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CLINICOPATHOLOGICAL SIGNIFICANCE OF EXOSOMAL PROTEINS CD9, CD63 AND DNA MISMATCH REPAIR PROTEINS IN PROSTATE ADENOCARCINOMA AND BENIGN HYPERPLASIA

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ANOTĀCIJA

Ievads

Pēdējā laikā ir pierādīts, ka eksosomu biomarķieriem un DNS bojājumu reparācijas proteīniem (MMR) varētu būt būtiska nozīme audzēja riska stratifikācijā un prognozes novērtēšanā.

Prostatas audzēja (PCa) diagnostikas zelta standarts ir biopsija un histopatoloģiskā izmeklēšana, kas tiek izmantota, lai noteiktu audzēja diagnozi.

Tādējādi eksosomu biomarķieru un DNS MMR novērtēšana paralēli standarta esošajai diagnostikai varētu būt specifiskāka un izmaksu efektīvāka metode.

Literatūrā ir maz datu par to, vai eksosomu biomarķieru un DNS MMR imūnhistoķīmiskā izmeklēšana ir atšķirīga pacientiem ar prostatas audzēju un labdabīgu prostatas hiperplāziju.

Darba mērķis un uzdevumi

Izvērtēt un salīdzināt eksosomu biomarķieru un DNS MMR ekspresiju un diagnostisko nozīmi pacientiem ar labdabīgu prostatas hiperplāziju un PCa.

Uzdevumi:

1. Salīdzināt eksosomālo biomarķieru (CD9, CD63) un DNS MMR (MSH2, MSH6, MLH1 un PMS2) ekspresiju audos pacientiem ar prostatas adenokarcinomu un labdabīgu prostatas hiperplāziju.
2. Analizēt DNS MMR un eksosomālo biomarķieru CD9, CD63 ekspresiju audos pacientiem ar prostatas adenokarcinomu atbilstoši *Gleason* vērtībai un Pasaules Veselības organizācijas 2016. gadā apstiprinātajai Prostatas karcinomas gradācijas sistēmai.
3. Analizēt DNS MMR un ekosomālo biomarķieru CD9, CD63 ekspresijas prognostisko nozīmi bezrecidīvu dzīvildzei pacientiem ar prostatas adenokarcinomu.

Metodes

Pētījums bija retrospektīvs. Kopumā pētījumā tika iekļauti 92 pacienti ar prostatas acināru adenokarcinomu un 20 pacienti ar prostatas labdabīgu hiperplāziju. CD63, CD9, MSH2, MSH6, MLH1 un PMS2 ekspresija tika analizēta ar imūnhistoķīmijas metodi. Katra prostatas karcinomas pacienta novērošana ilga līdz slimības progresēšanai un/ vai maksimāli 5 gadus.

Rezultāti

Kopumā pētījumā tika iekļauti 112 pacienti. 92 pacienti bija ar prostatas acināru adenokarcinomu un 20 pacienti ar labdabīgu prostatas hiperplāziju. Zemas pakāpes prostatas karcinoma (I un II pakāpe) tika novērota 56 pacientiem, augstas pakāpes prostatas karcinoma (III–V pakāpe) tika novērota 36 pacientiem. Kad zema un augsta riska prostatas karcinomas pacienti tika neatkarīgi analizēti, CD63 proteīna ekspresija bija ievērojami augstāka pacientiem ar prostatas karcinomu III–V pakāpē, salīdzinot ar I–II pakāpi, attiecīgi 6,24 (0–9) pret 1,57 (0–6), $P < 0,0001$. Audzēja bezrecidīva dzīvildze bija ievērojami ilgāka pacientiem ar zemu CD63 proteīna ekspresiju, salīdzinot ar augstu CD63 proteīna ekspresiju, attiecīgi, 42,50 un 26,50 mēneši [HR =1,73 (1,06–2,84); $P = 0,018$].

Iegūtie rezultāti parādīja, ka CD9 proteīna ekspresija ievērojami samazinājās prostatas acināras adenokarcinomas gadījumā, salīdzinot ar kontroles grupu: 1 (0–9) pret 6 (2–9), $P < 0,0001$. Audzēja bezrecidīva dzīvildze pacientiem ar augstu CD9 proteīna ekspresiju (4.–9. ekspresijas pakāpe) bija ievērojami ilgāka nekā pacientiem ar zemu CD9 proteīna ekspresiju (0–3) attiecīgi 43,00 un 28,50 mēneši [HR = 2,65 (1,15–4,28); $P = 0,016$].

DNS MMR ekspresija netika novērota 10 pacientiem (10,86%) no 92 pacientiem ar prostatas karcinomu (2 pacientiem ar II, 5 pacientiem ar IV un 3 pacientiem ar V pakāpes audzēju). Šis pētījums parādīja DNS MMR ekspresijas zudumu 8/36 (22,22%) pacientiem ar augstas pakāpes prostatas karcinomu un 2/56 (3,57%) – ar zemas pakāpes prostatas karcinomu. DNS MMR proteīni tika ekspresēti visos labdabīgas prostatas hiperplāzijas gadījumos (viegls vai mērens krāsojums). Pētījumā konstatēja negatīvu korelāciju starp DNS MMR zudumu un audzēja pakāpes grupām (Rho = – 0,25; $P < 0,02$).

Audzēja bezrecidīva dzīvildze pacientiem ar DNS MMR deficītu (vismaz viens DNS MMR proteīns) bija ievērojami īsāka nekā pacientiem ar saglabātu DNS MMR izteiksmi, attiecīgi 22,00 un 60,00 mēneši [HR = 4,18 (1,82–9,59); $P = 0,0007$].

Secinājumi

1. Prostatas audzēju raksturo paaugstināta CD63 proteīna ekspresija, bet samazināta CD9 proteīna ekspresija, un DNS MMR zudums, salīdzinot ar labdabīgu prostatas hiperplāziju audiem.
2. CD63 proteīna ekspresija ir palielināta, bet CD9 proteīna ekspresija ir samazināta augstas pakāpes prostatas audzēja audu paraugos.
3. DNS MMR ekspresijas zudums tika pierādīts 10.86% prostatas audzēja audu paraugos.
4. CD63 proteīna ekspresija korelēja ar prostatas audzēja pakāpes grupām.
5. DNS MMR zudums negatīvi korelē ar prostatas audzēja pakāpes grupām.
6. Bezrecidīvu dzīvildze ir ievērojami ilgāka pacientiem ar zemu CD63, augstu CD9 proteīna un saglabātu DNS MMR ekspresiju.

ABSTRACT

Introduction

Recently, it has been shown that exosome and DNA mismatch repair protein (MMR) biomarkers could play an important role in cancer risk stratification and prognosis assessment.

The gold standard for prostate carcinoma diagnosis is biopsy and histopathological examination, which is used to diagnose the cancer.

Thus, the evaluation of exosome- and DNA MMR-biomarkers in parallel with standard existing diagnostics could be a more specific and cost-effective method.

There is limited data in the literature on whether immunohistochemical examination of exosomes and DNA MMR is different in patients with prostate cancer (PCa) and benign prostate hyperplasia (BPH).

The aim and objektives of this study

To evaluate and compare the expression of exosomal biomarkers and DNA MMR in the tissue of patients with BPH and PCa.

The objectives were to:

1. To compare the expression of exosomal biomarkers (CD9, CD63) and DNA MMR (MSH2, MSH6, MLH1, and PMS2) by immunohistochemistry (IHC) in the tissue of patients with PCa and BPH.
2. To analyze DNA MMR and exosomal biomarkers CD9, CD63 expression according to the *Gleason* Grade and PCa grading system adopted by the WHO 2016 in patients with PCa.
3. To analyze the prognostic value of DNA MMR and exosomal biomarkers CD9, CD63 expression in disease progression free survival (PFS) in patients with PCa.

Methods

The study was retrospective. Altogether, 92 patients with prostate acinar adenocarcinoma and 20 patients with prostate benign hyperplasia were enrolled in the study. The CD63, CD9, MSH2, MSH6, MLH1, and PMS2 expression was analysed by immunohistochemistry. Follow up for each PCa patient in this study lasted till disease progression and/or maximal 5 years.

Results

A total of 112 patients were included in the study: 92 patients with acinar adenocarcinoma of the prostate and 20 patients with BPH. Low-grade prostate carcinoma (Grade I and II Grade group) was observed in 56 patients, and high-grade acinar prostate adenocarcinoma (Grade III–V Grade group) was observed in 36 patients. When low- and high-risk PCa patients were independently analyzed, CD63 expression was significantly higher in patients with grade III–V PCa compared with grade I–II, 6.24 (0–9) versus 1.57 (0–6), $P < 0.0001$. PFS was significantly longer in patients with low CD63 expression compared to high CD63 expression, respectively 42.50 and 26.50 months [HR = 1.73 (1.06–2.84); $P = 0.018$].

The obtained results showed that CD9 expression was significantly reduced in prostate adenocarcinoma patients compared to the control group: 1 (0–9) versus 6 (2–9), $P < 0.0001$. PFS in patients with high CD9 expression (expression levels 4 to 9) was significantly longer than in patients with low CD9 expression (0–3), respectively 43.00 versus 28.50 months, [HR = 2.65 (1.15–4.28); $P = 0.016$].

DNA MMR expression was absent in 10 patients (10.86%) from 92 PCa patients (two patients with Grade group II, five patients with Grade group IV and three patients with Grade group V). This study demonstrated loss of DNA MMR expression in 8/ 36 (22.22%) of high-grade PCa patients and 2/56 (3.57%) of low-grade PCa patients. DNA MMR was present in all cases of BPH (mild to moderate staining). The study found a negative correlation between DNA MMR loss and PCa WHO 2016 Grade groups (Rho = –0.25; $P = 0.02$).

PFS in patients with DNA MMR deficiency (at least one DNA MMR) was significantly shorter than in patients with maintained DNA MMR expression, 22.00 and 60.00 months, respectively [HR = 4.18 (1.82–9.59); $P = 0.0007$].

Conclusion

1. PCa is characterized by increased CD63 expression, but decreased CD9 expression and loss of DNA MMR compared to BPH tissues.
2. The CD63 expression is increased in patients in high grade PCa, whereas the CD9 expression was decreased.
3. The loss of DNA MMR expression was demonstrated in 10.86% of PCa patients.
4. CD63 expression correlated with PCa Grade groups.
5. The loss of DNA MMR negatively correlated with the Grade groups of PCa.
6. PFS is significantly longer in patients with low CD63 expression, but high CD9 expression and proficient DNA MMR expression.

ABSTRAKT

Einführung

Kürzlich wurde gezeigt, dass Exosomen- und DNA-Reparaturproteine- Biomarkern eine wichtige Rolle bei der Tumorrisikostratifizierung und Prognosebewertung des Prostatakarzinoms (PCa) spielen können.

Der Goldstandard für die Diagnose von Prostatakarzinom ist die Biopsie und histopathologische Untersuchung, die zur Diagnose des Karzinoms verwendet wird.

Daher könnte die Bewertung von Exosomen- und DNA-Reparaturproteine -Biomarkern parallel zur bestehenden Standarddiagnostik eine spezifischere und kostengünstigere Methode sein.

In der Literatur gibt es nur begrenzte Daten darüber, ob die immunhistochemische Untersuchung von Exosomen und DNA-Reparaturproteine bei Patienten mit Prostata-karzinom und gutartiger Prostatahyperplasie unterschiedlich ist.

Das Ziel und Objektive unserer Studie

Das Ziel der Studie war es, die Expression von exosomalen Biomarkern: CD63-, CD9- und DNA-Reparaturproteine (MMR) – MSH2, MSH6, MLH1 und PMS2 im Gewebe von Patienten mit gutartiger Prostata-Hyperplasie und Prostata-Adenokarzinom zu bewerten, und zu vergleichen.

Die Ziele waren:

1. Vergleich der Expression der exosomalen Biomarker CD9, CD63 und DNA-Reparaturproteine (MSH2, MSH6, MLH1 und PMS2) durch Immunhistochemie (IHC) in Prostatagewebe mit Prostata-Adenokarzinom und benigner Prostatahyperplasie;
2. Analyse der Expression von DNA-Reparaturproteinen und exosomalen Biomarkern CD9, CD63 gemäß dem *Gleason* Grade- und PCa- Grading- Bewertungssystem (WHO 2016) bei Patienten mit Prostata-Adenokarzinom;
3. Analyse des prognostischen Werts der Expression von DNA-Reparaturproteinen und exosomalen Biomarkern CD9, CD63 für das rezidivfreie Überleben bei Patienten mit Prostata-Adenokarzinom.

Methoden

Die Studie war retrospektiv. Insgesamt wurden 92 Patienten mit Prostata-Adenokarzinom und 20 Patienten mit benigner Prostata-Hyperplasie in die Studie aufgenommen. Die Expression von CD63, CD9, MSH2, MSH6, MLH1 und PMS2 wurde durch Immunhistochemie analysiert.

Ergebnisse

Insgesamt wurden 112 Patienten in die Studie eingeschlossen: 92 Patienten mit Azinaradenokarzinom der Prostata und 20 Patienten mit benigner Prostatahyperplasie. Bei 56 Patienten wurde niedriggradiges Prostatakarzinom (Grad I- und II-Gradgruppe) und bei 36 Patienten ein hochgradiges azinäres Prostata-Adenokarzinom (Grad III–V–Gradgruppe) diagnostiziert. Wenn PCa- Patienten mit niedrigem und hohem Risiko unabhängig voneinander analysiert wurden, war die CD63-Expression bei Patienten mit Prostatakarzinom vom Grad III–V signifikant höher als bei Patienten vom Grad I–II mit 6.24 (0–9) gegenüber 1.57 (0–6) $P < 0.0001$. Das rezidivfreie Überleben (PFS) war bei Patienten mit niedriger CD63- Expression signifikant länger als bei Patienten mit größerer CD63-Expression, bzw. 42.50 gegenüber 26.50 Monaten [$HR = 1.73$ (1.06–2.84); $P = 0.018$].

Die erhaltenen Ergebnisse zeigten, dass die CD9- Expression bei Patienten mit Prostata-Adenokarzinom im Vergleich zur Kontrollgruppe signifikant verringert war: Expressionsstufe 1 (0–9) gegenüber 6 (2–9), $P < 0.0001$. Das rezidivfreie Überleben (PFS) war bei Patienten mit hoher CD9- Expression (Expressionsniveaus 4 bis 9) signifikant länger als bei Patienten mit niedriger CD9- Expression (0–3), bzw. 43.00 versus 28.50 Monate [$HR = 2.65$ (1.15–4.28) $P = 0.016$].

Bei 10 Patienten (10,86%) von 92 PCa-Patienten (zwei Patienten mit Grad II, fünf Patienten mit Grad IV und drei Patienten mit Grad V) fehlte die MMR-Expression. Die Studie zeigte einen Verlust der MMR-Expression bei 8/36 (22.22%) der hochgradigen Prostatakrebspatienten und 2/56 (3.57%) der niedriggradigen Prostatakrebspatienten. MMRs wurden in allen Fällen von gutartiger Prostatahyperplasie aufrechterhalten. Die Studie fand eine negative Korrelation zwischen MMR- Proteinverlust und Gradgruppen des Prostatakarzinoms ($Rho = –0.25$; $P < 0.02$).

Das rezidivfreie Überleben (PFS) war bei Patienten mit MMR-Mangel (mindestens ein MMR-Protein) signifikant kurzer als bei Patienten mit aufrechterhaltener MMR-Expression (22.00 versus 60.00 Monate) [HR = 4.18 (1.82–9.59); P = 0.0007)].

Zusammenfassung

Die immunhistochemische Expression von CD63 und CD9 unterscheidet sich bei Patienten mit Prostatakarzinom von Patienten mit gutartiger Prostatahypeprlasie.

Die immunhistochemische CD63-Expression hängt eng mit der Gleason-Abstufung von Prostatakarzinom zusammen.

Das rezidivfreie Überleben war bei Patienten mit MMR-Mangel (mindestens ein fehlendes MMR-Protein) signifikant kurzer als bei Patienten mit beibehaltener MMR-Expression. CD63-Überexpression sowie reduzierter CD9-Expression waren charakteristisch für ein kürzeres rezidivfreies Überleben.

