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To my family

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KHIRUL ISLAM: Extracellular vesicle glycosylations as novel biomarkers of urological cancers
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ABSTRACT

Prostate and bladder cancer are common urological malignancies that are associated with major causes of morbidity and mortality worldwide. Current diagnosis options for prostate cancer (PCa) and bladder cancer (BlCa) suffer from a lack of sensitivity and specificity that lead to either over-diagnosis or missed cancers. Moreover, current methods are invasive and painful for patients. Therefore, there is an urgent need for the development of sensitive and specific assays involving non-invasive techniques.

The primary aim of this doctoral thesis was to explore possibilities to develop a simple assay for the detection of cancer-associated extracellular vesicles (EVs) directly from human urine without any extensive preprocessing. This project focused on the development of a europium chelate-doped nanoparticles (NPs)-aided immunocapture approach that uses protein and glycan-based markers and their potential combinations for the detection of urinary EVs of urological cancer patients. Several cancer-associated integrin markers in combination with a panel of lectin library were tested to find our best functional biomarkers and their corresponding potential assays. Then the functional biomarker combinations were characterized and validated using EVs-derived from cancer cell lines as well as urine of PCa and BlCa patients. The best biomarker assay, combining with integrin and lectin, ITGA3-UEA can significantly discriminate BlCa from PCa, benign, and healthy controls.

The results of this project emphasize the importance of systemically screening of lectin library in combination with different cancer-associated integrin markers for the advancement of an immunocapturing glycovariant assay to detect urinary EVs for the diagnosis of urological cancers from unprocessed samples. This assay concept could be used as an open platform for exploring glyco-isoform from the surface of EVs for novel biomarker discovery.

KEYWORDS: prostate cancer, bladder cancer, extracellular vesicles, integrin, lectin

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TIIVISTELMÄ

Eturauhas- ja virtsarakkosyöpä ovat yleisiä urologisia sairauksia, jotka aiheuttavat maailmanlaajuisesti merkittävää sairastuvuutta ja kuolleisuutta. Eturauhassyövän (PCa) ja virtsarakkosyövän (BlCa) nykyiset diagnosointivaihtoehdot kärsivät sekä herkkyyden että spesifisyyden puutteesta, mikä johtaa joko ylidiagnosointiin tai syöpien jäämiseen huomaamatta. Nykyiset menetelmät ovat invasiivisia ja kivuliaita potilaille, minkä takia on kiireellisesti kehitettävä herkkiä ja spesifisiä määrityksiä, joissa käytetään ei-invasiivisia tekniikoita.

Tämän väitöskirjan ensisijaisena tavoitteena oli tutkia mahdollisuuksia kehittää yksinkertainen immunomääritys syöpään liittyvien solunulkoisten vesikkeleiden (EV) havaitsemiseksi suoraan ihmisen virtsasta ilman mitään esikäsittelyä. Työssä keskityttiin kehittämään europiumkelaatteja sisältävien nanopartikkeleiden (NP:t) avustamaa immunokaappausmenetelmää, jossa hyödynnetään proteiini- ja glykaanipohjaisia biomarkkereita ja niiden mahdollisia yhdistelmiä syöpäpotilaiden virtsan EV:iden havaitsemiseksi. Syöpään liittyvien integriinimarkkereiden sekä lektiinikirjaston paneelin yhdistelmiä testattiin toimivien biomarkkereiden sekä niitä vastaavien mahdollisten määritysten löytämiseksi. Tämän jälkeen toimivien biomarkkereiden yhdistelmät karakterisoitiin ja validoitiin käyttämällä syöpäsolulinjoista sekä PCa- ja BlCa-potilaiden virtsasta peräisin olevia EV:itä. Paras määritys, joka yhdisti integriini ITGA3:n ja lektiini UEA:n, kykeni luotettavasti erottamaan BlCa:n PCa:sta, sekä hyvänlaatuisista- ja terveistä kontrolleista.

Tämän työn tulokset korostavat lektiinikirjaston ja syöpään liittyvien integriinimarkkereiden yhdistelmien systeemisen seulonnan tärkeyttä, erityisesti ajatellen immunokaappaukseen perustuvaa glykovariantti-määritystä virtsan EV:iden havaitsemiseksi ja urologisten syöpien diagnosoimiseksi käsittelemättömistä näytteistä. Kyseistä määrityskonseptia voisi käyttää avoimena alustana glykoisoformien tutkimiseen EV:iden pinnalta uusien biomarkkerien löytämiseksi.

AVAINSANAT: eturauhassyöpä, virtsarakkosyöpä, solunulkoiset vesikkelit, integriini, lektiini

Table of Contents

Abbreviations

BITC	biotin isothiocyanate
BlCa	bladder cancer
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
BTA	bladder tumor antigen
CA-125	cancer antigen-125
CEA	carcinoembryonic antigen
DRE	digital rectal exam
ELISA	enzyme-linked immunosorbent assays
EVs	extracellular vesicles
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
FISH	fluorescence in situ hybridization
ILVs	intraluminal vesicles
ITGs	integrins
LC	liquid chromatography
LoD	limit of detection
lnRNA	long non-coding RNA
Mab	monoclonal antibody
MS	mass spectrometry
MIBC	muscle-invasive bladder cancer
miRNA	micro-RNA
MISEV	minimal information for studies extracellular vesicles
MWCO	molecular weight cut-off
MVB	multivesicular body
NHS	N-hydroxysuccinimide
NMIBC	non-muscle-invasive bladder cancer
NMP22	nuclear matrix protein 22
NSCLC	non-small cell lung cancer
NTA	nanoparticle tracking analysis
PCa	prostate cancer
PCA3	prostate cancer antigen 3

PPV	positive predictive value
PSA	prostate specific antigen
PS	phosphatidylserine
PSMA	prostate-specific membrane antigen
RALP	robotic-assisted laparoscopic prostatectomy
ROC	receiver-operating characteristic curve
SEC	size exclusion chromatography
SP	soluble protein
TACSTD2	tumor-associated calcium-signal transducer 2
TEM	transmission electron microscopy
THP	tamm-horsfall protein
TRUS	transrectal ultrasonography
TRFIA	time-resolved fluorescence immunoassay
TRPRSS2-ERG	transmembrane protease, serine 2 and etv-related gene fusion
TURP	transurethral resection of the prostate
UC	ultracentrifugation
WB	western blotting

List of Original Publications

The thesis is based on the following original publications, referred to in the text by their Roman numbers **(I-III):**

I Islam MK, Syed P, Lehtinen L, Leivo J, Gidwani K, Wittfooth S, Pettersson K, Lamminmäki U. A nanoparticle-based approach for the detection of extracellular vesicles. *Scientific Reports.* 2019, 11; 9(1).

II Islam MK, Syed P, Dhondt B, Gidwani K, Pettersson K, Lamminmäki U, Leivo J. Bladder cancer detection with aberrantly fucosylated ITGA3. *Anal Biochem.* 2021, 11; 628:114283.

III Islam MK, Dhondt B, Syed P, Khan M, Gidwani K, Webber J, Hendrix A, Jenster G, Lamminen T, Boström P, Pettersson K, Lamminmäki U, Leivo J. Aberrantly fucosylated integrins are enriched in tumor derived urinary extracellular vesicles. (Manuscript).

1 Introduction

Cancer of prostate (PCa) and bladder (BlCa) are most lethal and frequent urological cancers, and their incidence and mortality are rapidly growing worldwide. Only in PCa, 1,276,106 new cases were diagnosed worldwide (representing 7.1% of all cancers in men) and caused 358,989 deaths (representing 3.8 % of all cancer related deaths) in 2018 (Rawla, 2019). Current diagnostic options for PCa are prostate specific antigen (PSA) measurement and digital rectal examination (DRE) check followed by elaborate approaches including risk assessment algorithms and image-guided needle biopsies. However, these tests are unable to discriminate indolent from aggressive cancers, which is still an important clinical problem in PCa diagnosis (Sharma et al., 2016). Moreover, PSA has low specificity that leads to overdiagnosis and overtreatment of PCa (Loeb et al., 2014). In case of BlCa, about 430,000 new cases were diagnosed and 165,000 deaths occurred worldwide in 2012 (Ferlay et al., 2015). Current diagnostic option for BlCa is cystoscopy and urine cytology. Cystoscopy is an invasive tool and unable to detect carcinoma in situ due to low sensitivity. Whereas urine cytology shows more specificity towards high grade tumors but low sensitivity for low-grade bladder tumor. Despite the search for urine-based biomarkers for the early diagnosis of BlCa as a non-invasive manner, none is used in daily clinical practice. Thus, the development of a sensitive assay for searching improved diagnostic biomarkers is still an essential research goal in both PCa and BlCa detection.

Urine is enriched in a wide variety of molecules. It may also contain prostatic secretions due to the proximity of the prostate gland to the bladder. Thus, urine may contain a wide range of molecular markers for PCa and BlCa. Urinary extracellular vesicles (EVs) being one of the emerging markers can be directly released into urine from the prostate and bladder. Several studies showed that EVs are found in all body fluids and contain a cargo of proteins, lipids, nucleic acids, and glycoproteins that could help in identifying novel tissue specific markers (Doyle and Wang, 2019; Zaborowski et al., 2015). Until now, several thousand proteins are discovered on the surface of EVs (Buzás et al., 2018; Raposo and Stoorvogel, 2013). Among other proteins, integrins, multi-functional cell-adhesion molecules, are abundantly found on EVs (Buzás et al., 2018; Hurwitz and Meckes, 2019). Integrins associated with

EVs play a vital role in extracellular matrix attachment and regulate cell growth, proliferation, and migration (Altei et al., 2020; Fuentes et al., 2020). Integrins are transmembrane glycoproteins composed of two sub-units, one α and another β.

The content of the EVs is exposed to different biochemical modifications such as glycosylation and ubiquitination. Alterations in glycan composition of glycoprotein or lipids on the cell surface are commonly observed in cancers. Since the surface of EVs mimic the cell surface, altered glycosylation known to exist on the surface of EVs. Hence, the detection of such altered glycans on EVs could be a viable diagnostic target for cancer and a way to get specificity in tumor detection. Unfortunately, detection of such altered glycans is quite challenging due to the lack of glycan-specific antibodies. Thus, there is an urgent need of a detection tool that can detect such altered glycans with an exceptional specificity and sensitivity. Thereby, lectins, glycan binding proteins, can be utilized as a valuable tool for glycosylated protein analysis. Studies demonstrate that the discrimination between control and cancer cases directly from cell lines and biofluids can be achieved by using lectins (Choi et al., 2021; Patwa et al., 2006; Turner, 1992). Usually, the strength of lectin-glycan interaction (dissociation constant, $K_d = 10^{-4}$-10^{-7} M) is relatively weaker in comparison to antibody-antigen interaction ($K_d = 10^{-8}$-10^{-12} M) (Syed et al., 2016). However, the problem of low affinity of lectins due to such weaker interactions can be compensated through the conjugation of lectins with europium-doped NPs which is embedded with approx. 30,000 europium chelates (Harma et al., 2001). This approach facilitates multivalent binding of lectins towards glycans of EVs and provides signal amplification. Previously, our group demonstrated that the use of macrophage galactose lectin (MGL) when coated with europium NPs, increases cancer specificity of a traditional cancer antigen-125 (CA-125) assay used for the detection of ovarian cancer (Gidwani et al., 2016).

In the present study, we have explored the possibilities to detect glycosylation changes of EVs from the urine of PCa and BlCa patients by constructing a unique and novel NPs-aided time resolved fluorescence immunoassay (NP-TRFIA). This NP-TRFIA uses various combination of cancer associated integrins and high-performance lectins for the discrimination of urological cancer from benign sources. This assay platform could be extended for the detection of other cancer biomarkers through the exploration of glyco-isoform presented on the surface of EVs.

2 Review of the Literature

2.1 Prostate cancer (PCa)

PCa is the most common cancer in men and the leading cause of cancer related death (Ferlay et al., 2015; Siegel et al., 2018). Advanced PCa can quickly metastasize to the lymph nodes adjacent to the prostate gland or in worse cases to the bone, central nervous system, or other organs. Although efficient methods for treatment of early disease stages are available, there is a lack of adequate diagnostic tools to discriminate aggressive PCa lesions from nonaggressive ones resulting in high PCa related mortality rate. Thereby, there is an urgent need for the development of specific and sensitive assays involving non-invasive techniques for the early detection of PCa.

Cancer statistics summarized that North American and Australian continents have comparatively highest incidence rate and mortality of PCa which are followed by Northern, Western and Caribbean countries. In an average, it is noticed that Asian countries have lower incidence and mortality rate of PCa. In Finland, according to the Finnish Cancer Registry in 2019, in total 934 men died from PCa. Due to the extended life span, prostate cancer incidence rate is also getting higher every year in Finland.

2.1.1 Current detection method of PCa

Detection of prostate cancer in the early stage can significantly reduce the mortality rate (Grubb, 2018). Currently, several approaches are available for PCa detection. DRE was earlier used as an initial check for PCa and other health problems such as abnormal mass in the rectum. Unfortunately, this method is ineffective for detecting asymptomatic cancer. Another technique for prostate cancer detection is transrectal ultrasonography (TRUS) which is associated with video imaging of the prostate gland. However, PCa detection with TRUS guided needle biopsy has a risk of 10-30% false negative and sometimes misses PCa (Shariat and Roehrborn, 2008). Recently, for the management of PCa, a multiparametric magnetic resonance imaging (mpMRI) technique has been introduced to discriminate life-threatening cancer from non-life-threatening one (Gaunay et al., 2017).

Prostate specific antigen (PSA) measurement is the most popular screening method for PCa which was approved in 1994 by the United States Food and Drug Administration (FDA). PSA measurement gives a better outcome than both TRUS and DRE approaches for early detection of PCa. Unfortunately, PSA has its own set of drawbacks. One of the major problems with PSA is its false positive results without any incidence of cancer. PSA level can elevate in any disease conditions of prostate gland, for example, prostatitis or benign prostate hyperplasia (BPH). Along with other current detection approaches of PCa, to some extent PSA is still used as a sensitive test in clinics (Schröder et al., 2014). Nevertheless, the use of this test is highly controversial for population screening since excessive use of PSA test leads to unnecessary biopsies of non-aggressive cancers with adverse effects. Thus, PSA test is not suggested for wide population-based screening (Heidenreich et al., 2014).

Another approach for PCa test is prostate cancer antigen 3 (PCA3) score measurement. The PCA3 is a prostate-specific long noncoding RNA (lncRNA) which is abundantly overexpressed in prostate cancer compared to normal condition (Wang et al., 2014). In some PCA3 score measurement studies, the urinary PCA3 lncRNA was measured and then the score was normalized with the value from urinary PSA mRNA. The sensitivity of PCA3 test depends on the cutoff value of PCA3 score (Luo et al., 2014). FDA has approved the PCA3 test for men aged 50 years or more with a previous negative biopsy to help in decision making whether a patient needs repeated biopsies (Tombal et al., 2013).

Another test for PCa detection includes TMPRSS2-ERG fusion gene detection in urine specimens. It has resulted in significant predictive value for PCa diagnosis. This TMPRSS2-ERG fusion gene is found to be 40-80% of PCa (Tomlins et al., 2005). The transcripts of TMPRSS2-ERG gene are found in localized PCa patients after DRE and show a high specificity of 93% with a positive predictive value (PPV) of 94%, unfortunately this gene has a problem of low sensitivity of 37% for the detection of PCa (Hessels and Schalken, 2013; Sartori and Chan, 2014). Due to the high specificity, TMPRSS2-ERG could serve as a viable biomarker in clinic for investigating the absence or presence of PCa (Perner et al., 2007).

Among above mentioned approaches, no single test is enough to correctly detect PCa in early stage. Several elaborate approaches in a combination and algorithm are widely used for PCa detection. Thus, more research is required to search for simple and effective non-invasive tests for the early detection of PCa.

