain reaction

Validation of potential PCa miRNA biomarkers was done using RT-qPCR, which is based on detection of DNA fragment amount in sample. During reaction, fluorescent marker binds to DNA fragment and each fragment is amplified proportionally to its initial amount, resulting in higher fluorescent signal if more fragments are present. Master mixes were prepared using 1µl of selected primer mix (containing reverse and forward primer) or UniSp6 primer (containing reverse and forward primer) and 5µl of ExiLENT SYBR® Green master mix for each reaction. Afterwards, they were vortexed, spun down and aliquoted in 96 well plate (6µl master mix per well). 4µl (diluted 1:40 in DEPC-treated water) of cDNA sample was then added to each well according to previously made experiment setup template. Plate was sealed with optical adhesive sheet, vortexed and spun down for 15 minutes at RT at 1500x*g* to remove any bubbles. RT-qPCR was performed on ViiA 7 Real-Time PCR System with following settings: 10 minutes at +95°C (1x cycle); 10 seconds at 95°C and 1 minute at +60°C (45x cycles); passive reference – none. As there is no known internal control for plasma or urinary EVs, all RT-qPCR assay data was normalized against input RNA amount and UniSp6 RNA spike-in. Relative quantification (RQ) values were calculated using $2^{\Delta Ct}$ method.

3. RESULTS AND DISCUSSION

3.1 Workflow

In order to characterize the sRNA cargo in plasma and urinary EVs and to compare it with the sRNA content in matching tumor and non-tumor prostate tissues, EVs were isolated from four patients' plasma and urine samples collected before and after radical prostatectomy. Complete workflow has been summarized in Figure 9.

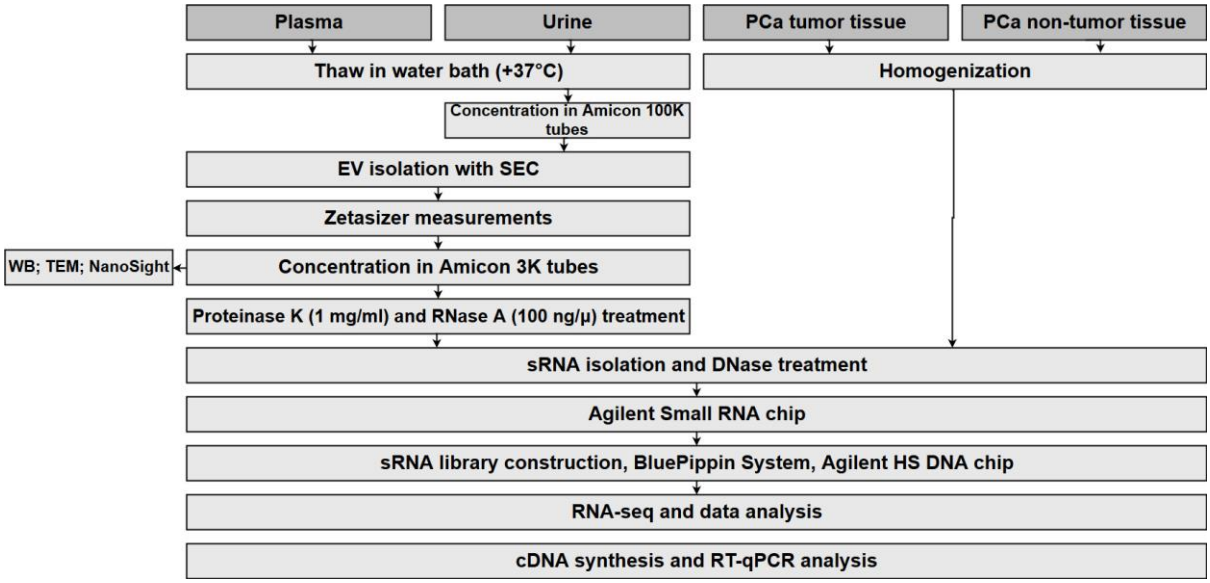

Figure 9. Complete workflow summary.

9. attēls. Pilnas darba gaitas apkopojums.

Abbreviations that are used for samples throughout chapter "Results and discussion" are listed in Table 8, therefore they are not mentioned repeatedly in Figure descriptions.

Table 8
Sample abbreviations.
8. tabula
Paraugu apzīmējumi.

Abbreviation	Explanation
PreOP P/U	Plasma/urine sample collected one day before operation and/or on operation day
PostOP P/U	Plasma/urine sample collected seven days after operation
PostOP 3m P/U	Plasma/urine sample collected three months after operation
N tissue	Non-tumor prostate tissue
T tissue	Tumor PCa tissue

3.2 Isolation and characterization of extracellular vesicles

Characterization of EVs was done using WB, TEM and NanoSight particle tracking system. WB was used to confirm that EV isolation from both urine and plasma samples using SEC has been successful and to confirm different subpopulation presence in EV samples. EVs were assessed visually using TEM as it is among the few methods allowing to determine particle size and structure at the same time (van der Pol *et al.* 2010). Usage of NanoSight allowed to determine EV size and total concentration. Summary of EV samples used for EV characterization is given in Table 9.

Table 9
Summary of samples used for EV characterization.
9. tabula
EV raksturošanai izmantotie paraugi.

Characterization method	**WB**	**TEM**	**Particle tracking analysis**
Patient	P38, P56, P57, P60	P38, P56, P57, P60	P56
Proteinase K treatment	No	No	Yes
RNase A treatment	No	No	No
Urinary EV samples	Urinary EVs isolated from urine collected before and after operation pooled together		Each urinary EV sample analyzed separately
Plasma EV samples	EVs isolated from plasma collected before and after operation pooled together		Each plasma EV sample analyzed separately

3.2.1 Western blot result analysis

Total EV protein amount was analyzed by BCA; based on results, 10µg of protein per sample was loaded on polyacrylamide gel for WB analysis. Antibodies CD9, Alix, Vimentin and Tsg101 were used to confirm EV presence in the samples. Usage of Calnexin and PC3 PCa cell lysates as negative and positive controls respectively was done in order to confirm that cell debris was not present in the isolated EV samples and antibodies worked properly. As Calnexin is a protein located in endoplasmic reticulum, it should be absent in EVs and only present in the cells (Théry *et al.* 2006; Lötvall *et al.* 2014). All used markers, except for Calnexin, were found to be positive in urine and plasma samples (see Figure 10).

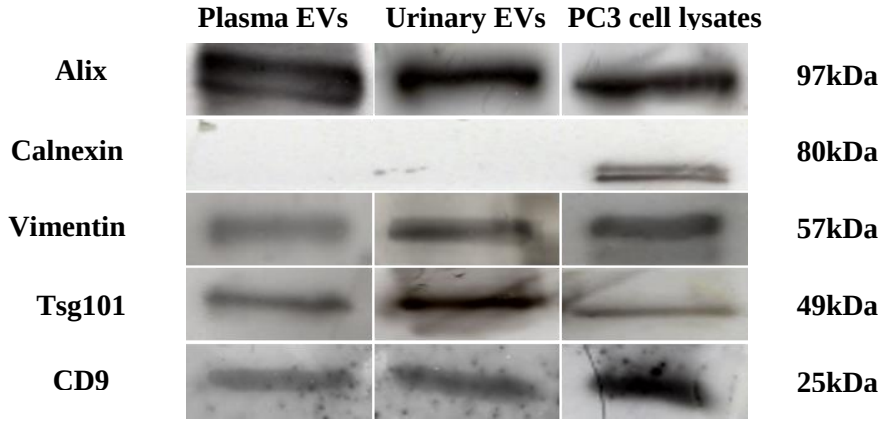

Figure 10. Western blot results for urinary EV, plasma EV and PC3 cell lysate samples. Alix was detected at 97kDa, Vimentin at 57kDa, Tsg101 at 49kDa, and CD9 at 25kDa. Calnexin was only detected in positive control (PC3 cell lysates) at 80kDa.

10. attēls. Vesterna blota rezultāti urīna EV, plazmas EV un PC3 šūnu lizātu paraugiem. Alix tika detektēts pie 97kDa, Vimentīns pie 57kDa, Tsg101 pie 49kDa, CD9 pie 25kDa. Kalneksīns tika detektēts tikai pozitīvajā kontrolē (PC3 šūnu lizātos) pie 80kDa.

It was concluded that EV isolation using SEC has been successful and there are no cells debris present in EV samples. Furthermore, it can be hypothesized that samples contain a mixture of different EV subpopulations (see Table 2, page 16) as Vimentin has been described inside MVs and apoptotic bodies, but not in exosomes (Keerthikumar *et al.* 2015). Similarly, Tsg101 and Alix should be present only in exosomes and MVs and absent in apoptotic bodies (Lee *et al.* 2011). To obtain better quality WB images, digital imagers instead of image development by hand using fixer and developer, should be used.

3.2.2 Transmission electron microscopy result analysis

Positive TEM imaging EV pictures were obtained (see Figures 11 and 12; Appendix 4). In both, plasma and urine samples, EVs, varying in size from approximately 40 to 230nm (depending on sample), were detected. All of them appeared to have EV characteristic cup-shaped morphology. Even though this peculiarity is an artifact related to sample fixation for TEM, it is still commonly used to confirm EV presence (Théry *et al.* 2006). Furthermore, it could be seen that there is a relatively large background signal caused by other particles, most likely, proteins, which have been shown co-isolated with EVs during SEC (Böing *et al.* 2014). Sample treatment with proteinase K prior obtaining TEM images was averted in order to avoid increasing already large background signal.

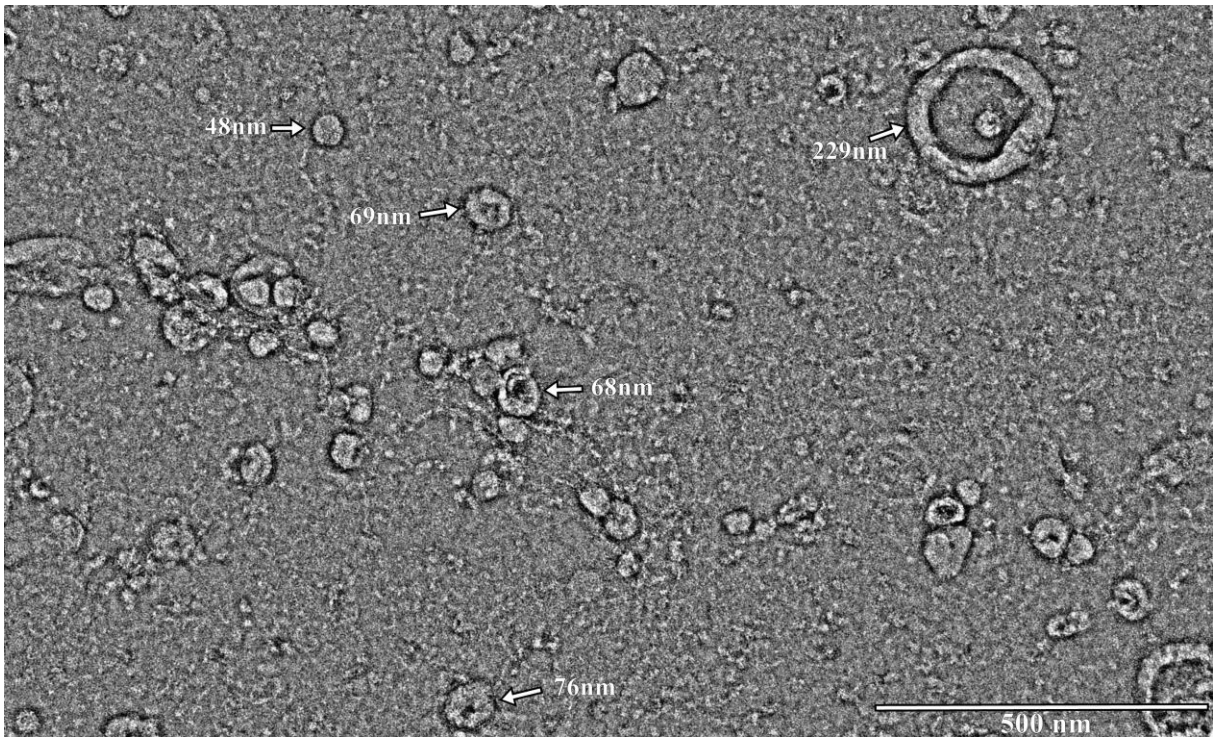

Figure 11. TEM image of urinary EVs. Presence of different size EVs in urine samples was confirmed based on their cup-shaped morphology.