ABBREVIATIONS

Abbreviation	Description
ADT	Androgen deprivation therapy
AR	Androgen receptore
ARTA	androgen receptor targeted agents
ANOVA	Analysis of variance
AS	Androgen supression
BMI	Body mass index
BPH	Benign prostatic hyperplasy
BRCA1/2	Breast cancer type 1/2
CD9	CD9 cell-surface glycoprotein
CD44	CD44 cell-surface glycoprotein
CD63	CD63 cell-surface glycoprotein
CD81	CD81 cell-surface glycoprotein
CRPC	Castration reistant prostate cancer
CT	Computer tomography
dMMR	Mismatch repair deficient
DNA	Desoxyribonucleic acid
DRE	Digital rectal examination
EBRT	External beam radiotherapy
ECM	Extracellular matrix
eLND	Extended LND
EMA	European Medicines Agency
EPE	Extra-prostatic extension
ER	Endoplasmatic reticulum
ERG	ETS (erythroblast transformation-specific) related gene
FAK	Focal adhesin kinase
FASN	Fatty Acid Synthase
FDA	Food and Drug Administration
FSH	Follicle stimulationg hormone
GS	Gleason score

HDL	High-density lipoprotein cholesterol
HER-2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HIFU	high-intensity focused ultrasound
HPV	Human papilloma virus
HT	Hormonal therapy
HSPs	Heat shock proteins
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IPSS	International Prostate Symptom Score
ISEV	International Society for Extracellular Vesicles
ISUP	International Society of Urological Pathology
KAI1	KAI 1 tumorsupressorgene on human Chromosome 11
LDL	Low-density lipoprotein cholesterol
LDR	Low-dose-rate
LH-RH	Luteinizing hormone-releasing hormone
LNs	Lymph nodes
LVI	Lymphovascular invasion
MBVs	Matrix-bound nanovesicles
mCRPC	Metastatic castration-resistant prostate cancer
MetS	Metabolic syndrome
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
miRNA	Micro RNA
MMR	Mismatch repair protein
mpMRI	multiparametric magnetic resonance imaging
mRNA	Messenger RNA
MRI	Magnetic resonance imaging
MSC	Major histocompatibility complex

MSH2	DNA mismatch repair protein, also known as MutS homolog 2
MSI	Microsatellite instability
MSI-H	Microsatellite instability-high
MVB	Multivascular bodies
NGS	Next Generation Sequencing
NCCN	National Comprehensive Cancer Network
NEPC	Neuroendocrine prostate carcinoma
nFC	Nanoscale flow cytometry
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NGF	Nerve growth factor
NHT	Neoadjuvant hormonal therapy
OS	Overall survival
PARP	Poly adenosine diphosphate ribose polymerase
PCa	Prostate cancer
PCA3	Prostate cancer gene 3
PC3	Human prostate cancer cell line
PD-1	Programmed cell death protein 1
PET	Positron emission tomography
PFS	Progression free survival
PHI	Prostate Health Index
PIN	Prostatic intraepithelial neoplasia
PINATYP	High-grade PIN with atypical glands, suspicious for adenocarcinoma
PMS2	Mismatch repair endonuclease
PI3K	Phosphoinositide 3-kinases
PSA	Prostate specific antigen
PSAV	PSA velocity
PSA-DT	PSA doubling time
PSMA	Prostate specific membrane antigen

PTEN	Phosphatase and tensin homologe
RECIST	Response Evaluation Criteria in Solid Tumours
REDUCE	Reduction by Dutasteride of Prostate Cancer Events
RNA	Ribonucleic acid
RP	Radical prostatectomy
RT	Radiation therapy
STAT3	Signal transducer and activator of transcription 3
TC	Total cholesterol
TGN	Trans Golgi network
TMPRSS2-ERG	Gene fusions (predominant molecular subtype of prostate cancer)
TM9SF4	Transmembrane 9 Superfamily Member 4
TNM	Tumor Node Metastasis
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TRUS	Transrectal ultrasound
TURP	Transurethral resection of the prostate
UICC	Union for International Cancer Control
US	Ultrasound
UTI	Urinary tract infections
WW	Watchful waiting
ZIP1	Zinc transporter 1 protein
4K	Four kallikrein
5-ARIs	5-alpha-reductase inhibitors

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1. INTRODUCTION

Prostate cancer (PCa) remains the second most commonly diagnosed cancer in men, with an estimated 1.1 million diagnoses worldwide in 2012, accounting for 15% of all cancers diagnosed [1]. In Latvia in 2017 PCa was the most commonly diagnosed cancer in men with an incidence of 1286 cases per year [2, 3].

Present diagnostic markers such as prostate specific antigen (PSA) and its isoforms have substantial drawbacks such as false-negatives, false-positives and lack of tumor-type specificity [1].

Tumor biopsy and histopathological evaluation is still the only definitive method of diagnosis, but it is invasive.

Different risk classification tools have been developed to distinguish patients with early PCa according to the prognosis, including the D'Amico classification system, the Cancer of the Prostate Risk Assessment score, and the National Comprehensive Cancer Network (NCCN) risk groups classification [1,4–21]. All these systems recognize a low risk of progression for patients with a biopsy Gleason score ≤ 6.

Liquid biopsies, circulating tumor cells, exosomes and circulating nucleic acids have been developed as minimally invasive assays to monitor PCa patients. Recently, there has been remarkable progress in understanding the role of exosomes in cancer development and progression. Previous studies showed that exosomes from cancer cells might be associated with intracellular communications involved in the development of the tumor microenvironment, such as metastatic niche formation and angiogenesis, resulting in the progression of carcinoma [22–46].

Exosomes are vesicles of 30–150 nm diameter, loaded with unique cargo, including proteins, nucleic acids, lipids, and metabolites, that could predict the cells of their origin [46].

Exosomal membranes are enriched with endosome-specific tetraspanins such as CD9, CD63, and CD81 [46].

Several authors suggested that exosomes obtained from blood and urine are a consistent source of miRNA for disease biomarker detection, although some doubts have been presented by other researchers underlying that exosomes in standard preparations do not carry a biologically significant amount of miRNAs. Moreover, according to *Arroyo et al.*, vesicle associated miRNAs only represent a minority, while around 90% of miRNAs in the

circulation are present in a non-membrane-bound form. Instead, *Gallo et al.* showed that the majority of miRNAs detectable in serum and saliva are concentrated in exosomes. Moreover, *Cheng et al.* showed that in urine the highest proportion of miRNA was extracted from exosomes [46–85].

Recently it has been shown that CD63 concentration isolated from plasma exosomes in patients with PCa was significantly higher compared to patients with benign hyperplasia [86] In addition, it was showed that CD63 level in urine samples was significantly increased in patients with PCa [87].

However, the significance of CD63 in patients with PCa tissue has not been fully investigated [86, 87].

Furthermore, exosomes have been shown to be crucial for the development of drug resistance in patients with prostate carcinoma. Exosome-derived microRNAs also contribute to PCa chemoresistance and can act as surrogate biomarkers of tumor response to taxanes [88].

CD9 protein is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. Tetraspanins are cell surface glycoproteins with four transmembrane domains that form multimeric complexes with other cell surface proteins. The encoded protein functions in many cellular processes including differentiation, adhesion, and signal transduction, and expression of this gene plays a critical role in the suppression of cancer cell motility and metastasis. It is found on the surface of exosomes and is considered exosome housekeeping protein for the quantitative analysis of plasma derived nanovesicles [90].

PCa related exosomes from clinical samples in general show the presence of some cancer related proteins such as CD9, CD81, and TSG101, Annexin A2, Fatty Acid Synthase (FASN) and a PCa specific biomarker, FOLH1 (Prostate Specific Membrane Antigen or PSMA) [46, 65, 66, 90].

Some reports indicate the trend of exosome markers in cancer progression. For example, in clinical studies, reduced expression levels of the tetraspanin CD9 are correlated with tumor progression in a range of cancers. Crossing a CD92/2 (KO) murine model with transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (a PCa mouse model showing de novo development of spontaneously metastasising PCa) shows that ablation of CD9 had no detectable effect on de novo primary tumor onset, but increased metastasis to the liver [46, 72, 90].

Failure of effective DNA damage repair is a hallmark of cancer. Mismatch repair (MMR) pathway recognizes and repairs insertions, deletions and base–base mismatches that occur on single-stranded DNA during replication. However previous studies showed that the prevalence of mismatch repair pathway deficiency is about 15% of PCa cases. The most commonly altered MMR genes are MSH2 and MSH6, which can be inactivated by intronic structural rearrangements that are undetectable by exon-limited sequencing approaches [91–93].

However, it is not yet completely clear whether MMR defects are enriched in metastatic disease relative to localized disease [91–93].

A recent study of MSH2 protein expression in 1133 primary prostatic adenocarcinomas identified loss in 1.1% of cases, which is similar to metastatic disease, but significantly enriched in Gleason 9–10 tumors, which implies an association with disease progression [93, 94].

To conclude, it has been previously demonstrated the value of exosomal biomarkers CD63 and CD9 in patients with prostate carcinoma in blood serum, plasma and urine and the significant correlation with disease prognosis with the level of exosomes in blood plasma has been observed. Furthermore, it has been recently, shown the role of DNA mismatch repair protein in the high-grade cancer.

However, the tissue expression of exosomal biomarkers CD63 and CD9 has not been previously investigated, and it is still unclear whether the CD9 and CD63 expression increased also in the tissue of PCa and whether it correlated with clinical behavior, histopathological characteristics and disease prognosis.

Novelty, scientific and practical importance of the study

PCa remains the second most commonly diagnosed cancer in men, with an estimated 1.1 million diagnoses worldwide, accounting for 15% of all cancers diagnosed. In Latvia in year 2017 PCa was the most commonly diagnosed cancer in men with an incidence of 1286 cases per year.

Present diagnostic markers such as prostate specific antigen (PSA) and its isoforms have substantial drawbacks such as false-negatives, false-positives and lack of tumor-type specificity.

The gold standard for prostate carcinoma diagnosis is biopsy and histopathological examination, which is used to diagnose the cancer, but these are invasive methods, oft to been done within general anesthesia, with appropriate procedure associated risk and complication as well as cost-bearing setting.

Recently, it has been shown that exosome and DNA repair biomarkers could play an important role in cancer risk stratification and prognosis assessment. But there exists limited and controversial information in literature about the role of these markers in diagnostical and therapeutically setting in relation to PCa.

Relatively limited clinicomorphological research has been done yet on the detection of exosome- and DNA repair-biomarker expression in prostate tissue to the relation of BPH and PCa, especially within its complicated and wide-ranging diagnostical and therapeutically setting in the world.

To date, no studies have been performed in Latvia on the correlation of exosomal and DNA MMR biomarkers with the histopathological finding of PCa and disease progression free survival.

Thus, the evaluation of exosome- and DNA repair-biomarkers in parallel with standard existing diagnostics could be a more specific, with a disease progression free survival prognostic role, thus also associated with lower treatment costs.

2. REVIEW OF LITERATURE

Prostate cancer

Epidemiology

Prostate cancer (PCa) remains the second most common diagnosed cancer in males, with an estimated 1.1 million diagnoses worldwide in 2012, accounting for 15% of all cancers. The frequency of autopsy detected PCa is roughly the same worldwide. Series of autopsy studies showed a prevalence of PCa at age < 30 years of 5% (95% CI: 3–8%), increasing by an odds ratio of 1.7 (1.6–1.8) per decade, to a prevalence of 59% (48–71%) by age > 79 years [1].

The incidence of PCa, however, varies widely between different geographical areas, being highest in Australia, New Zealand and Northern America (age-standardized rates (ASR) of 111.6 and 97.2 per 100,000, respectively), and in Western and Northern Europe (ASR 94.9 and 85), largely due to the use of PSA testing and the aging population. The incidence is low in Eastern and South-Central Asia (ASR 10.5 and 4.5), whilst rates in Eastern and Southern Europe, which were low, have showed a steady increase [1].

There is relatively less variation in mortality rates worldwide, although rates are generally high in populations of African descent (Caribbean, 29 per 100,000 and Sub-Saharan Africa, ASRs 19–24 per 100,000), intermediate in the USA and very low in Asia (2.9 per 100,000 in South-Central Asia) [1].

In Latvia in 2017 the PCa in males was the most common cancer. There was new 1286 cases of PCa in 2017. Furthermore, 427 patients died due to PCa in 2017. By the end of 2017, 8,665 men were registered with a diagnosis of PCa. 48% of those registered have lived for more than 5 years since the diagnosis. In Latvia PCa in 43% of cases was detected in stage II, but in 25% of cases in stage I [2, 3].

Etiology of prostate cancer

Multiple risk factors have been implemented in the development of PCa.

Both inherited and acquired risk factors had the significant role in the progression of cancer.

Genetical risk factors

Family history and racial/ethnic background are associated with an increased PCa incidence suggesting a genetic predisposition. However, only a small subpopulation of men with PCa (~ 9%) have true hereditary disease. This is defined as three or more affected relatives, or at least two relatives who have developed early-onset PCa (< 55 years). Males with African ethnicity origin show a higher incidence of PCa and generally have a more lethal course of disease [1].

Genome-wide association studies have identified 100 common susceptibility loci contributing to the risk for PCa, explaining ~ 38.9% of the familial risk for this disease. Furthermore, an incidence was found of 11.8% of germline mutations in genes mediating DNA-repair processes among men with metastatic PCa. Germline mutations in genes such as HOXB13 and BRCA1/2 have been associated with an increased risk of PCa, targeted genomic analysis of these genes could offer options to identify families at high risk. Trials of screening for PCa-targeting BRCA mutation carriers are ongoing [1].

Acquired and nutritional risk factors

A wide variety of exogenous/environmental factors have been discussed as being etiologically important for the risk of progression from latent to clinical PCa. Results of ecological studies suggest that PCa is associated with western lifestyle and in particular, diet that includes a high intake of fat, meat, and dairy products [1].

Although the results of these studies are contradictory, but some dietary components are associated with prostate cancer e.g., high intakes of α-linolenic acid (a polyunsaturated fatty acid in vegetables and dairy products) and calcium [1].

Several large studies have been conducted in recent years to demonstrate the role of insulin-like growth factor (IGF) -I in increasing the risk of prostate cancer. Insulin-like growth factor is a peptide growth factor that is easily measured in the circulation, regulates proliferation, differentiation, and apoptosis of cancer cells. The IGF system might be the link

between the sedentary western lifestyle and prostate cancer: consumption of large amounts of fat result in raised production of insulin that in turn increase production of IGF, thus explaining how IGF could be a risk factor for prostate cancer [1].

The single components hypertension and waist circumference > 102 cm of metabolic syndrome (MetS) have been associated with a significantly greater risk of PCa, but conversely, having > 3 components of MetS is associated with a reduced [1]. In addition, the diabetes, obesity, fat rich diet, hormone treatment increased the risk of PCa development [1].

Prostate cancer theoretically may result from an increase in oxidative stress, but supportive evidence is limited. Clinical studies indicate that intake of antioxidants, such as selenium, tocopherol (vitamin E), and lycopene (a carotenoid) offers protection against PCa [1, 120].

However, currently there are no effective preventative dietary or pharmacological interventions [1].

Pathology of prostate cancer

The most common histopathological form of PCa is classified as an adenocarcinoma, or glandular cancer, that begins when normal semen-secreting prostate gland cells mutate into cancer cells. The region of prostate gland where the adenocarcinoma is most common is the peripheral zone [4–7].

PCa most commonly metastasizes to the bones, lymph nodes, and may invade rectum, bladder and lower ureters after local progression. The route of metastasis to bone is thought to be venous as the prostatic venous plexus draining the prostate connects with the vertebral veins [7].

PCa cells are generally devoid of zinc. This allows PCa cells to save energy not making citrate and utilize the new abundance of energy to grow and spread. The absence of zinc is thought to occur via a silencing of the gene that produces the transporter protein ZIP1. Zinc inhibits NF-κB (nuclear factor kappa light chain enhancer of activated B cells) pathways, is anti-proliferative and induces apoptosis in abnormal cells. Unfortunately, oral ingestion of zinc is ineffective since high concentrations of zinc into prostate cells is not possible without the active transporter, ZIP1 [8]. Loss of cancer suppressor genes, early in the prostatic carcinogenesis, have been localized to chromosomes 8p, 10q, 13q, and 16q [9].

p53 mutations in the primary PCa are relatively low and are more frequently seen in metastatic settings, hence, p53 mutations are a late event in the pathology of PCa. Other tumor suppressor genes that are thought to play a role in PCa include phospathase and tensin homolog (PTEN) gene and KAI1 tumorsupressorgene on human Chromosome 11 (KAI1). Up to 70 percent of men with PCa have lost one copy of the PTEN gene at the time of diagnosis [9]. Relative frequency of loss of E-cadherin and CD44 has also been observed [9].

RUNX2 is a transcription factor that prevents cancer cells from undergoing apoptosis thereby contributing to the development of PCa [10].

The PI3k/Akt signaling cascade works with the transforming growth factor beta/SMAD signaling cascade to ensure PCa cell survival and protection against apoptosis [11]. X-linked inhibitor of apoptosis (XIAP) is hypothesized to promote PCa cell survival and growth and is a target of research because if this inhibitor can be shut down then the apoptosis cascade can carry on its function in preventing cancer cell proliferation [12]. Macrophage inhibitory cytokine-1 (MIC-1) stimulates the focal adhesion kinase (FAK) signaling pathway which leads to PCa cell growth and survival [13].

The androgen receptor (AR) helps PCa cells to survive and is a target for many anti-cancer research studies; so far, inhibiting the AR has only proven to be effective in mouse studies [14].

Prostate specific membrane antigen (PSMA) stimulates the development of PCa by increasing folate levels for the cancer cells to use to survive and grow; PSMA increases available folates for use by hydrolyzing glutamated folate [15].

A series of studies involved introduced viruses known to cause cancerous mutation in prostate cells: Serine/threonine-*protein* kinase *AKT (AKT),* erythroblast transformation-specific related gene (ERG), and AR into isolated samples of basal and luminal cells and grafted the treated tissue into mice. After 16 weeks, none of the luminal samples had undergone malignant mutation, while the basal samples had mutated into prostate-like tubules which had then developed malignancy and formed cancerous tumors, which appeared identical to human samples under magnification. This led to the conclusion that the prostate basal cell may be the most likely “site of origin” of PCa [16].

Arachidonate 5-lipoxygenase has been identified as playing a significant role in the survival of PCa cells [17–19]. Medications which target this enzyme may be an effective therapy for limiting tumor growth and cancer metastasis as well as inducing programmed

cell death in cancer cells [17–19]. In particular, arachidonate 5-lipoxygenase inhibitors produce massive, rapid programmed cell death in PCa cells [17–20].

Classification and staging

The Gleason grading system for prostate adenocarcinoma has evolved from its original scheme established in the 1960s–1970s, to a significantly modified system after three major consensus meetings conducted by the International Society of Urologic Pathology (ISUP) in 2005, 2014 and 2019, respectively. The Gleason grading system has been incorporated into the World Health Organisation (WHO) classification of PCa, the American Joint Committee on Cancer / the International Union against Cancer (AJCC/UICC) staging system, and the National Comprehensive Cancer Network (NCCN) guidelines as one of the key factors in treatment decision [21].

Gleason grading system, which is based on architectural patterns of prostate adenocarcinoma demonstrated on hematoxylin-eosin (H & E) sections, rather than cellular features [21].

The Gleason score is the sum of the primary (most predominant in terms of surface area of involvement) Gleason grade and the secondary (second most predominant) Gleason grade. Where no secondary Gleason grade exists, the primary Gleason grade is doubled to obtain a goal at a Gleason score. The primary and secondary grades should be reported in addition to the Gleason score, that is, Gleason score 7(3 + 4) or 7(4 + 3) [21].

The Gleason scores (from 2 to 10) have been variably lumped into different groups for prognosis and patient management purposes. It has been proposed the grouping scores into 5 prognostic categories, grade groups, 1–5 [21]. This grade grouping strongly correlate with biochemical recurrence [21].