2.2 Bladder cancer (BlCa)

BlCa is the ninth most common cancer in developed countries, and its occurrence rate is second among urogenital cancers (Siegel et al., 2017). Men constitute nearly three-quarters of the BlCa cases and the mortality rates among European men were

the highest compared to the rest of the world (Antoni et al., 2017). Due to a high recurrence rate (50-70% within 5 years) and lifelong examinations, BlCa has the highest cost per patient among all malignancies (Darwiche et al., 2015). In 2012, BlCa cost EU alone €4.9 billion, which roughly accounted for 3% of all cancer costs in EU (Leal et al., 2016). Current diagnostic options for BlCa have limited sensitivity and specificity. Moreover, most of them are costly and invasive in nature (Avritscher et al., 2006). Thus, there is a great need and anticipation to develop novel non-invasive tools for BlCa diagnosis and monitoring.

2.2.1 Current detection method of BlCa

Currently, there are different modalities available for BlCa diagnosis. The most common method for BlCa diagnosis is cystoscopy, which is used to examine the lining of the bladder. This test allows the physician to observe the inside of the body with a thin flexible tube known as a cystoscope. This process can detect the growth in the bladder and helps to decide whether the patient needs to go for biopsy or surgery. However, this procedure is invasive leading to increased patient anxiety and discomfort. Since lifelong monitoring of BlCa patients is required, such an approach puts a significant strain on healthcare resources incurring substantial costs to the society (Malmström et al., 2017).

Another approach, urine cytology observes abnormal cells in urine in a non-invasive manner, which is considered as a very effective tool for high-grade BlCa detection. However, the performance of urine cytology is better at finding larger and more aggressive cancers than low-grade BlCa (Antoni et al., 2017). The collection of cells by cytology is easier than tissue collection by biopsy, which causes less risk and less discomfort to the patient. Sometimes biopsy or other test is required when urine cytology fails to clarify a diagnosis.

ImmunoCyt/uCyt+ test is an adjunct to urine cytology and involves the detection of carcinoembryonic antigen (CEA) and two mucins using fluorescently labeled antibodies (Fradet and Lockhard, 1997; Mian et al., 1999). By combining ImmunoCyt/uCyt+ test with urine cytology, the sensitivity for detecting BlCa can range between 50-90%.

Nuclear matrix protein (NMP22) test involves immunoassay-based measurement of NMP22 in urine, which is overexpressed in urothelial cells and is used for BlCa diagnosis with a sensitivity of 51-85% and specificity of 77-96%. Healthy controls have low levels of urinary NMP22 whereas BlCa patients contain levels that are 25-fold higher (Tsai et al., 2018).

Bladder Tumor Antigen (BTA) test detects antigens that are directly coming from the bladder tumor. BTA TRAK, a quantitative ELISA assay, whereas BTA stat assay, a qualitative POC approach, has shown a significant potential as an adjunct to

cystoscopy. The BTA TRAK test showed better performance than cytology, but AUC and specificity are relatively low. BTA stat has a relatively high sensitivity of 85% but has an average specificity of 62.6% (Guo et al., 2014; Oeyen et al., 2019).

Furthermore, a test called fluorescence in situ hybridization (FISH), involves the analysis of exfoliated urothelial cells for aneuploidy of chromosomes 3, 7 and 17 and loss of the 9p21 locus for detecting BlCa. However, FISH test displays the sensitivity of only 36-57% for the detection of low-grade BlCa (Vrooman and Witjes, 2009).

Above mentioned tests have been approved by FDA, but all have their own set of limitations such as low specificity, false positive indication. Despite their high demand in clinical settings, none of these markers have been fixed for routine diagnosis of BlCa (Babjuk et al., 2017). Thus, more research is essential to find out better non-invasive urine-associated tests to help in the diagnosis and monitoring of BlCa.

2.3 Urine composition and its biomarker potential in cancer

Urine has been examined for centuries as a potential source of important information for the investigation of different diseases. Compared to most other biological fluids, urine is much simpler in composition, and also has the advantage of being inexpensive, available in large volumes, easy to handle, and rich in metabolites. Moreover, urine can be collected continuously in large quantities through a non-invasive process. Additionally, collection and storage of urine have become much more simple, more stable, and also more reliable that facilitates urine-based biomarker discovery. As urine is in direct contact with urogenital organs (renal systems, ureters, urothelium lining, bladder, and urethra), broad variety of disease markers can directly be released into urine (Birder and Andersson, 2013; Linxweiler and Junker, 2020). One of the emerging sources of biomarkers, urinary EVs, are also directly released into urine from urogenital organs (Nawaz et al., 2014; Yanez-Mo et al., 2015). Thus, organ-specific markers could be detected in such urinary EVs, as a concept of liquid biopsy, providing a promising source of biomarkers for urogenital cancer diagnosis, too.

2.4 Extracellular vesicles (EVs)

Almost all cells in the human body release nano-sized membranous vesicles, known as extracellular vesicles (EVs). There are three types of cell-derived vesicles (40-5000 nm diameter) such as exosomes (40-150 nm), microvesicles (100-1000 nm), and apoptotic bodies (1000-5000 nm). Evidence gathered during the last 10 years

shows that EVs have been found in almost all body fluids, including urine (Street et al., 2017), blood (Baranyai et al., 2015) breast milk (Qin et al., 2016), saliva (Machida et al., 2015), cerebrospinal fluid (Yagi et al., 2017), ascites (Peng et al., 2011), semen (Madison et al., 2015), and amniotic fluid (Keller et al., 2007).

2.4.1 Subtypes of extracellular vesicles

The EVs were first recognized as distinct entities 50 years ago, but at that time these membranous vesicles were assumed as waste products released by plasma membranes. The recognition by name of exosomes came in 1983 when studies showed that exosomes were released by exocytosis of multivesicular endosomes (Harding and Stahl, 1983; Pan and Johnstone, 1983). Studies on the morphological and physiological structure of exosomes have disclosed that they are spherical in shape and contain lipid bilayer membranes. Over time, many studies have proven that exosomes contain a cargo of proteins, lipids, and nucleic acids and play important roles in intercellular communication (Théry, 2011).

Microvesicles are usually larger than exosomes, but the size ranges overlap between these two types of vesicles (Akers et al., 2013). Microvesicles are distinguished themselves by their mode of biogenesis, which are generated by direct budding, and fission of the plasma membrane (Cocucci et al., 2009). Like exosomes, a subset of proteins is highly enriched on microvesicles. For instance, beta-1 integrin receptor and vesicles–associated membrane protein 3 (VAMP3) are found on microvesicles derived from melanoma cancer cells (Muralidharan-Chari et al., 2009).

Apoptotic bodies are another category of EVs that are generated by cells undergoing apoptosis. Apoptosis is a major mechanism of cell death. Apoptotic bodies are formed during programmed cell death. Apoptotic bodies are exclusively released from the plasma membrane during the late stage of apoptosis.

2.4.2 Biogenesis of extracellular vesicles

Exosomes are major sub-types of EVs having a unique generation pathway. Exosomes originate through the endosomal pathway (Figure 1). In the beginning of the exosome biogenesis, an early endosome is formed through the invagination of the plasma membrane that allows the internalization of specific proteins present on the cell surface (Scott et al., 2014). The early endosome matures to late endosome by acidification and then intraluminal vesicles (ILVs) form by reverse budding from cytoplasm into the lumen of the endosome. When ILVs bud inward into the late endosome, proteins, lipids, and RNA are packed inside these vesicles. After budding of ILVs, the late endosome is called a multivesicular body (MVB) (Johnstone et al.,

1987). When the MVB fuses with a lysosome, the whole MVB degrade (degraded MVB). On the other hand, when MVB fuses with the plasma membrane, the ILVs of exocytic MVB release into the extracellular space and afterwards, these are called exosomes (Edgar, 2016; Johnstone et al., 1987). The mode of exosomes biogenesis is different than those vesicles that are generated through the direct budding and fusion of the plasma membrane called microvesicles (Figure 1).

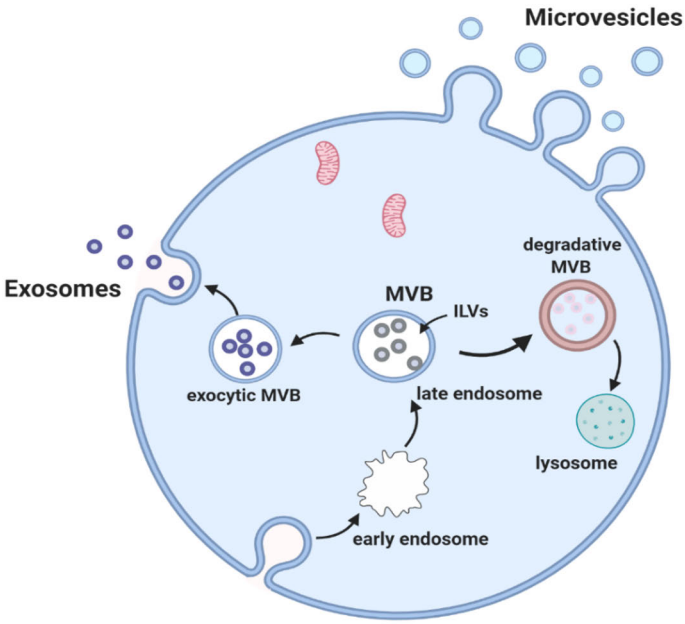

Figure 1: Schematic representation of the EVs release into the extracellular space. In early endosomes, proteins are sequestered in ILVs of the larger MVB. ILVs of MVBs are formed by budding into the lumen of endosomes. In biogenesis, MVBs can either be degradative that are directed into lysosomes or be exocytic, fusing with the plasma membrane and releasing their contents that are called exosomes. The vesicles that generate through direct fusion of the plasma membrane called microvesicles. *This figure is created with BioRender.com (2021).*

2.4.3 Molecular composition of extracellular vesicles

Over time, the molecular content of EVs has been the center of attention to scientists. To explore the molecular composition of EVs, a wide range of profiling tools have been used including proteomics, lipidomics, transcriptomics, and glycomics. Proteomic studies have revealed the diversity of proteins in or on EVs. Among various types of EV-associated proteins reported, some frequently found include tetraspanins (CD9, CD81, CD82, and CD63), ESCRT machineries proteins (ALIX, TSG101), heat-shock proteins (HSP70, HSP90), adhesion proteins (EpCAM,

ITGA3), endosomal proteins (flotillin-1, LAMP-1), and small GTPases (Rab proteins) (Figure 2). The composition of these proteins, in some instances, is related to the cell types and mode of biogenesis.

Exosomes consist of luminal cargo (for instance proteins, peptides, nucleic acids, lipid derivatives) covered by a lipid bilayer membrane that assists as a transport vehicle and protects the luminal cargo from the tough extracellular environment. Based on the statistics of Exocarta database, exosomes contain 9,769 proteins so far found in multiple organisms (Mathivanan and Simpson, 2009). Apart from proteins, exosomes also contain RNA including mRNAs, miRNAs, and some non-coding RNAs (Valadi et al., 2007). Exosomes are also enriched with lipids such as ceramide, phospholipid phosphatidylserine (PS), and cholesterol (Mathivanan and Simpson, 2009; Subra et al., 2007). Additionally, exosomes contain carbohydrates on their outer surface, predominantly consisting of mannose, complex N-linked glycans, fucose, sialic acids, and polylactosamine (Batista et al., 2011; Saunderson et al., 2014).

Microvesicles are relatively heterogeneous considering their composition and size. Microvesicles have similar types of cargo like exosomes, but the molecular content of microvesicles is less defined. Different proteins are enriched in microvesicles including matrix metalloproteinases (MMPs) (Li et al., 2013; Martínez de Lizarrondo et al., 2012), receptors (EGFRvIII) (Al-Nedawi et al., 2008), integrins (Pluskota et al., 2008), cytoskeletal components (β-actin and α-actinin-4) (Bernimoulin et al., 2009), and glycoproteins (P-selectin, GPIIb-IIIa, GBIb) (Del Conde et al., 2005; Mezouar et al., 2015) (Figure 2).

Little is known about the apoptotic bodies due to their diverse cell origins and size heterogeneity. Moreover, apoptotic bodies are often referred to as cellular debris or garbage bags. Nonetheless, some studies have highlighted the fact that apoptotic bodies contain annexin A5, coagulation factor III, and PS (Mallat et al., 1999). Recent proteomic studies showed that apoptotic bodies are also enriched with annexin A6, heat shock protein, RAB11A, and low-density lipoprotein receptor-related protein 1 (Lleo et al., 2014; Turiák et al., 2011).

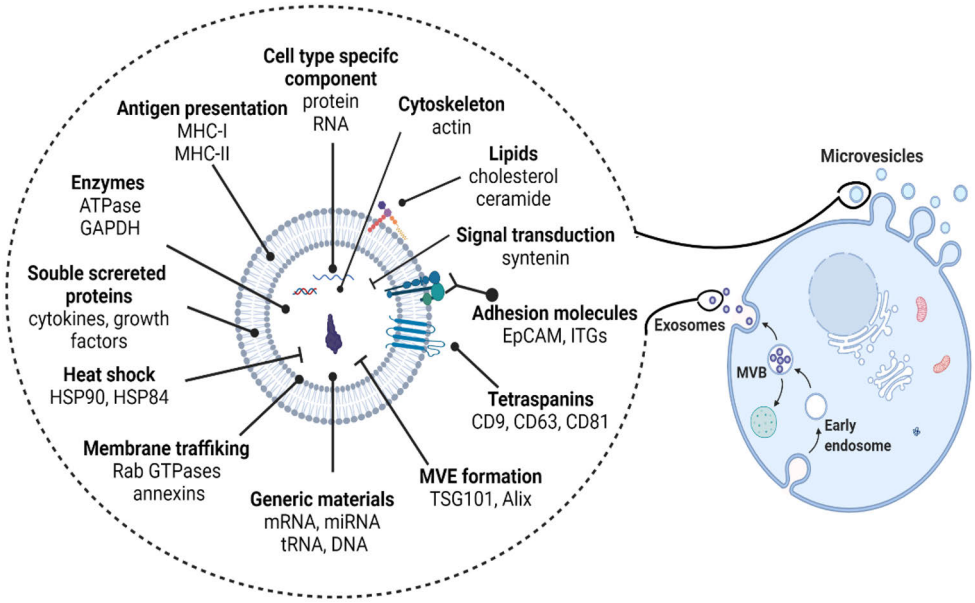

Figure 2: A schematic presentation of molecular composition of a typical extracellular vesicle. A typical EV contains proteins, lipids, carbohydrates, and genetic materials. The proteins in EVs include transmembrane proteins (e.g.-tetraspanin, cell adhesion molecules), cytosolic proteins, intracellular and extracellular proteins. This figure is created with BioRender.com (2021).

Expanding research on EVs has led to the rapid accumulation of information on their composition. Therefore, different databases have been established to facilitate the sharing of this information. For example, information on the molecular composition of EVs is continuously deposited in two databases- Exocarta and Vesiclepedia. Exocarta database lists the proteins, lipids, and nucleic acids that are identified in exosomes (Simpson et al., 2012) and Vesiclepedia is a community annotation compendium for all EVs (Kalra et al., 2012).