11. attēls. Urīna EV TEM attēls. Dažādu izmēru EV klātbūtne urīna paraugos tika apstiprināta balstoties uz EV raksturīgo tases formas morfoloģiju.

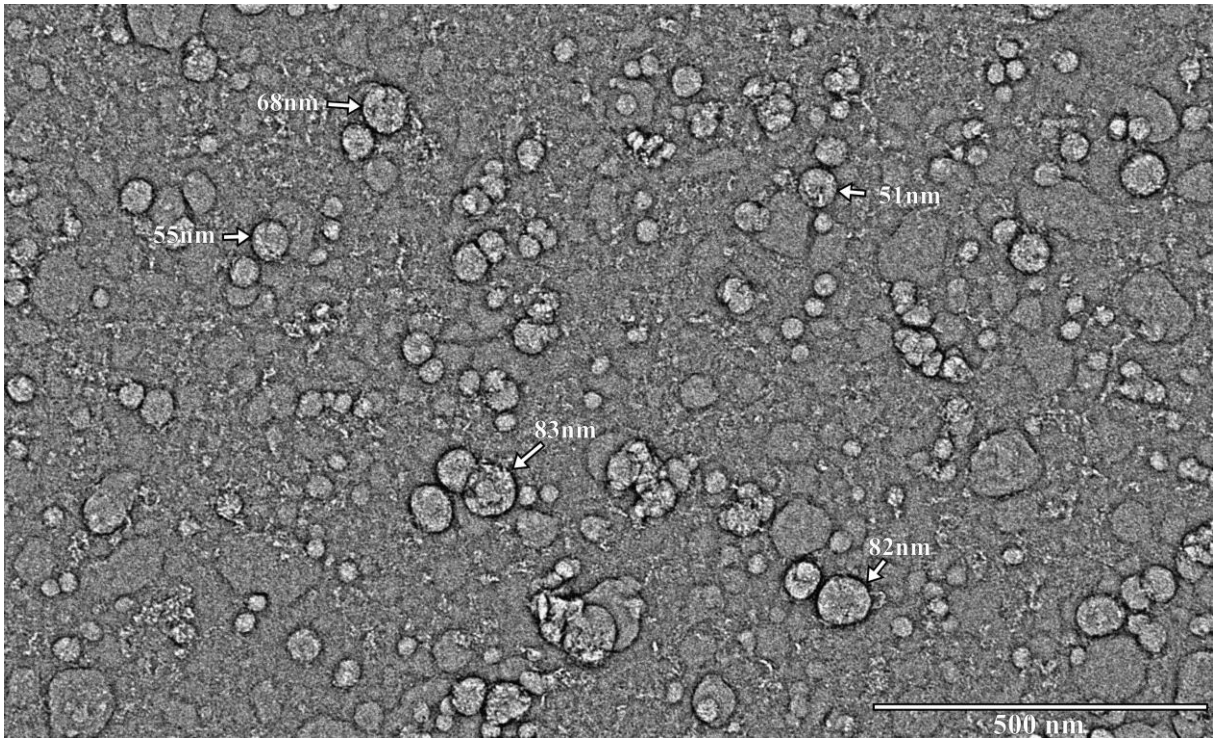

Figure 12. TEM image of plasma EVs. Presence of different size EVs in plasma samples was confirmed based on their cup-shaped morphology.

12. attēls. Plazmas EV TEM attēls. Dažādu izmēru EV klātbūtne plazmas paraugos tika apstiprināta balstoties uz EV raksturīgo tases formas morfoloģiju.

3.2.3 Nanoparticle tracking data analysis

EV samples from patient P56 were characterized using NanoSight, allowing to determine EV size and total concentration. Prior to analyzing samples, they were treated with proteinase K to eliminate protein complexes as they can affect measurement results and reliability (Sódar *et al*. 2016).

Data (Figure 13A) showed that in plasma samples there were two times more particles than in urine samples. However, such difference could have occurred due to different initial biofluid volumes, thus further conclusions regarding this variability will not be drawn. Noteworthy, in both postOP EV samples there were more particles than in preOP EV samples. To the best of author's knowledge, it has not been reported yet, therefore it can be hypothesized that this difference might be because of wound healing process caused as a result of radical prostatectomy. Nevertheless, analysis of more samples is needed to obtain more reliable data.

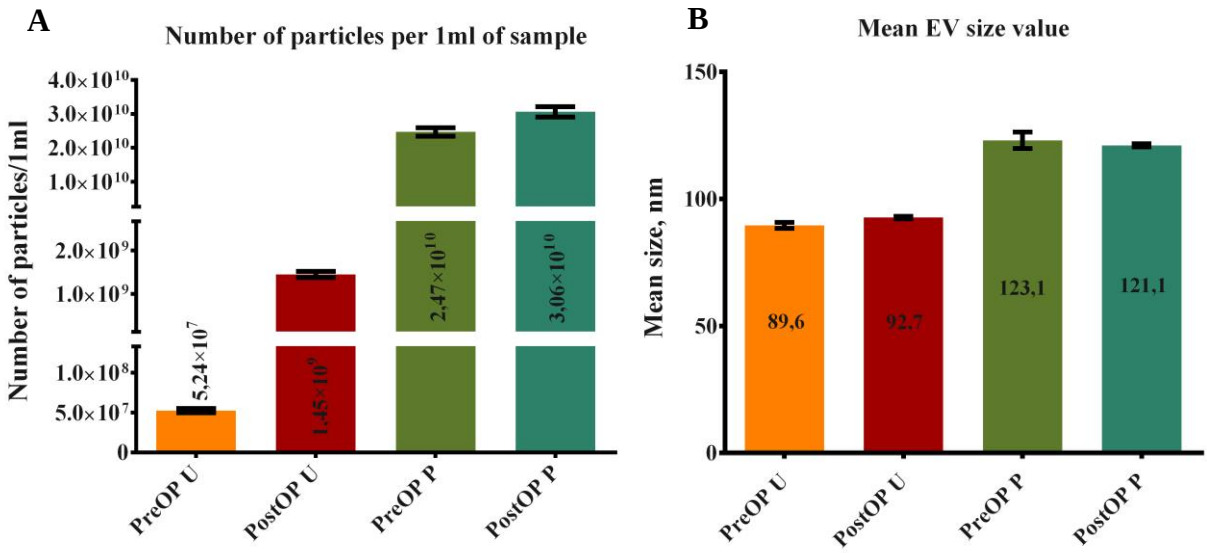

Figure 13. Number of particles per 1ml of sample (A) and mean EV size values (B).

13. attēls. Daļiņu skaits 1ml parauga (A) un vidējās EV lieluma vērtības (B).

Mean particle size values (Figure 13B) ranged from 89.6 to 123.1nm, which corresponds to exosome and smaller MV size (György *et al*. 2011). However, samples also contained particles larger than 400nm (see Appendix 5) suggesting a mixture of different EVs present in the samples. Additionally, previous experiments carried out by author's colleagues in the same laboratory have shown that particle mean size in proteinase K untreated plasma EV samples was approximately 50nm (data not shown) instead of 122nm; as seen in this experiment using proteinase K treated samples. Results confirm that proteinase K treatment eliminates protein complexes as particle mean size has increased and is not lipoprotein size range (~25-80nm) (Sódar *et al.* 2016). A shift in EV size was seen when comparing both biofluids. Plasma samples

seemed to have more exosome-like particles as their size was below 100nm (Figure 13B). On the contrary, urine samples contained larger particles (>120nm), which were in the size range of MVs (György *et al*. 2011). Difference could be explained by the fact that plasma EVs should not be able to pass through the glomerular filtration machinery as described in literature (Ranghino *et al*. 2015).

3.3 Small RNA quantification

sRNA isolated from plasma and urinary EVs, PCa tumor and non-tumor tissues was analyzed using Agilent Small RNA chip in order to check its profile and quantity. Prior to RNA isolation from EVs, they were treated with proteinase K and RNase A to ensure that only RNA enclosed inside the vesicles would be analyzed. Results were displayed as sRNA concentration, RNA electropherogram and gel electrophoresis image (see Figure 14). Obtained data indicated that all tissue and EV samples contained sRNA. Furthermore, it proves that RNA has not been lost during EV enzymatic treatment, which correlates with Shelke *et al.* data published in 2014 (Shelke *et al*. 2014). The main peak in EV RNA samples was detected around 40nt, but in tissue RNA samples around 60nt, corresponding to sRNA size range, which is 18 to 200nt (reviewed in Fritz *et al*. 2016). In all samples marker peak was detected at 4nt.

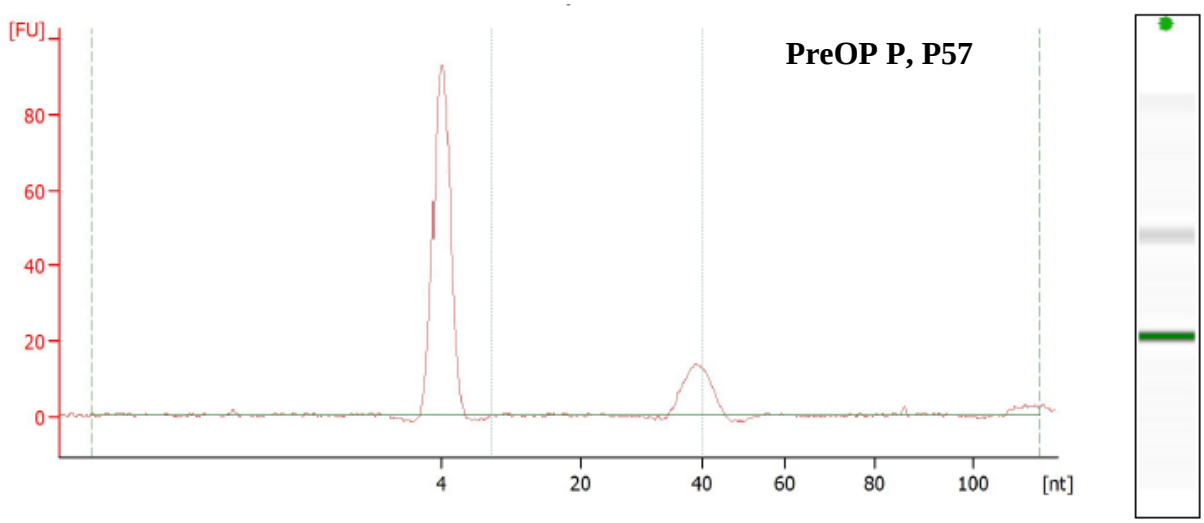

Figure 14. A representative image of Agilent Small RNA chip electropherogram and gel for sample PreOP P from patient P57. Main peak was seen at around 40nt, corresponding with sRNA size range (18-200nt).