At the 2014 ISUP Consensus Conference, details of this prognostic system were clarified, and it was recommended for usage together with the Gleason system. This grade grouping has also been subsequently validated by other independent studies in surgical and radiation cohorts show significant correlation with survival [21]. The new grade grouping has been endorsed in the 2016 WHO classification [21].

The grade grouping has also been endorsed by ISUP and is referred to as ISUP grade in literature [1, 21]. Like Gleason scoring in needle biopsies, the grade group can be applied at core, specimen, or case levels [21] (*Table 2.1.*).

Table 2.1. **International Society of Urological Pathology 2014 grades (adapted from [21])**

Gleason score	**ISUP grade**
2–6	1
7 (3 + 4)	2
7 (4 + 3)	3
8 (4 + 4 or 3 + 5 or 5 + 3)	4
9–10	5

The PCa clinical classification is based on current 8th Edition of AJCC Staging Manual [21].

Based on clinical TNM staging, PSA value and Grade groups the risk group for PCa biochemical recurrence has been elaborated (*Table 2.2.*) (adapted from [1]).

Table 2.2. **EAU risk groups for biochemical recurrence of localised and locally advanced prostate cancer (adapted from [1]. GS = Gleason score; ISUP = International Society for Urologcal Pathology; PSA = prostate-specific antigen**

Definition			
Low-risk	**Intermediate-risk**	**High-risk**	
PSA < 10 ng/mL and GS < 7 (ISUP grade 1) and cT1-2a	PSA 10–20 ng/mL or GS 7 (ISUP grade 2/3) or cT2b	PSA > 20 ng/mL or GS > 7 (ISUP grade 4/5) or cT2c	any PSA any GS cT3-4 or cN+ Any ISUP grade
Localised			Locally advanced

Diagnosis of PCa is based on histology. The diagnostic criteria include features pathognomonic of cancer, major and minor features favouring cancer and features against cancer. Ancillary staining and additional (deeper) sections should be considered if a suspect lesion is identified. Diagnostic uncertainty is resolved by intradepartmental or external consultation. *Table 2.3.* reviewed the recommended terminology for reporting prostate biopsies [1, 21].

Table 2.3. **Recommended terminology for reporting prostate biopsies [1]**

Benign/negative for malignancy; if appropriate, with included ancillary description
Active inflammation
Granulomatous inflammation
High-grade prostatic intraepithelial neoplasia (PIN)
High-grade PIN with atypical glands, suspicious for adenocarcinoma (PINATYP)
Focus of atypical glands/lesion suspicious for adenocarcinoma/atypical small acinar proliferation, suspicious for cancer
Adenocarcinoma
Intraductal carcinoma

Each biopsy site should be reported individually, including its location (in accordance with the sampling site) and histopathological findings, which include the histological type and the ISUP 2019 Gleason grading system. A global Gleason score comprising all biopsies is also reported according to the ISUP 2014 grade. Intraductal carcinoma, lymphovascular invasion (LVI) and extra-prostatic extension (EPE) must each be reported, if identified. More recently, expansile cribriform pattern of PCa as well as intraductal carcinoma in biopsies were identified as independent prognosticators of metastatic disease [1, 21].

The proportion of carcinoma-positive cores as well as the extent of tumour involvement per biopsy core correlate with the Gleason score, tumour volume, surgical margins and pathologic stage in radical prostatectomy (RP) specimens and predicts biochemical recurrence (BCR), post-prostatectomy progression and radiation therapy (RT) failure. These parameters are included in nomograms created to predict pathologic stage and seminal vesicle invasion after RP and RT failure. A pathology report should therefore provide both the proportion of carcinoma-positive cores and the extent of cancer involvement for each

core. The length in millimeters and percentage of carcinoma in the biopsy have equal prognostic impact. An extent of > 50% of adenocarcinoma in a single core is used in some active surveillance (AS) protocols as a cut off triggering immediate treatment *vs.* AS in patients with Gleason score 6 [1, 21].

A prostate biopsy that does not contain glandular tissue should be reported as diagnostically inadequate. Mandatory elements to be reported for a carcinoma-positive prostate biopsy are:

- type of carcinoma;
- primary and secondary/worst Gleason grade (per biopsy site and global);
- percentage high-grade carcinoma (global);
- extent of carcinoma (in millimeters or percentage) (at least per biopsy site);
- if present: extraprostatic extension (EPE), seminal vesicle invasion, lymphovascular invasion (LVI), intraductal carcinoma/cribriform pattern, peri-neural invasion [1, 21].

The role of exosomes in the pathogenesis and diagnosis

Exosomes

Exosomes are cell-derived vesicles that are present in many and perhaps all eukaryotic fluids, including blood, urine, and cultured medium of cell cultures [22, 23].

A sub-type of exosomes, defined as Matrix-bound nanovesicles (MBVs), was reported to be present in extracellular matrix (ECM) bio-scaffolds (non-fluid). The reported diameter of exosomes is between 30 and 100 nm, which is larger than low-density lipoproteins (LDL) but much smaller than, for example, red blood cells [22, 23].

Exosomes are either released from the cell when multivesicular bodies fuse with the plasma membrane or released directly from the plasma membrane [23, 24]. Evidence is accumulating that exosomes have specialized functions and play a key role in processes such as coagulation, intercellular signaling, and waste management [22–24]. Consequently, there is a growing interest in the clinical applications of exosomes. Exosomes can potentially be used for prognosis, for therapy, and as biomarkers for health and disease [25–26].

Exosomes contain various molecular constituents of their cell of origin, including proteins and RNA. Although the exosomal protein composition varies with the cell and tissue of origin, most exosomes contain an evolutionarily conserved common set of protein molecules.

The protein content of a single exosome, given certain assumptions of protein size and configuration, and packing parameters, can be about 20,000 molecules [27–31]. The cargo of mRNA and miRNA in exosomes was first discovered at the University of Gothenburg in Sweden [32].

Exosomes can transfer molecules from one cell to another via membrane vesicle trafficking, thereby influencing the immune system, such as dendritic cells and B cells, and may play a functional role in mediating adaptive immune responses to pathogens and tumors. mRNA in exosomes has been suggested to affect protein production in the recipient cell [33–45].

Up to date pitfalls of current available isolation methodologies have been discussed in the scientific community and recently have been reported in detail. They range from classical methodologies such as ultracentrifugation, as well as immunoprecipitation, gel-filtration, size exclusion chromatography, size exclusion filters, to microfluidic devices. Identification is probably less problematic than purification as a few proteins are widely accepted as exosomal markers including: Alix, tumor susceptibility gene 101 (TSG101) (component of the Endosomal Sorting Complex Required for Transport, ESCRTs), CD9, CD63, CD81, and transferrin receptor (TfR) [46].

Exosomes released from tumors into the blood may also have diagnostic potential [46–48].

Exosomes are remarkably stable in body fluids strengthening their utility as reservoirs for disease biomarkers [49].

Urinary exosomes have also proven to be useful in the detection of many pathologies, such as mineralocorticoid hypertension, through their protein and miRNA cargo [50].

Increasingly, exosomes are being recognized as potential therapeutics as they have the ability to elicit potent cellular responses in vitro and in vivo. Exosomes mediate regenerative outcomes in injury and disease that recapitulate observed bioactivity of stem cell populations. Mesenchymal stem cell exosomes were found to activate several signaling pathways important in wound healing (protein kinase B (Akt), extracellular signal-regulated kinase (ERK), and signal transducer and activator of transcription 3 (STAT3)) and bone fracture repair. They induce the expression of a number of growth factors (hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF1), nerve growth factor (NGF), and stromal-derived growth factor-1 (SDF1)). Exosomes secreted by human circulating fibrocytes, a population of mesenchymal progenitors involved in normal wound healing via paracrine signaling,

exhibited in-vitro proangiogenic properties, activated diabetic dermal fibroblasts, induced the migration and proliferation of diabetic keratinocytes, and accelerated wound closure in diabetic mice in vivo. Important components of the exosomal cargo were heat shock protein-90α, total and activated signal transducer and activator of transcription 3, proangiogenic (miR-126, miR-130a, miR-132) and anti-inflammatory (miR-124a, miR-125b) microRNAs, and a microRNA regulating collagen deposition (miR-21). Exosomes can be considered a promising carrier for effective delivery of small interfering RNA due to their existence in body's endogenous system and high tolerance. Patient-derived exosomes have been employed as a novel cancer immunotherapy in several clinical trials [22, 23, 26, 31, 34, 38, 48, 51].

Exosomes contain RNA, proteins, lipids and metabolites that is reflective of the cell type of origin. As exosomes contain numerous proteins, RNA and lipids, large scale analysis including proteomics and transcriptomics is often performed [52].

Exosomes offer distinct advantages that uniquely position them as highly effective drug carriers. Composed of cellular membranes with multiple adhesive proteins on their surface, exosomes are known to specialize in cell–cell communications and provide an exclusive approach for the delivery of various therapeutic agents to target cells. For example, researchers used exosomes as a vehicle for the delivery of cancer drug paclitaxel. They placed the drug inside exosomes derived from white blood cells, which were then injected into mice with drug-resistant lung cancer. Importantly, incorporation of paclitaxel into exosomes increased cytotoxicity more than 50 times as a result of nearly complete co-localization of airway-delivered exosomes with lung cancer cells [53].

The role of exosomes in different malignant tumors

A recent investigation showed that exosome release positively correlates with the invasiveness of ovarian cancer [47].

One of potential surrogate method to detect exosomes in the tissue is the immunohistochemical method. There are only few studies investigated the role of exosomal biomarkers in the tissue model. In pancreatic cancer, the expression of CD63 has been reported to be higher in cancerous tissues than in normal tissues [54].

Furthermore, it has been shown that CD63 expression in gastric cancer cells was a significant independent prognostic factor in patients with gastric cancer [55].

In breast cancer it has been demonstrated that patients with CD9 expression had worse overall survival and disease-free survival compared to patients without CD9 expression [56].

In addition, it was revealed that high CD9 expression correlated with breast cancer bone metastasis [57].

At contrast in tissue of colorectal carcinoma it has been observed that high expression of CD9 was associated with a favorable disease-free survival, especially in left-sided colorectal carcinoma [58].

Furthermore, recently for the first time the difference in exosomal marker (CD63) expression pattern and its prognostic significance in patients with right side colorectal carcinoma and left side colorectal carcinoma has been demonstrated. It has been observed that there was no difference in progression free survival (PFS) between patients with right and left side colorectal carcinoma. In all patients, there was no difference in PFS in patients with CD63 expression. However, among patients with right side colorectal carcinoma, there was a significantly lower PFS in patients with CD63 expression. At contrast, among patients with left side colorectal carcinoma, there was no difference in PFS in patients with CD63 expression [59].

Exosomes as Prostate Cancer Biomarkers

Studies of exosomes relating to PCa have also been confused with terminology, in this case, “prostasomes”. The prostasomes are vesicles isolated from semen. *Stridsberg et al.* reported the prostasomes as neuroendocrine-like vesicles, as they contain neuroendocrine markers, chromogranin B, neuropeptide Y, and vasoactive intestinal polypeptide [60]. Prostasomes have a mean diameter of 150 nm, even though some authors report a broader range of 40–500 nm vesicles; they contain CD38, PSMA, and Anx1, have a complex membrane composition (cholesterol/phospholipid ratio 2:1), as well as expressing exosomal markers, CD9 and CD63, and other markers such as CD46, CD55 and CD59. Prostasome specific markers are commonly found to be located at the plasma membrane [60].

Images of prostasomes show a multiplicity of vesicular structures, with smaller vesicles inside larger vesicles. The prostasomes have a trilamellar membrane structure, are round or egg shaped and some have “cauliflower-like” protrusions. The prostasomes also vary in electron density [46, 60].

This description is significantly different from exosomes, which are usually described, according to *Bijaya et al*. as round, cup-shaped vesicles [60, 81].

However, nanosized vesicles secreted by prostate cancer cell line 3 (PC3) cells, have also been previously reported as prostasomes, presumably due to the discrepancy in vesicle size as discussed above. PC3 derived vesicles have a diameter of 30–150 nm with a round, cup shape morphology and they contain exosome markers TSG101 and CD63. In a paper by *Llorente et al.*, electron microscope (EM) images of secreted vesicles, some with diameters around 150 nm, and the CD63 (one of many exosome markers)-labelled nanovesicles were found in organelles that resemble the multivesicular bodies (MVB). *Llorente et al.* have also shown an apparent release of these vesicles. While the paper named the vesicles as "microvesicles", the MVB origin of these vesicles suggests that these vesicles are exosomes and that exosomes from PC3 can be bigger than 100 nm [61, 62].

Another PCa literature analysis shows an EM image of isolated exosome, secreted from the PC346C, labeled with CD9, which is also bigger than 100 nm [63, 64].

Validation of exosomes in the provision of PCa biomarkers can only be achieved by understanding the exosome biogenesis in PCa. Some authors have suggested that vesicles isolated from biofluids should be termed extracellular vesicles since their biogenesis is unknown. However, publications related to this data call these exosomes based on their purification methodology, markers and size. Given the current literature, it has been continued to use the term "exosomes" for vesicles that harbor currently accepted exosome specific markers. Proteins isolated from circulating exosomes are not reflective of the proteins that are highly expressed in the respective cells, or in other cells as well as PCa cells [63, 64], demonstrating that the content of exosomes is selectively screened intracellularly from cytoplasmic proteins through the MVB sorting process. This process of exosome biogenesis provides a source of biomarkers that do not rely on a single protein or molecule, but a group of proteins, ribonucleic acid (RNAs), deoxyribonucleic acid (DNAs) and lipids in a population of vesicles, all of which in combination may help to stage heterogeneous diseases such as PCa. As drugs used to treat cancer would also affect the intracellular dynamic, the exosomes may also provide improved biomarkers to indicate treatment responsiveness or resistance [46, 63, 64].

Proteomic studies of PCa exosomes have been reported on various PCa cell lines and clinical samples. PCa related exosomes from clinical samples in general show the presence of some cancer related proteins such as CD9, CD81, and TSG101, Annexin A2, FASN and a PCa specific biomarker folate hydrolase 1 (FOLH1), PSA or PSMA [46, 65, 66].

PSMA is a transmembrane glycoprotein that is expressed in normal prostatic epithelial cells and elevated in androgen deprived PCa, and confirmed to be highly upregulated in poorly differentiated, metastatic, and hormone refractory carcinomas [65, 66]. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays with primers specific for PSMA have been shown to be more effective than PSA-specific primers in detecting PCa cells [65, 66, 67]. PSMA is also used in immunotherapy of PCa [67]. PSMA was currently utilized as an immunoscintigraphic target using the antibody conjugate *ProstaScint* to detect occult PCa, but the antibody used for this detection could only recognized an internal epitope; as such it would detect dead PCa cells. New tests have been developed using an antibody against an external epitope [68]. PSMA expression has been evaluated as predictive marker of PSA recurrence; even though PSMA is independently associated with PSA recurrence in a high-risk cohort [65–68]. *Mitchell et al.* have reported a pilot study (10 PCa patients and 10 healthy donors) in which five of eight PCa patients had PSMA in their urine derived exosomes, whilst three of eight PCa patients expressed PSA in the exosomes as indicated by western blot analysis [45, 67–69]. The presence of PSA in exosomes from this study might suggest that the exosome isolation procedure from urine may be contaminated by soluble proteins as it has been reported *Jansen et al*., that PSA is not found in the exosomes [70].

PCa proliferation and survival are greatly influenced by phosphoinositidine 3-kinases (PI3K) pathways. In PCa, loss of phosphatase and tensin homolog (PTEN), a lipid phosphatase and negative regulator of PI3K, will cause a more severe disease, as hemizygous mutation of PTEN increases the risk and biochemical relapse, while homozygous deletion of PTEN increase the incidence of metastasis of PCa [69, 71]. Recently, *Gabriel et al.* reported that PTEN is found in exosomes isolated from DU145 cells and these exosomes can influence cell proliferation in PTEN negative cells such as DU145Kd (knock down PTEN DU145 cells) and PC3. PTEN is also found in exosomes isolated from the blood of PCa patients, while normal individuals have no PTEN in their exosomes. In this study however, PSA was also found in exosomes from both PCa patients and normal individuals [71].

Some reports indicate the trend of exosome markers in cancer progression. For example, in clinical studies, reduced expression levels of the tetraspanin CD9 are correlated with tumor progression in a range of cancers [46]. Crossing a CD92/2 knockout (KO) murine model with (TRAMP) mice (a PCa mouse model showing de novo development of spontaneously metastasising PCa) shows that ablation of CD9 had no detectable effect on de novo primary tumor onset, but increased metastasis to the liver [72].

Comparison of exosomal proteins secreted by some AR positive and AR negative PCa cell lines grown in serum free medium has been reported. However, it was not very clear whether the exosome pathways of each PCa cell line studied were affected by the growth conditions used in the study [73].

Apart from the protein content, the RNA species found in exosomes could also provide an indication for cancer progression. For example, despite the lack of PSA protein in exosomes, the RNA was present in exosomes isolated from both VCaP and PC346C (an androgen dependent cell) [70, 74].

In PCa, specific gene fusions such as the TMPRSS2-ERG (predominant subtype of PCa) gene fusion event occur in a subset of patients and are associated with lethal PCa. TMPRSS2-ERG gene fusion is androgen regulated and found in 50% of clinically localised PCa, and in 90% of PCa over-expressing ERG. The TMPRSS2-ERG RNA is found in exosomes isolated from VCaP (an androgen responsive cell) [46, 74].

Both TMPRSS2-ERG and a PCa biomarker, prostate cancer antigen (PCA-3) mRNA were also reported in urine derived exosomes (CD63-labelled vesicles), showing the mRNA content of exosomes is informative and can provide potential biomarkers for PCa [45, 74].

Mitchell et al. have reported that expression of miR-141, the first miRNA reported as a potential diagnostic marker in PCa, correlates significantly with serum PSA levels and could detect individuals with advanced metastatic PCa with 60% sensitivity and 100% specificity [74].