EVs are highly enriched with membrane proteins particularly tetraspanins and integrins (Andreu and Yanez-Mo, 2014). The members of tetraspanin super-family often organizes membrane microdomains called tetraspanin-enriched microdomains (TEMs) by forming clusters that interact with a large variety of transmembrane proteins around them, including integrins (Charrin et al., 2009; Yáñez-Mó et al., 2009). Several studies have shown that tetraspanins can be used for the immunoaffinity isolation/capture of EVs from biofluids, forming a major part in the context of EV-associated diagnostic assays (Duijvesz et al., 2015; Islam et al., 2019; Shao et al., 2018). However, EV-integrins have been previously shown to play a vital role in cancer progression and metastasis (Hamidi and Ivaska, 2018; Hurwitz and Meckes, 2019). Integrins are transmembrane glycoproteins and consist of two

heterodimer sub-units (α and β). Functional integrins play a major role in extracellular matrix attachment, cell growth, proliferation and signal transduction (Hamidi and Ivaska, 2018; Hou et al., 2016; Huttenlocher and Horwitz, 2011). Recently, several studies showed that different sub-units of integrin family are found on EVs-derived from cancer cells as well as biofluids (Fedele et al., 2015; Hoshino et al., 2015; Hurwitz et al., 2016; Kawakami et al., 2015; Paolillo and Schinelli, 2017; Rana et al., 2012; Singh et al., 2016). Furthermore, numerous studies have demonstrated that different integrin sub-units are highly enriched on EVs-derived from cancer patients compared to controls (Bijnsdorp et al., 2013; Hurwitz and Meckes, 2019; Welton et al., 2010). Thus, analysis and characterization of EV-integrins could serve as a potential source for cancer biomarker discovery.

2.4.4 Isolation of extracellular vesicles

Several approaches have been applied for the isolation of EVs from cell culture supernatants and biological fluids, but no single technique alone has proven to be universally superior. Method independent considerations need to be counted in selecting an EVs-isolation protocol including equipment availability, biological source, sample number and volume, types of EVs, extent of EVs purity, downstream analysis application, processing time and cost (Witwer et al., 2013). The isolation of EVs could be achieved by using a variety of methods, including ultracentrifugation-, size exclusion chromatography-, filtration- and affinity- based techniques (Konoshenko et al., 2018).

2.4.4.1 Ultracentrifugation

Ultracentrifugation (UC) is a widely used technique for EVs isolation from culture medium and biological fluids. This classical technique for EVs isolation utilizes the separation of particles based on their buoyant density during centrifugation. Centrifugation at speed above 100,000 x g is frequently used (Fernández-Llama et al., 2010; Théry et al., 2006). The efficacy of EVs isolation by ultracentrifugation depends on several parameters including the time, viscosity of the sample, and rotor speed (Konoshenko et al., 2018). EVs isolation from cell culture media needs 100,000 x g for 90 minutes whereas urinary EVs need 200,000 x g for 150 minutes (Konoshenko et al., 2018; Merchant et al., 2017). For further purification and for better separation of diverse EV sub-types, UC method is combined with sucrose density gradient centrifugation. Unfortunately, UC method has some shortcomings such as being costly, time-consuming and unsuitable for large sample volumes (Hogan et al., 2014; Van Deun et al., 2014; Wang and Sun, 2014). Despite the

limitations, it is still considered as the gold standard protocol of EVs isolation for many basic and functional EV research studies.

2.4.4.2 Size exclusion chromatography

The size exclusion chromatography (SEC) is another approach for isolation of EVs. In a comparison study, SEC has been reported as a method to produce highly pure urinary EVs (Rood et al., 2010). SEC can separate EVs from the additional components such as lipoproteins and other proteins. SEC can produce high quality purified EVs from tissue culture supernatants (Lobb et al., 2015; Nordin et al., 2015). However, SEC is not suitable for larger sample volume (Oeyen et al., 2018; Yamamoto et al., 2019b).

2.4.4.3 Ultrafiltration

Due to the easy availability, ultrafiltration is widely used and known as common isolation method for EVs. Different types of centrifugal filter device have been used for this approach. The most widely used filter has the pore size molecular cut-off (MWCO) of 100 kilodaltons (KDa) (Cheruvanky et al., 2007; Nordin et al., 2015). The main reasons why researchers are widely using this approach include commercial availability, low cost, shorter isolation period, convenient to use, and suitable for large sample volume compared to SEC (Cheruvanky et al., 2007). But this approach has its own set of drawbacks such as aggregation of EVs in pressure-driven cells, loss of smaller EVs, lower sample quality, and co-enrichment of non-EV proteins (Cheruvanky et al., 2007; Lobb et al., 2015).

2.4.4.4 Affinity capture

Affinity based approach has been commonly used to get highly pure individual EVs sub-populations by targeting and binding with the specific markers presented on the surface of EVs. In this technique, magnetic beads coated with different affinity reagents such as antibodies or lectins are used for capturing EVs. Then, bead bound EVs are separated by placing them in a magnetic field using a magnetic separator (Kalra et al., 2013; Théry et al., 2006). In the case of antibody-based affinity isolation of EVs, the most commonly targeted surface markers are tetraspanins CD63, CD9, CD81 (Jorgensen et al., 2013). The other markers used for EVs isolation include heat shock proteins (Ghosh et al., 2014) epithelial cellular adhesion molecules (EpCAM) (Kalra et al., 2013), heparin (Balaj et al., 2015), and chondroitin sulfate peptidoglycan 4 (Sharma et al., 2018). In addition to antibodies, lectins have been explored for affinity capturing of EVs by targeting specific glycans on the surface of

EVs. For example, lectin arrays for affinity-based EVs-isolation from urine matrix have been developed with the aim of obtaining a cheap and easy technique for EVs isolation and purification (Echevarria et al., 2014). However, affinity capturing of EVs has some drawbacks such as the reagents are comparatively expensive. Moreover, due to the lack of truly specific EV-markers, yield and purity can be compromised with the non-EVs proteins and non-specific membrane fragments (Raposo and Stoorvogel, 2013).

2.4.4.5 Kit-based precipitation

Recently, there are several commercially available EV isolation kits are available in the market. Researchers usually order these kits based on their EV downstream applications. Among several kits, Total EVs Isolation (Invitrogen), ExoQuick (System Bioscience), and Exo-spin (Cell Guidance System) are often noticed to be extensively used for isolation of EVs. However, EVs isolated by various kits may co-precipitate with non-EVs protein, which can compromise the efficacy and purity of EVs yields (Van Deun et al., 2014).

2.5 Extracellular vesicles in PCa diagnosis

As described above, EVs contain a diverse set of molecules such as proteins, RNA, and lipids. Due to the diverse molecular contents, EVs are considered as hidden treasures for cancer detection. Indeed, EVs released in urine from urogenital glands can provide a potential source of biomarkers for urogenital cancer diagnosis. In a recent study, transmembrane proteins CD9 and CD63 were found to be highly enriched on urinary EVs (uEVs)-derived from men with PCa compared to subjects without PCa after DRE (Duijvesz et al., 2015). In another study, a group of proteins on uEVs was also identified as potential biomarkers for PCa by proteome analysis (Fujita et al., 2017; Øverbye et al., 2015). Similarly, the presence of well-known PCa markers such as PSA and PSMA and several other cancer–associated markers have also been reported in uEVs of PCa (Mitchell et al., 2009). Furthermore, another proteomics analysis study by Welton et al., showed differential expression of protein markers in plasma derived-EVs compared to uEVs from PCa patients using a multiplex, aptamer-based protein array (Welton et al., 2016). Recently, several studies demonstrated that integrin sub-units are highly enriched on EVs from PCa compared to healthy sources (Bijnsdorp et al., 2013; Kawakami et al., 2015; Krishn et al., 2019). For example, integrin α3 and β1 are abundantly found on uEVs of metastatic PCa patients compared to non-metastatic PCa and benign sources (Bijnsdorp et al., 2013). Similarly, other integrin sub-units such as αvβ3 and β4 are

also enriched on EVs of PCa that could be utilized as a prognostic marker for PCa diagnosis (Hurwitz and Meckes, 2019; Kawakami et al., 2015; Krishn et al., 2019).

In addition to proteins, the surface of EVs is enriched with glycans. As explained in the chapter 2.7.1, alterations of the glycans in a tumor cell derived EVs can reflect the clinical status of cancer. For instance, in a recent study, changes in glycosylation as detected on uEVs-derived from prostatic fluids are associated with PCa progression (Nyalwidhe et al., 2013).

EVs also contain various genetic materials such as DNA and RNA. In a study, several miRNAs have been explored in urinary EVs, which have potential diagnostic value in distinguishing PCa cases (Bryzgunova et al., 2016). Study by Isin et al., revealed that long intergenic (linc) RNA-p21 may help to discriminate PCa from benign disease (Işın et al., 2015). Furthermore, numerous miRNAs, especially miRNA-21 and miR-375, derived from uEVs have been implicated as a potential biomarker candidate for PCa diagnosis (Foj et al., 2017). In another study, the miR-145 levels in uEVs were found to be significantly higher in PCa patients compared to BPH patients (Xu et al., 2017). In addition, a next generation sequencing based analysis demonstrated that mature miRNAs and their isomiRs show different expression in urinary EVs of patients with PCa compared to healthy men which disclose the possibility of using miRNA isoforms in PCa diagnosis (Koppers-Lalic et al., 2016).

PCA3 (PCa antigen 3) and ERG (also called TRPRSS2-ERG gene fusion transcript) represent well-known PCa-specific biomarkers. In a recent study, uEVs were isolated from non-DRE urine of men after prostate biopsy and then RNA from the uEVs was extracted to predict initial biopsy result for high-grade PCa. This study identified PCA3 lncRNA and ERG mRNA signatures that were potentially useful for the diagnosis of high-grade PCa on a subsequent biopsy (Donovan et al., 2015). Using the finding from this study, a test called ExoDx Prostate Intelliscore was developed (http://www.exosomedx.com /prostate-cancer). The test demonstrates consistent discriminative potential of high-grade PCa from low-grade and benign disease (McKiernan et al., 2016).

Lipid contents of EVs are another potential source for PCa biomarker discovery. A molecular lipidomic analysis study revealed that several lipid species, for instance, diacylglycerol and triacylglycerol, are differentially expressed in uEVs of PCa patients compared to healthy controls (Skotland et al., 2017). Similarly, lipidomic profiling of uEVs-derived from PCa patients highlighted the potential for developing biomarkers for PCa diagnosis (Yang et al., 2017).

2.6 Extracellular vesicles in BlCa diagnosis

Just like PCa-EVs described in section 2.5, BlCa-derived EVs are also directly released into urine since urine is in direct contact with the walls of the bladder. Thereby, urine-EVs, coming from bladder tumor lining, can hold the promises as a unique source of potential biomarker discovery for BlCa. The idea of using EVs concentration as a biomarker in BlCa, has been explored in a study by Liang et al., which showed that high concentration of CD63-positive uEVs was found in patients with BlCa compared to healthy controls (Liang et al., 2017). Several proteomics studies have demonstrated the potential of urinary EVs in the non-invasive diagnosis of BlCa (Chen et al., 2012; Welton et al., 2010). One of these studies revealed a strong association of tumor-associated calcium-signal transducer 2 (TACSTD2) with BlCa and highlighted the potential of uEVs in BlCa detection (Chen et al., 2012). A proteomics analysis showed that bioactive molecule EDIL-3/del1 is significantly higher in urinary EVs-derived from BlCa patients than healthy individuals (Beckham et al., 2014). Similarly, another study identified histone H2B1K and alpha 1-antitrypsin in urinary EVs as potential biomarkers that could facilitate rapid diagnosis as well as prognosis of BlCa (Lin et al., 2016). Furthermore, the proteomics study by Silvers et al., successfully identified several proteins in urinary EVs associated with muscle-invasive BlCa that were not found in healthy individuals (Silvers et al., 2017). Similarly, another proteomics study has shown that various integrin proteins such as α3, α6, αV, β1, β4 are highly enriched on EVs from BlCa sources compared to controls (Hurwitz and Meckes, 2019; Welton et al., 2010).

Different classes of RNAs found in urine-EVs such as lncRNAs, miRNAs and mRNAs are also considered as a promising source of biomarkers for BlCa diagnosis. A pilot study by Perez et al., investigated potential gene expression in uEVs from BlCa patients and healthy controls and found four differentially expressed genes. Among these four genes, GALNT1 and LASS2 were observed in BlCa patients, and these two genes were associated with tumor progression. On the other hand, ARHGEF39 and FOXO3 were only observed in controls and these two genes were associated with tumor supersession (Perez et al., 2014). Another study showed that certain types of lncRNA are highly expressed in MIBC than healthy individuals (Berrondo et al., 2016). Additionally, a wide range of miRNAs have been found to be differentially expressed in BlCa patients compared to controls (Andreu et al., 2017; Armstrong et al., 2015; Matsuzaki et al., 2017).

2.7 Glycosylation

Glycosylation is a process of attachment of glycans to proteins and lipids that occur in all living beings. Protein glycosylation is the most common post-translational modification and about 80 percent of all human proteins are glycosylated.

Glycosylation plays important role in many biological processes or functions including protein folding, trafficking, cell-cell and cell-matrix interactions, cell adhesion, differentiation, and immune response (Marth and Grewal, 2008; Reily et al., 2019).

Biogenesis of glycans is processed by a myriad of enzymes, which play a vital role in processing, assembly, and turnover of glycans (Varki et al.,2015). Glycosidases and glycosyltransferases are primary enzymes responsible for the biosynthesis of glycans.

Glycosylation can result in diverse forms of glycans which can vary from linear to complex or highly branched structures. Major classes of glycans are N-glycans, O-glycans, glycosphingolipids, glycosylphosphatidylinositol (GPI)-anchors, and glycosaminoglycans (Varki et al.,2015; Varki, 2017). Among major types glycans, most observed N- and O-glycans are shown in figure 3. The oligosaccharide of N-glycan is transferred from dolichol to Asparagine. Similarly, oligosaccharide of O-glycan is transferred from dolichol to Serine (Figure 3).

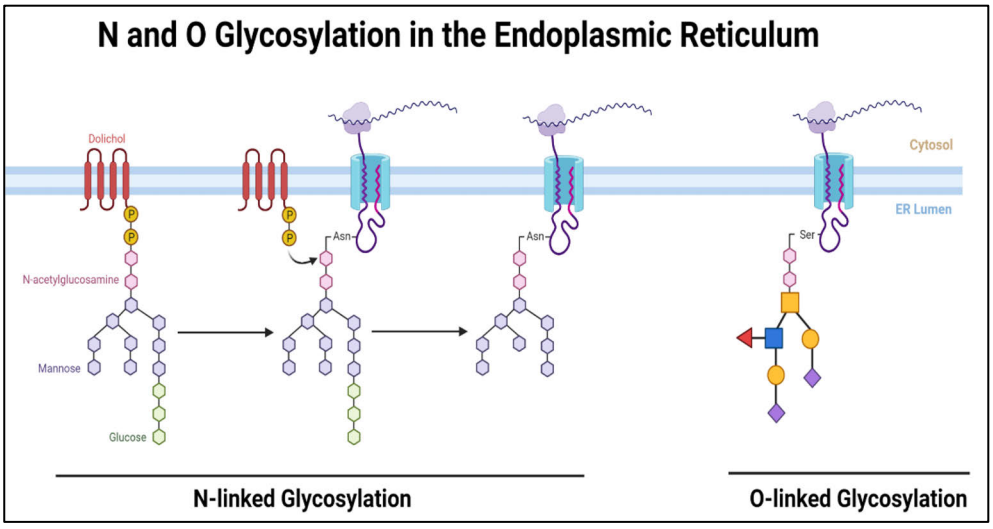

Figure 3: Formation of N- and O- linked glycosylation in the endoplasmic reticulum (ER). This figure is created with BioRender.com (2022) following the original idea described by (Varki, 2017; Varki et al., 2015).

N-and O-glycosylation are the most common forms of protein glycosylation. The difference between N- and O-linked glycosylation is where the sugar molecule is attached (Figure 4). In N-linked glycosylation, glycan is attached to an asparagine residue via the side chain nitrogen atom. The N-glycans can be broadly classified into several classes: a) complex oligosaccharides that contain multiple sugar types such as fucose, galactose and N-acetylneuraminic acid; b) high-mannose

oligosaccharides that contain mannose and GlcNAc residues; as well as c) hybrid branches of both complex oligosaccharides and high mannose.