14. attēls. Reprezentatīvs Agilent Mazo RNS čipa elektroferogrammas un gēla attēls paraugam PreOP P no pacienta P57. Galvenā smaile bija redzama pie 40nt, kas sakrīt ar mazo RNS izmēru robežām (18-200nt).

sRNA concentrations for EV samples are shown in Figure 15 and for tissue samples in Figure 16. In EV RNA samples it varied from 75.1 to 304.5 pg/µl with exception of two samples from patient P56, in which sRNA concentrations were notably higher (557.5 pg/µl in preOP P and 958.9 pg/µl in postOP 3m U). An immense inter-individual variability is observed, although such difference could be implemented due to varying amount of RNA enclosed inside EVs (Chevillet *et al*. 2014). In tissue samples sRNA concentration ranged from 9745 to 57 030 pg/µl. Data for postOP U sample from patient P57 was not obtained due to technical difficulties.

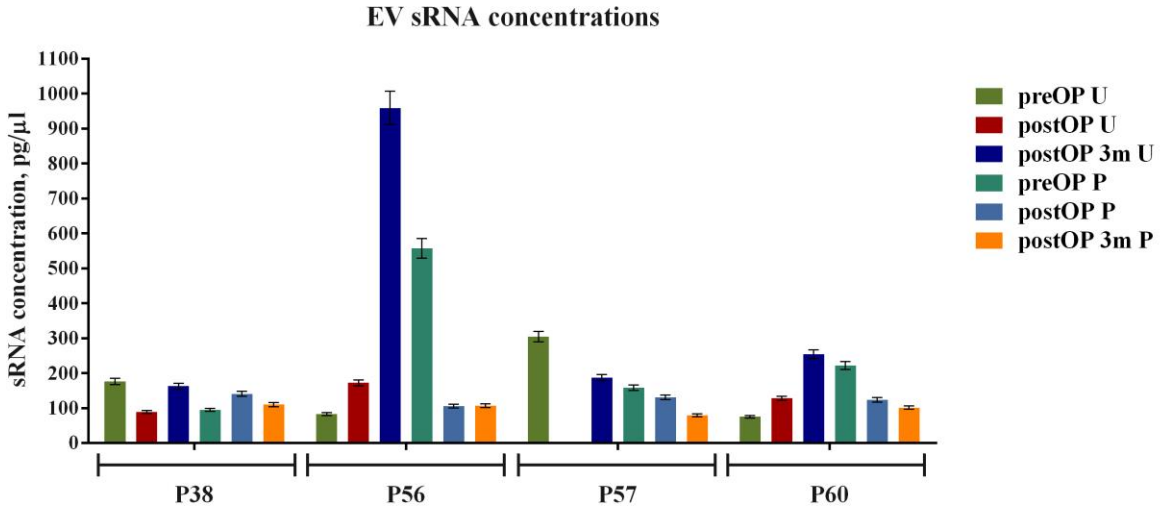

Figure 15. EV sRNA concentrations. sRNA concentration for PostOP U EV sample from patient P57 was not obtained due to technical difficulties.

15. attēls. EV sRNS koncentrācijas. Mazo RNS koncentrācija PostOP U EV paraugam no pacient P57 netika iegūti tehnisku problēmu dēļ.

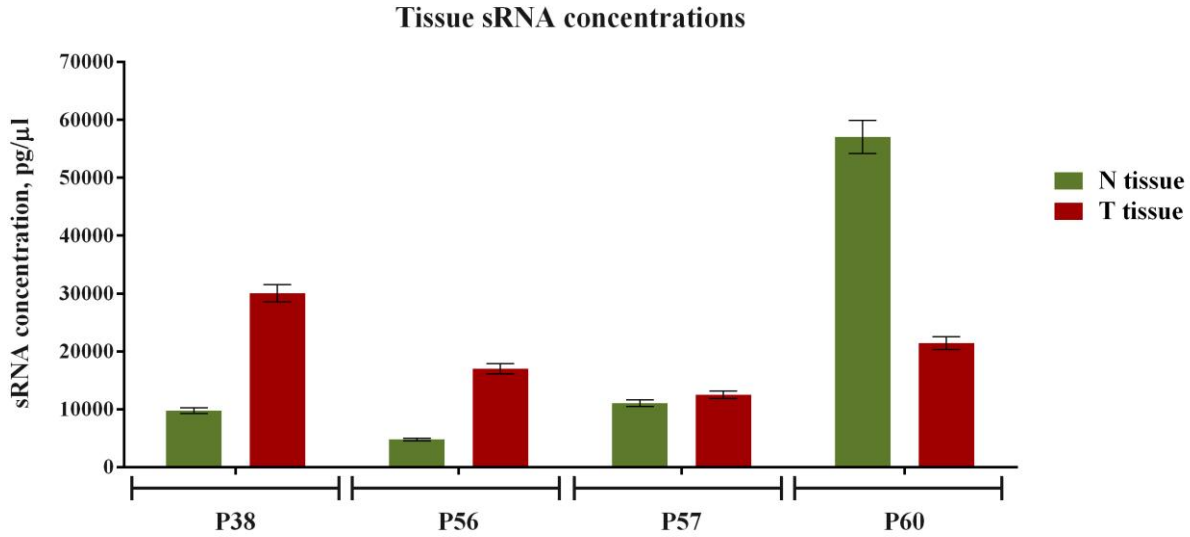

Figure 16. Non-tumor and tumor PCa tissue sRNA concentrations.

16. attēls. Normālu prostatas un prostatas vēža audu sRNS koncentrācijas.

3.4 Assessment of small RNA library quality

In order to assess sRNA library quality, samples were analyzed using Agilent High Sensitivity DNA chips. Based on obtained results, it was concluded that size selection step was needed as multiple peaks below 87bp, corresponding with primer-dimer size, were found. Library size selection was successful, as indicated by the absence of primer-dimer peaks (<87bp) in sample electropherograms after using BluePippin System (Figure 17A&B). After size selection step, a main peak in all samples was located at 95 - 108bp size range, confirming that desired length fragments (87 – 130bp) have been obtained. Positive outcome during this step was crucial for further analysis, as too many primer-dimers could lead to insufficient amount of sample of interest being sequenced. In all samples, lower marker was detected at 35bp, higher marker at 10380bp.

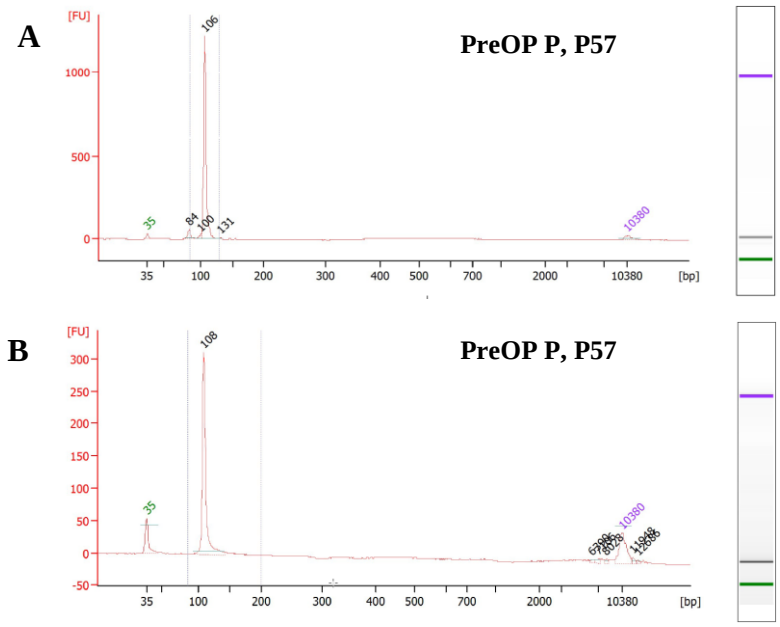

Figure 17. Representative images of Agilent High Sensitivity DNA chip electropherograms for sample PreOP P from patient P57 before (A) and after (B) size selection step using BluePippin System. Before performing size selection, a peak below 87bp was seen, suggesting primer-dimer presence in the sample, however, afterwards main peak was at 108bp, confirming that size selection had been successful.

17. attēls. Reprezentatīvi Agilent Augstas Jutības DNS čipu elektroferogrammas un gēlu attēli PreOP P paraugam no pacienta P57, kas iegūti pirms (A) un pēc (B) lieluma izvēlēšanās soļa, lietojot BluePippin sistēmu. Pirms lieluma izvēlēšanās soļa, smaile bija redzama zem 87bp robežas, norādot uz praimeru-daimeru klātbūtni paraugos, taču pēc lieluma izvēlēšanās soļa, galvenā smaile bija redzama pie 108bp, apstiprinot to, ka soļa izpilde noritējusi veiksmīgi.

3.5 RNA sequencing results

In order to find potential PCa biomarkers and compare their expression levels between different samples, RNA-seq was performed using constructed libraries. Patient P38, P56 and P60 samples were sequenced in the laboratories of Latvian Biomedical Research and Study Centre (Riga, Latvia) using Ion Torrent technology, but patient's P57 samples were sequenced in Oslo, Norway by project's collaborators using Illumina technology. RNA-seq data analysis was done following the pipeline shown in Figure 8 on page 37.

Total number of mapped reads (MR) to hg38 (Ensembl 2017) varied between samples and was higher in tissue samples compared to EVs (Table 10). In tissues samples, 84 to 97% reads were mapped to hg38, with exception of patient's P56 N tissue sample, where it was only 54%. However, in EV samples amount varied from 12% (postOP P, P60) to 75% (postOP U, P38). Similarly to total MR, MR to features (different RNA species) varied among individuals, as well as EV and tissue samples (Table 10).

Table 10
RNA-seq library mapped reads to hg38.
10. tabula
Sekvencēto RNS bibliotēku lasījumu daudzums, kas piekārtoti hg38.