In 2009, *Nilsson et al*. reported that prostate cancer-derived exosomes, which contain the prostate cancer-specific markers of PCA3 and TMPRSS-ERG, were detected in urine after prostate massage of patients with PCa [75].

Llorente et al. have characterized other miRNAs as PCa biomarkers from biofluids and have shown several promising candidates depending on the stage of PCa [61, 62, 74, 75].

Bryant et al. reported on 11 miRNAs derived from microvesicles which were present in a significantly higher amount in plasma samples of PCa patients compared to normal individuals. Ten miRNAs were also identified to differ quantitatively between patients with distant metastasis in comparison with normal individuals [76]. In this study, miR-375 and miR-141 were significantly increased in microvesicles from sera of metastatic patients compared with non-recurrent PCa patients. The microvesicles were isolated using 1.2 µm filter and concentrated using a filter concentration (150 kDa cut off). This purification

strategy is not able to differentiate exosomes from other microvesicles, thus it is not clear whether the miRNAs studies were exosomally derived miRNAs [76].

In cell line models, functional studies on miRNA exosomes using DU145 (AR negative) and CW22RV1 (AR positive) cell lines were recently published. The PCa cells were infected with exosomes derived from docetaxel resistant cells, and exosome-exposed cells became capable of conferring the resistance toward docetaxel [77]. Cells exposed to exosomes isolated from patients after docetaxel treatment have also shown an increased level of docetaxel resistance, indicating the potential of exosomes to indicate docetaxel resistance [77].

Exosomes are enriched in glycosphingolipids, sphingomyelin, cholesterol, and phosphatidylserine in various PCa cell lines, irrespective of the presence of AR. Interestingly, the lipid species found to be enriched in exosomes, similar to proteins and miRNA, are also not the ones mostly found in cell lysates. In PC3 cells, there is an 8-fold enrichment of lipids/mg protein in exosomes, in comparison with the cell lysates [46, 77].

Some studies show the role of lipids is to influence the amount of exosomal proteins being secreted. As well a particular lipid species, the sphingolipid, has a role in exosome biogenesis. Calveolin-1 (cav-1), an integral membrane protein which binds to lipid rafts, has been shown to be upregulated in metastatic PCa and its expression is correlated with Gleason Score [78], indicating the potential of cav-1 as biomarker for advanced PCa. *Llorente et al*. have reported that the amount of cav-1 in isolated exosomes from PC3 cells increases with time [61, 62, 78]. Treatment using membrane-impermeable, cholesterol-extracting drug (MBCD) increased cav-1 levels in exosomes, but not in the cell lysates, suggesting that the cholesterol/lipid raft is involved in regulating exosomal protein secretion. Cholesterol, however, does not seem to influence exosome size [61, 62, 78].

Due to the micellar nature of extracellular vesicles such as exosomes, some biomolecules present in these vesicles can be detected without lysing the vesicles because they reside on the membrane, whereas some others may only be detected after lysis of the vesicles because they are located within the vesicle [79].

TM9SF4 is a recently described transmembrane protein highly expressed in melanoma, colon carcinoma cell lines and acute myeloid leukemia and myelodysplastic syndromes. Recent studies have demonstrated that TM9SF4 is an autophagic marker involved in tumor cells cannibalism, a phenomenon often related to poor prognosis. TM9SF4 is mainly detectable in intracellular vesicles such as endosomes and other vesicles, where aberrantly

activates the H^{+}-Vacuolar ATPase, a proton pump involved in the tumor pH gradient alterations associated with drug resistance and invasiveness of cancer cells [79].

TM9SF4 protein is a recently described transmembrane protein that belongs to Transmembrane-9 Superfamily (TM9SF), a well-defined family of proteins characterized by a large hydrophylic N-terminal domain followed by nine transmembrane domains. This protein is known to be overexpressed in melanoma and in acute myeloid leukemia and myelodysplastic syndromes, latter due to a three to tenfold amplification of a chromosome 20 fragment (20q11.21) bearing the entire TM9SF4 gene. TM9SF4 is involved in phagocytosis of bacteria and in the cannibal phenotype of metastatic melanoma cells, a phenomenon often related with poor prognosis. Cannibal cancer cells have been frequently detected in gastric and colon cancers [79].

It has been recently shown that TM9SF4 binds to V-ATPase, a pH regulating proton pump overexpressed in several tumors. This interaction aberrantly stabilizes the proton pump in its active state with the consequent pH gradient alterations that in turn is associated with drug resistance and invasiveness of colon cancer cells [79].

CD9 protein is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. Tetraspanins are cell surface glycoproteins with four transmembrane domains that form multimeric complexes with other cell surface proteins. The encoded protein functions in many cellular processes including differentiation, adhesion, and signal transduction, and expression of this gene plays a critical role in the suppression of cancer cell motility and metastasis. It is found on the surface of exosomes and is considered exosome housekeeping protein for the quantitative analysis of plasma derived nanovesicles [79].

CD 63 and CD9 exosomal protein and prostate cancer

Recently in scientific literature *Panigrahi et al.* demonstrated the potential role of exosomal protein value in the diagnosis in risk stratification of PCa by assessment of the level of exosomal proteins in plasma [80].

Bijaya et al. proved the hypothesis that CD9 surface marker is less expressed compared to CD63 in serum exosomes from PCa patients [81].

This, according to *Gould et al.* paper may also indicate the exosomal sub-population theory regarding their concentration, heterogeneous surface markers, and contents are influenced by multiple factors (e.g. clinical phenotypes) [82].

In contrast, study of *Mizutani et al.* have also found exosomes representing higher amount of CD9 surface marker in advanced and chemo resistant PCa compared to others [83].

Such variation showing selective enrichment of exosomes can also be due to methodological variation used for their isolation and processing, according to *Panigrahi et al.* and *Yamashita et al.* [80, 84].

By using nanoscale flow cytometry, *Ranjit et al.* found that prostate derived extracellular vesicles are primarily of cell membrane origin, microparticles/microvesicles, and not all PSMA expressing extracellular vesicles co-express exosomal markers such as CD9, CD63, and CD81. CD9 was the most abundant exosomal marker on prostate derived extracellular vesicles [85].

CD63-positive extracellular vesicles (EVs) were also present but not as abundant as CD9-positive EVs. CD81 positive extracellular vesicles were notably absent in PCa patient plasma samples. Extracellular vesicles from healthy/PCa patient plasmas showed the same degree of CD9/CD63 positivity without CD81, suggesting that the CD9 antigen is broadly present on different types of extracellular vesicles [85].

The results from *Ranjit et al.* study group revealed that the CD81 did not have utility in identifying exosomes nor did it bind to any similar magnitude as CD9 or CD63 on extracellular vesicles. In the same vein, CD9 proved to be a more "ubiquitous" exosomal marker whereas CD63 was observed insignificantly lower numbers of extracellular vesicles and PSMA + extracellular vesicles. This may be due to the fact that mRNA levels of CD9 are higher than CD63 levels in the prostate. The differences in CD9 and CD63 expression in plasma, according to patient groups: BPH versus PCa with appropriate Gleason grading did not reach statistical significance [85]. These statistically not significant results were one of corner points, which made us to extend the research in this field, using the same exosomal biomarkers CD63 and CD9), and using resembling patients' grouping, only with the exception that in this study the research substance would be histological patients' tissue with the rationale more stable findings to gain, which would be expected in comparison with liquid biopsies.

Park et al. showed that CD63 concentration isolated from plasma exosomes in patients with PCa was significantly higher compared to patients with benign hyperplasia [86].

In addition, *Duijvesz et al.* showed that CD63 and CD9 level in urine samples was significantly increased in patients with PCa [87].

Furthermore, according to *Huang et al.* – exosomes have been shown to be crucial for the development of drug resistance in patients with prostate carcinoma and the situation that higher levels of exosomal miR-1290 and miR-375 were significantly associated with poor overall survival in patients with castration-resistant PCa [88].

But, within the profound literature analysis, and the fact that the scientific literature provides a lot of information about CD63 and CD9 expression in other malignancies besides PCa [54–59, 86–88] – it's noticeable that, the significance of CD63 and CD9 protein expression in patients with PCa and benign hyperplasia tissue has not been fully investigated.

One of potential surrogate method to detect exosomes in the tissue is the immuno-histochemical method. Since the prostate biopsy and histopathological examination is a gold standard of PCa diagnosis, the parallel immunohistochemical examination of exosomal biomarkers could be cost-effective and time effective.

There are only few studies investigated the role of exosomal biomarkers in the tissue model. For example *Khushman et al.* found out that in pancreatic cancer, the expression of CD63 and CD9 has been reported to be higher in cancerous tissues than in normal tissues [54, 89].

Furthermore, recently within the study of *McCormick et al.* for the first time the difference in exosomal markers CD63 and CD9 expression pattern and their prognostic significance in patients with right side colorectal carcinoma and left side colorectal carcinoma has been demonstrated [59, 89]. It has been observed that among patients with right side colorectal carcinoma, there was a significantly lower progression – free survival (PFS) in patients with higher CD63 expression. At contrast, among patients with left side colorectal carcinoma, there was no difference in PFS in patients with maintained CD63 expression [59, 89].

Only some studies investigated the expression of CD9 in PCa tissue by immuno-histochemistry. *Bijaya et al*. showed that CD9 expression is significantly reduced and even lost during PCa progression. Moreover, deletions and mutations of the CD9 mRNA may be associated with loss of protein expression observed in tumor cells [81, 90].

The data of *Bijaya et al.* and *Wang at al.* suggest that CD9 inactivation may play an important role in PCa progression. Furthermore, CD9 was well expressed in nonmetastatic disease but less expressed or absent in metastatic PCa [81, 89, 90].

In contrast, as already mentioned-study of *Mizutani et al.* found higher amount of CD9 surface marker expression, respectively overexpression, in advanced and chemo resistant PCa [83, 89].

According to *Uhlén et al.* these differences in exosomal biomarker CD63 and CD9 expression are due to the fact that mRNA levels of CD9 differ with those of CD63 levels in the prostate [89].

Therefore, the profound literature analysis showed, that in some cancer type the overexpression of exosomal biomarkers correlated with the metastatic disease and disease progression, whereas other studies revealed that downregulation of CD9 correlated with disease progression.

However, the most important point is that-there is lack of data in the literature about CD63 and CD9 expression in PCa tissue by immunohistochemistry, because the majority of studies showed exosomal biomarker expression in biological fluids of patients.

The Role of mismatch repair (MMR) in prostate cancer

Mismatch repair (MMR) is an excision-resynthesis system that acts as a DNA damage sensor, correcting mismatches generated during DNA replication. The best-known complexes are MutSα and MutLα, formed by MSH2 and MSH6, and MLH1 and PMS2, respectively defects in DNA MMR proteins are permissive for carcinogenesis, giving rise to microsatellite instability (MSI) and conferring a hypermutated status. The role of MMR genes and its proteins has been extensively studied in colorectal and endometrial cancer. There are recent studies on MMR protein expression in prostate tumors [91–99], but the biologic and clinical meaning in this setting is not fully understood. The literature analysis describes a hypermutated microsatellite – unstable form of advanced PCa, associated with MSH2 and MSH6 structural rearrangements [100]. In contrast to most studies in which loss of MMR proteins expression is associated with cancer development [92, 93], some data have suggested that genomic damage could trigger their upregulation, and overexpression of these proteins has been linked to higher tumor aggressiveness. It is showed that MMR gene overexpression is associated to poor outcome, and this relationship was more prevalent in neoplasms lacking the TMPRSS2-ERG fusion [94–97].

Approximately 10% of advanced/metastatic prostate tumors have a markedly elevated rate of single nucleotide mutations, almost always due to underlying somatic and/or germline inactivation of genes in the MMR family [92]. (*MSH2*, *MSH6*, *MLH1* or *PMS2*) and often accompanied by microsatellite instability (MSI), similar to what has been observed in colorectal carcinoma. Similarly, a significant fraction of the commonly used PCa cell lines

have bi-allelic loss of MMR genes, including DU145, LNCaP, CWR22RV1, and VCaP cells [92–97].

Importantly, advanced prostate tumors with MMR gene loss and hypermutation may respond favorably to immunotherapies targeted to PD-1 and/or CTLA-4, similar to what has been seen in colorectal carcinoma, due to the generation of neoepitopes and resulting immune recognition of “non-self” tumor antigens [92].

Though previous studies have focused on MMR defects in advanced PCa, the relative frequency and clinical significance of MMR alterations in primary PCa is less certain [92].

Most studies describing the prevalence of microsatellite instability in primary PCa were performed more than a decade ago and a wide range of MSI frequency (2 to 65%) has been reported. The numbers and types of microsatellite markers used to define MSI in these older studies differed significantly from international standardized guidelines subsequently developed for MSI testing in colorectal carcinomas. When current MSI definitions are super-imposed on these earlier studies, the MSI prevalence in prostate PCa is rarely higher than 10% overall [92].

Indeed, more recent work using the previously recommended mono- and di-nucleotide marker panels from the Bethesda Consensus Panel has suggested that the rate of MSI in primary prostate cancers is < 4% similar to recent genomic profiling studies of primary PCa where the rate of MMR gene loss was even lower (< 3%) [92].

Recent studies of Lynch syndrome, an autosomal dominant condition associated with increased incidence of early colorectal and endometrial carcinomas due to germline MMR gene inactivation, have suggested that increased risk of prostate carcinoma is likely part of the syndrome, though not all studies are consistent. Small series of Lynch-associated PCa patients have found that some, though notably not all, prostate cancers arising in this setting are associated with MSI and there may be an association with increased cancer-infiltrating lymphocytes and higher pathologic grade. Given the relative rarity of MSI and MMR gene alterations in primary PCa, few studies have characterized primary prostate cancers with MMR gene inactivation outside of Lynch syndrome [92].

This is of particular interest and clinical relevance with the recent FDA-approval of the PD-1 inhibitor pembrolizumab to treat metastatic cancers of all histologic types with MMR deficiency or MSI [98].

According to data basis of *Guedes et al.* it has been shown that MSH2 loss appears more common among very-high-grade prostatic primary cancers, with rates approaching 10%

among cancers with primary Gleason pattern 5 in series. If validated in subsequent studies, these data argue for routine clinical screening of very-high-risk patients for germline and sporadic MMR gene loss using IHC or other techniques. The high Gleason grade of most cancers with MSH2 loss, combined with the overall enrichment of MMR defects among metastatic compared to primary cases, suggests that these cancers may behave aggressively from the outset, in contrast to what has been observed in MMR defective colorectal cancers [92].

Many of the prostate cancers with MSH2 loss in the study of *Guedes et al.* had significantly increased CD8+ lymphocyte density. The presence of a marked lymphocytic infiltrate, which is also frequently seen in colorectal cancers with MMR loss, may contribute to the undifferentiated, high-grade appearance of the cancer in some cases [92].

This phenomenon is also commonly seen in lymphoepithelioma-like carcinomas and medullary cancers of the breast, which are not associated with MMR defects and in all of these cases, the presence of high-grade carcinoma may not always be well-correlated with aggressive cancer progression [92].

However, beyond the appearance of high histologic grade, the potentially aggressive behaviour of primary prostate cancers with MSH2 loss was also supported by their generally high pathological stage in the current series. It may also be consistent with the relatively higher rate of MMR defects among advanced or metastatic PCa cases compared to primary cancers, as well as the enrichment of MMR defects observed in aggressive variants of PCa, including ductal adenocarcinoma and potentially neuroendocrine prostate carcinoma (NEPC) [92–97].

There are few studies dealing with MMR defects outside the spectrum of Lynch Syndrome. This research field has become of relevance with the recent approval of an immunotherapy-based PD-1 inhibitor cancer treatment (Pembrolizumab) by the U.S. Food and Drug Administration (FDA) for patients with metastatic or unresectable solid tumors with MMR deficiency or MSI regardless of the histology [98, 99, 100].

The role of DNA MMR genes in PCa is controversial, as genetic alterations leading to microsatellite instability are incompletely defined. ERG (ETS- related gene), which is oncogene, rearrangements and PTEN (phosphatase and tensin homolog), which is tumor suppressor-gene, loss are concomitant events in PCa. It was recently observed that PTEN expression loss was statistically more frequent in high grade cancers. PMS2 loss was an infrequent event, but it was statistically associated with shorter time to PSA recurrence [101].

The role of MMR genes and their respective proteins has been extensively addressed in colorectal and endometrial cancer [102, 103]. For both cancers, a considerable group of hypermutated tumors was associated with MSI, most frequently due to MLH1 epigenetic silencing. However, there is controversy about the role of MMR genes in the development and progression of PCa, as genetic alterations leading to MSI are less well defined in this type of cancer. There are few reports of defects in MMR protein expression in PCa, and the underlying mechanisms conditioning these deficiencies and their clinical-pathological impact deserve further investigation [92–97].

Recently, according to *Guedes et al.* MSH2 protein loss has been reported in primary prostate tumors, more commonly in high-grade tumors [92].

In addition, a significantly lower expression of MSH2, MSH6, and MLH1 genes in prostate cancers, proposing that this deficiency would be a hallmark distinguishing PCa from benign prostatic hyperplasia [104].

Interestingly, while some studies, for example *Guedes et al. and Langeberg et al.*, reported an association between loss of function of MMR genes and less favorable PCa features [92; 105], other authors, for example, *Burger et al., Norris et al. and Wilczak et al.*, hypothesized that genomic damage could trigger MMR gene upregulation, linking overexpression to higher tumor aggressiveness and poor outcome [95–97].

In PCa it's been reported (*Chen et al.*) reduction or absence of MMR protein expression (MLH1, MSH2, PMS2) in the epithelium of prostate carcinoma foci compared to normal adjacent prostate tissue. The reduction or absence of the PMS2 and MSH2 (but not MLH1) protein was correlated to the differentiation of the tumor. Poorly differentiated tumors showed greater loss of these two proteins than the well differentiated tumors ($P < 0.05$) [93].

It's been also documented that increased PMS2 was a prognostic marker in pre-neoplastic and PCa tissue [94].