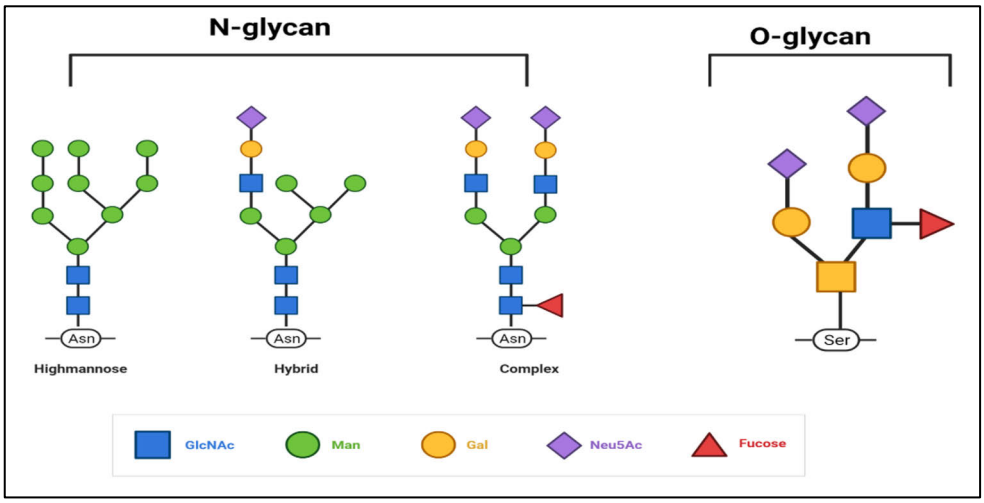

Figure 4: Model examples of branched structures in N-and O-linked glycans. This figure is created with BioRender.com (2022) following the idea described by (Varki et al., 2015).

In O-linked glycosylation, glycan is attached to side chain of serine or threonine, via oxygen atom. Several different sugars can be attached to the threonine or serine which can affect the protein in different ways by changing protein activity and stability. One of the common types of O-linked glycosylation is O-GalNAc which helps the biosynthesis of mucins, a family of high molecular weight with heavily O-glycosylated proteins. Glycoproteins that contain hundreds of O-GalNAc glycans are called mucin-type O-glycans which was first reported in 1980 (Torres and Hart, 1984). Changes in O-glycosylation are usually common in cancer. Understanding such changes in O-glycosylation of cancer cells can lead to potential diagnostic approaches (Van den Steen et al., 1998; Varki, 2017).

2.7.1 Glycosylation in cancer

Glycan changes are commonly observed in cancers (Pinho and Reis, 2015). In cancer cells, aberrant glycosylation happens for various reasons such as attaching to the endothelium, avoiding immune system, and creating new adhesions for metastasis (Stowell et al., 2015; Varki, 2017).

In many cancers, inflammation is considered as one of the key stimulators for glycosylation changes. It results in secretions of several cytokines such as TNFα, IL-6, and IL-1β that can modify the levels of glycosides and glycosyltransferases,

hereby affecting the biogenesis of glycan structures and glycan expression in the cancer cells (Dewald et al., 2016; Reily et al., 2019).

In cancer cells, one common modification is the rise of β1,6 branching of N-glycans caused by augmented action of β1,6-N-acetylglucosaminyltransferase V. This kind of modification can contribute the formation of metastasis (Pinho and Reis, 2015). Another common phenomenon is an increase in fucosylation. Addition of α2,3/4-linked fucose residues needed in the formation of N- and O-glycan associated Lewis blood group antigens, whose expression is elevated in different cancers such as colon and breast cancer (Christiansen et al., 2014).

Altered glycosylation in cancer involves both up-regulation and down-regulation of glycans. In cancer cell, one of the common features is increased branching of N-glycans, which results in more sites for attachment of terminal sialic acid residues and, hereby, extensive increase of total sialylation (Torii et al., 2014). Another feature is abnormal O-glycosylation which is associated with poor prognosis and unfavorable outcome from the cancer treatment (Scott and Drake, 2019). The incomplete or partial glycosylation of cancerous mucins leads to the expression of T, Tn, and sialyl-Tn (STn) antigens. The differential expression of such mucins in normal and tumor tissues makes them interesting diagnostic target in many cancers (Gidwani et al., 2019; Julien et al., 2012).

2.7.2 Glycosylation of Extracellular vesicles

Surface of EVs is highly enriched with N- and O-glycans (Batista et al., 2011; Stowell et al., 2015; Surman et al., 2019; Williams et al., 2018). Moreover, many cancer-associated glycan alterations have been identified in cancer EVs (Figure 5), a feature that might be of use in the development of novel EV-based cancer biomarkers. Interestingly, majority of cancer biomarker proteins used in clinics are glycoproteins (Badr et al., 2014). Studies showed that EVs are highly enriched with mannose and complex type N-glycans, polylactosamine, and 2,6-linked sialic acids (Batista et al., 2011; Surman et al., 2019). It has also been demonstrated that sialoglycoproteins and N-glycans are highly enriched in EVs (Escrevente et al., 2013).

Mucins are found on EVs and the attachment of O-linked glycans to mucins is described in several EV-studies (Kesimer and Gupta, 2015; Stowell et al., 2015). Furthermore, fucosylation is commonly seen in EVs (Williams et al., 2019). Studies demonstrated that core fucosylated N-glycans and terminal fucosylated glycans are differentially expressed on EVs-derived from cancer cell lines and biofluids (Costa et al., 2018; Feng et al., 2018; Freitas et al., 2019; Surman et al., 2018; Zhang et al., 2018). These fucosylation changes on the surface of EVs open the door for targeting by fucose binding reporter molecules.

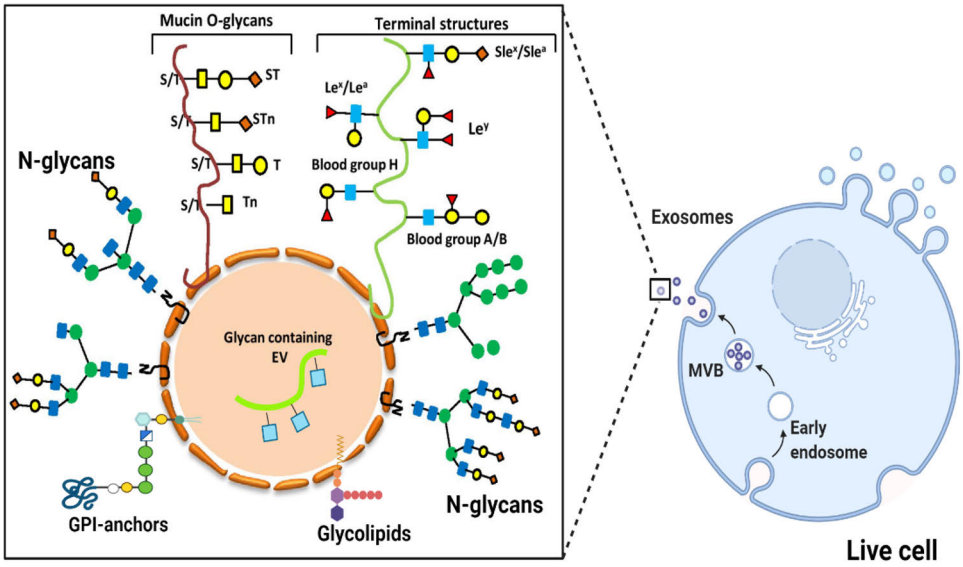

Figure 5: A schematic representation of glycosylation of extracellular vesicles. EVs hold a wide variety of potential functional molecules, including glycoproteins and glycolipids. *This figure is created with BioRender.com (2021).*

2.7.3 Glycosylation of extracellular vesicles as novel biomarker

Patterns of glycosylation on EVs reflect their cells of origin, and the glycosylation patterns of EVs from cancer significantly differ from non-cancer sources (Pinho and Reis, 2015). Recently, several studies have revealed cancer associated EV-glycosignatures that could be considered for the development of potentially improved biomarkers for cancer (Table 1). For example, O-GlcNAcylation is a post-translational modification that is recently found in proteins of EVs derived from colorectal cancer cells. Furthermore, the O-GlcNAc modification of many EV proteins was significantly increased in colorectal metastatic cells, thereby, suggesting its potential as a biomarker (Chaiyawat et al., 2016). Similarly, LGALS3BP glycoprotein is found heavily glycosylated and highly enriched in EVs, which could be served as a potential biomarker for ovarian cancer. As summarized in Table 1, several other glycans of interest in terms of their biomarker potential have been identified on EVs.

Table 1: An overview of cancer associated glycans studied on EV research.

Glycans/molecules	Sources	Cancers	Reference
O-GlcNAc	SW480 and SW620 cells	colorectal	(Chaiyawat et al., 2016)
ADAM10, LGALS3BP, specific glycosignatures	SKOV3, OvMz cells	ovarian	(Escrevente et al., 2013; Escrevente et al., 2011; Escrevente et al., 2008; Gomes et al., 2015)
EpCAM, CD24	cancer cells, ascites fluids	ovarian	(Im et al., 2014; Runz et al., 2007)
highly glycosylated EMMPRIN	MCF-7 cells	breast	(Menck et al., 2015)
EWI-2 N-glycan	SK-Mel-5 cells	melanoma	(Liang et al., 2014)
GlcNAc-containing glycans	urine	-	(Echevarria et al., 2014)
N-glycoproteins	urine	-	(Saraswat et al., 2015)
N-glycosylation profiling of uEVs-associated PSA	urine	prostate	(Vermassen et al., 2017)
tetra-antennary glycans	urine	prostate	(Nyalwidhe et al., 2013)
leucine-rich α-2-glycoprotein	urine	NSCLC	(Li et al., 2011)
MUC1	plasma	NSCLC	(Pan et al., 2019)
highly glycosylated CD133	ascites	pancreatic	(Sakaue et al., 2019)

NSCLC- non-small cell lung cancer

2.8 Lectins and their applications in detection of EVs glycosylation

Lectins are a diverse group of carbohydrate-binding proteins that are found abundantly in nature. Lectins can be divided into structurally different groups, and they are found in various life forms. Both plant and mammalian lectins have been used as a significant tool to study glycosylation, particularly for identifying aberrant glycans during tumorigenesis. As an example of mammalian lectins, the family of C-type lectin receptors can be mentioned. Many members of this family are found on the surface of immune cells such as macrophages and dendritic cells. In the field of glycobiology, lectins are historically most frequently and still widely used biorecognition agents for glycans (Sharon, 2007).

The heterogeneity of glycan structures in glycosylation progression has limited the utility of glycan biomarkers. Long branches and diverse structures of glycans create challenges in analyzing glycans with other conventional methods like LC and MS. On the other hand, lectin-based tools reveal rapid and sensitive profiling of complex glycan structures on the surface of EVs. Krishnamoorthy and his co-workers first introduced the lectin microarray concept for EV glycome analysis in

their studies on intact HIV-1 virions and EVs-derived from T-cells (Krishnamoorthy et al., 2009). In a similar study, the same group demonstrated EVs-glycosylation profiling on different cancer cells (Batista et al., 2011). Further studies with lectin microarrays demonstrated that lectins can be successfully applied for detecting differential glycosylation on EVs surface from patients with different diseases such as autosomal dominant polycystic kidney disease (Gerlach et al., 2013) and classical galactosemia (Staubach et al., 2012).

Lectin blotting is also widely used in EV glycan profiling. In this approach, selective lectins conjugated with a reporter molecule such as enzyme or fluorophore is used as a label to detect glycan signatures on EVs. Due to the simplicity, this technique has gained popularity in extracellular vesicles study.

Highly pure EVs are required to understand their biogenesis and roles in disease pathogenesis. Unfortunately, current approaches for pure EVs isolation have several shortcomings including co-isolation of unwanted proteins and often low yields (Boriachek et al., 2018). Thus, EVs isolation by lectin-based affinity approach has been introduced as an alternative method. Several studies have described EVs isolation by lectin aggregation process from different biological fluids including urine and different cancer cells (Samsonov et al., 2016; Shtam et al., 2017; Yamamoto et al., 2019a). There is also a progressive increase in published literature on the use of lectin aggregation technique as a method for isolation of pure EVs.

Many studies have also shown that a broad range of lectins are capable of selectively bind with different EVs which make lectins interesting tools in terms of the development of assays for specific detection of EVs (Kosanovic and Jankovic, 2014). Lectin can easily be attached with nanoparticles which helps them to make easy tools for selective detection of biomolecules. As an example, Choi and co-workers developed nanoparticle aided microfluidic tool that can selectively detect EVs from pancreatic cancer compared to controls following lectin-glycan interaction (Choi et al., 2021).

Plant and human lectins have frequently been used as tools for exploring glycosylation which can help in cancer diagnosis. For example, a plant lectin called *Lens culinaris agglutinin* can react with liver cancer associated forms of alpha fetoprotein (AFP), which can discriminate AFP-produced by liver cancer patients from that of originating from non-liver cancer patients (Taketa et al., 1993). However, lectin-glycan affinity is generally weaker, compared to that of antibodies. To overcome this low affinity of lectins, assay constructs of highly improved performance are needed where the binding affinity of lectins is highly enhanced without adversely affecting the binding specificity. Our research group previously showed a Europium (Eu^{3+}) Nanoparticles (NPs)-assisted approach whereby the binding affinity of lectins can be greatly enhanced (Gidwani et al., 2016; Kekki et al., 2017; Terävä et al., 2019). The uses of lectin conjugated NPs compensate the

weaker lectin-glycan binding and enable the identification of cancer associated glycans. As the Eu^{3+}-NPs are packed with approx. 30,000 Eu^{3+} chelates, thereby these NPs have a high specific activity (Kokko et al., 2007). In this study, these NPs were used to construct immunoassays possibly applicable for the detection of urological cancers. Special interest was focused to explore a wide panel of lectin aided NPs in combination with integrin antibodies for the detection of cancer EVs derived from the urine of PCa and BlCa patients.

Eventually, to launch the potential benefit of lectins in EVs based cancer research, a versatile panel of lectins has been explored in this thesis for improved cancer biomarker discovery. Particularly, testing of novel lectins or antibodies for the diagnosis of urological cancers has been conducted, which can lead to the identification of novel biomarkers as well as unique assay concept for their detection.

2.9 Time resolved fluorescence immunoassay (TRFIA)

Rosalyn Yalow and Solomon Berson are credited with the concept for the development of the first immunoassays in late 1950s (Yalow and Berson, 1959). Since then, many changes and advancements have taken place in the immunoassays. Many immunoassay reagents have been developed such as antibodies, automated-devices, and reporter molecules. Among them, antibody production through the hybridoma technology in the 1970s, was the most influential that provides a continuous source of monoclonal antibodies having specificity towards a certain epitope (Köhler and Milstein, 1975). During the last decade, the availability of personal computers for data analysis, development of automated plate-reading systems and advancement of fluorescence technology have brought immunoassay tools to everyday clinical practice.

Time-resolved fluorescence immunoassay (TRFIA) is a sensitive analytical approach based on the affinity reaction of antibody-antigen by time-resolved fluorescence measurement. This TRFIA concept utilizes lanthanide ions and their chelating system as tracers with unique fluorescence properties (Hagan and Zuchner, 2011; Sy et al., 2016). TRFIA is considered as one of the most promising immunoassays compared to other traditional immunoassays as it has wider detection range, multi-label detection, less sample interference, more affordable, less time consuming, amenable to automation as well as higher sensitivity. In this thesis, lanthanide-doped polystyrene nanoparticles have been used as labels, hereby the following section concisely describes lanthanide chelate technology and its use with polystyrene nanoparticles.

2.9.1 Lanthanide-doped polystyrene nanoparticles

In the late 1930s, it has been discussed that salts of certain lanthanides are fluorescent compound which can bind with nucleic acids. Attention increased greatly in the 1970s when Finnish research group proposed that lanthanide ions (Eu^{3+}, Tb^{3+}, Sm^{3+}, Dy^{3+}) can be used as luminescent sensors in the time-resolved luminescent immunoassays (Bünzli, 2010; Soini and Hemmilä, 1979). Nowadays, these lanthanide nanomaterials can be used for different biomedical applications including bio-detection, bio-labeling, fluorescence imaging, drug delivery, cancer therapy and detection (Bagheri et al., 2018; Chan and Liu, 2017).