Patient / EV sample	P38			P56		
	Library size after trimming, millions	**MR, millions; %**	**MR to features, millions; %**	**Library size after trimming, millions**	**MR, millions; %**	**MR to features, millions; %**
PreOP U	2.76	1.72; 62%	0.25; 8.9%	1.77	0.70; 39%	0.069; 3.9%
PostOP U	3.75	2.82; 75%	0.52; 14%	0.12	0.04; 34%	0.006; 5.5%
PreOP P	3.34	0.92; 27%	0.12; 3.6%	1.39	0.44; 31%	0.046; 3.3%
PostOP P	1.54	0.48; 31%	0.06; 3.7%	0.86	0.27; 31%	0.027; 3.1%
N tissue	3.06	2.94; 96%	1.02; 33%	1.28	0.70; 54%	0.22; 18%
T tissue	3.59	3.43; 96%	0.63; 18%	7.69	7.12; 92%	2.67; 34%
Patient / EV sample	**P57**			**P60**		
	Library size after trimming, millions	**MR, millions; %**	**MR to features, millions; %**	**Library size after trimming, millions**	**MR, millions; %**	**MR to features, millions; %**
PreOP U	38.88	23.71; 61%	5.82; 15%	0.73	0.28; 39%	0.056; 7.6%
PostOP U	23.48	17.19; 73%	11.12; 47%	0.22	0.06; 28%	0.012; 5.5%
PreOP P	23.51	9.70; 41%	1.44; 6,1%	0.25	0.09; 36%	0.009; 5.1%
PostOP P	18.97	7.83; 41%	0.98; 5.1%	0.16	0.02; 12%	0.002; 1.0%
N tissue	18.94	17.60; 93%	10.46; 55%	3.38	3.01; 89%	2.20; 65%
T tissue	19.22	16.74; 97%	9.20; 48%	3.78	3.18; 84%	1.65; 44%

To better understand the differences in samples and explain them, reads that could not be mapped to human genome from patient's P38 samples, were mapped against microbial genomes (Ensembl Bacteria release 35 2017). Results (data not shown) revealed that most of them correspond to bacterial genome, more specifically to *Pseudomonas* genome, thus showing that we had sequenced bacterial EV RNA content from human biofluids. This correlates with literature where it has been described that gram-negative bacteria are capable of forming outer membrane vesicles and gram-positive bacteria can form membrane vesicles (Manning and Kuehn 2013). Obtained preliminary data should be studied further, including more patient samples and deeper analysis as it could contribute to already ongoing studies regarding EV use to study human microbiota and its effect on various health conditions (Kang *et al.* 2013; Choi *et al*. 2017; Kim *et al.* 2017).

Next step of analyzing RNA-seq data included calculating how many different RNA species are presented in EV and tissue samples (see Figure 18). We obtained that in three patients, the patterns were relatively similar, whilst in patient's P38 samples, pattern was completely different, suggesting a real variability between individuals. The major RNA species in EVs were mRNAs fragments (average 48,2%), miRNAs (24,3%), followed by lncRNA fragments (13,6%), processed (transcribed) pseudogenes (4,7%), rRNAs (3,6%), Y RNAs (3,4%), mtRNAs (1,6%), snRNAs (0,3%), snoRNAs (0,1%), vRNAs (0,004%) and piRNAs (0,0005%). In both T and N tissues dominant RNA species were miRNAs (70,6%), whilst only a minor part was composed of mRNAs (8,9%) and lncRNAs (3,8%). As mRNAs and lncRNAs are usually longer than 200nt (Mercer *et al.* 2009), they are outside our sequenced sRNA size range (15 - 200nt), therefore only fragments of them were being sequenced.

RNA-seq analysis regarding EV content is still controversial thus comparison between author's and other people studies is challenging. For example, data published by Huang *et al*. in 2013 showed that miRNA composes more than 75% of plasma-derived EV sRNA content, suggesting that EVs are enriched in miRNAs (Huang *et al.* 2013). However, in this study the average miRNA amount calculated based on all plasma EV samples, was only 24,3%, correlating with data published by other authors, showing that EVs are not enriched in miRNAs (Arroyo *et al*. 2011; Chevillet *et al*. 2014). Furthermore, in urine EV samples miRNA average amount was 51,5%, similar to have it has been described in literature review (Cheng *et al.* 2014). However, our data and that of Cheng *et al.* contradict with findings of Weber *et al*. in 2010, stating that cell-free urine is a rather poor miRNA carrier (Weber *et al.* 2010; Cheng *et al*. 2014). Surprisingly, all EV samples contained very low amounts (<0,004%) of piRNAs, which have been previously described to be present in EVs, contributing to approximately 1 to 5% of EV sRNA content (Huang *et al*. 2013; Ogawa *et al*. 2013). Reason for shift in these

values could occur because most piRNAs are unannotated in the hg38 human genome version. So, numbers regarding piRNA levels might be inaccurate.

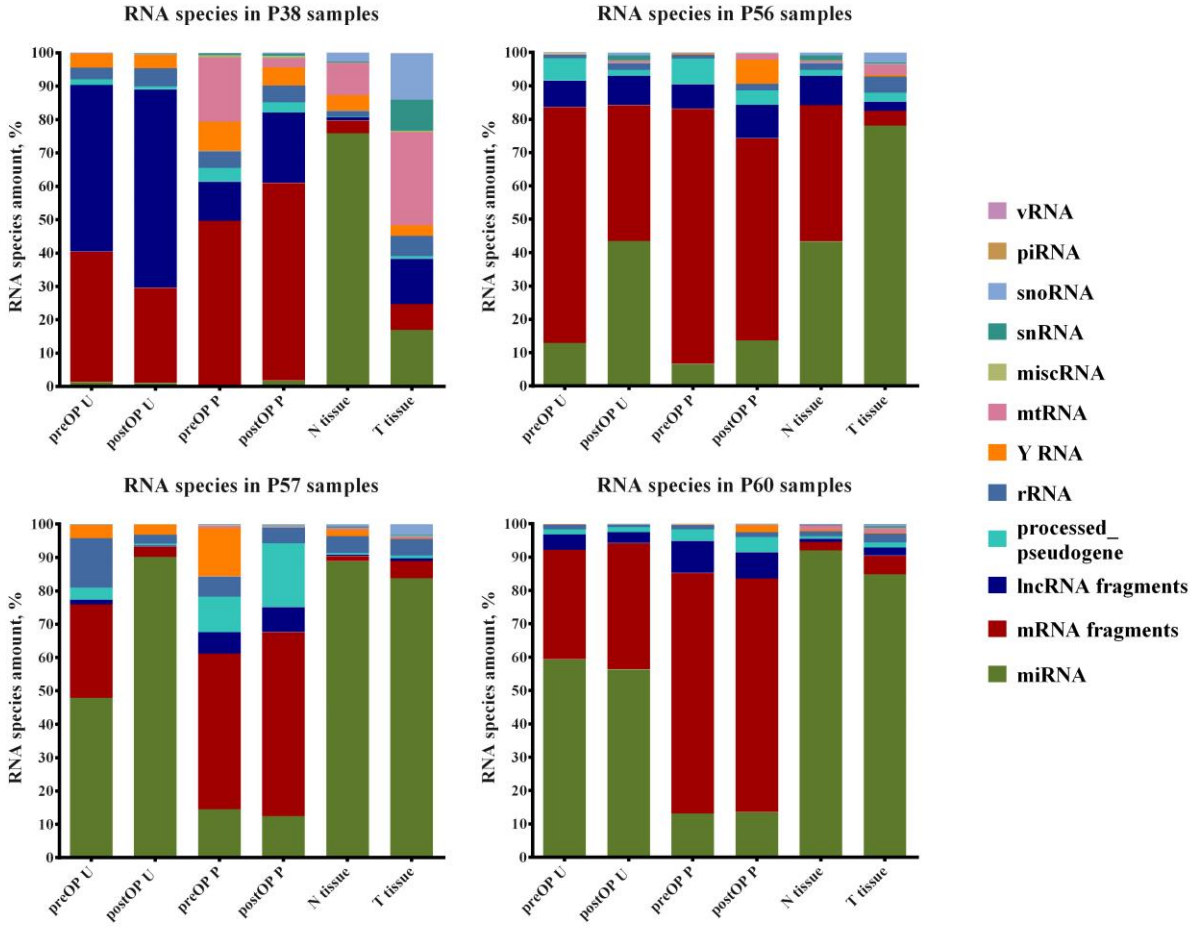

Figure 18. Proportions of various RNA species in EV and tissue samples P38, P56, P57, P60. Proportions of RNA species among P56, P57 and P60 samples were similar whilst they completely differed from patient's P38 samples; most likely representing a real variability between individuals.

18. attēls. Dažādu RNS tipu proporcionālais daudzums EV un audu paraugos, kas iegūti no P38, P56, P57 un P60 paraugiem. RNS tipu daudzums pacientu P56, P57 un P60 paraugos ir līdzīgs, taču tas pilnībā atšķiras no pacienta P38 paraugiem; visticamākais tādēļ, ka pastāv ievērojamas atšķirības starp indivīdiem.

3.5.1 Micro RNA, piwi-interacting RNA and long non-coding RNA content in extracellular vesicles

Three RNA species (miRNAs, piRNAs and lncRNAs) were chosen to identify biomarkers among them. Based on literature review, these specific RNAs classes have been shown to circulate in human biofluids enclosed inside EV (Huang *et al*. 2013). Furthermore, all of them have been shown to play a role in progression of various cancers (Di Leva *et al*. 2014; Assumpção *et al*. 2015; Fatima *et al*. 2015). As previously mentioned, human genome annotation file is not really accurate regarding small non-coding regions, therefore for

identification of miRNA and piRNA potential biomarkers, specific databases, piRBase and miRBase respectively, were used (miRbase release 21 2014; piRBase v1.0 2014). As for lncRNA biomarker discovery, the initial hg38 annotation file was used (Ensembl 2017).

miRNAs have been described to serve as promising cancer biomarkers, especially, because many of them are often dysregulated in cancers (Lu *et al.* 2005; Volinia *et al.* 2006; Ozen *et al.* 2008). Plasma and urinary EV miRNA content analysis revealed a total of 822 different mature miRNAs found in either pre-operation plasma/urine, post-operation plasma/urine or both (Figure 19A). Notably, PCa patient plasma EV RNA-seq analysis carried out by Huang *et al.* in 2015, revealed 375 known miRNAs, correlating with our findings for PreOP P samples where we identified a total of 352 miRNAs (Huang *et al.* 2015). Only miRNAs that had at least ten reads in RNA-seq from tissue samples and two or more reads in biofluids, were included. Further, 194 of them were overexpressed (logFC>1) in PCa tumor tissue compared to non-tumor tissue and present in at least one of the biofluid samples (Figure 19B). Comparison between tumor and non-tumor tissues was done in order to determine the possible content that could be directly related to PCa as collected EVs could be derived from various tissues not only PCa.