Recent study of *Fraune et al.* showed that MMR status suggests that MSI occurs early in PCa. It was concluded also that MMR analysis on limited biopsy material by IHC is sufficient to estimate the MMR status of the entire cancer mass [106].

Immune checkpoint blockade has shown limited benefit in PCa. Nonetheless, durable objective responses have been reported, suggesting that patients with molecularly defined subsets of PCa may benefit from this therapeutic approach. Pembrolizumab, an antibody targeting the programmed cell death protein 1 (PD-1) receptor, recently earned accelerated approval by the US Food and Drug Administration for the treatment of microsatellite

instability–high (MSI-H) or mismatch repair deficient (dMMR) solid tumors, independent of site of origin. Detection of MSI thus represents the first clinical indication for prospective tumor profiling in patients with PCa. However, the optimal method for determining MSI-H/dMMR status in patients with PCa and the clinical implications of broader screening for this phenotype remain unknown [107].

According to *Abida et al.* literature analysis- the prevalence of MSI-H/dMMR in PCa is unclear, with frequencies ranging from 1.2% to 12.0%. It's been showed that 2% to 3% of tumors have a higher mutation burden that is often associated with genomic alterations in MMR-associated genes, suggesting that tumor sequencing may be an efficient method for identifying MSI-H/dMMR PCa [107].

The National Comprehensive Cancer Network guidelines for PCa were recently amended to include consideration of MSI-H/dMMR testing and pembrolizumab treatment for MSI-H/dMMR mCRPC in the second line setting or beyond. However, in its approval of pembrolizumab, the US Food and Drug Administration did not define how MSI-H/dMMR status should be evaluated, and detailed guidance is not provided as part of national guidelines. Some next-generation sequencing assays can assess for MSI-H/dMMR status by interrogating microsatellite loci for evidence of MSI, by identifying mutations and copy number alterations in_MMR-associated genes. Next-generation sequencing–based tumor genomic profiling may therefore represent a robust and efficient strategy to identify the subset of patients with PCa who may benefit from anti–PD-1/PD-L1 therapy [107].

According to *Abida et al.* – because the MSI-H/dMMR phenotype is uncommon in PCa, data describing responses to immune checkpoint blockade in this disease subset remain limited. Overall, 45.5% of patients with MSI-H/dMMR mCRPC derived durable clinical benefit, in line with other MSI-H/dMMR malignant neoplasms. Because approximately half of patients with MSI-H/dMMR had no response to immunotherapy, future studies should explore mechanisms of resistance in this population, which may involve alterations in the tumor antigen–presenting machinery and tumor-extrinsic factors, including inadequate T-cell activation [107].

Therefore, it could be summarized that the detection of exosomal protein CD9 and CD63 as well as MMR could be potentially benefit for the diagnosis and prognosis of PCa.

However, the immunohistochemical evaluation of CD63 exosomal protein expression in prostate tissue have not been previously adequate described. In addition, there is a little evidence about the role of CD9 and DNA mismatch repair proteins expression in PCa and BPH.

The newest trends in advanced prostate cancer medicinal setting

Checkpoint inhibitor therapy

Checkpoint inhibitor therapy is a form of cancer immunotherapy. The therapy targets immune checkpoints, key regulators of the immune system that when stimulated can dampen the immune response to an immunologic stimulus. Some cancers can protect themselves from attack by stimulating immune checkpoint targets. Checkpoint therapy can block inhibitory checkpoints, restoring immune system function [121].

The first anti-cancer drug targeting an immune checkpoint was ipilimumab, a cytotoxic T-lymphocyte-associated Protein 4 (CTLA4) blocker approved in the United States in 2011 [122].

Currently approved checkpoint inhibitors target the molecules CTLA4, PD-1, and PD-L1. PD-1 is the transmembrane programmed cell death 1 protein (also called PDCD1 and CD279), which interacts with PD-L1 (PD-1 ligand 1, or CD274). PD-L1 on the cell surface binds to PD1 on an immune cell surface, which inhibits immune cell activity. Among PD-L1 functions is a key regulatory role on T cell activities [123].

It appears that (cancer-mediated) upregulation of PD-L1 on the cell surface may inhibit T cells that might otherwise attack. Antibodies that bind to either PD-1 or PD-L1 and therefore block the interaction may allow the T-cells to attack the tumor 124].

The discoveries in basic science allowing checkpoint inhibitor therapies led to James P. Allison and Tasuku Honjo winning the Tang Prize in Biopharmaceutical Science and the Nobel Prize of Physiology or Medicine in 2018 [125].

Therapy of CRPC

The guidelines have a clear definition for the CRPC. Respectively according laboratory findings Castrate serum testosterone level should be < 50 ng/dL or 1.7 nmol/L plus either-laboratory findings should proof biochemical progression (three consecutive rises in PSA at least one week apart resulting in two 50% increases over the nadir, and a PSA > 2 ng/mL) or a radiological progression should be diagnosed and verified in the means of appearance of new lesions: either two or more new bone lesions on bone scan or a soft tissue lesion using RECIST (Response Evaluation Criteria in Solid Tumours). Symptomatic progression alone must be questioned and subject to further investigation. It is not sufficient to diagnose CRPC [126].

The general aspects within management of mCRPC include multifactorial analysis on patient selection and in general dependent on previous treatment for metastatic hormone sensible PCa and for non-metastatic hormone sensible PCa; previous treatment for metastatic CRPC; quality of response and pace of progression on previous treatment; known cross resistance between androgen receptor targeted agents (ARTA); co-medication and known drug interactions; known genetic alterations; known histological variants and DNA repair deficiency (consider platinum or targeted therapy like poly-ADP ribose); local approval status of drugs and reimbursement situation; available clinical trials; the patient and his co-morbidities [126].

Molecular diagnostics

All metastatic patients should be offered somatic genomic testing for homologous repair and mismatch repair (MMR) defects, preferably on metastatic carcinoma tissue but testing on primary tumour may also be performed. Alternatively, but still less common, genetic testing on circulating tumour DNA is an option and has been used in some trials. One test, the FoundationOne® Liquid CDx has been Food and Drug Administration approved [126]. Defective MMR assessment can be performed by immunohistochemistry for MMR proteins (MSH2, MSH6, MLH1 and PMS2) and or by next-generation sequencing assays [127].

Germline testing for BRCA1/2 and MMR is recommended for high risk and particularly for metastatic PCa if clinically indicated. Molecular diagnostics should be performed by a certified (accredited) institution using a standard NGS (Next Generation Sequencing) multiplication procedure (minimum depth of coverage of 200 X). The genes and respective exons should be listed; not only DNA for mutations but RNA needs to be examined for fusions and protein expression to obtain all clinically relevant information. A critical asset is the decision support helping to rate the mutations according to their clinical relevance [128].

Level 1 evidence for the use of pharmacological inhibitors of the enzyme poly ADP ribose polymerase (PARP-inhibitors) has been reported [129].

Microsatellite instability (MSI)-high or mismatch repair deficiency is rare in PCa, but for those patients, pembrolizumab has been approved by the FDA and could be a valuable additional treatment option [92, 98, 99, 100, 101].

Targeted therapies for for metastatic CRPC

The use of PARP (poly adenosine diphosphate ribose polymerase) inhibitors (for example, olaparib, rucaparib, talazoparib or niraparib) was investigated in various studies in patients with metastatic CRPC and mutations in DNA repair genes, and there were promising response rates [130]. Recently, data from a prospectively randomized phase III study (PROfound study) have also been available. The study treated patients who were progressing under or after treatment with a new type of anti-hormonal therapy (abiraterone or enzalutamide) and who had received or refused docetaxel chemotherapy. The study only treated patients in whom a defect in a DNA repair gene was found within the preliminary examination (cohort A: mutation in one of the genes Breast Cancer 1 early-onset (BRCA1), breast cancer type 2 susceptibility protein (BRCA2) or “Ataxia teleangiectasia mutated gene” (ATM); Cohort 2: Mutation in another DNA repair gene. The proportion of patients with metastatic CRPC who have a mutation in one of the genes is estimated to be around 20%. The patients treated in the study were treated either with olaparib or with abiraterone or enzalutamide (depending on the decision of the investigator). Of the 387 patients included in the study, 45% had already received docetaxel and 20% had received both docetaxel and cabazitaxel. Of the patients included in the study, just 19.1% had received both abiraterone and enzalutamide before inclusion in the study. The primary endpoint of the study was the radiological verified PFS of the patients in cohort A (mutation in BRCA1, BRCA2 or ATM). With a median radiological verified PFS of 7.4 versus 3.6 months, there was a significant advantage for the patients treated with olaparib (HR: 0.34; $p < 0.0001$). Despite a crossover of 81% of the patients treated in the control arm, the interim analysis also showed an advantage in overall survival (18.5 vs. 15.1 months; HR: 0.64; $p = 0.02$). The final data from the definite overall survival analysis are still pending [130, 131].

The results of the PROfound study led to the approval of olaparib in patients with metastatic CRPC and a mutation in a DNA repair gene. In addition to olaparib, rucaparib, a second active therapeutic agent of the PARP inhibitors for the same indication, was already approved in the USA. The approval is based on the data from the TRITON2 study, a phase II study in which patients were treated with the PARP inhibitor rucaparib after previous treatment with chemotherapy and a new generation hormonal therapy. In the patients with a mutation of the BRCA1 or the BRCA2 gene, 41.5% showed an objective tumor response on imaging [130, 131].

The optimal use of PARP inhibitors in patients with PCa is currently being discussed (before or after chemotherapy, qualifying gene mutations etc.). The use of PARP inhibitors in the first line in patients with metastatic CRPC is currently being investigated in numerous combination studies (for example abiraterone plus olaparib, abiraterone plus niraparib or enzalutamide plus talazoparib). These studies also treat patients who do not have a proven mutation in a DNA repair gene [130, 131]. For example the study of *Clarke et al.* is based on a phase II combination study in which the combination of olaparib and abiraterone showed a tendency towards improved radiological verified PFS compared to abiraterone. There was also an insignificant trend towards improved PFS for the combination in patients without a proven DNA repair gene defect [131, 132].

As another alternative sequence after treatment after docetaxel and one line of hormonal treatment for metastatic CRPC counts the immune checkpoint inhibitor pembrolizumab. It was approved by the FDA for all MMR–deficient cancers or in those with instable microsatellite status (MSI-high). This also applies to PCa but is a very rare finding in this tumour entity. In all other PCa patients pembrolizumab monotherapy is still experimental. It shows limited anti-tumour activity with an acceptable safety profile [100, 101, 133].

A phase II trial enrolled 258 patients treated with pembrolizumab [4,50]. The objective response rate was around 4%, but those responses were durable [133].

3. AIM OF THE STUDY

The aim of this study was to evaluate and compare the expression of exosomal biomarkers and DNA MMR in the tissue of patients with prostate benign hyperplasia and prostate adenocarcinoma.

4. WORK TASKS

1. To compare the expression of exosomal biomarkers (CD9, CD63) and DNA MMR (MSH2, MSH6, MLH1, and PMS2) by IHC in the tissue of patients with PCa and BPH.
2. To analyze DNA MMR proteins and exosomal biomarkers CD9, CD63 expression according to the *Gleason* Grade and PCa grading system adopted by the WHO 2016 in patients with PCa.
3. To analyze the prognostic value of DNA MMR and exosomal biomarkers CD9, CD63 expression in disease progression free survival (PFS) in patients with PCa.

5. PATIENTS CHARACTERISTICS AND METHODS

5.1. Patients characteristics

The study was retrospective. Altogether, 92 patients with prostate acinar adenocarcinoma undergoing radical prostatectomy and 20 patients undergoing fine needle biopsy with histopathologically confirmed prostate benign hyperplasia (control group) diagnosis in 2013–2015 year were enrolled in the study. All of PCa patients of this study have been followed up till disease progression and/or for maximal 5 years of timeframe.

The study was performed in accordance with the *Declaration of Helsinki*.

The study was approved by the *Ethics Committee of Institute of Cardiology and Regenerative Medicine, Riga, Latvia*. Study approval number: July, 2018, No. 42/2018.

The clinical samples for research were approved by the comity of Biomedical ethics of Riga East Clinical University Hospital (decision No. 7-A/15, 04.06.2015). The study was registered in Riga East Clinical University Hospital (Nr. AP-02/13).

All patients given written consent to participate in scientific research.

5.2. Histological and immunohistochemical examination

Histopathological examination

The histopathological evaluation of PCa tissue was performed according to the guidelines of current WHO classification of Tumors of the Urinary System and Male Genital Organs and CAP (*College of American Pathologist*) PCa protocol. Briefly, the tumor type, Gleason grading, Grade group and cancer invasiveness was assessed.

The tumor TNM staging was performed according to the 8th American Joint Committee on Cancer cancer staging manual.

Tissue processing and Immunohistochemistry

Paraffin embedded tissue specimens were cut in 3-µm-thick sections and slides were stained with haematoxylin and eosin to evaluate histopathological changes. Antigen retrieval was achieved by incubating the slides with *Tris/EDTA buffer* at pH=9.0 for 30 min in a scientific microwave. The slides were then incubated overnight at 4 ºC with mouse monoclonal CD9 (*AbCam, ab215*), mouse monoclonal CD63 antibody (*AbCam, ab215891*),

rabbit monoclonal antibody MSH2 (*AbCam, ab227941*, dilution 1:500), rabbit monoclonal antibody MSH6 (*AbCam, ab273076*), rabbit monoclonal antibody MLH1 (*AbCam, ab23844*, dilution 1:500), and rabbit monoclonal antibody PMS2 (*AbCam, ab110630*, dilution 1:100). Antibody binding was detected using the *EnVision reagent* following the manufacturer's instructions (*DAKO*).

Immunostained slides of each histology sample were scanned with at magnification x20. The whole-area scanned of each slide was analyzed with Image Analysis *QuantCenter* (*3DHistech*).

CD63, CD9 expression and mismatch repair protein- expression (MSH2, MSH6, MLH1, and PMS2) was evaluated by intensity of staining and percentage of stained cancer cells and stromal cells respectively: intensity was given scores 0–3 (0 = no, 1 = weak, 2 = moderate, 3 = intense), and the percentage of immunopositive cells was given scores 0–3 (0 = 0%, 1 = 10%, 2 = 20–30%, 3 = 40%–100%).

The two scores were multiplied to obtain the decisive result of 0–9. Expressions were considered positive in tumor cells when scores were 2 or more and negative when scores were 0–1. Evaluation was made by two double-blinded independent observers who were unaware of clinical data and outcome.

5.3. Statistical analysis

Values were expressed as mean (range). *The Fisher exact test* or *chi-square test* was used to evaluate the association between categorized variables.

Associations between CD63, CD9 MSH2, MSH6, MLH1, and PMS2 expression and clinicopathological findings were analyzed using the *chi-square test*. Progression free survival (PFS) was defined as the time from operation to the time of disease progression.

PFS curves were estimated by the *Kaplan-Meier method* and compared using the *log-rank test*. Multivariate analysis was carried out using *cox proportional hazard model*.

The estimated PSA value decrease within 6 weeks post RP was according to guidelines defined as < 0.1 ng/ml. According to guidelines we used PSA value > 0.4 ng/ml as the threshold post RP, that best predicts metastasis, and in this study is defined as PSA relapse or biochemical recurrence. The local or distant metastasis would be detected, using imaging diagnostics (for example bone scan, abdominopelvic CT or MRI).

P values less than 0.05 were statistically significant. SPSS 21.0 version software was used for the statistical analysis.

6. RESULTS

Altogether, 112 patients were enrolled in the study (*Table 6.1.*). 92 patients were with PCa and 20 patients with BPH. The median age of study subjects with PCa and BPH respectively was 63.23 years (43–85) vs 61.65 years (45–81). The low-grade PCa (Grade group I and II) was observed in 56 patients, the high-grade cancer (Grade group III–V) were observed in 36 patients with PCa. Histologically all tumor- patients had prostate acinar adenocarcinoma.
pT2 stage was observed in 66 (71.74%) patients, pT3 stages was observed in 24 (28.26%) patients. The regional lymph node metastasis was found in 8 (8.69%) patients. None of the patients had distant metastasis. Representative photomicrographs of BPH and PCa were demonstrated (*Fig. 6.1, 6.2, 6.3, 6.4*). By all the PCa patients within the follow up PSA value > 0.4 ng/ml as threshold post RP primary and/or imaging data would be used as indicator for tumor progression. Using imaging diagnostics subsequently the local and/or distant metastasis secondary have been detected within maximal 5 year follow up in 18 patients (19.5%). The median time of disease progression was 34.5 months.