For the construction of reporter molecules (labels/probes), chelated lanthanide ions (Eu^{3+}, Tb^{3+}, Sm^{3+}, Dy^{3+}) can be excited with a series wavelength of light and after energy transfer, a luminescence signal can be measured from the lanthanide ions. Compared to the conventional labels, lanthanide luminescence produces long decay times, in a range of µs to ms, which enables its use in time-resolved model and thereby resulting in lower interference from background fluorescence. Since lanthanide labels display unique fluorescence properties such as long lifetime of fluorescence, large stokes shift and narrow emission peak. Thus, they can overcome self-quenching problem, which is not possible with conventional labels (Hagan and Zuchner, 2011).

2.9.2 Production of lanthanide-doped polystyrene nanoparticles

General concept for the development of a sensitive assay is the attachment of several chelates into a single biomolecule to improve limit of detection of label reactants (Diamandis et al., 1989). Unfortunately, this process causes ultimate non-specific binding which negatively affects immunoassay result (Laukkanen et al., 1995). As only a few certain chelates are allowed to attach into a single analyte, thereby the lanthanide chelates have been incorporated into a protecting shell. However, to produce lanthanide-doped polystyrene nanoparticles, polymerization of styrene monomer is a well-established approach. One easy way to produce nanoparticles doped with lanthanide chelates is their manufacture in aqueous solutions. Since lanthanide chelates are hydrophobic, these chelates are forced into a less hydrophilic environment in the polymer capsules (Huhtinen et al., 2005). Approximately, 30,000 of these chelates can be incorporated into a nanoscale shell to generate a protected label, which is called nanoparticle (Härmä et al., 2001). Nowadays, commercial lanthanide-doped polystyrene nanoparticles are widely available in the market.

2.9.3 Lanthanide-doped polystyrene nanoparticles aided immunoassays

The use of lanthanide-doped polystyrene nanoparticles as a reporter molecule has been proven in different bioaffinity assays for nucleic acids and protein diagnostics (Soukka et al., 2001). In the utilization of polystyrene nanoparticles for protein diagnostics, Soukka et al., have shown that by optimizing protein amount and activation reagents in the conjugation reaction, around 200 active binding sites can be generated onto a single 107 nm particle (Soukka et al., 2001). This phenomenon creates an increase in the binding area of the labels and enhances the association rate of the detection antibody used. In this concept, polystyrene nanoparticles coated with antibodies act as highly fluorescent labels enabling efficient binding to multiple epitopes though avidity effect. Signal amplification through this avidity-effect assists in the development of highly sensitive assays with simple test designs. Thereby, lanthanide-doped polystyrene nanoparticles aided immunoassays have been continuously used as a sensitive test for various bioanalytical applications (Järvenpää et al., 2012; Liu et al., 2013; Soukka et al., 2001).

3 Aims and Significance

The goal of this thesis was to explore the possibilities of using urinary-EVs for the detection of PCa and BlCa. The specific interest of this study was to develop a sensitive, simple and non-invasive immunoassays enabling the capture and detection of uEVs using a panel of lectins or antibodies for improved cancer biomarker discovery. For this purpose, captured EVs were detected with europium-doped nanoparticles conjugated either with antibodies targeting cancer-specific antigens or lectins binding with glycan moieties on EVs surface.

The main objectives were included as follows:

I. Develop a simple, non-invasive, and highly sensitive assay-platform for the detection of EVs from urine and cell culture supernatants without the need for any isolation and purification steps.

II. Detection of ITGA3-glycoisoforms directly from urine for the diagnosis of bladder cancer using lectin nanoparticle assays.

III. Evaluate the potential of lectins and integrins based combinatorial biomarkers in EV based detection of BlCa using EVs derived from tumor cells as well as uEVs of urological cancer patients.

4 Summary of Materials and Methods

A summary of methods and materials with some additional information is addressed here. More detailed information is available in the original publications (**I-III**)

4.1 Samples

The summary of sample cohorts used in the study is compiled in table 2. All samples were collected following the normal laboratory guidelines. A written informed consent was taken from all the participants. The study design and protocol were approved by the local ethics committee, and it was in accordance with the Helsinki Declaration as revised in 2006. UGent urine samples were included here with the approval of Ghent University Hospital ethics committee (B670201420715). Similarly, Turku Prostate Cancer Consortium (TPCC) urine samples were used in this study following the guidelines of University of Turku ethics committee (ETMK Dnro: 3/1801/2013).

Table 2: Description of the samples used in this study.

Collection	Population	Number of samples	Sample matrix	Used in publication
DBUT	Healthy male and female	18	Urine	I & III
UGent	Male and female with BPH & BlCa	22	Urine	II
UGent	uEVs from BPH, PCa and BlCa	15	uEVs isolated from urine	III
TPCC	Male with BPH and PCa, Male and female with BlCa	94	Urine	III

DBUT: Division of Biotechnology, University of Turku, Turku, Finland; UGent: Ghent University, Ghent, Belgium; TPCC: Turku Prostate Cancer Consortium, Turku, Finland

4.1.1 Healthy urine from male and female (DBUT, I)

First morning mid-stream urine samples were collected from 6 male and 6 female healthy volunteers. Similarly, second morning urine samples were collected from 6 male healthy individuals. Samples were stored at +4 ℃ for one day and then prepressing and centrifugation was conducted to remove cells and cell-debris. After that, cell-free urine samples were stored at -80 ℃ until further use.

4.1.2 Urine samples from BlCa patients (UGent cohort, II)

Urine samples were collected from 13 BlCa patients through catheterization before surgical treatment by transurethral resection of the bladder tumor (TURB). Similarly, age-matched non-BlCa 9-urine samples were collected from patients with benign prostatic hyperplasia (BPH) before transurethral resection of the prostate (TURP). All patients were in a fasting condition. Both groups of samples (bladder and non-bladder cancer) were historically classified including the information of urological co-morbidities.

4.1.3 Urine samples from BlCa and PCa patients (TPCC cohort, III)

Urine samples were collected from 32 BlCa patients through catheterization before surgical treatment by transurethral resection of the bladder tumor (TURB). Similarly, Urine samples were collected from 32 PCa patients through catheterization before surgical treatment by robotic-assisted laparoscopic prostatectomy (RALP). Age-matched 30-urine samples were collected from patients with benign prostatic hyperplasia (BPH). Three groups of samples (BlCa, PCa and BPH) were historically classified including the information of urological co-morbidities.

4.2 Reagents

4.2.1 Antibodies

A panel of monoclonal antibodies used in this study is listed in table 3. According to the specification of the manufacturer, these anti-antibodies are specific for their corresponding antigens presented on the surface of EVs. In this study, listed antibodies are either biotinylated or conjugated on nanoparticles to capture or detect EVs. The 10B5 and 5E4 antibodies were developed at the University of Turku Biotechnology department. Sheep anti-mouse horseradish peroxidase-linked antibody (1:3000) (NA931V) and donkey anti-rabbit horseradish peroxidase-linked

antibody (1:4000) (NA934V), GE Healthcare sciences, Uppsala Sweden) were used for immunostaining of urinary EVs.

Table 3: Antibodies used in this doctoral study.

Antibody name	specificity	Clone name	manufacturer	Publication
10B5-antibody	10B5-antigen	-	UTU/BT	I
5E4-antibody	5E4-antigen	-	UTU/BT	I, III
CD9-antibody	CD9-antigen	209306	R&D systems	I, III
CD81-antibody	CD81-antigen	555675	BD bioscience	I
CD63-antibody	CD63-antigen	5566019	BD bioscience	I, III
EpCAM-antibody	EpCAM antigen	158210	R&D systems	I
ITGA3-anibody	ITGA3-antigen	IA3	R&D systems	I, II, III
ITGA1 -antibody	ITGA1-antigen	639508	R&D systems	III
ITGA2-antibody	ITGA2-antigen	HAS3	R&D systems	III
ITGA5-antibody	ITGA5-antigen	612557	R&D systems	III
ITGA6-antibody	ITGA6-antigen	MP4F10	R&D systems	III
ITGAV-antibody	ITGAV-antigen	273210	R&D systems	III
ITGB1-antibody	ITGB1-antigen	4B7R	R&D systems	III
ITGB2-antibody	ITGB2-antigen	6.7	BD bioscience	III
ITGB3-antibody	ITGB3-antigen	VI-PL2	BD bioscience	III
ITGB4-antibody	ITGB4-antigen	422325	R&D systems	III
ITGAM-antibody	ITGAM-antigen	ICRF44	BD bioscience	III
ITGAX-antibody	ITGAX-antigen	3.9	BD bioscience	III
Alix antibody	Alix antigen	3A9	Cell Signaling Technology	III
TSG101 antibody	TSG101 antigen	sc-7964	Santa Cruz Biotechnology	III
CD9 antibody	CD9 antigen	D3H4P	Cell Signaling Technology	III
Flotillin-1 antibody	Flotillin-1 antigen	610820	BD bioscience	III

4.2.2 Lectins

A panel of lectins used in this study is listed in table 4. For the glycan profiling of EVs, the lectins were labeled with Eu^{3+}-chelates or conjugated with NPs. Lectins are carbohydrate binding proteins that have specificity to the carbohydrate moieties presented on the surface of EVs.

Table 4: Lectins used in this doctoral study.

Lectin name	Abbreviation/ synonym	Major carbohydrate binding specificity
Aleuria aurantia lectin	AAL	α1-6 Fuc
Aspergillus oryzae lectin	AOL	Core Fucα1,2 and Fucα1-6
Bauhinia purpurea lectin	BPL	N-acetylglucosamine
Concanavalin A	ConA	Mannose
Dolichos biflorus agglutinin	DBA	N-Acetylgalaxtosamine
Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin	DC-SIGN	Nonsialylated Lewis antigens and high mannose-type structures
C-type lectin	Dectin-1	Sialic acid
Datura stramonium lectin	DSL	(β-1,4) linked N-acetylglucosamine oligomers
β Galectin-3	Gal-3	galactomannans, mannan
Galectin-7	Gal-7	β-galactoside binding lectins
Griffonia (Bandeiraea) *simplicifolia*-1 lectin	GSL-1B/BSL-1B	N-acetylgalactosamine, galactose
Helix pomatia agglutinin	HPA	GalNAc (Tn antigen)
Artocarpus integrifolia lectin	Jacalin	Galactose
Lens culinaris aggulutinin	LCA	Mannose
Lotus tetragonolobus agglutinin	LTA	β-GalNAc (α-linked L-fucose) GlcNAc
Maackia amurensis agglutinin II	MAA-II/ MAH	α2-3-linked sialic acids
Mannose-binding lectin	MBL	Mannose
Phaseolus vulgaris agglutinin-erythroagglutinin	PHA-E	Bisecting GlcNAc
Peanut agglutinin	PNA	Galβ1-3 GalNAc (terminal)
Pisum sativum agglutinin	PSA	α-Mannose (partially α-Fucose)
Pinellia ternata agglutinin	PTA	Mannose
Phytolacca americana (pokeweed) lectin	PWM/Pokeweed mitogen	di-N-acetylchitobiose
Ricinus communis agglutinin	RCA	Gal-β1-4GlcNAc
Recombinant prokaryotic lectin-Fuc 1	RPL-Fuc1	α-Fucose residues
Soybean agglutinin	SBA	Terminal α-or β-linked GalNAc
Sia-recognizing Ig-superfamily lectin 9	Siglec-9	Sia-> α3Gal-> β4GlcNac-> βR, 6-sulfated sLe^x
Sia-recognizing Ig-superfamily lectin 5	Siglec-5	Sia-> α3Gal-> β4GlcNac-> βR
Sambucus nigra agglutinin	SNA	Sialic acid α2-6Gal
Trichosanthes japonica agglutinin-II	TJA-II	Fucα1-2 Gal and β-GalNAc
Ulex europaeus agglutinin-I	UEA-1	Fucα1-2Gal
Vicia villosa lectin	VVL	Terminal α-or β-linked GalNAc (Tn antigen)
Wisteria floribunda agglutinin	WFA/WFL	GalNAcα or β- 3 or 6 position of galactose
Wheat germ agglutinin	WGA	Terminal N-acetylglucosamine

4.3 Preparation of assay reagents

4.3.1 Nanoparticles-conjugation with antibody and lectin

For the preparation of nanoparticle-bioconjugate with antibody or lectin, europium-fluorescent Fluoro-Max™ polystyrene particles (Seradyn Indianapolis IN, USA) were used throughout the study. These nanoparticles (NPs) have activated carboxyl groups that can be covalently coupled with antibodies and lectins. The concentration of antibodies and lectins in the coupling reaction was 0.1 mg/mL. The NPs (5×10^{11} - 1×10^{12} units) were washed with phosphate buffer (10 mmol/L, pH 7.0) using a Nanosep 300 kDa cut-off microfiltration centrifugal device (Pall Corp., MI, USA). The surface of these NPs was activated for 15 mins with 10 mmol/L sulfo-NHS (Sigma-Aldrich) and 0.75 mmol/L sulfo-EDC (Sigma-Aldrich) in phosphate buffer (10 mmol/L, pH 7.0). After activation of NPs, antibodies and lectins were coupled to the NPs under vigorous shaking for 2 hours at RT. Then the bioconjugated antibody-NPs and lectin-NPs were washed with Tris-based buffer (10 mM, pH 7.8, 0.05% NaN_3) and they were stored overnight at +4 ˚C with a supplement of 2 g/L BSA buffer for blocking of the remaining active sites on the NPs. Next day, bioconjugated-NPs mixture was again washed, resuspended, and then stored at +4 ˚C for two days for the removal of aggregates. After that, bioconjugated-NPs mixture was centrifuged (350-x g, 5 min) to remove noncolloidal aggregates. Then aggregates-free supernatant was transferred to a new tube. The particle concentration was determined by diluting the particles with 0.1% Triton X100 solution and by comparing with a known standard particle stock concentration. The measurements were performed with 1420 Victor™ Multilabel Counter (PerkinElmer, Finland).

4.3.2 Labeling of antibody and lectin

Europium (Eu^{3+}) –chelates were labelled with a group of antibodies and lectins in this study. Briefly, 100-fold molar excess of Eu^{3+}–chelates was added in the mixture of antibodies and lectins containing 50 mmol/L carbonate buffer (pH 9.8). The reaction solution was incubated overnight at +4 ˚C. From the mixture, unconjugated chelates were removed using gel filtration with a NAP-5, and NAP-10 columns. Then the labelled antibodies and lectins solutions were stabilized by adding the supplement of 0.1 g/L BSA buffer. This solution was stored at +4 ˚C and ready to use.

4.3.3 Biotinylation of antibody

A group of monoclonal antibodies were biotinylated in this study. Briefly, the pH of antibody solution was adjusted up to 9.8 in a carbonate buffer (50 mM). The biotin isothiocyanate (BITC) was dissolved in dimethyl formamide (DMF) to a final concentration of 10 mM. A 40-fold molar excess of biotin was added over antibody and the reaction was incubated for 4 h at RT. In the final reaction volume, antibody concentration should be closed to 2 mg/mL. After the biotinylation reactions, all the antibodies were purified twice with a NAP-5, and NAP-10 columns (GE healthcare life science, Belgium) into 50 mmol/L Tris-HCL (pH 7.5), containing 150 mmol/L NaCl and 0.5 g/L NaN_3. The biotinylated antibodies were stabilized with 1 g/L BSA and stored at +4 ˚C.