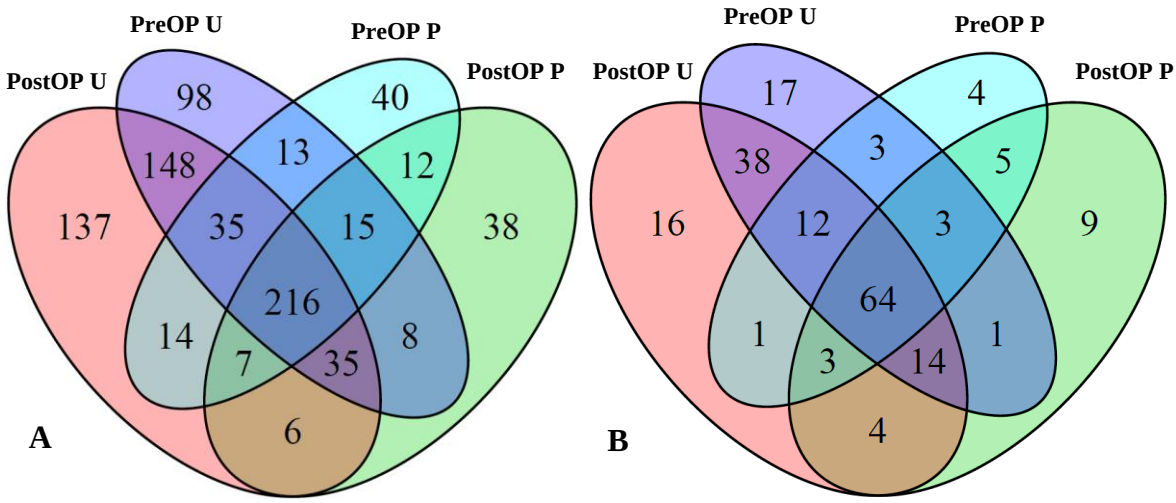

Figure 19. Total number of miRNAs identified in plasma and/or urinary EVs (A) and total number of miRNAs that are overexpressed (logFC>1) in PCa tumor tissue and found in plasma and/or urinary EVs (B).

19. attēls. Kopējais plazmas un/vai urīna EV ietverto un identificēto miRNS skaits (A) un kopējais to miRNS skaits, kuras ir augsti ekspresētas (logFC>1) PCa audzēja audos un atrodamas plazmas un/vai urīna EVs (B).

Next, each sample was analyzed separately in order to see how many of these miRNAs that are overexpressed (logFC>1) in tumor versus non-tumor tissue are increased (logFC>1) in pre-operation EVs samples relative to post-operation EV samples (Figure 20). These miRNAs are likely to be released by PCa cells and may represent PCa biomarker candidates. Number of miRNAs that met previously described criteria varied from patient to patient and only in one

sample set it was possible to identify miRNAs that were overexpressed in tumor, and in both biofluids (P57=7). Results also showed that there were more miRNAs (average of 16) that were increased in pre-operation urine EVs samples and less (average of 4) that were increased in pre-operation plasma EV samples. Those could also be used as specific biofluid biomarkers. However, differences could occur due to individual variability between patients. Nevertheless, miRNAs that are not overexpressed in tumor tissue, but are decreased in post-operation samples could still be used as valid biomarkers, although these miRNAs might be associated to cancer-related inflammation and are most likely not released by cancer cells.

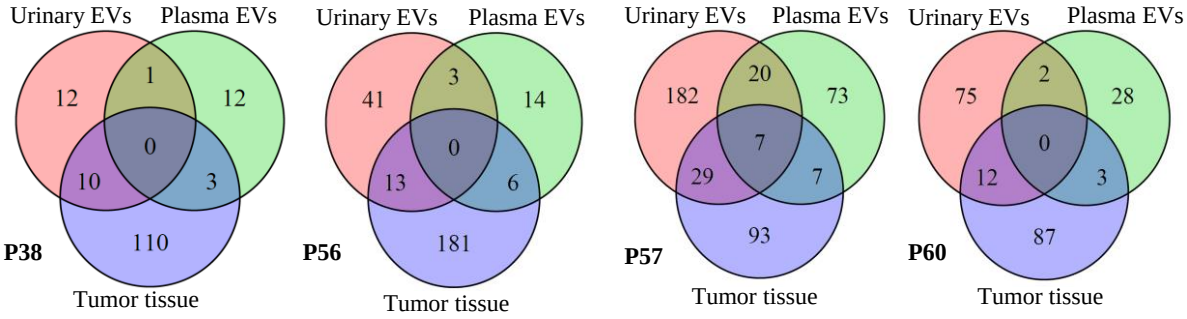

Figure 20. Number of miRNAs in different patient sample sets that are overexpressed (logFC>1) in PCa tumor tissue and upregulated (logFC>1) in pre-operation urinary and/or plasma EVs relative to post-operation urinary and/or plasma EVs.

20. attēls. Dažādu pacientu paraugos esošu miRNS skaits, kuras ir augsti ekspresētas (logFC>1) PCa audos audos un, kuru ekspresija ir paaugstināta (logFC>1) pirms operācijas urīna un/vai plazmas EVs, salīdzinot ar pēc operācijas urīna un/vai plazmas EVs.

Same analysis as described before, was performed in search for biomarkers among piRNAs. However, as previously the amount of piRNAs found in EVs based on RNA-seq data was low, we now used piRBase instead of hg38 annotation file (piRBase v1.0 2014; Ensembl 2017). Based on literature, piRNAs have been suggested to affect tumorigenesis thus making them worthy novel biomarker candidates (reviewed in Assumpção *et al*. 2015). A total of 2761 piRNAs were identified in all EV samples and 313 of them were overexpressed in tumor tissue versus non-tumor and present in at least one biofluid sample (Figure 21A&B). Similarly to miRNAs, piRNA numbers differ among individuals, although in each patient's sample set, there was at least one piRNA overexpressed in tumor tissue and increased in pre-operation biofluids (Figure 22). Notably, in comparison to miRNAs, in each sample set, there were 7 to 31 piRNAs, depending on patient, that were decreased in plasma or urine post-operation samples, whilst there were slightly less (3 to 29) such miRNAs.

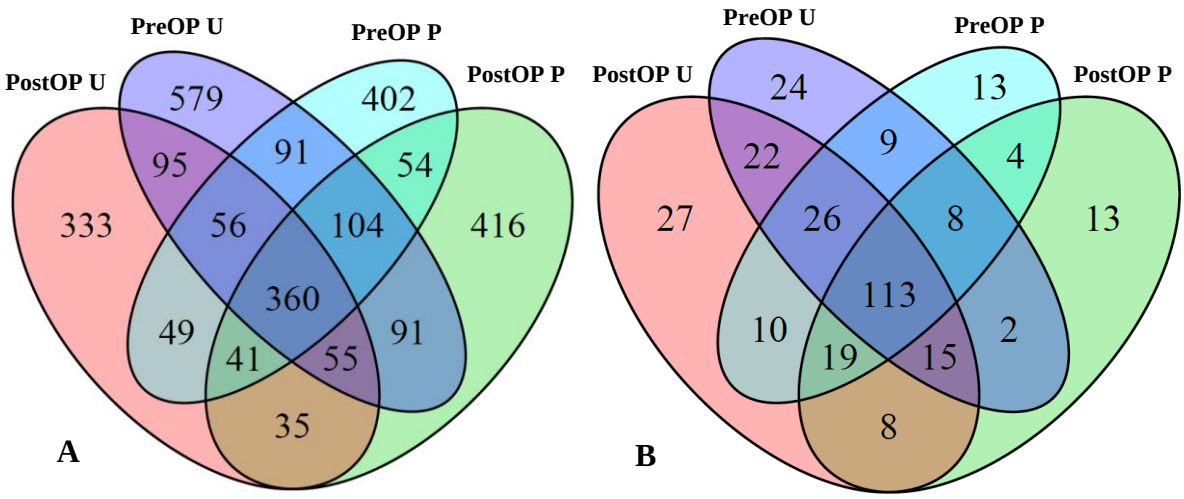

Figure 21. Total number of piRNAs identified in plasma and/or urinary EVs (A) and total number of piRNAs that are overexpressed (logFC>1) in PCa tumor tissue and found in plasma and/or urinary EVs (B).

21. attēls. Kopējais plazmas un/vai urīna EV ietverto un identificēto piRNS skaits (A) un kopējais to piRNS skaits, kuras ir augsti ekspresētas (logFC>1) PCa audzēja audos un atrodamas plazmas un/vai urīna EVs (B).

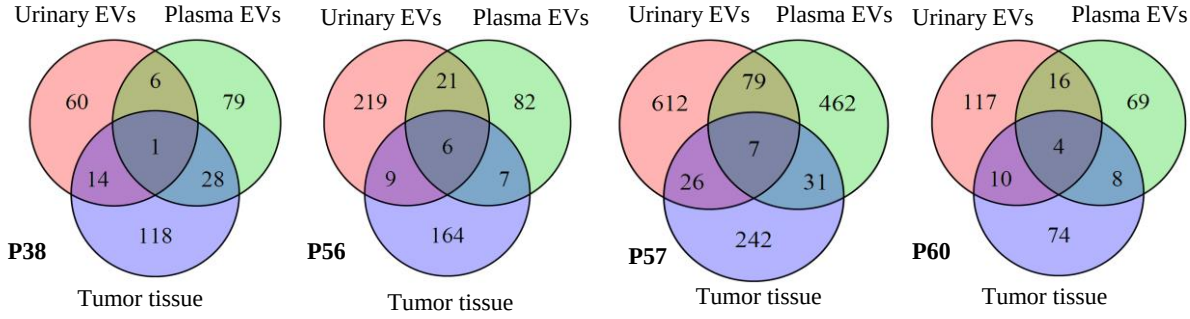

Figure 22. Number of piRNAs in different patient sample sets that are overexpressed (logFC>1) in PCa tumor tissue and upregulated (logFC>1) in pre-operation urinary and/or plasma EVs relative to post-operation urinary and/or plasma EVs.

22. attēls. Dažādu pacientu paraugos esošu piRNS skaits, kuras ir augsti ekspresētas (logFC>1) PCa audzēja audos un, kuru ekspresija ir paaugstināta (logFC>1) pirms operācijas urīna un/vai plazmas EVs, salīdzinot ar pēc operācijas urīna un/vai plazmas EVs.

Last but not least, a total of 2548 different lncRNA fragments were found in EV samples with only minority of them (86 total) being overexpressed in tumor tissue versus non-tumor and present in at least one biofluid sample (Figure 23A&B). Nonetheless, these few lncRNAs could still be of use as biomarkers as lncRNAs have been found in EVs connected to different cancers and are proposed to be related with poor prognosis and contribute to cancer drug resistance (Kogure *et al.* 2013; Işın *et al*. 2015; Berrondo *et al*. 2016; Deng *et al*. 2016).