Table 6.1. **Clinical characteristics of patients**

Age	**63.23 (43–85) years**
Grade group I	24 patients
Grade group II	32 patients
Grade group III	18 patients
Grade group IV	10 patients
Grade group V	8 patients
pT2	66 patients
pT3a	18 patients
pT3b	6 patients
pN0	81 patients
pN1	8 patients

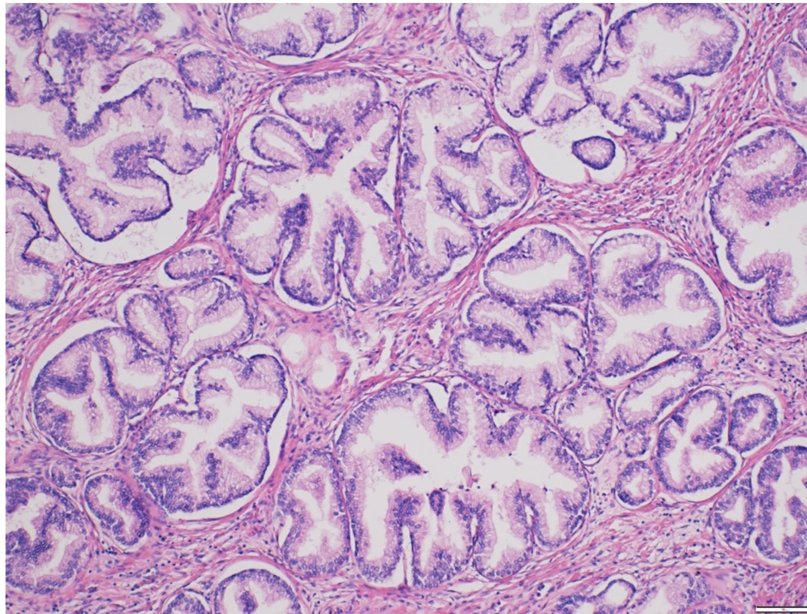

Figure 6.1. **Prostate benign hyperplasia. Hematoxylin eosin staining method, magnification x100, scale bar-200 µm**

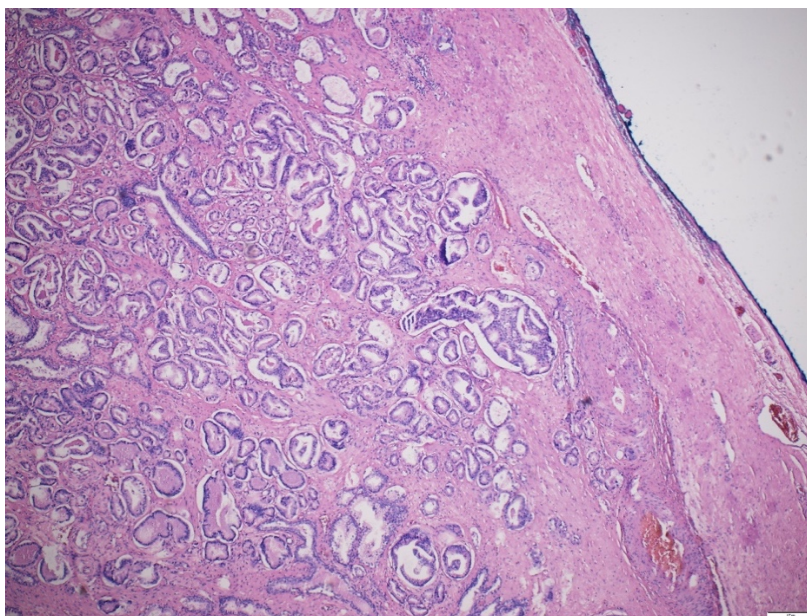

Figure 6.2. **Prostate acinar adenocarcinoma, Gleason 3 + 4 = 7, Grade group II. Hematoxylin eosin staining method, magnification x100, scale bar-200 µm**

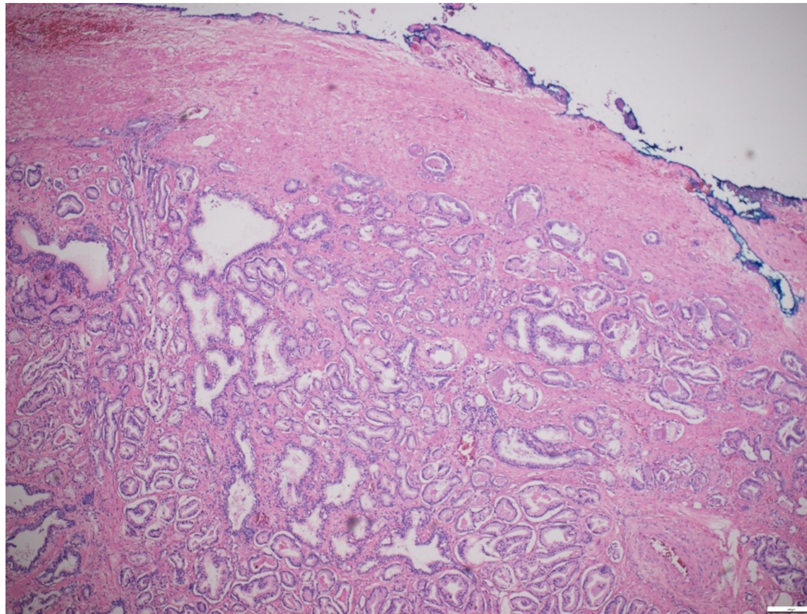

Figure 6.3. **Prostate acinar adenocarcinoma, Gleason 3 + 3 = 6, Grade group I. Hematoxylin eosin staining method, magnification x100, scale bar-200 µm**

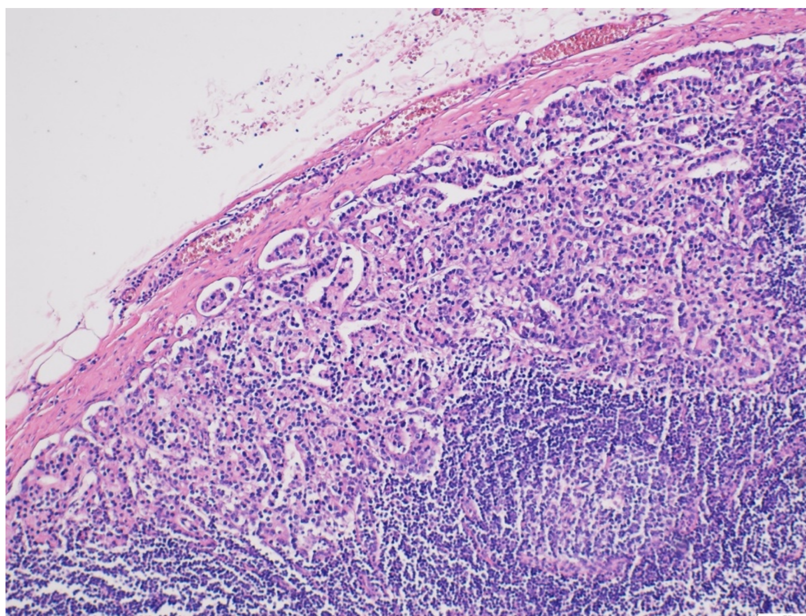

Figure 6.4. **Prostate acinar adenocarcinoma metastasis to lymph node. Hematoxylin eosin staining method, magnification x200, scale bar-100 µm.**

CD63 expression in prostate tissue

CD63 was mainly expressed on the cell membrane of PCa cells, with a smaller amount in the cytoplasm. Representative photomicrographs of CD63 expression in high Grade prostate cancer and benign prostate hyperplasia tissue were demonstrated (*Fig. 6.6., 6.7., 6.8.*).

Obtained results showed that there was no statistically significant difference between CD63 expression between benign prostate hyperplasia and prostate carcinoma when all patients were analyzed together, however the tendency of increased CD63 expression was observed in patients with PCa (4.21 (0–9) vs. 2.55 (0–9) score, $P = 0.09$, score) (*Fig. 6.5.*).

However, when the low and high- grade patients were independently analyzed, the CD63 expression was significantly higher in patients with PCa Grade III–V compared to Grade I–II, respectively, 6.24 (0–9) vs 1.57 (0–6) score, $P < 0.0001$ (*Fig. 6.5.*).

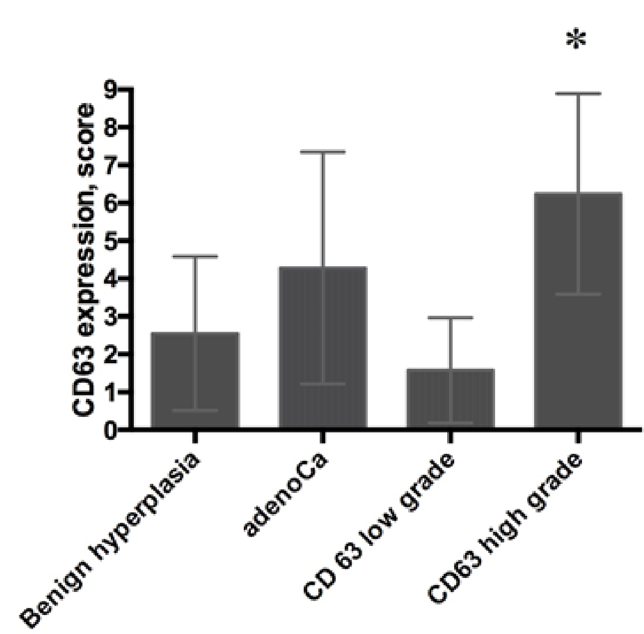

Figure 6.5. **CD63 expression in prostate benign hyperplasia, acinar adenocarcinoma and low grade (Grade group I–II) and high- grade (Grade III–V) prostate acinar adenocarcinoma, compared high- grade to low- grade adenocarcinoma $P < 0.0001$, one way ANOVA followed by Bonferroni test**

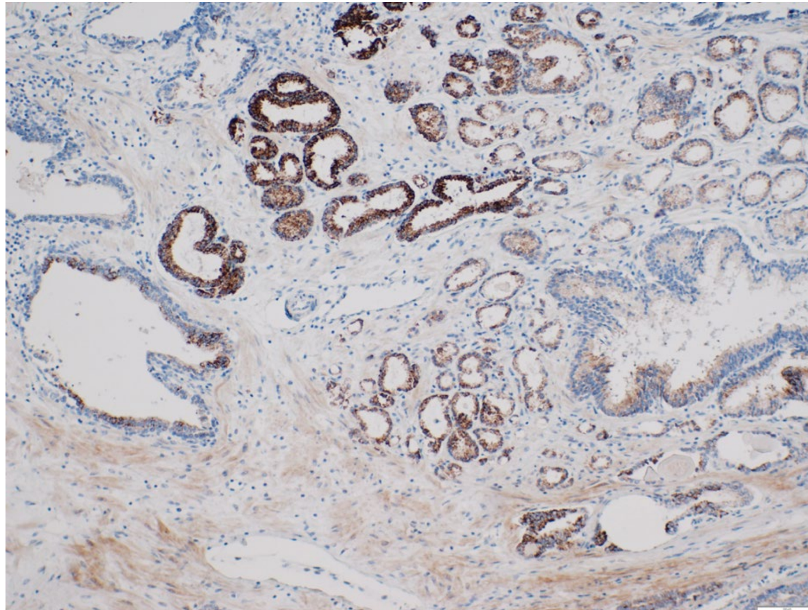

Figure 6.6. **Representative photomicrographs of CD63 expression in high grade prostate cancer. Immunohistochemical staining method, magnification x200, scale bar 100 µm**

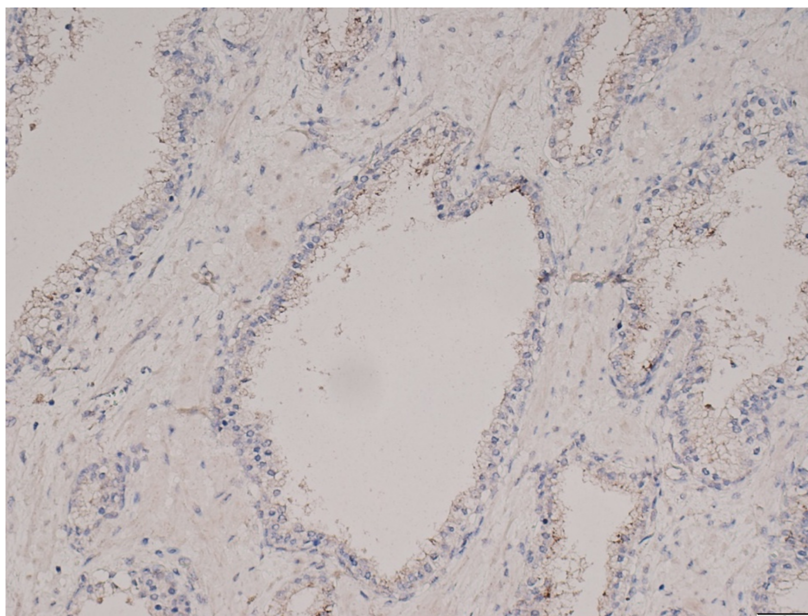

Figure 6.7. **Representative photomicrographs of CD63 expression in benign prostate hyperplasia. Immunohistochemical staining method, magnification x200, scale bar 100 µm**

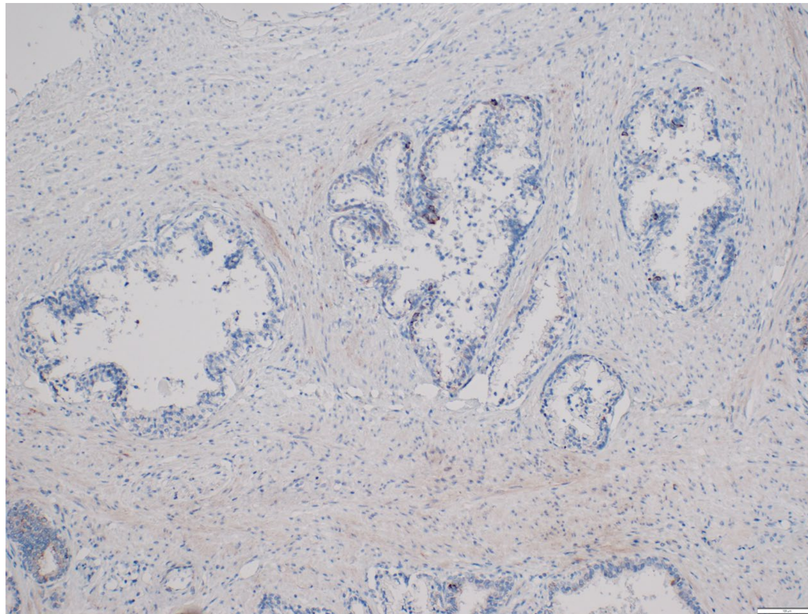

Figure 6.8. **Representative photomicrographs of CD63 expression in benign prostate hyperplasia. Immunohistochemical staining method, magnification x200, scale bar 100 µm**

In addition, the significant positive correlation between the CD63 expression and Grade groups was revealed (Rho = +0.76; P < 0.0001) (*Fig. 6.9.*).

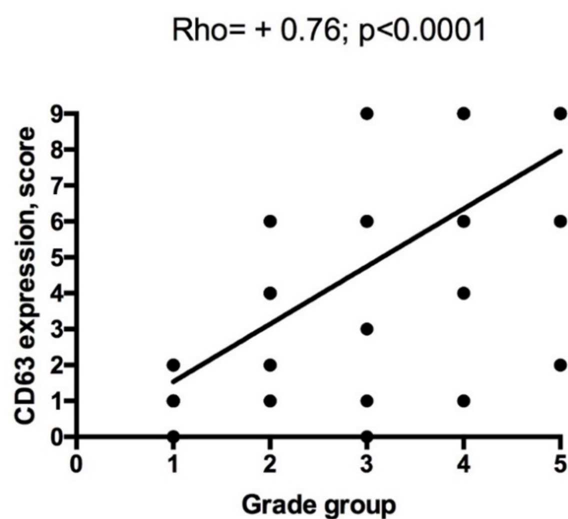

Figure 6.9. **The correlation between CD63 expression and Grade groups, Chi squared, Rho = +0.76; P < 0.0001**

The median progression free survival (PFS) was significantly longer in patients with low CD63 expression compared to high CD63 expression, respectively 42.50 and 26.50 months, (P = 0.018) (*Fig. 6.10.*).

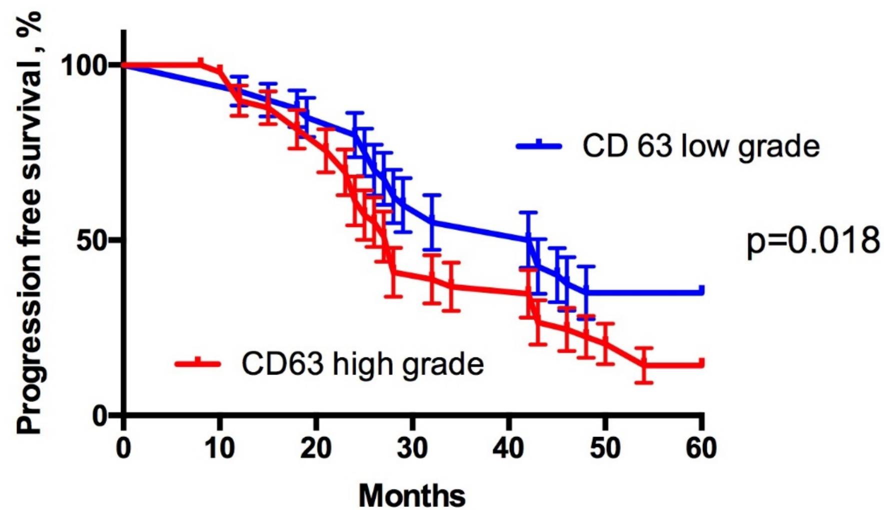

Figure 6.10. **Progression free survival of prostate cancer with low CD63 (blue line) and high CD63 (red line) expression. Kaplan-Meier method using the log-rank (Mantel-Cox) test, P = 0.018**

CD9 expression in prostate tissue

CD9 staining was cytoplasmic, vesicular, predominantly focal, mainly located apically. In benign hyperplasia mild, moderate or intense staining was observed, whereas the expression of CD9 in cancer tissue was almost mild or absent. Representative photomicrographs of CD9 expression in prostate cancer and benign prostate hyperplasia tissue were demonstrated (*Fig. 6.13., 6.14., 6.15.*).