4.4 Isolation of extracellular vesicles for this study

4.4.1 EVs isolation from cell culture medium

Non-cancer (HEK293 and MCF10A), PCa (DU145, LNCaP, PC3, and VCaP), and BlCa (T24 and J82) cell-lines were purchased (ATCC, Teddington, UK) and cultured in T175 culture flask (Greiner Bio-One, Frickenhausen Germany) and Integra bioreactor flask (Integra Biosciences Corp, Hudson, USA) in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) (Mitchell et al., 2008). Cell conditioned media was collected upon reaching the confluency 80-100%. To remove cells and cell-debris, cell culture medium was centrifuged at 400 g for 10 min followed by 2000 g for 15 min. Then conditioned media was ultracentrifuged at 100,000 g for 75 min at 4 ^{0}C using a SW-28 rotator (Beckman Coulter, Fullerton, California, USA) (Lamparski et al., 2002; Théry et al., 2006). Further, EVs-associated samples were separated by floatation within a 30% sucrose/D_2O cushion (Théry et al., 2006). Then EVs sample were collected with a subsequent PBS wash. Further, pelleted EVs were resuspended in PBS and quantified using BCA-protein assay. EVs were stored at -80 ^{0}C until further use.

4.4.2 EVs isolation from urine

Urinary EVs were isolated from the urine of three groups of patients such as BPH (considered as benign control), PCa and BlCa following the previous method (Dhondt et al., 2020b). Urine samples (starting volume 50 mL) were centrifuged at 1000 g for 10 min at 4 ^{0}C to remove cell and cell-debris. Then cell-free urine was collected and concentrated to 800 µL using 10 kDa centrifugal filter (Centricon Plus-70, Merck Millipore, USA). Then 800 µL urine was resuspended in 3.2 mL working

solution buffer (0.25 M sucrose, 6 mM EDTA, 10 mM Tris-HCL, pH 7.4), obtaining a 40% iodixanol suspension, and placed on the bottom of a 17 mL Thinwall Polypropylene Tube (Beckman Coulter, Fullerton, California, USA). Urine solution was then layered into a discontinuous bottom up OptiPrep density gradient (DG) with 4 mL 20%, 4 mL 10%, and 3.5 mL 5% iodixanol solutions and 1 mL PBS, respectively. The DG was centrifuged at 100,000 g for 18 h at 4 ^{0}C using a SW 32.1 Ti rotor (Beckman Coulter). Then 1 mL DG fraction was collected from the top of the gradient. EV-enriched fractions 9-10 and protein-enriched fractions 14-16 were pooled and diluted to 16 mL in PBS in a 17 mL Thinwall Polypropylene Tube (Beckman Coulter). Both pooled fractions were again centrifuged at 100,000 g for 3 h at 4 ^{0}C using a SW 32.1 Ti rotor (Beckman Coulter). Pelleted EV and protein enriched fractions were resuspended in 100 µL PBS and stored at -80 ^{0}C until further use.

4.5 Characterization of extracellular vesicles

4.5.1 Nanoparticle Tracking Analysis

EVs particles were measured by nanoparticle tracking analysis (NTA) using a NanoSignt LM10 system equipped with 405 nm and 488 nm laser (Malvern Instruments Ltd, Amesbury, UK) according to the published articles (Webber and Clayton, 2013), (Dhondt et al., 2020a). This system used highly sensitive sCMOS camera. For each sample, three-30s and -60s videos were recorded at camera for cell-line derived EVs and urinary EVs, respectively. Temperature was controlled during recording. Recorded videos were analyzed by NTA Software (version 3.3) to determine the size distribution and concentration of EVs particles. All size distributions measured with NTA system correspond to the hydrodynamic diameters of the particles in solution. To get optimal concentration of EVs particles, sample was diluted in PBS until particle concentration was within the concentration range of the NTA system (3 x 10^8-1 x 10^9). The NTA measurements were performed through the collaboration with Cardiff University, UK and Ghent University, Belgium.

4.5.2 Transmission Electron Microscopy

Urinary EVs were assessed by transmission electron microscopy (TEM) following the published articles (Dhondt et al., 2020a). EVs were adsorbed to an ultra-thin formvar coated grids that were glow discharged and stabilized by evaporated carbon film before sample application. The grids were negatively stained using neutral uranyl acetate (2% in AD). Then the grids were coated with 2% methyl

cellulose/uranyl acetate (0.4%) solution. The grids were visualized using a Tecnai G2 Spirit TEM (FEI, Eindhoven, The Netherlands) operating at 100 kV. All images were captured using a Quemesa charge-coupled device camera (GMBH, Munster, Germany). Similarly, cancer cell derived EVs were visualized in a JEM-1400 plus TEM (Jeol, Tokyo, Japan) at 80 kV with the cooperation of laboratory of electron microscopy, University of Turku, Finland.

4.5.3 Western Blot

Assessment of EV protein markers and non-EV protein markers was performed by western blot (WB) as described previously (Dhondt et al., 2020a). Samples were dissolved in reducing buffer (40% glycerol, 9.2% SDS, 3% 2-mercaptoethanol, 0.005% bromophenol blue and 0.5 M Tris-HCl at pH 6.8) and boiled for 5 min at 95 ^{0}C. Proteins were separated by SDS-polyacrylamide gel electrophoresis and placed into a nitrocellulose membrane (Bio-Rad Laboratories). After blocking the membranes, blots were incubated overnight with relevant primary antibodies. Then the membranes were extensively washed in a PBS buffer containing 0.5% Tween 20. The blots were then incubated with relevant secondary antibodies. After final wash, chemiluminescence substrate (WesternBright Sirius, Advansta, California, USA) was added and images were taken using Proxima 2850 Imager (IsoGen Life Sciences, De Meern, The Netherlands).

4.6 Immunoassays (I-III)

Immunoassays were performed in this study as shown in Figure 6. All the assays were conducted as a heterogenous non-competitive immunoassay-format. Immunoassays were used in all the settings to capture and detect EVs-derived from cell lines and urine. In the publication (I), EVs were first captured with biotinylated antibodies against tetraspanins or cancer-associated proteins. Then, the captured EVs were detected targeting tetraspanins and glycans presented on their surface by anti-tetraspanin antibodies or lectins coated on nanoparticles. In the publication (II), Integrin-glycoisoforms were captured with anti-integrin antibodies and subsequently detected with either anti-integrin antibodies or lectins coated on NPs. In the publication (III), Isolated EVs derived from cancer cell lines and urine were directly incubated in the solid surface of a 96-well maxisorp plate. After washing, non-specific sites were blocked by 2% BSA (from a stock of 7.5% BSA-TSA). Then the presence of glycan and integrin on EVs was confirmed by either lectin or anti-integrin-antibody coated on NPs. Assay constructing antibody and lectin were also evaluated using a 96-well streptavidin plate in a sandwich assay format, where urine-EVs were captured with anti-integrin-antibody and detected with lectin-NPs.

Unbound materials were washed-away using DELFIA series instrument and reagents in all the assays. Then the time-resolved fluorescence (TRF) counts were measured with 1420 Victor Multilabel Counter (Perkin-Elmer, Finland).

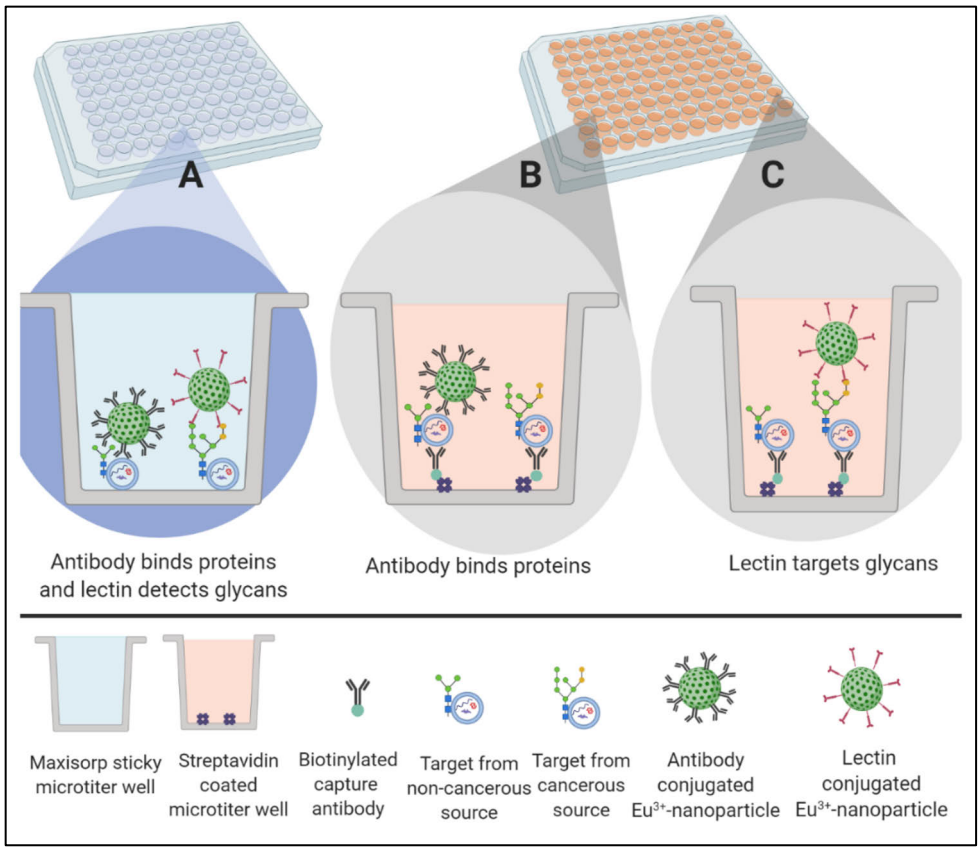

Figure 6: Schematic presentation of immunoassay concept. A) Direct assay: EVs were passively immobolized on maxisorp plate and then detected with either antibody or lectin coated on europium NPs. (B and C) Sandwich assay; Biotinylated antibodies were immobilized in the streptavidin-coated wells to capture EVs from samples. The captured EVs were detected consisting either antibody or lectin coated on europium NPs.

4.7 Immunoassay LoD evaluation

The limit of detection (LoD) of the ITGA3-ITGA3 and ITGA3-UEA assays were evaluated with the use of cancer cell DU145-derived EVs spiked in urine. In the experiment, a series of EVs, 1 x10^6, 1 x10^7, 1 x10^8, 1 x10^9, 1 x10^{10}, and 1 x10^{11} EVs/mL, were used. For optimal average value, calibrator was measured in a range of 6-60 replicates. The blank calibrator was measured in 60 replicates. Then the calibrator 1 x10^6, 1 x10^7, 1 x10^8, 1 x10^9 EVs/mL were measured in 20 replicates. The

calibrator 1 $x10^{10}$, and 1 $x10^{11}$ EVs/mL were measured in 12 and 6 replicates, respectively. The LoD of the ITGA3-ITGA3 and ITGA3-UEA assays were calculated by fitting the data to Sigmoidal and 4PL (four parameters logistic) regression of Origin 2016 using the values from standard curves. The LoD was determined following the formula:

$$\text{LoD} = \mu_\beta + 1645\sigma_\beta + 1.645\sigma_s$$

Where, μ_β is the mean measurement of blank calibrator, σ_β is the standard deviation measurement of blank, and σ_s is the standard deviation measurement of low concentration calibrators.

4.8 Statistical analyses (I-III)

Statistical analyses were performed using GraphPad Prism, lnc (version 6) and R-software. The limit of detection for the assays (LoD) was calculated using OriginLab 2016 (version 2016Sr2). Mann-Whitney U test was used to compare immunoassay results from cancer patients and benign controls. A two-sided P value < 0.05 indicated statistical significance.

5 Summary of Results and Discussions

EVs have gathered a lot of attention in the past decade due to increasing understanding on their multiple biological functions and their abundance in various biological fluids including urine. The analysis and characterization of EVs typically require a time-consuming and labor-intensive isolation process. In this work, we have focused on the development of a lanthanide chelate-doped nanoparticle assisted immunocapture method for the detection of urinary EVs without the need of any isolation process. In this approach, the EVs were first captured using biotinylated anti-tetraspanin or anti-integrin antibodies immobilized on a 96-well microtiter plate. Then the captured EVs were detected with either anti-tetraspanin and anti–integrin antibodies or lectins coated on nanoparticles. The signals were then counted with time-resolved fluorescence-based detection. The developed assays were further characterized with EVs-derived from cancer cells and urine of cancer patients.

A previous study by Duijvesz et al., showed that the double monoclonal immunoassay using tetraspanin specific antibody can capture and detect urinary EVs without the need of any isolation steps (Duijvesz et al., 2015). In their TRFIA assay, they used Eu^{3+}-chelate as a reporter. In our developed TRFIA assay, we used Eu^{3+}-nanoparticles (Eu^{3+}-NPs) to further increase our assay sensitivity. Each Eu^{3+}-NP contains approximately 30,000 Eu^{3+}-chelate that can provide signal amplification through a specific activity (Kokko et al., 2007).

The majority of protein biomarkers for cancer are glycoprotein. Interestingly, changes of their glycan part can often be noticed in the early stages of cancer progression. Unfortunately, conventional double monoclonal immunoassays are blind to detect such glycosylation changes, as they are based on the peptide epitope recognition (Gidwani et al., 2016). Herein, we have used a wide selection of lectins with different glycan specificities to recognize changes in glycan composition on the surface of cancer EVs. We found that fucosylated ITGA3 is enriched on the surface of EVs, which can be extensively detected by fucose-binding lectin UEA. We have also characterized our biomarker combination on EVs from cancer cells and urine of urological cancer patients. Eventually, we have found that biomarker combination, ITGA3-UEA, can significantly discriminate BlCa patients from benign conditions.

In this section, results of this project are summarized and discussed. More detailed information is available in the original publications (I-III). The thesis also includes some additional information.

5.1 Performance of NP and Eu^{3+}-chelate based tracers to detect EVs

In this study, a time-resolved fluorescence immunoassay (TRFIA) was developed to detect EVs from minimally preprocessed urine and cell culture supernatants. Two types of tracers were used in this TRFIA and to check the performance of these tracers, different configurations were set up. In all these assays, an immobilized anti-CD9 antibody was applied for capturing EVs, whereas tracers varied being either NP or Eu^{3+}-chelate conjugated to various lectins (lectin-NP and lectin-Eu assays). These assays were used to detect EVs from the LNCaP medium and urine of two healthy individuals. The signal-to-background (S/B) ratios obtained from the assays were compared. The S/B ratios of different lectin-NP tracers were 2-10-fold higher compared to the corresponding lectin-Eu tracers (Figure 7 a, b & c). Importantly, few lectins like GSL-1 & MAA-II were able to detect EVs only when conjugated with NP, not with Eu^{3+}-chelate. Besides lectin tracers, anti-CD9 antibodies were also conjugated with either NP or Eu^{3+}-chelate (antibody-NP and antibody-Eu assays). The antibody-NP and antibody-Eu tracers showed similar performance in the detection of EVs from the LNCaP medium and urine of two healthy volunteers (data not shown).