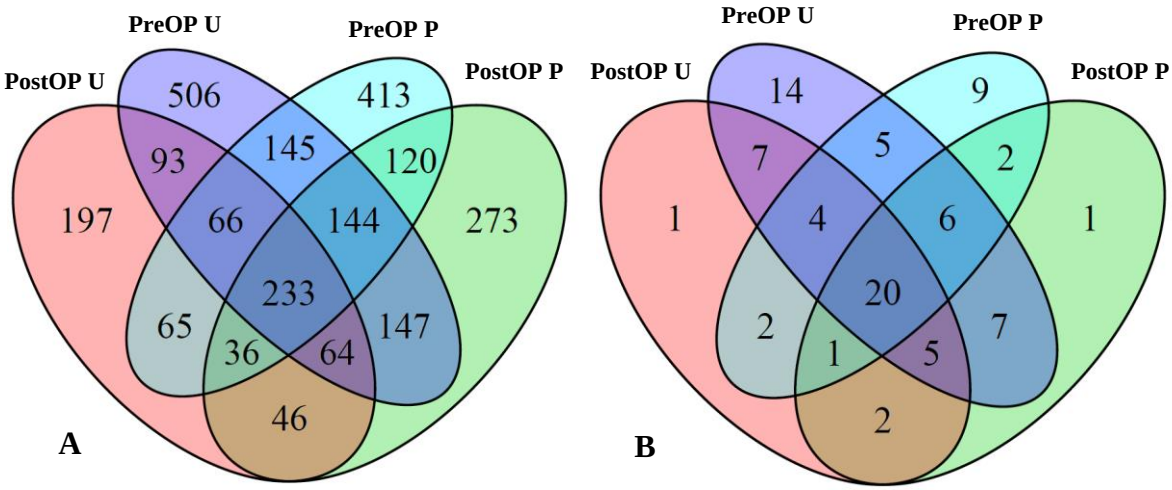

Figure 23. Total number of lncRNAs identified in plasma and/or urinary EVs (A) and total number of lncRNAs that are overexpressed (logFC>1) in PCa tumor tissue and found in plasma and/or urinary EVs (B).

23. attēls. Kopējais plazmas un/vai urīna EV ietverto un identificēto lncRNS skaits (A) un kopējais to lncRNS skaits, kuras ir augsti ekspresētas (logFC>1) PCa audzēja audos un atrodamas plazmas un/vai urīna EVs (B).

When comparing amount of lncRNAs overexpressed in tissues and upregulated in pre-operation samples, there was no consistency among patients and, unfortunately, there were fewer lncRNAs that are common for both biofluids (Figure 24). Additionaly, number of lncRNAs that were present in at least one biofluid, had also decreased (1 to 19) compared to miRNAs (3 to 29) and piRNAs (7 to 31). However, increasing amount of patient samples sets could contribute to these numbers.

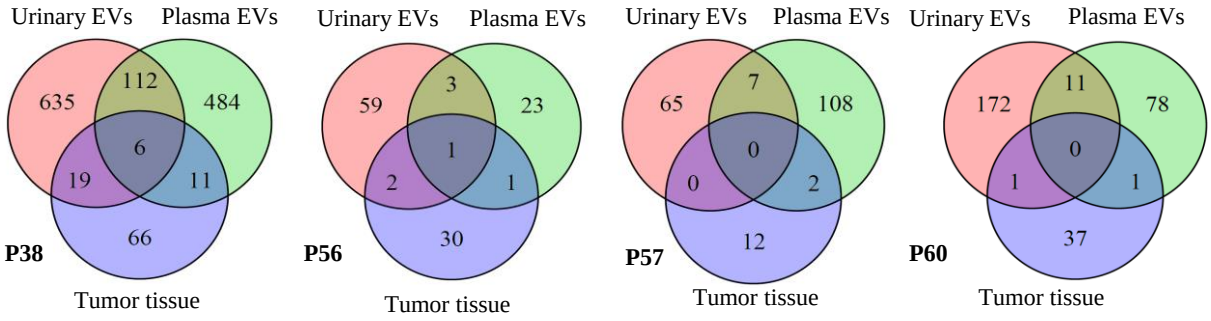

Figure 24. Number of lncRNAs in different patient sample sets that are overexpressed (logFC>1) in PCa tumor tissue and upregulated (logFC>1) in pre-operation urinary and/or plasma EVs relative to post-operation urinary and/or plasma EVs.

24. attēls. Dažādu pacientu paraugos esošu lncRNS skaits, kuras ir augsti ekspresētas (logFC>1) PCa audzēja audos un, kuru ekspresija ir paaugstināta (logFC>1) pirms operācijas urīna un/vai plazmas EVs, salīdzinot ar pēc operācijas urīna un/vai plazmas EVs.

In summary, there is a certain number of miRNAs (9), piRNAs (22) and lncRNAs (7) that are overxpressed in PCa tumor tissue (logFC>1) and upregulated (logFC>1) in EVs isolated from pre-operation versus post-operation plasma and urine samples, thus they could

serve as PCa biomarker candidates (Figure 25). Furthermore, there were total of 57 miRNAs, 53 piRNAs and 21 lncRNAs in urinary EVs and 17 miRNAs, 65 piRNAs and 15 lncRNAs in plasma EVs that were overxpressed in tumor tissue and upregulated in corespoding pre-operation samples, making also these RNAs potential PCa biomarker candidates.

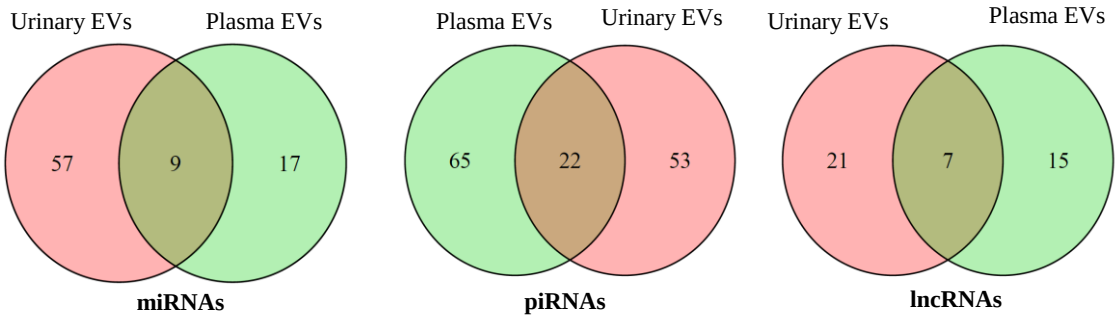

Figure 25. Numbers of potential miRNA, piRNA and lncRNA PCa biomarker candidates. Only number of those RNAs that are overexpressed (logFC>1) in PCa tumor tissue in comparison to normal prostate tissue are shown.

25. attēls. Potenciālo miRNS, piRNAs un lncRNS prostatas vēža biomarķieru kandidātu skaits. Aprēķinos iekļautas tikai tās RNS, kas ir augsti ekspresētas (logFC>1) PCa audzēja audos salīdzinājumā ar normāliem prostatas audiem.

Variability between individuals should be take into account and based on only these four patient datasets it is not possible to speculate, which biofluid could serve as a better source of biomarkers or whether there would be a significant difference between patients in case more samples would be analyzed.

3.6 Validation of potential micro RNA biomarker candidates

Biomarker selection and validation is still in progress, however the preliminary results revealed two promising biomarker candidates, which were selected for validation in either plasma or urine EV samples using RT-qPCR. These miRNAs were overexpressed (logFC>1) in PCa tumor tissue in all four patients and present in preOP EV samples at relatively high levels. However, their amount had decreased in postOP EV samples suggesting their potential use as PCa biomarkers. Due to disclosure policy, selected miRNA names are not being revealed and no references to articles related to them are shown.

miR-xxx is a multifunctional cancer related miRNA, which has been shown to be dysregulated in various human cancers. Depending on cancer type, it has been proposed to function as both tumor suppressor gene and oncogene play a role in tumorigenesis by moderating cell proliferation, apoptosis, autophagy and invasion. Furthermore, in some cancers, low expression levels of miR-xxx contribute to metastasis development and poor outcome, whilst in other cancers, including PCa, downregulation of miR-xxx correlates with

positive outcome. In PCa, miR-xxx has been reported to have oncogenic properties as it represses tumor suppressor genes and also promotes tumor progression by enhancing cell proliferation. Additionally, reduced survival rates and cancer drug resistance correlates with high miR-xxx expression levels.

As seen in Figure 26A, miR-xxx is significantly overexpressed in PCa tumor tissue in comparison to non-tumor tissue in all samples. Data correlates with other reports, showing high expression of miR-xxx in PCa tissues. When performing RT-qPCR assay on EVs obtained from urine, a decrease of miR-xxx was seen already in PostOP U samples collected only seven days after operation, suggesting that miR-xxx could be directly derived mainly from PCa and lower over time as a consequence to prostate removal (Figure 26B). As of author's knowledge, data regarding miR-xxx expression in urinary EVs from PCa patients has not been previously described, making these findings particularly appealing.

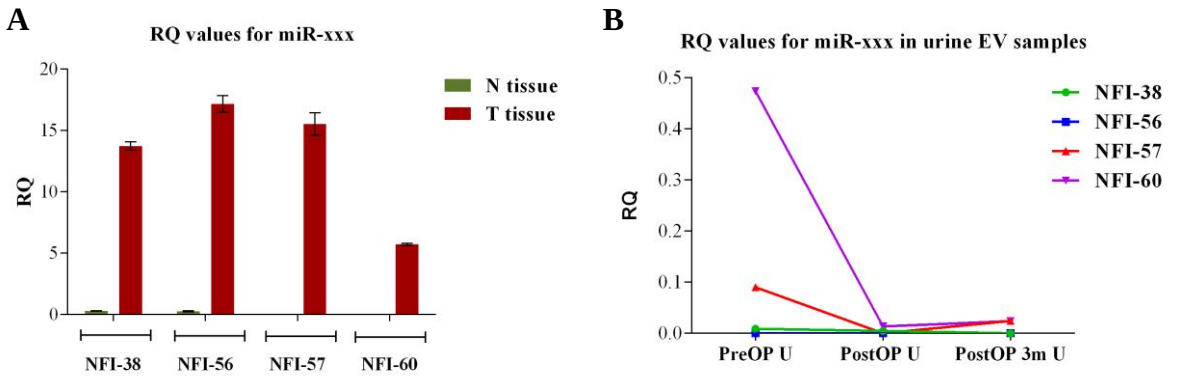

Figure 26. RQ values of miR-xxx in tissue (A) and urine EV (B) samples. *Abbreviations:* RQ – relative quantification.

26. attēls. Relatīvā miR-xxx ekspresija audu (A) un urīna EV (B) paraugos. *Apzīmējumi*: RQ – relatīvā ekspresija.

As next, miR-yyy, another cancer related miRNA's expression was assessed by performing RT-qPCR and compared among different plasma EV and tissue samples. Similar to miR-xxx, miR-yyy has been described as tumor suppressor in several cancers, such as gastric, pancreatic and breast, suggesting that its downregulation promotes tumorigenesis. In contrast, multiple other reports show miR-yyy to be upregulated in PCa and to correlate with high GS, metastatic or castration resistant cancer. Furthermore, recent discovery shows that miR-yyy promotes PCa cell proliferation. RT-qPCR analysis for miR-yyy revealed that it is highly overexpressed in PCa tumor tissue in comparison to matching normal prostate tissue (see Figure 27A), correlating with data found in the literature. Furthermore, expression levels of miR-yyy in plasma EVs (see Figure 27B) had decreased over time in three out of four sample sets, suggesting its potential use as PCa biomarker.