Obtained results showed that CD9 expression was significantly decreased in prostate acinar adenocarcinoma compared to control group: expression score 1 (0–9) vs 6 (2–9), P < 0.0001, *Fig. 6.11.*

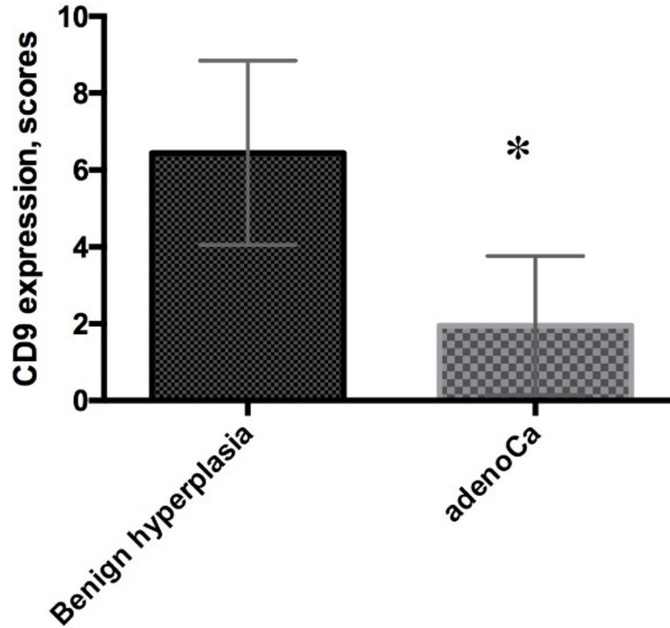

Figure 6.11. **CD9 expression in prostate benign hyperplasia and acinar adenocarcinoma, P < 0.0001, compared patients with benign hyperplasia to adenocarcinoma, Mann Whitney U test**

When all patients with adenocarcinoma were analyzed together, the correlation between CD9 expression and Grade groups has not been observed (P = 0.36), *Fig. 6.12.*

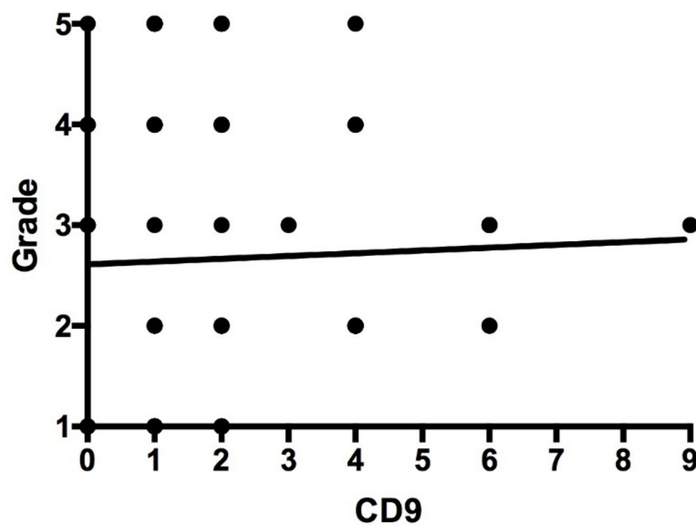

Figure 6.12. **The correlation between CD9 expression and Grade groups, Chi squared test, P = 0.36**

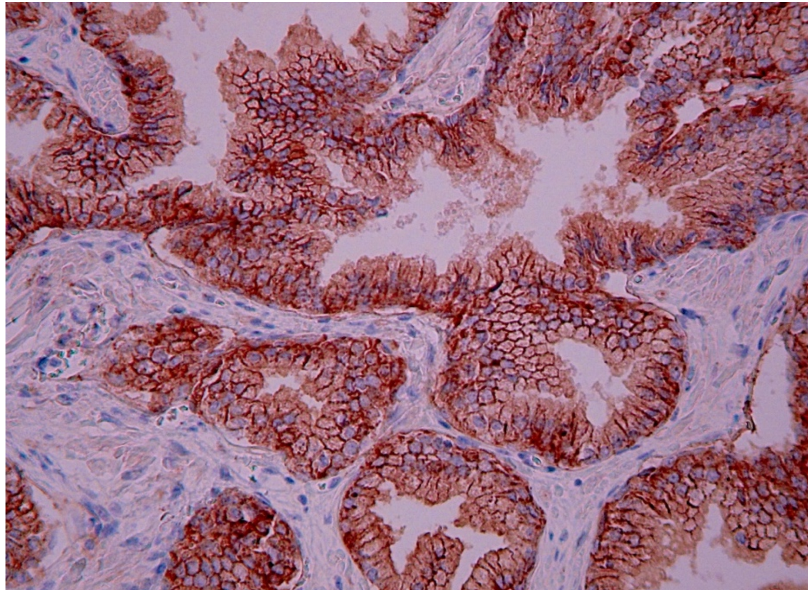

Figure 6.13. **CD9 expression in benign prostate hyperplasia. Immunohistochemical staining method, magnification x200**

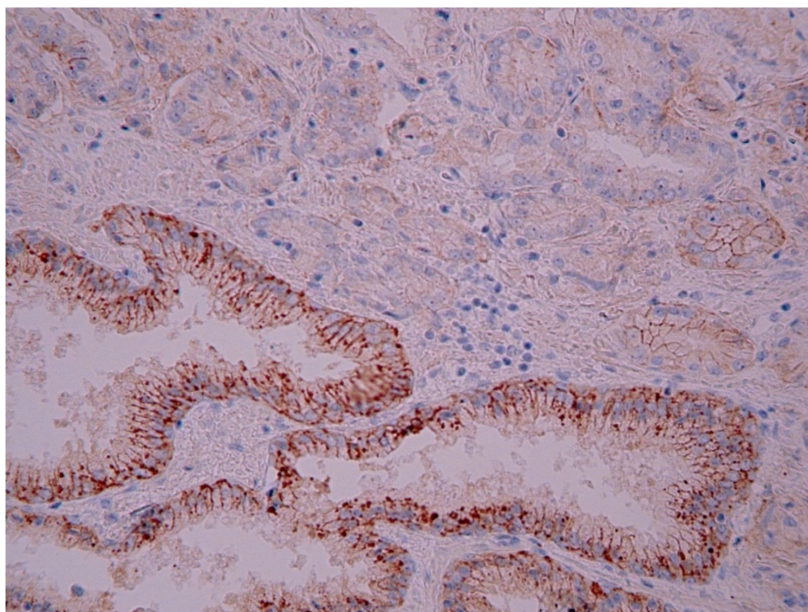

Figure 6.14. **Loss of CD9 expression in prostate acinar adenocarcinoma. Immunohistochemical staining method, magnification x200**

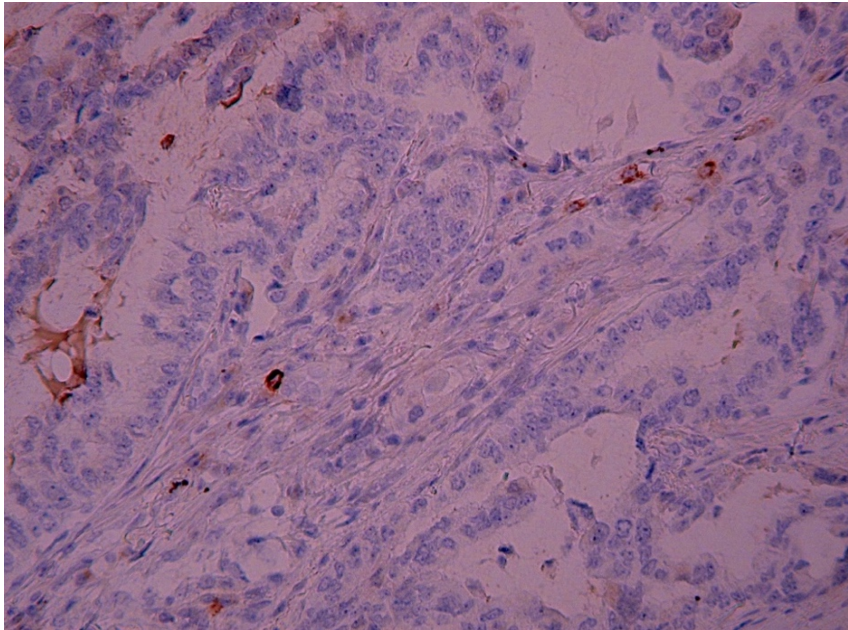

Figure 6.15. **Loss of CD9 expression in prostate acinar adenocarcinoma. Immunohistochemical staining method, magnification x400**

The median progression free survival (PFS) in patients with high CD9 expression (Score 4–9) was significantly longer compared to patients with low CD9 expression (score 0–3) respectively 43.00 and 28.50 months (P = 0.016), *Fig. 6.16.*

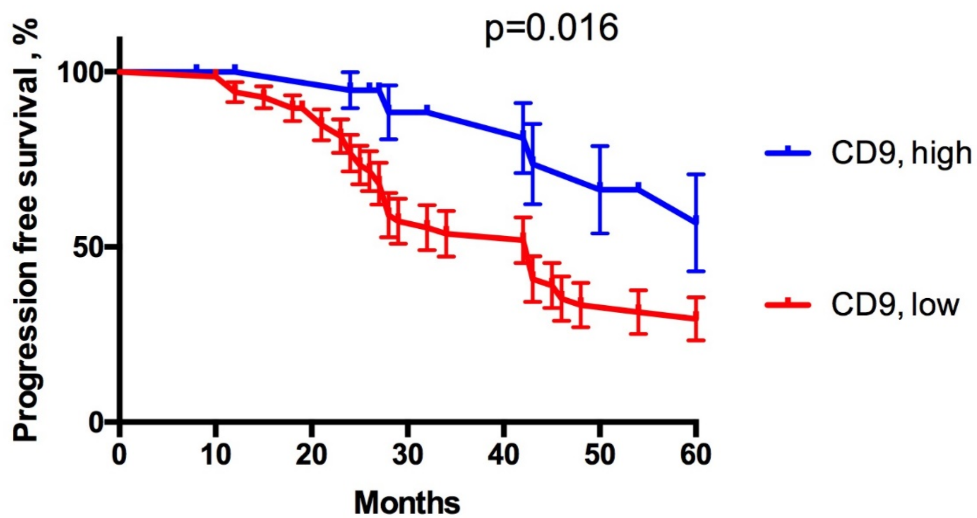

Figure 6.16. **Progression free survival of prostate cancer with high CD9 (blue line) and low CD9 (red line) expression. Kaplan-Meier method using the log-rank (Mantel-Cox) test, P = 0.016**

DNA MMR (MSH2, MSH6, MLH1, and PMS2) expression in prostate tissue

Four MMR proteins were assessed immunohistochemically – MSH-2, MSH-6, MLH-1 and PMS-2.

MMR expression was absent in 10 patients (10,86%) from 92 PCa patients (two patients with Grade group II, five patients with Grade group IV and three patients with Grade group V). This study demonstrated loss of MMR expression in 8/36 (22.22%) of high-grade prostate cancer patients and 2/56 (3.57%) of low-grade prostate cancer patients.

MMR were present in all cases of benign prostate hyperplasia (mild to moderate staining).

Overall, MLH1, MSH2, MSH6, and PMS2 were lost in 2 (2.17%), 4 (4.34%), 4 (4.34%), and 6 (6.52%) prostate cancer patients. It was observed that all cases with MLH1 loss concurrently lost other 3 proteins, while all cases with MSH2 loss showed concurrent MSH6 loss. Representative photomicrograph demonstrates MSH-2 expression in prostate acinar adenocarcinoma (*Fig. 6.17.*).

Thus, loss of at least 1 DNA MMR protein was identified in 10 (10.86%) cases. From these patients 8 patients had Grade III–V cancer, but 2 patients had Grade I–II cancer.

The study revealed negative correlation between loss of DNA MMR proteins and Grade groups, *Fig. 6.18*.

The median progression free survival (PFS) in patients with DNA MMR deficiency (at least one DNA MMR) was significantly shorter compared to in patients with preserved DNA MMR expression, respectively 22.00 and 60.00 months ($P = 0.0007$), *Fig. 6.19.*

The loss of MSH2, MSH6, MLH1, and PMS2 was mainly characteristics in high grade cancer (Grade group III, IV and V). In addition, the loss of all four DNA MMR concomitantly – MSH2, MSH6, MLH1, and PMS2 was not observed in patients with Grade group I–II.

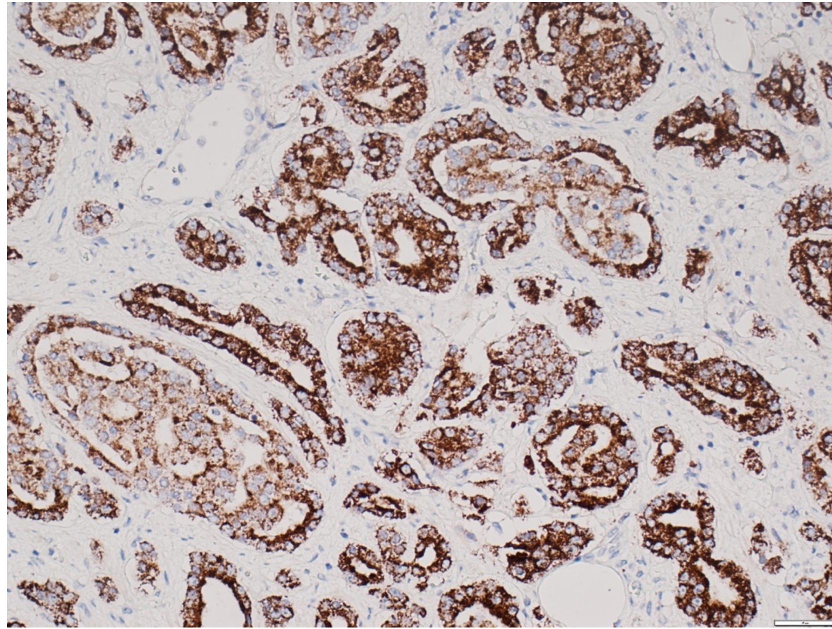

Figure 6.17. **MSH-2 expression in prostate acinar adenocarcinoma. Immunohistochemical staining method, magnification x200, scale bar – 100 µm**

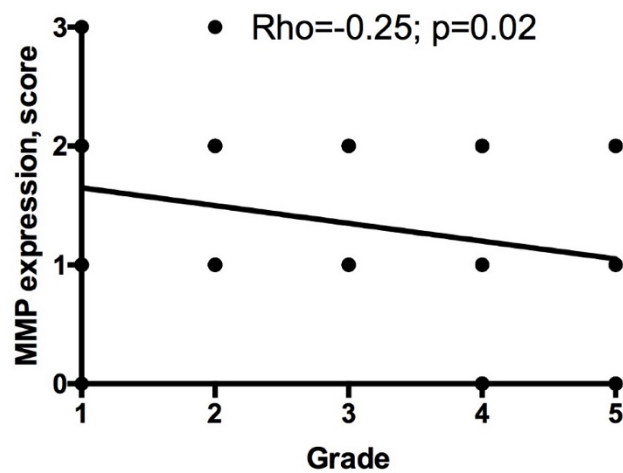

Figure 6.18. **The negative correlation between DNA MMR expression and Grade groups, Chi squared, Rho = - 0.25; P = 0.02**

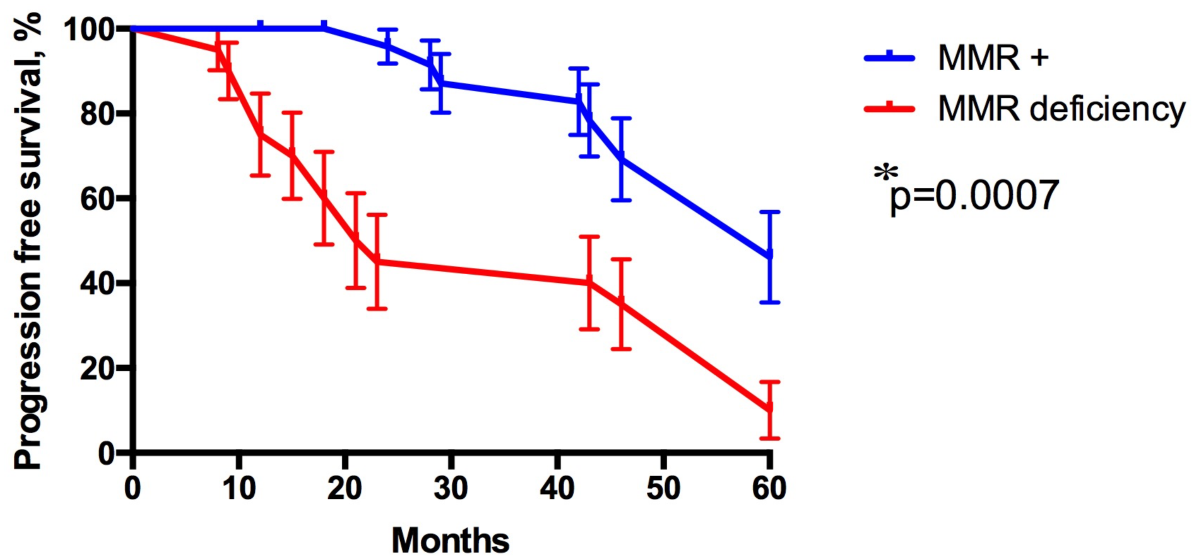

Figure 6.19. **Progression free survival of prostate cancer patients with MMR deficiency (red line) and maintained MMR expression (blue line). Kaplan-Meier method using the log-rank (Mantel-Cox) test, P = 0.0007**

7. DISCUSSION

Prostate cancer is one of the most common malignancies in developed countries and the second cause of cancer death for men [1].

Most PCa cases are latent, which remain locally disease, which never progress to metastatic disease. It is of utmost importance to identify which PCa are destined to progress and which would benefit from an early radical treatment [1, 7].

Prostate-specific antigen (PSA) currently is the widely used testing to detect PCa. However, its limited specificity and high rate of overdiagnosis led to extensive scientific research of novel biomarkers and testing methods [1, 7, 20].

There are urgently need of biomarkers for the disease progression risk stratification and prognosis for the patients with prostate cancer. The conventional prostate cancer predictive criteria, which stratify the risk for disease progression are such histopathological criteria like cancer histopathological subtype, Gleason grading system, Grade groups, which are assessed during the prostate biopsy by pathologist [1, 7, 15, 20, 21].

The progress in personalized treatment of cancer aimed on molecular biomarkers led to increased demand of novel specific predictive tools for the early diagnosis, risk stratification and prognosis [21].

Exosomes are cell-derived vesicles that are present in many and perhaps all eukaryotic fluids, including blood, urine, and cultured medium of cell cultures [22, 23].

In malignancies the regulatory circuit which guards exosome homeostasis is coopted to promote cancer cell survival and metastasis [37, 48]. Tumor cells exposed to hypoxia secrete exosomes with enhanced angiogenic and metastatic potential, suggesting that tumor cells adapt to a hypoxic microenvironment by secreting exosomes to stimulate angiogenesis or facilitate metastasis to more favorable environment [40].