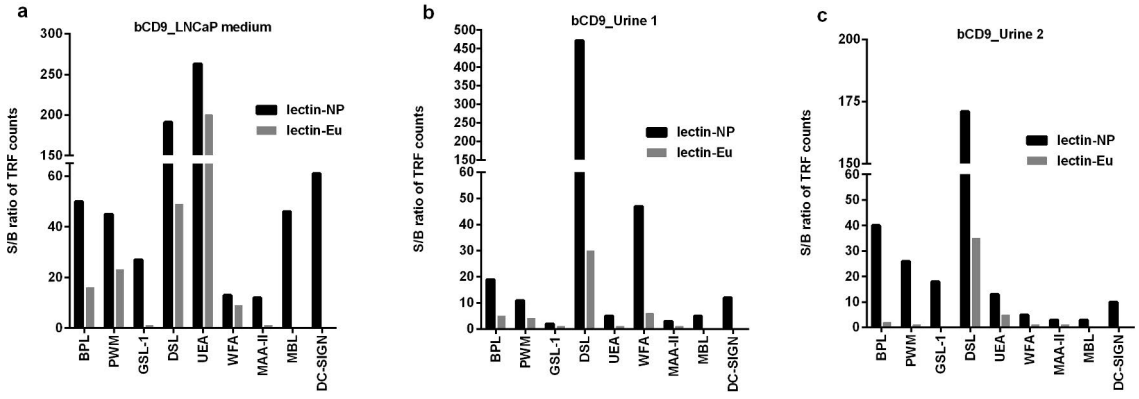

Figure 7: Comparison of Eu^{3+}-nanoparticles (Eu-NP) vs Eu^{3+}-chelate (Eu-chelate) conjugated with lectin as tracers. LNCaP medium and urine-derived EVs were captured with biotinylated anti-CD9 antibody immobilized on streptavidin-coated microtiter wells and detected with either Eu-NP or Eu-chelate based tracers. a) comparison of lectin-NP and lectin-Eu assays performance in LNCaP medium in terms of S/B ratio. b) and c) comparison of S/B ratio obtained from lectin-NP and lectin-Eu assays in two individual urine samples, respectively. Herein, for the lectin DC-SIGN and MBL, only NP tracer was used.

5.2 Specificity of nanoparticle assisted TRFIA (NP-TRFIA)

To further study the specificity of EV capture, biotinylated anti-CD9 antibody was immobilized on microtiter wells for capturing EVs from LNCaP medium and then the captured EVs were detected by NPs assisted tracers conjugated either with tetraspanin antibodies (CD9-, CD81-, CD63-NP) or lectins (MBL-NP and DC-SIGN-NP). The capture and tracer specificity of the assay were evaluated using non-EVs specific antibodies. For instance, the capture mAb 10B5 (specific for mycotoxin) and tracer mAb 5E4 (specific for kallikrein 2) used in the assay, are not usually present on the surface of EVs. Interestingly, countable signals only obtained from the assays using anti-CD9 antibody for capturing EVs followed by the detection with either anti-tetraspanin (CD9, CD81, and CD63)-NPs or lectin (MBL and DC-SIGN)-NPs. The signals achieved from the non-EVs specific antibodies (10B5 and 5E4) was almost like the signals found from the control without any capture (No Capture) and tracer (no-Ab-NP) (Figure 8).

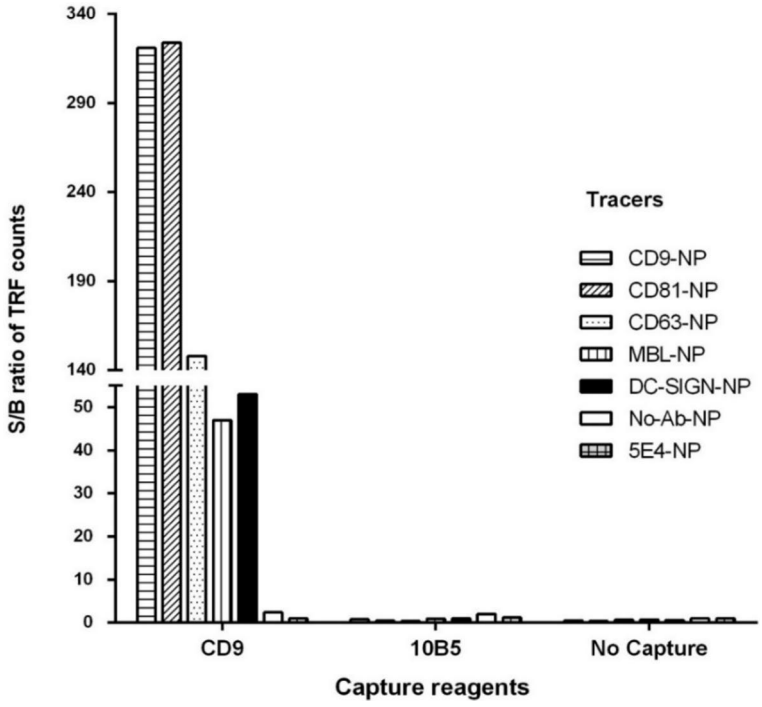

Figure 8: Capture and tracer specificity of nanoparticle assisted TRFIA (NP-TRFIA). EVs from LNCaP medium were captured with immobilized anti-CD9 antibody and detected with several different tracers (CD9-, CD81-, CD63-, MBL- and DC-SIGN-NP). The non-EVs-specific antibodies 10B5 and 5E4 were used to rule out the specificity of capture and tracer, respectively. Additional controls included capture without any antibody (No Capture) and NPs without any antibody or lectin (No-Ab-NP).

5.3 Sensitivity of NP-TRFIA

To assess the sensitivity and linearity of the NP-TRFIA, the detection limit of the assay was calculated with serial dilutions of both culture medium and isolated-EVs from LNCaP supernatant. Gradual increase of sample amount from LNCaP medium and isolated LNCaP-EVs in per well show the linear increase of S/B ratio (Figure 9 a & b). The detection limit of EVs isolated from LNCaP using MBL-NP and DC-SIGN-NP was 1-2 ng/mL, while the corresponding value for CD9-and CD81-NP tracers were 4-5 ng/mL (Figure 9 b). Similarly, the detection limit of isolated-EVs using CD63-NP was 13 ng/mL.

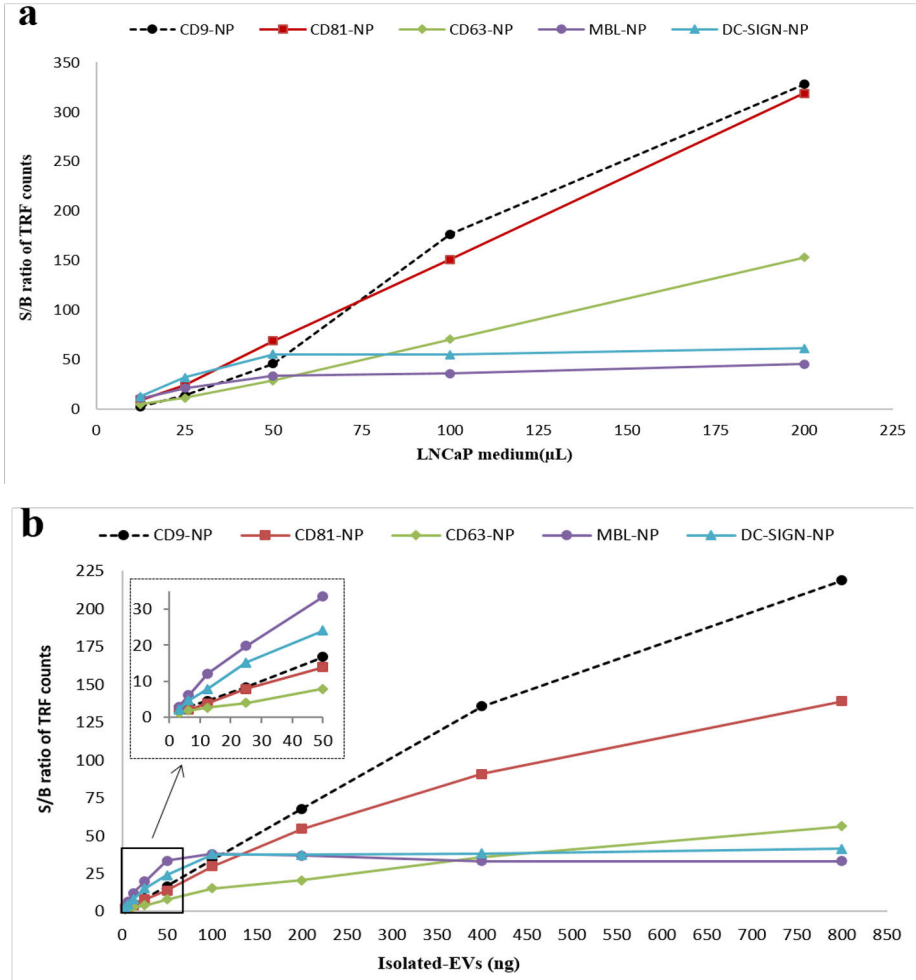

Figure 9: Sensitivity and linearity response of NP-TRFIA assay using different NP based tracers. a) LNCaP medium dilution series, and b) isolated EVs from LNCaP supernatant were captured using biotinylated anti-CD9 and detected with several anti-CD9-, anti CD81-, anti-CD63-, MBL- and DC-SIGN-NP tracers.

5.4 Tetraspanin and glycan profiling of urinary EVs

The various NP-TRFIA assay configurations were tested for the profiling of tetraspanins and glycans of urinary EVs. The assays were constructed using all possible combinations of three anti-tetraspanin (CD9, CD81, and CD63) antibodies and their corresponding NP-based tracers as well as a panel of lectin-NP tracers. Most of the tetraspanin and lectin tracers-based assays were able to detect uEVs in terms of S/B ratios from minimally preprocessed 12 individual urine samples. In tetraspanin profiling of uEVs, the S/B ratios achieved from the assays constructed with CD63-NP showed higher S/B ratios upon using biotinylated (bCD9, bCD81, and bCD63) antibodies (Figure 10 a). In glycoprofiling of uEVs, signal levels achieved from different lectin-NP tracers showed distinct variation from each other. The highest S/B ratios were obtained from the assays involving lectins DC-SIGN-NP (with bCD63 combination), DSL-NP (with bCD9 combination), and WFA-NP (with bCD9 combination) (Figure 10 b).

The Tamm-Horsfall protein (THP) is abundantly found in urine, which creates a common problem in the evaluation of uEVs. In order to remove THP, we treated urine with NaCl salt, which showed a partial removal of THP. In order to study the potential interference of THP with our NP-based assays, we then compared NaCl treated and untreated urine samples which resulted in very similar S/B ratios, thereby indicating that the presence of THP does not have impact on the assays (data shown in original publication I).

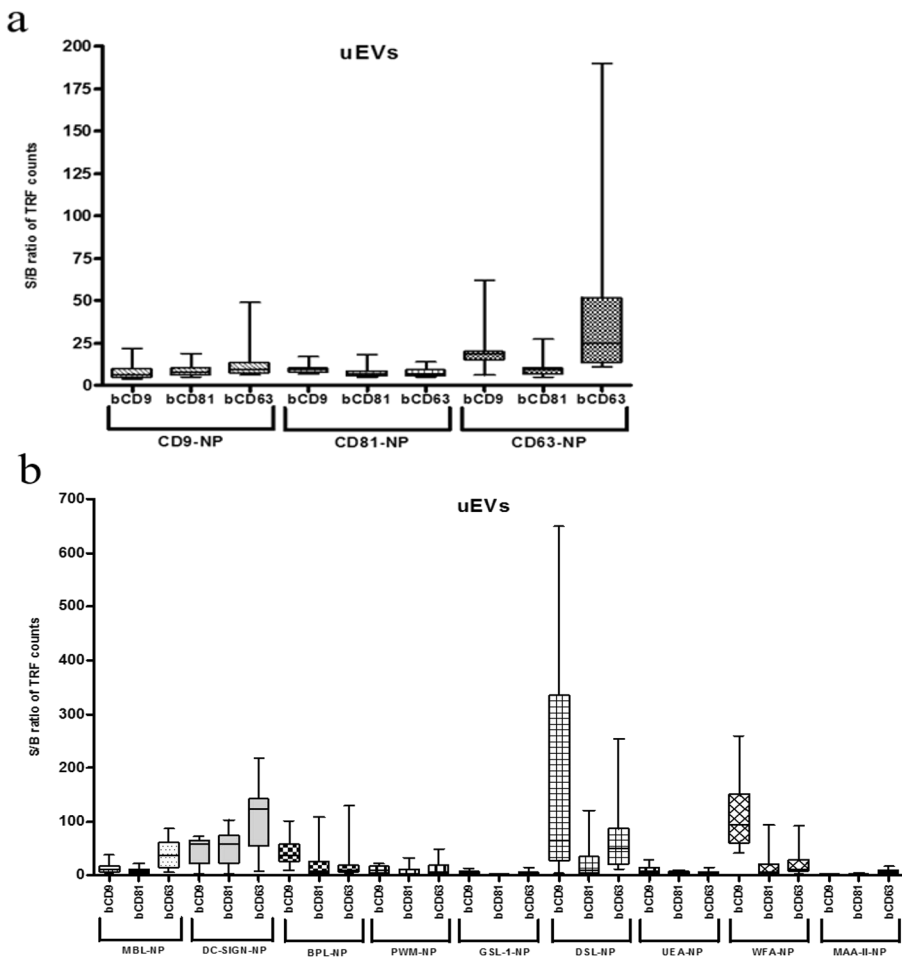

Figure 10: Protein and glycan profiling of urinary EVs with NP-based assays using various antibody-NPs and lectin-NPs. Urine EVs from healthy individuals (*n*=12) were first captured with biotinylated antibodies (bCD9, bCD81, and CD63) and then detected with a. antibody-NPs (CD9, CD81, and CD63) and b. lectin-NPs (MBL, DC-SIGN, BP-, GSL-1, DSL, UEA, WFA, and MAA-II).

5.5 Assay performance through the capturing of cancer-associated proteins on the surface of EVs

We also evaluated the potential of the developed NP-TRFIA assay concept in measuring the abundance of tumor-associated proteins (EpCAM and ITGA3) on the surface of EVs. The assays were constructed using biotinylated anti-EpCAM, anti-ITGA3 or anti-CD63 antibodies for capturing EVs from cell culture medium of prostate cancer cell lines LNCaP, DU145, PC3, and one of the normal healthy cell-line HEK293. Then the

captured EVs were detected using CD63-NPs. The S/B ratio obtained by the EpCAM-CD63 and ITGA3-CD63 assays were normalized by the S/B ratio of CD63-CD63 assay. The results suggest that the levels of EpCAM-EVs were 6-7-fold higher in hormone-dependent LNCaP compared to hormone-independent DU145 and PC3 (Figure 11 a). These results are consistent with previous observations showing that the overexpression of EpCAM is associated with the hormone (androgen)-dependency of prostate cancer (Massoner et al., 2014). On the other hand, the level of ITGA3-EVs was 10-15-fold higher in more aggressive and hormone-independent DU145 and PC3 cell lines compared to less aggressive and hormone-dependent LNCaP cell lines (Figure 11 b). These results are in line with the study reported by Bijnsdorp et al., which showed an elevated level of ITGA3 on urine-EVs of metastatic prostate cancer patients compared to benign and less aggressive cancer (Bijnsdorp et al., 2013). The results obtained through this simple NP-based assay suggest that the assay concept could be utilized for the evaluation of also other tumor-associated surface proteins.

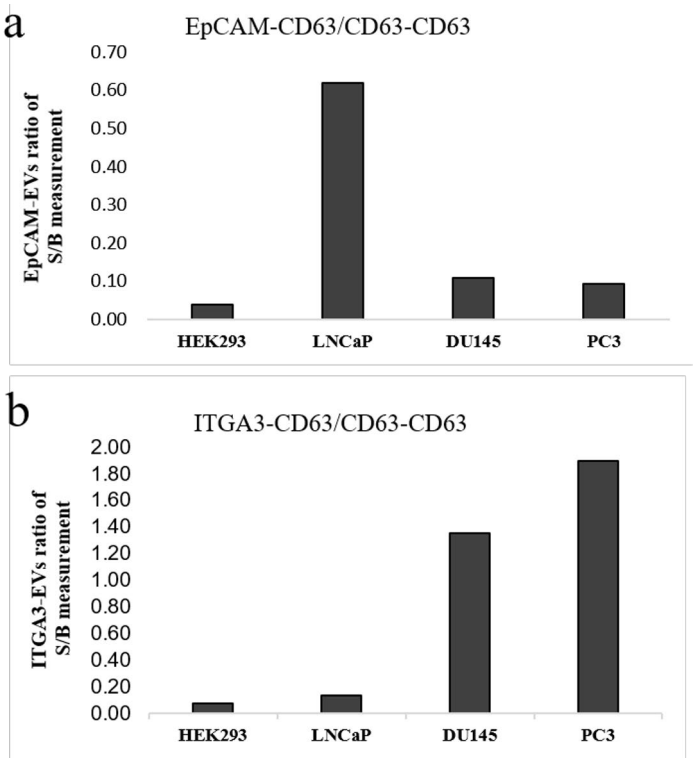

Figure 11: Detection of EVs based on the capture of tumor-associated proteins. Biotinylated antibodies against tumor-associated proteins EpCAM and ITGA3 were used to capture EVs from cell culture supernatant of LNCaP, DU145, and PC3 prostate cancer cell line and one control HEK293 cell line. The captured a. EpCAM-EVs and b. ITGA3-EVs were detected by CD63-NPs. The S/B ratios for EpCAM-CD63 and ITGA3-CD63 assays were normalized by the corresponding value from the CD63-CD63 assay.