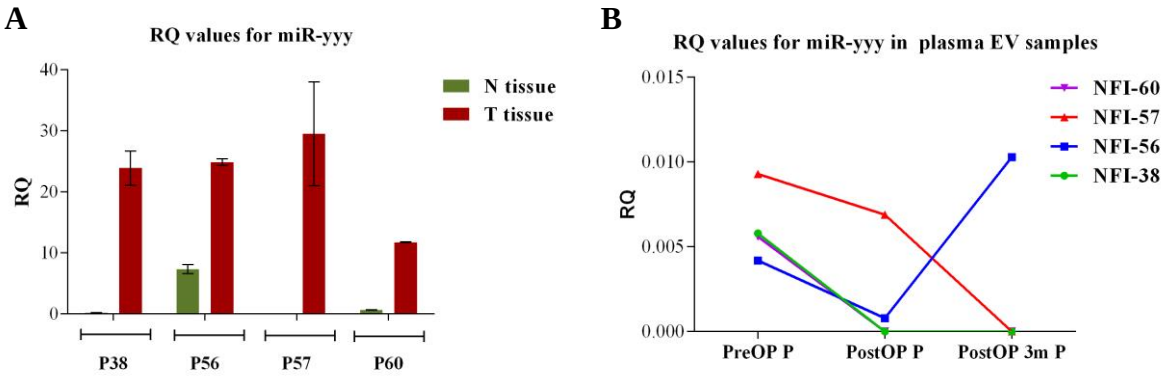

Figure 27. RQ values of miR-yyy in tissue (A) and plasma EV (B) samples. *Abbreviations:* RQ – relative quantification.

27. attēls. Relatīvā miR-xxx ekspresija audu (A) un plazmas EV (B) paraugos. *Apzīmējumi*: RQ – relatīvā ekspresija.

Based on obtained preliminary results, miR-xxx and miR-yyy show a potential to serve as PCa biomarkers. miR-xxx has previously been found only in blood-derived EVs, however these data show miR-xxx presence in urinary EVs and expression decrease over time after PCa has been removed. Furthermore, a decrease in miR-yyy's expression in plasma EVs has also been shown, therefore only contributing to the hypothesis that it could be used as PCa biomarker.

As the validation process of biomarker candidates is still in progress, it is not yet possible to confirm how many of miRNAs could be used as prognostic or diagnostic PCa tools. Further studies will include validation of these and other miRNA, piRNA and lncRNA biomarker candidates in a larger cohort of patients, thus making the data more reliable. Also, data will be compared not only among samples obtained before operation, seven days and three months after it, but also with samples obtained nine months after radical prostatectomy.

In summary, experimental data shows that it has been possible to successfully isolate EVs from plasma and urine samples. The major species found in EVs are mRNAs, miRNAs, lncRNAs and piRNAs. Additionally, plasma and urinary EV RNA content differs immensely. Nevertheless, there are multiple potential miRNA, piRNA and lncRNA biomarker candidates, which are overexpressed in PCa tissue and upregulated in pre-operation plasma and urinary EVs. Two of such miRNAs were shown to serve as promising PCa biomarker candidates. Finally, considering the inter-individual variability, a combination of various RNA species would be beneficial for PCa surveillance.

CONCLUSIONS

1. Based on WB, TEM and particle tracking analysis data it has been confirmed that EV isolation from plasma and urine samples using SEC was successful.
2. The major RNA species found in the plasma and urinary EV small RNA fraction were mRNAs, miRNAs, lncRNAs and piRNAs. However, their proportions varied greatly among individuals and biofluids.
3. RNA-seq analysis revealed a total of 822 different mature miRNAs, 2761 piRNAs and 2548 lncRNAs found in plasma and urinary EVs.
4. Among identified RNAs, 83 miRNAs, 140 piRNAs and 43 lncRNAs were overexpressed in PCa tumor in comparison to matching non-tumor prostate tissue and upregulated in either pre-operation urine and/or plasma EVs relatively to post-operation urine and/or plasma EVs, making them promising PCa biomarker candidates.
5. So far, two miRNAs were shown to have a potential to serve as tools for PCa monitoring.

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APPENDICES

Appendix 1

Summary of the different RNAs classes identified in human blood circulation or extracellular fluids (Fritz *et al*. 2016, table has been modified)

Name	Abbreviation	Size range, nt	Definition or function	Reference
MicroRNA	miRNA	18-25	Epigenetic control of gene expression; mRNA degradation; suppression of mRNA translation	Krol *et al*. 2004; Carthew and Sontheimer 2009
Small interfering RNA	siRNA	20-25	mRNA degradation; overlapping with miRNAs	Carthew and Sontheimer 2009
Small nucleolar RNA	snoRNA	70-240	Chemical modification of RNA; telomere maintenance; pre-ribosomal RNA processing; regulatory role in alternative splicing	Marz *et al*. 2011
Piwi-interacting RNA	piRNA	24-31	Repression of transposons; mRNA silencing	Weick and Miska 2014
Small nuclear RNA	snRNA	~150	Modulation of the activity of RNA polymerase II; involvement in splicing	Nguyen *et al*. 2001
Transfer RNA fragments	tRFs	10-45	Translation control; RNA silencing	Gebetsberger and Polacek 2013
Vault RNA	vRNA	80-50	Constitutive component of the vaults, which are RNP complexes involved in transport, signal transmission, and immune responses	Lara *et al*. 2011
Y RNA	Y RNA	83-112	Required for initiation step of chromosomal RNA replication	Kheir and Krude 2017
yRNA fragments	yRFS	20-1600	Apoptosis	Chakrabortty *et al.* 2015
Long non-coding RNA	lncRNA	>200	Chromatin remodeling; transcription stability maintenance; posttranscriptional modifications; translation repression	Hu *et al*. 2012; Keniry *et al.* 2012; Fatica and Bozzoni 2014; Ha *et al.* 2014
Circular RNA	circRNA	80–90000	Templates for viroid and viral replication; intermediate in RNA processing reactions; regulator of transcription; function as miRNA sponges	Memczak *et al.* 2013; Lasda and Parker 2014; Panda *et al.* 2017

Appendix 2-1

Examples of tumor suppressor miRNAs (Di Leva *et al*. 2014, table has been modified)

miRNA	Chromosome	Cancer	Target gene	Note
miR-15/16	13q14.2 3q25.33	Chronic lymphocytic leukemia	*BCL2*	miR-15/16 target *BCL2* inducing apoptosis in a leukemic cell line model
		Colon	*COX-2*	miR-16 targets *COX-2*; elevated levels of HuR antagonize miR-16 function
		Follicolar lymphoma	*CHEK1*	miRNA profiles are associated with an increased proliferation and a "late" germinal center B-cell phenotype
		Myltiple myeloma	*FGFR1, PI3KCa, MDM4, VEGFa*	Deletion of miR-15/16 is observed in early stages of multiple myeloma
		Breast	*WIP1*	miR-16 regulates Wip1 phosphatase in the DNA damage response and mammary tumorigenesis
		Ovarian	*BMI-1*	miR-15a/16 target Bmi-1 is leading to low proliferation and clonal growth
		Lung	*CCND1, CCND2, CCNE1*	Overexpression of miR-15/16 induces arrest in G(1)-G(0)
miR-34	1p36.22 11q23.1	Colon	*SIRT1, SNAIL*	miR-34 targets SIRT1 leading to apoptosis only in p53 wildtype context; a new link between p53, miR-34, and Snail1 in the regulation of cancer cell EMT programs
		Gastric	*BCL2, NOTCH, HMGA2*	miR-34 targets Bcl-2, Notch, and HMGA2
		Lung	*AXL*	miR-34a and -199a/b target Axl receptor; both miRs are silenced by promoter methylation
		Ovarian	*MET*	miR-34 targets MET

Appendix 2-2

Examples of tumor suppressor miRNAs (Di Leva *et al*. 2014, table has been modified)

miRNA	**Chromosome**	**Cancer**	**Target gene**	**Note**
Let-7 family	9q22.32 Xp11.22 22q13.31 21q21.1 19q13.41 11q24.1 3q21.1 12q14.1	Lung	*KRAS*	The let-7 family negatively regulates let-60/RAS in *C. elegans* and lung tumors
		Breast	*IL6*	Inflammation activates a positive feedback loop that maintains the epigenetic transformed state
		Prostate	*E2F2, CCND2*	Let-7a targets *E2F2* and *CCND2* acting as a tumor suppressor in prostate cancer
		Liver	*BCL-XL*	Let-7 targets Bcl-xL and potentiates sorafenib-induced apoptosis
miR-200 family	1p36.33 12p13.31	Breast	*ZEB1, ZEB2, FAP1, SUZ12*	Downregulation of the miR-200 family may be an important step in tumor progression; miR-200c sensitizes cells to apoptosis mediated by CD95; miR-200b-Suz12-cadherin pathway sustains cancer stem cell growth and invasiveness
		Bladder	*ERRFI-1*	miR-200 restores EGFR dependency
		Nasopharyngeal	*BMI-1*	miR-200a inhibits cell growth, migration and invasion
		Lung	*FLT1/VEGFR1*	miR-200 suppresses metastasis by targeting Flt1
		Ovarian	*P38α*	miR200a-dependent stress signature correlates with improved survival and response to treatment

Appendix 3-1

Examples of oncogenic miRNAs (Di Leva *et al*. 2014, table has been modified)

miRNA	**Chromosome**	**Cancer**	**Target gene**	**Note**
miR-17-92	13q23.1	Colon	*TSP-1, CTGF*	Upregulated in colonocytes coexpressing K-Ras, c-Myc and p53 impaired activity
		Prostate	*E2F2, E2F3, MnSOD, GPX2, TRXR2*	Autoregulatory feedback loop between E2F factors and miR17/92; miR-17/92 suppress tumorigenicity by inhibiting mitochondrial antioxidant enzymes
		Lung	*HIF1α*	Intricate and finely tuned circuit involving c-myc, miR-17/92, and HIF1α
		Breast	*HBP1*	miR-17/92 inhibits HBP1 regulates invasion activating Wnt/β-catenin.
miR-222/221	Xp11.3	Prostate	*P27(Kip1)*	High miR-222/221 maintain low p27(Kip1) and stimulate proliferation
		Breast	*FOXO3A, ESR1, TRSP1, DICER*	miR-222/221 target FOXO3A to suppress p27(Kip1) also at a transcriptional level; modulation of ERα is associated with anti-estrogen therapy; miR-221/222 promote EMT contributing to the more aggressive clinical behavior of basal- like breast cancers.
		Lung	*PTEN, TIMP3, APAF1*	miR-222/221 target PTEN and TIMP3 induce TRAIL resistance and enhance cellular migration; MET oncogene activates miR-222/221 through the c-Jun transcription factor; miR-221/222 are activated by EGFR and MET; by targeting APAF1, miR-221/222 are responsible of gefitinib resistance