Exosomes have been shown to act as mediators for cell to cell communication and as a potential source of biomarkers for many diseases, including prostate cancer [46].

Moreover, the expression of serum exosomal miRNAs induced by radiotherapy may have potential value as prognostic and predictive biomarkers PCa [46, 81].

Exosomal proteins might be the potential biomarkers for the prostate carcinoma and could be detected in blood plasma and urine (46, 81). However, there is a little evidence about exosomal protein expression in tissue of prostate benign hyperplasia and adenocarcinoma.

This study demonstrated that CD63, but not CD9 expression in prostate cancer was an independent significant prognostic factor correlating with Grade group and disease progression free survival. Since CD63 is a surface protein marker of exosomes, data in this study might suggest that exosomes derived from prostate cancer cells are enrolled in cancer growth and progression. In addition, in patients with high CD9 expression (Score 4–9) progression free survival was significantly longer compared to patients with low CD9 expression (score 0–3).

This study is one of first research investigated the clinico-pathological significance of CD63, CD9 expression in prostate cancer tissue, since previous studies focused on the role of exosomes and CD63, CD9 in plasma and urine samples.

Recently it has been shown that CD63, CD9 concentration isolated from plasma exosomes in patients with prostate cancer was significantly higher compared to patients with benign hyperplasia [85, 86]. In addition, it was showed that CD63, CD9 level in urine samples was significantly increased in patients with prostate cancer [87].

This study demonstrated that CD63, but not CD9 expression was significantly higher in patients with prostate cancer Grade III–V compared to Grade I–II. In addition, the significant positive correlation between the CD63, but not CD9 expression and Grade groups was revealed. Furthermore, the progression-free survival was significantly longer in patients with low CD63, but not CD9 expression, compared to high CD63, but not CD9 expression.

Previous studies showed that patients with lower plasma CD63 concentration had greater prostate volume and lower pathologic Gleason score [85, 86], which, in relation to prostate cancer grading, inclusive, Gleason scoring is consistent with this study's findings in tissue model.

Previous studies showed that urinary exosomes markers can aid in the decision-making process regarding whether to carry out a prostate biopsy and in the design of a therapeutic strategy [87]. Urinary exosomes and their cargo, especially miR-21 and miR-375, have become an emerging source of biomarkers in the detection and prognosis of PCa [87]. Moreover, the expression of serum exosomal miRNAs induced by radiotherapy may have potential value as prognostic and predictive biomarkers PCa [81].

Furthermore, it has been demonstrated that exosomes from prostate cancer are highly enriched with PSA, representing characteristics of the original PCa cells [46].

In prostate cancer and normal prostatic epithelium, both exosomes and prostasomes, micro-particles, microvesicles are produced and released into semen and blood respectively

but a multi-parametric and high throughput means of making this distinction was not possible until now. The use of nanoscale flow cytometry for prostate extracellular vesicles analysis as pioneered by *Ranjit et al.* study group sought to address the longstanding issue of the origin of prostate derived extracellular vesicles as being released by exocytosis as exosomes, or if they were released at the cell membrane as microparticles, microvesicles [85], but there were no studies in literature to show exosome marker CD63 and CD9 and MMR comparison simultaneously, using derivation from postoperative tissue sampling- that's why this study under estimation of aforesaid criterion could be supposed as pioneering in this research field.

Both CD9 and CD63 are the exosomal biomarkers. However, according to the findings- it is very intriguing why the CD63 is upregulated, but CD9 downregulated in prostate cancer.

Bijaya et al. have found that CD9 surface marker is less expressed compared to CD63 in serum exosomes from PCa patients [81].

This may also indicate the exosomal sub-population theory regarding their concentration, heterogeneous surface markers, and contents are influenced by multiple factors (e.g. clinical phenotypes) [81].

In contrast, studies for example *Mizutani et al.* have also found exosomes representing higher amount of CD9 surface marker in advanced and chemo resistant PCa compared to others [83].

By using nanoscale flow cytometry (*Ranjit et al.*), it was determinated that prostate derived extracellular vesicles are primarily of cell membrane origin, microparticles, microvesicles, and not all extracellular vesicles co-express exosomal markers such as CD9, CD63 parallel. CD9 was the most abundant exosomal marker on prostate derived extracellular vesicles (12–19%) [85].

Previous findings reveled that CD63-positive extracellular vesicles were present but not as abundant as CD9-positive extracellular vesicles. Extracellular vesicles from healthy and prostate cancer patient plasmas showed similar CD9 and CD63 positivity, suggesting that both proteins could be detected in different types of extracellular vesicles, which could in part explain the differences of CD9 and CD63 expression in the tissue of prostate cancer different [85].

Approximately 10% of advanced, metastatic prostate carcinomas have single nucleotide mutations, almost always due to underlying somatic and or germline inactivation of genes in the mismatch repair (MMR) family (*MSH2*, *MSH6*, *MLH1* or *PMS2*) and often accompanied

by microsatellite instability (MSI). Significant proportion of the commonly used prostate cancer cell lines have bi-allelic loss of MMR genes. This is of particular interest and clinical relevance with the recent FDA-approval of the PD-1 inhibitor pembrolizumab to treat metastatic tumors of all histologic types with MMR deficiency or MSI [92, 98, 99, 100].

The most interesting described phenotypic correlation discovered that MSH2 loss appears more common among very-high-grade prostatic primary tumors, with rates approaching 10% among tumors with primary Gleason pattern 5 in series [92].

This study demonstrated that MMR expression was absent in 10 patients (10.86%) from 92 PCa patients (two patients with Grade group II, five patients with Grade group IV and three patients with Grade group V). This study demonstrated loss of MMR expression in 8/36 (22.22%) of high-grade prostate cancer patients and 2/56 (3.57%) of low-grade prostate cancer patients.

In addition, a negative correlation between the loss of MMR expression and Grade groups was revealed in this study, according with the results of previous studies in literature [92, 93, 104, 105].

Only a few immunohistochemical studies have reported the incidence of MMR deficiency in prostate cancer ranges from 1.2 to to 22.7% [92, 101, 106].

MSH2 loss was significantly more often seen in tumors with Gleason score 9–10, Grade Group 5 than in those with Gleason score ≤8, Grade Group ≤ 4 [92]. At contrast other studies did not demonstrated significant associations between MLH, MSH2, PMS2 loss and Gleason and Grade Groups [94–97].

This research field has become of particular interest since recently immunotherapy-based PD-1 inhibitor (Pembrolizumab) treatment has been approved by the U.S. Food and Drug Administration (FDA) for patients with metastatic or unresectable solid tumors with MMR deficiency or MSI regardless of the histology [98, 99, 100].

The role of DNA MMR genes in PCa is still controversial, as genetic alterations leading to microsatellite instability are incompletely defined in PCa. In the study of *Gonzalez et al.* MSH2, MLH1, PMS2 losses were documented in 8%, 5%, 2%. MSH6 showed an increase of expression in 42.1% of the cases. Furthermore, an association between MSH6 overexpression and tumor differentiation was found. PMS2 loss was an infrequent event, but it was related to shorter time of PSA recurrence. It has been suggested that MSH6 overexpression could be a marker of aggressiveness in PCa. The IHC assessment of DNA

MMR proteins could be the surrogate markers for the assessment of prostate cancer prognosis and risk stratification [101].

This study's findings confirmed and extent previous demonstrated observations that the loss of MSH2, MSH6, MLH1, and PMS2 was statistically proved characteristics in high grade cancer (Grade group 3, 4 and 5). The loss of MSH2, MSH6, MLH1, and PMS2 was observed in patients with Grade group 1–2 statistically distinctly less expressed. In addition, the loss of MSH2, MSH6, MLH1, and PMS2 concomitantly- was not observed in patients with Grade group 1–2. Additionally, MMR was present in all cases of benign prostate hyperplasia, supporting the previous literature analysis [104].

The prevalence of MMR deficiency in prostate cancer ranges from 1.2% to 12.0%. Recent studies of metastatic castration–resistant prostate cancer (mCRPC) showed that 2% to 3% of tumors have a higher mutation burden that is often associated with genomic alterations in MMR-associated genes, suggesting that MMR detection may be an efficient method for identifying loss of DNA mismatch repair proteins in prostate cancer [107].

Some studies showed that in 21.9% of patients with MSI-H/dMMR prostate cancer had a germline mutation in an MMR gene, suggesting that germline testing should be considered for all patients with MSI-H/dMMR prostate cancer [107].

In the study of *Gonzalez et al*., loss of protein expression was detected in 8% for MSH2, 5% for MLH1, and 2% for PMS2, with no statistical differences among the group grading categories [101], opposing the trend with this study's results: overall, MLH1, MSH2, MSH6, and PMS2 were lost in 2 (2.17%), 4 (4.34%), 4 (4.34%), and 6 (6.52%) prostate cancer patients, with statistical differences among the group grading and the control group- respectively, in keeping up with previous literature analysis [92, 93, 104, 105], which intensify, according the literature [101] the thesis, that MMR nature in PCa is seen as controversial and need further to be researched.

In the study from *Fraune et al.* MMR deficiency/MSI was observed in 3.5%. In this study it was concluded that the absence of intratumoral heterogeneity for the MMR status suggests that MSI occurs early in prostate cancer. It was concluded also that MMR analysis on limited biopsy material by IHC is sufficient to estimate the MMR status of the entire cancer mass [106].

Recently it has been demonstrated that MSH2 protein loss more commonly occur in high-grade tumors [92].

It's been reported a significantly lower expression of MSH2, MSH6, and MLH1 genes in prostate tumors might be a significant hallmark distinguishing high grade PCa from benign prostatic hyperplasia [104], which completely matches with this study's results, because in cases of benign prostatic hyperplasia- the MMR expression was maintained.

In addition, in PCa it has been observed that higher staining intensity of MSH2 in prostate tumor samples compared to normal glands and benign prostatic hyperplasia [108].

Interestingly, while some studies reported an association between loss of function of MMR genes and less favorable PCa features [92, 105], other authors hypothesized that genomic damage could trigger MMR gene upregulation, linking overexpression to higher tumor aggressiveness and poor outcome [95, 96, 97].

In this regard, *Gonzalez et al.* found MSH6 overexpression in about 42% of prostate tumors, with an association with the more aggressive cases [101]. It's been speculated that MSH6 protein would be overexpressed in this setting because the increased DNA replication disarrangements require an efficient DNA repair system [101].

However, the same impact on MMR overexpression has not been observed in this study, which could be explained by genomic polymorphism. In addition, in this study only a small numbers of patients (8 patients) had locally advanced or primary metastatic disease.

It has been suggested that MSH6 could be a biomarker of genomic damage and aggressiveness in PCa [101].

This study's findings in part support this observation, by demonstrated that loss of MSH6 expression was predominantly observed with patients with Grade group III–V cancer.

However, some studies demonstrated that MSH6 expression could be downregulated not only in prostate cancer, but also in benign hyperplasia, leading to the hypothesis about inherited cases [95, 96, 97, 101]. The loss of MSH6 expression in benign hyperplasia have not been demonstrated in this study.

Recently it has been found that loss MSH2 correlated with ERG (erythroblast transformation specific – related gene (oncogene) overexpression. Moreover, concomitant loss of MSH2, MLH1, or the single loss of one of them, was also statistically associated with ERG status [101].

This study demonstrated that loss of MMR correlated with CD63 overexpression but not with CD9 overexpression.

Previous studies demonstrated that loss of PMS2 expression associated with PSA progression free survival [101]. This study demonstrated that the median progression free

survival in patients with MMR deficiency (at least one DNA MMR) was significantly shorter compared to in patients with preserved MMR expression.

In the current setting of personalized medicine, a better understanding of MMR deficiency leading to MSI is becoming more important with the recent approval of an immunotherapy-based PD-1 inhibitor (Pembrolizumab) by the FDA, which could be beneficial in cancers with these alterations [101].

Though the recent reports have described favorable response rates for immune checkpoint inhibitors in prostate cancers with microsatellite instability (MSI), it is still unclear whether MSI affects the entire tumor mass or is distributed heterogeneously, the latter potentially impairing treatment efficiency [106].

Immune checkpoint blockade has shown limited benefit in prostate cancer. Nonetheless, durable objective responses have been reported, suggesting that patients with molecularly defined subsets of prostate cancer may benefit from this therapeutic approach. Pembrolizumab, an antibody targeting the programmed cell death protein 1 (PD-1) receptor, recently earned accelerated approval by the US Food and Drug Administration for the treatment of microsatellite instability–high (MSI-H) or mismatch repair deficient (dMMR) solid tumors, independent of site of origin. Detection of MSI thus represents the first clinical indication for prospective tumor profiling in patients with prostate cancer [106].

The National Comprehensive Cancer Network guidelines for prostate cancer were recently amended to include consideration of MSI-H, dMMR testing and pembrolizumab treatment for MSI-H, dMMR mCRPC in the second-line setting or beyond. However, in its approval of pembrolizumab, the US Food and Drug Administration did not define how MSI-H, dMMR status should be evaluated, and detailed guidance is not provided as part of national guidelines. Some diagnostic assays can assess for MSI-H, dMMR status by interrogating microsatellite loci for evidence of MSI, by identifying mutations and copy number alterations in DNS MMR-associated genes. DNA MMR profiling may therefore represent a robust and efficient strategy to identify the subset of patients with prostate cancer who may benefit from anti–PD-1/PD-L1 therapy [101, 106, 107].

In conclusion, high grade prostate cancer characterized by increased expression of CD63, and downregulation of MMR, compared to low grade cancer, which correlated with progression free survival. The loss of MSH2, MSH6, MLH1, and PMS2 was statistically proved characteristics in high grade cancer (Grade group 3, 4 and 5). In addition, the loss of MSH2, MSH6, MLH1, and PMS2 was observed in patients with Grade group 1–2

statistically distinctly less expressed. MMR protein expression was present in all cases of benign prostate hyperplasia.

CD63, CD9 and MMR routine tissue immunohistochemical detection might be a prognostic marker for patients with prostate cancer.

8. THESIS TO DEFEND

The thesis to defend of this PhD thesis is-the expression of CD9 and CD63 and DNA MMR differed between prostate benign hyperplasia and prostate cancer and correlated with clinical behavior and disease prognosis.

9. CONCLUSIONS

1. Prostate cancer is characterized by increased CD63 expression, but decreased CD9 expression and loss of DNA MMR compared to benign prostate hyperplasia tissues.
2. The CD63 expression is increased in patients in high grade prostate cancer, whereas the CD9 expression was decreased.
3. The loss of DNA MMR expression was demonstrated in 10.86% of prostate cancer patients.
4. CD63 expression correlated with PCa Grade groups.
5. The loss of DNA MMR negatively correlated with the Grade groups of prostate cancer.
6. The progression free survival is significantly longer in patients with low CD63 expression, but high CD9 expression and proficient DNA MMR expression.

10. LIST OF PUBLICATIONS

10.1. Internationaly citated publications (Web of Science, PubMed, SCOPUS, ERIH PLUS)

1. Submitted and accepted: Folkmanis, K.; Eglitis, J.; Jakubovskis, M.; Lietuvietis, V.; Folkmane, I; Isajevs, S. CD63 and DNA mismatch repair protein expression in prostate cancer. *Proceedings of the Latvian Academy of Sciences, Section B.* **March 2021**.
2. Submitted and accepted: Folkmanis, K.; Junk E.; Merdane, E.; Folkmane, I.; Folkmanis, V.; Isajevs, S. Current trends in prostate cancer etiology, pathogenesis and diagnostics. *Proceedings of the Latvian Academy of Sciences*, Section B. **2021.**
3. Submitted and accepted: Folkmanis, K.; Junk., E; Merdane, E.; Folkmanis, V.; Folkmane, I.; Isajevs, S. "New kids on the block" – the gamechangers. The role of immune check point blockade in personalized treatment of prostate, urinary bladder and kidney cancer. *Proceedings of the Latvian Academy of Sciences*, Section B. **2021.**
4. Submitted and accepted: Folkmanis, K.; Hajjar, A.; Junk., E; Merdane, E.; Folkmanis, V.; Folkmane, I.; Isajevs, S. Prostate specific antigen dynamics and features in prostate cancer. *Proceedings of the Latvian Academy of Sciences*, Section B. **2021.**

10.2. Presenting author and/or thesis in international congresses

1. 1. Participation as presenting author at LU MF plenarium in international congress of Latvian doctors 22.9.2017 (presentation of clinical case in poster session- "Primary or secondary metastatic pulmonal neoplastic leasion-clinical case in urological praxis).
2. 2. Participation as presenting author at RSU scientifical conference at the section of medicine year 2018 (VII section on thesis bundle-Exosomal biomarker expression in prostate cancer and benign hyperplasia).
3. Participation as presenting author at LU MF international scientific conference of year 2018 (oncology session-Increased CD9 expression in PCa compared to BPH, poster session, page 173 abstract book).

4. Participation as presenting author at IV international Latvian scientific congress of year 2018 (section of medicine-Increased CD9 expression in PCa compared to BPH, poster session Nr. 4).
5. Participation as presenting author at Nordkongress Urologie 2018, Rheinsberg (Posterbegehung 2, Seite 53 PS 2.2 Erhöhte CD9 Expression bei Patienten mit Prostatakarzinom).
6. Participation as presenting author at EAU Congress Baltic session 2019 with poster presentations: Loss of DNA mismatch repair proteins characterizes Grade group 3–5 prostate carcinoma and did not correlate with serum PSA level (Topic 7, Abstract number 46); Downregulation of DNA mismatch repair proteins correlated with increased CD9 expression in high grade prostate cancer (Topic 7, Abstract number 47).
7. Participation as presenting author of oral presentation at LU MF international scientific conference of year 2021(23. april) (oncology session- CD63 and DNA mismatch repair protein expression in prostate cancer, oral presentation session).

11. ACKNOWLEDGEMENTS

I would like to thank the following people, without whom I would not have been able to complete this research, and would not have made it through my PhD degree.

The Professor Sergejs Isajevs' team at University of Latvia and Pathology Centre of Riga East Clinical University Hospital, especially to my supervisor and mentor Professor Sergejs Isajevs himself.

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