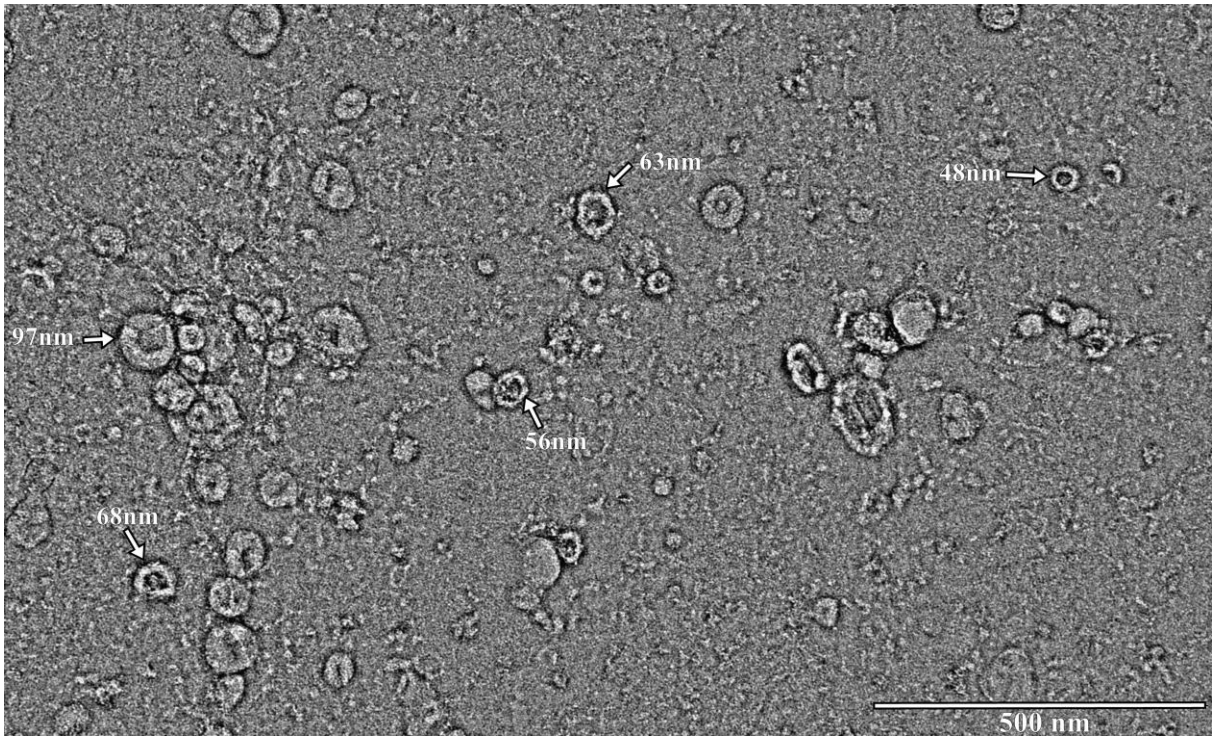
Appendix 3-2

Examples of oncogenic miRNAs (Di Leva *et al*. 2014, table has been modified)

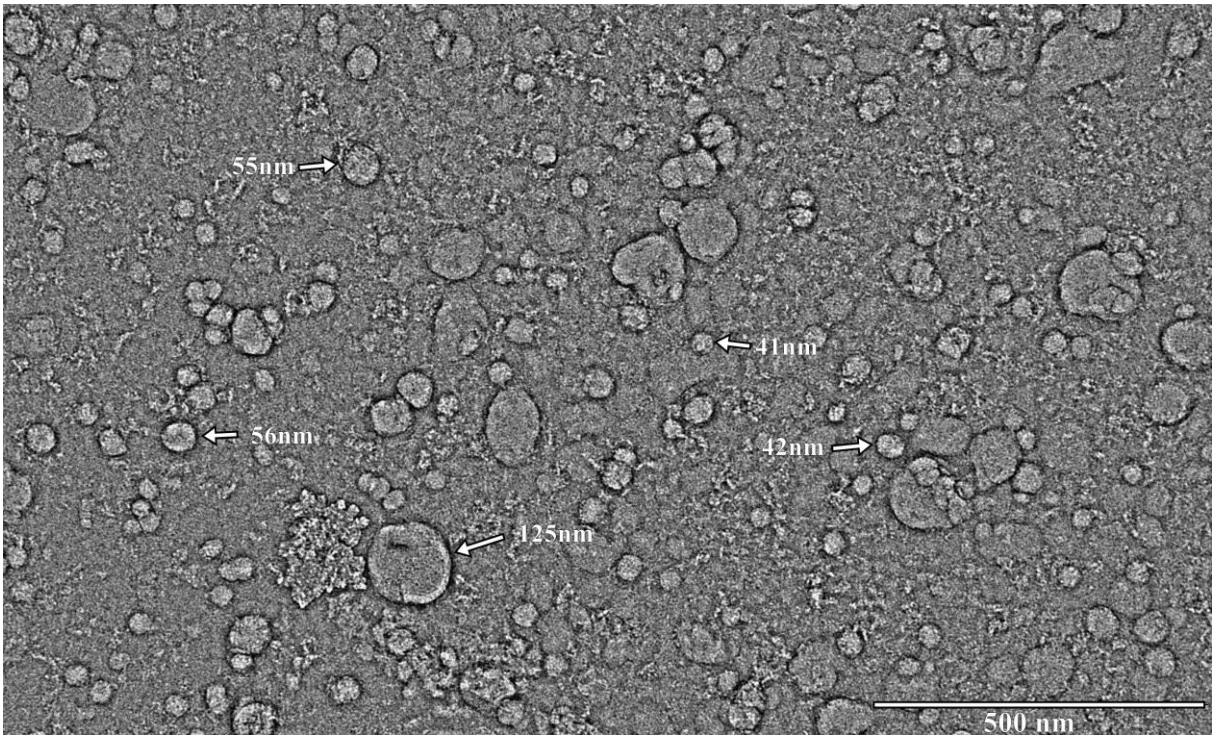
miRNA	**Chromosome**	**Cancer**	**Target gene**	**Note**
miR-21	17q23.1	Breast	*TPM1, PDCD4*	Suppression of mir-21 inhibits tumor growth; miR-21 suppresses PDCD4 to control apoptosis.
		Glioblastoma	*RECK, TIMP3, p63, JMY, TOPORS, TP53BP2, DAXX, HNRPK, TGFβRII*	Inhibition of miR-21 provides a novel therapeutic approach for "physiological" modulation of multiple proteins whose expression is deregulated in cancer; miR-21 targets multiple important components of p53, TGF-β and mitochondrial apoptosis tumor-suppressive pathways
		Prostate	*MARKS, ANP32A, SMARCA4*	miR-21 promotes apoptosis resistance, motility, and invasion
		Bile duct	*PTEN*	miR-21 modulates gemcitabine-induced apoptosis by PTEN-dependent activation of PI3K
miR-155	21q21.3	Breast	*SOCS1, WEE1, FOXO3a*	miR-155 may serve as a bridge between inflammation and cancer; miR-155 targets WEE1 and enhances mutation rates by decreasing the efficiency of DNA safeguard mechanisms
		Nasopharyngeal	*JMJD1A*	LMP1 and LMP2A activates miR-155 to repress JMJD1A
		Acute myeloid leukemia	*CEBPB, PU1, CULT*	miR-155 as a contributor to physiological GM expansion during inflammation and to certain pathological features associated with AML

Appendix 4

TEM image of urinary and plasma EVs



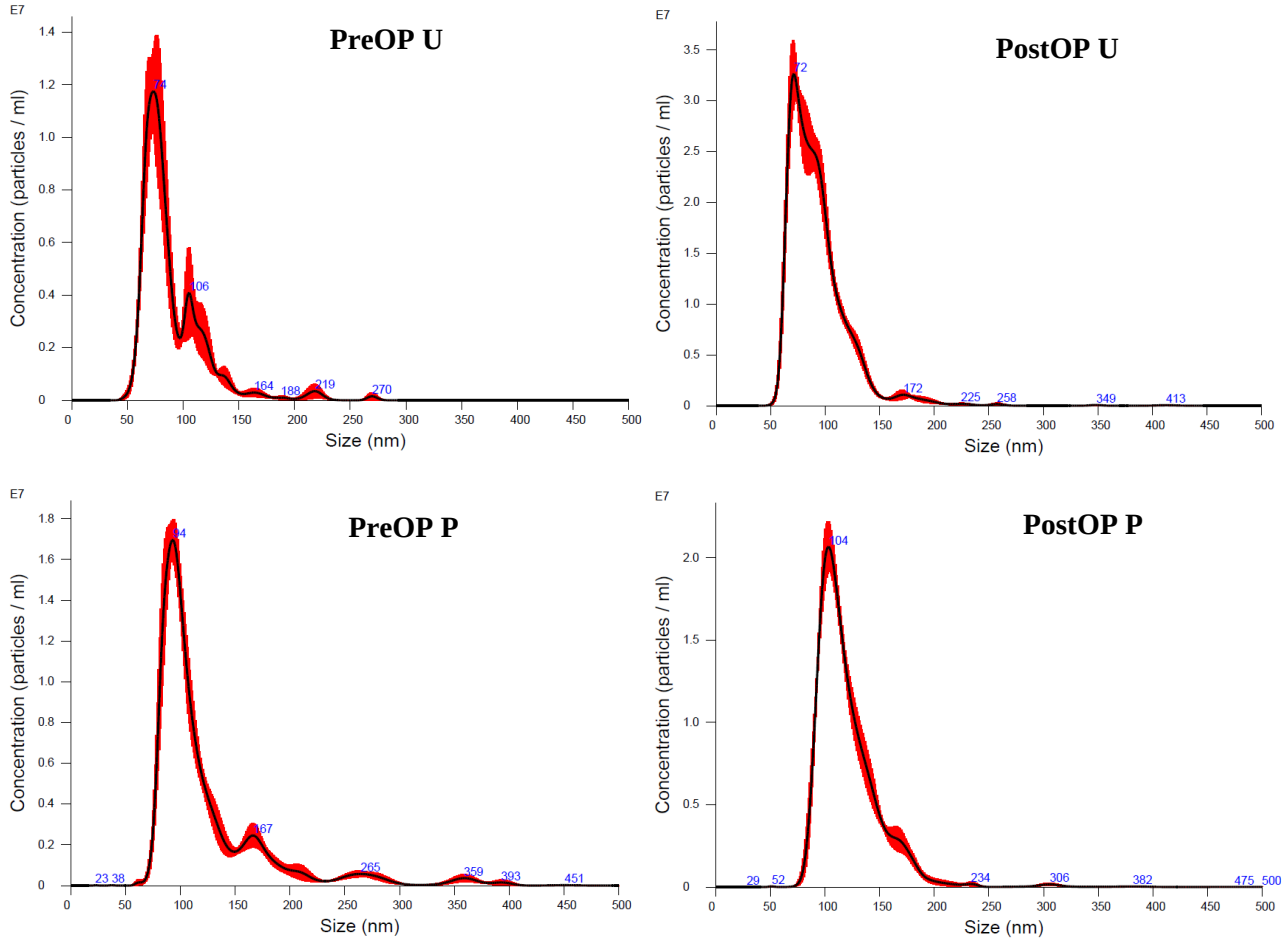
Urinary EVs



Plasma EVs

Appendix 5

Nanoparticle tracking analysis (NanoSight) data for EVs isolated from patient P56 samples



Bakalaura darbs „Characterization of small RNA content in urinary and plasma extracellular vesicles from prostate cancer patients” izstrādāts LU Bioloģijas fakultātē.

Ar savu parakstu apliecinu, ka pētījums veikts patstāvīgi, izmantoti tikai tajā norādītie informācijas avoti un iesniegtā darba elektroniskā kopija atbilst izdrukai.

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