5.6 Screening of ITGA3-lectin-NP based assay

The promising results obtained with ITGA3 in PCa cell lines, encouraged us to explore further the potential of ITGA3 based capture using lectin tracer-based assays. Moreover, ITGA3 is glycosylated and known as a cancer associated biomarker and also has a recognized role in cancer progression (Huang et al., 2018; Ren et al., 2014). This time we focused on BlCa, another urological track cancer, where the role of ITGA3 has also been previously implicated in a study by Sakaguchi et al., (Sakaguchi et al., 2017), and for which we had representative patient samples. Thus, anti-ITGA3 was used as a capture for the assay in combination with a panel of 9 lectin-NPs tracers with various binding specificities. The control assay (ITGA3-ITGA3) having the anti-ITGA3 both as a capture and tracer showed 2-fold higher S/B ratio with pooled samples of BlCa compared to those of benign sources (blue box), whereas the assay constructed with ITGA3 as a capture and lectin UEA as a tracer showed a 6-fold higher S/B ratio (red box) (Figure 12). The rest of the lectin-NPs showed poor discrimination of BlCa compared to benign samples. The results suggest that ITGA3 is overexpressed in the case of BlCa compared to benign cases. They also suggest that as compared to the detection based on the mere ITGA3 protein epitopes, the combined detection with the protein epitope-based capture (anti-ITGA3) and a glyco-isoform targeting tracer (UEF-NP) enables more efficient differentiation between the cancer and benign samples. The blue box represents the total amount of ITGA3 determined in the urine of benign and BlCa patients, whereas the red box indicates the binding of UEA lectin to ITGA3-glycan in the urine.

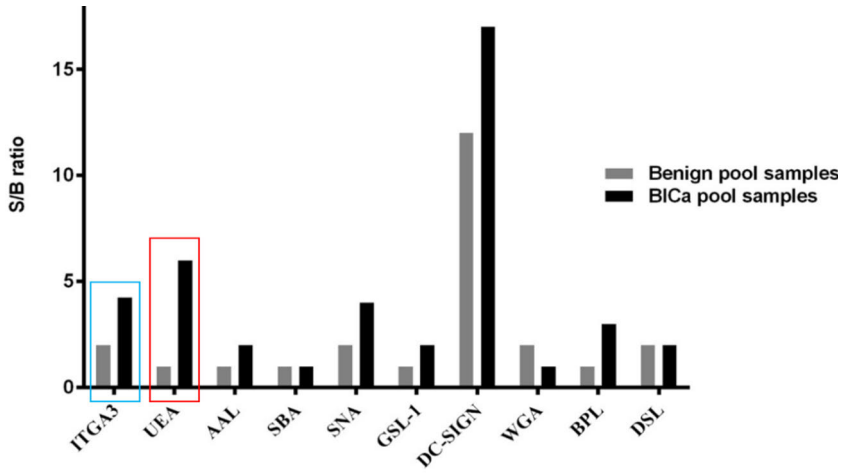

Figure 12: Assays using anti-ITGA3 capture in combination with a panel of lectin-NPs tracers. Biotinylated anti-ITGA3 antibody was used for capture from the pooled urine of BlCa and benign condition. The binding intensity was represented by S/B ratios. Herein, blue box indicates ITGA3-ITGA3 assay, whereas red box represents ITGA3-UEA assay.

5.7 Evaluation of ITGA3-UEA assay for bladder cancer detection

For closer study of the potentially enhanced cancer specificity of ITGA3-UEA assay, the assay was applied to the analysis of individual urine samples from bladder cancer patients and for comparison samples from patients with benign urological condition like BPH. For comparison, also the total ITGA3 assay (ITGA3-ITGA3) was performed. The ITGA3-ITGA3 showed limited discrimination of BlCa compared to benign sources (Figure 13 a, p=0.023), whereas the ITGA3-UEA assay demonstrated significant discrimination of BlCa compared to benign condition (Figure 13 b, p=0.007). The assay was considered statistically significant when the two-tailed p value was <0.05.

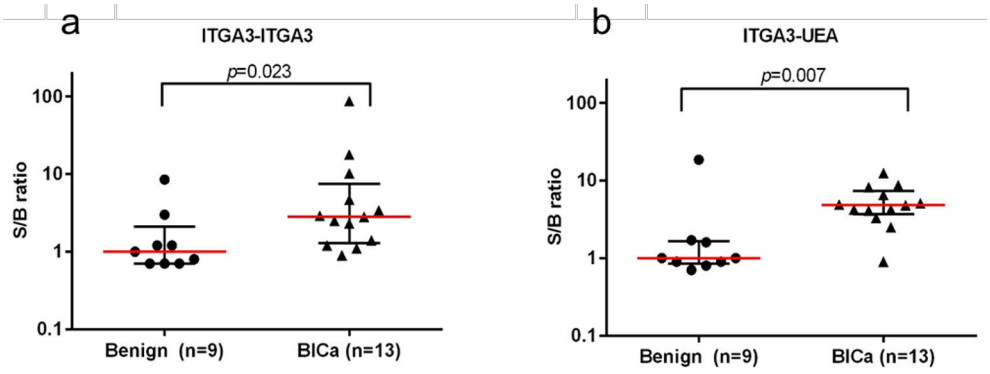

Figure 13: Detection of BlCa patients (n=13) compared to benign individuals (n=9) using (a) ITGA3-ITGA3 and (b) ITGA3-UEA assay. Mann-Whitney U test was applied for the calculation of p-values from signal to background (S/B) values.

It has been reported that several integrin subunits (α and β) are found on the surface of cells and EVs (Anderson et al., 2014; Bijnsdorp et al., 2013; Clayton et al., 2004; Hurwitz and Meckes, 2019). Some integrin subunits, for example, α1 can also be found in urine as a soluble protein. Moreover, integrins themselves are glycosylated. Based on the obtained results, we were unable to infer whether our investigated biomarker ITGA3 is present on EVs or as soluble protein in urine, or both. Thus, this motivated further studies to explore in what extent our investigated integrin-glycoform biomarker combination (detected by the ITGA3-UEA assay) is present on uEVs in the patients of urological cancers.

5.8 EVs characterization and quality control check

5.8.1 Cancer cell-line derived EVs characterization

In order to investigate biomarkers, EVs were derived from four PCa cell-lines. These PCa-EVs were isolated through the collaboration with Cardiff University, UK. As a quality control for cancer cell-line derived EVs, the vesicles were analyzed for tetraspanin expression, total particle counts, and structural integrity following the previously published procedures (Webber et al., 2014). Tetraspanins (CD9, CD63 and CD81) were used to confirm their enrichment on EVs from DU145, LNCaP, PC3, and VCaP using immunofluorescent assay (Figure 14 A). The particle size and concentration were measured by nanoparticle tracking analysis (NTA) using the Nanosignt NS300 (Figure 14 B). The NTA demonstrated 1.27 x 10^{13}-1.11 x 10^{14} EV particles per mL of sample from PC3, DU145, VCaP, and LNCaP cells. Moreover, transmission electron microscopy (TEM) was performed to check the morphological characteristics such as structure and integrity of EVs. The TEM showed that EVs had a typical lipid bilayer membrane structure and were approximately 200 nm in diameter. (Figure 14 C).

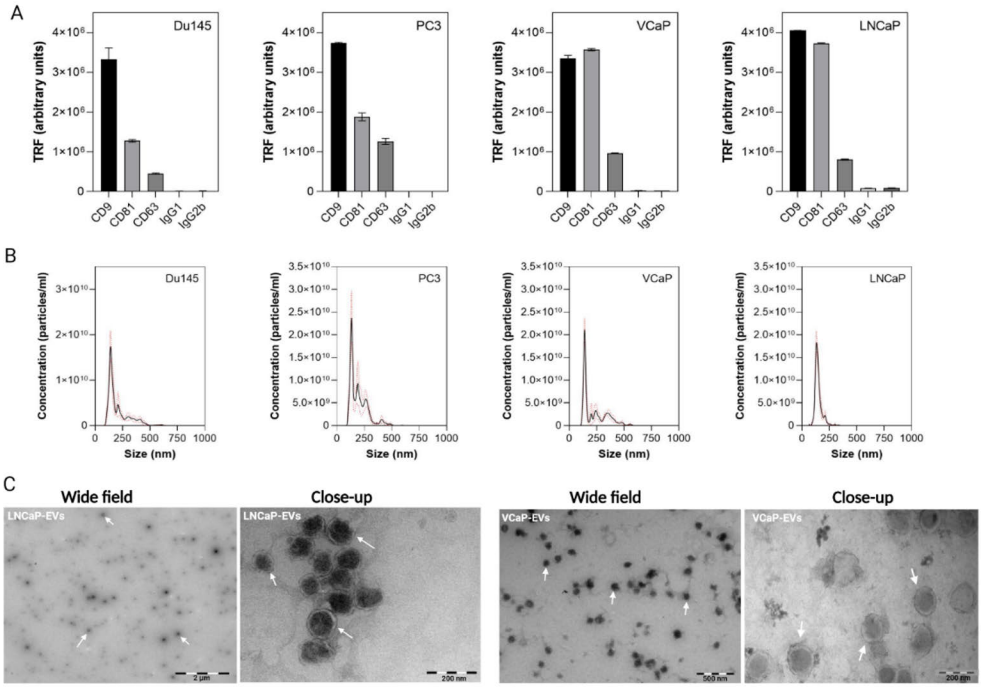

Figure 14: Characterization of EVs derived from cancer cell-lines. Cancer cell-line derived EVs were analyzed with A) tetraspanin expression profiles, B) NTA for particles count, C) TEM for vesicles integrity.

5.8.2 Urine derived EVs characterization

Urinary EVs were isolated and collected from Ghent University, Belgium through the collaboration with EV-group of Professor An Hendrix. However, to assess the quality of urine derived EVs, western blot (WB), NTA, and electron microscopy (EM) were performed following the previously published article (Dhondt et al., 2020a). WB demonstrated the presence of EV-enriched proteins such as Alix, Flotillin-1, TSG-101, CD9 and depletion of non-EV-enriched proteins such as THP (Figure 15 A). The NTA demonstrated 1.24 x 10^{11} EV particles on per mL of EV-enriched protein fractions and 5.92 x 10^{9} particles on per mL of non-EV-enriched protein fractions (Figure 15 B). Moreover, EM was used to confirm the structure and integrity of EV-enriched and non-EV-enriched proteins. The EM revealed vesicles with lipid bilayer membranes within EV-enriched proteins having a size range of approximately 200 nm, whereas the non-EV-enriched fractions demonstrated a complex network of protein complexes and THP (Figure 15 C).

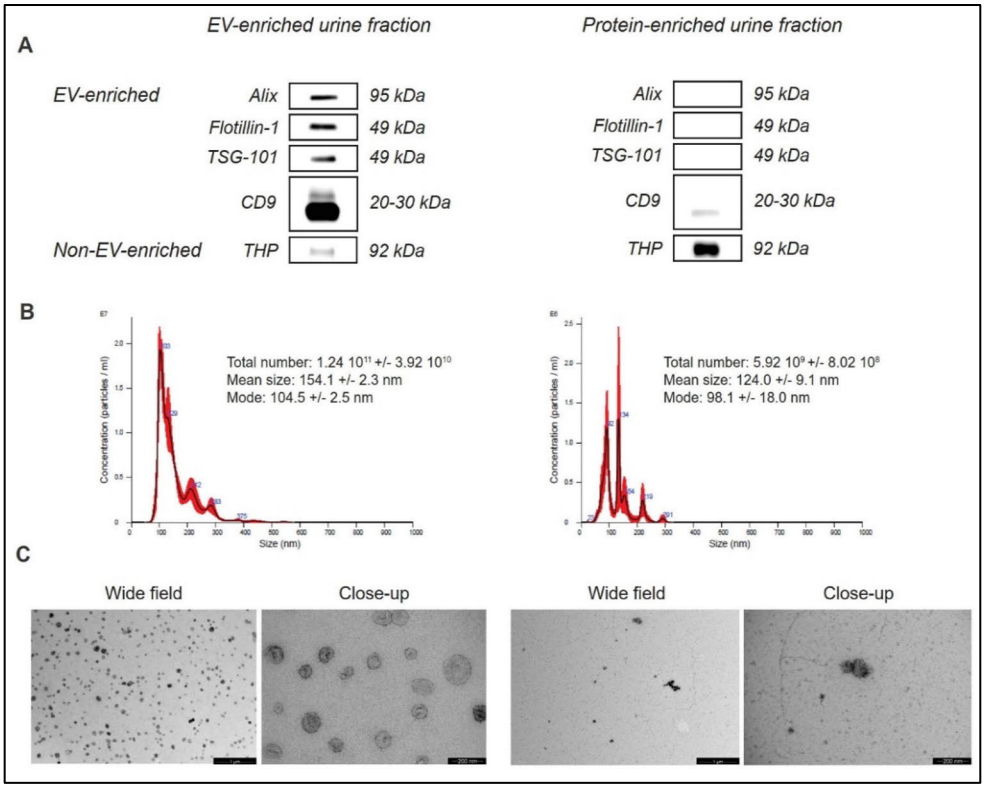

Figure 15: Characterization of EV-enriched and non-EV-enriched protein fractions from urine. EV-enriched and protein-enriched urine fractions were analyzed with A) WB B) NTA, and C) EM.

5.9 Characterization of cancer cell derived EVs by direct assay

Many studies highlighted the potential of lectins as tools for disclosing the glycosylation patterns presented on the surface of EVs derived from different sources (Freitas et al., 2019; Gerlach and Griffin, 2016; Williams et al., 2018). Herein, we used a rich library with 34 lectins for the glycan profiling of EVs derived from PCa cell lines (DU145, LNCaP, PC3, and VCaP). In the experiment, EVs were first immobilized on the wells of a sticky microtiter plate and then detected with lectin-NPs as tracers using time-resolved fluorometry-based direct immunoassays. The 34 tested lectins with a capacity to recognize six different glycan groups e.g., fucose, galactose, GalNAc, mannose, GlcNAc, and sialic acid with different binding specificities. Results showed that mostly lectins have a strong binding intensity targeting different glycan moieties on EVs-derived from cancer cell-lines (Figure 16 A). Particularly, we observed that 12 lectins out of 34 candidates, namely UEA, TJA-II, SBA, PNA, Galectin 3, WFA, BPL, WGA, PWM, DC-SIGN, ConA, and MAA displayed strong binding profiles towards cancer EVs.

Similarly, we also characterized the expression profile of the integrins on EVs derived from same PCa cell lines using direct immunoassay. We tested the binding of 12 anti-integrin antibodies coated on nanoparticles using cell line derived purified EVs passively coated on the microtiter wells (Figure 16 B). As expected, different anti-integrin antibodies showed distinct binding intensities towards cancer cell line derived EVs. Among 12 anti-integrin antibodies, we observed that ITGA3 shows high binding intensities with PCa cell-lines derived EVs (Figure 16 B).

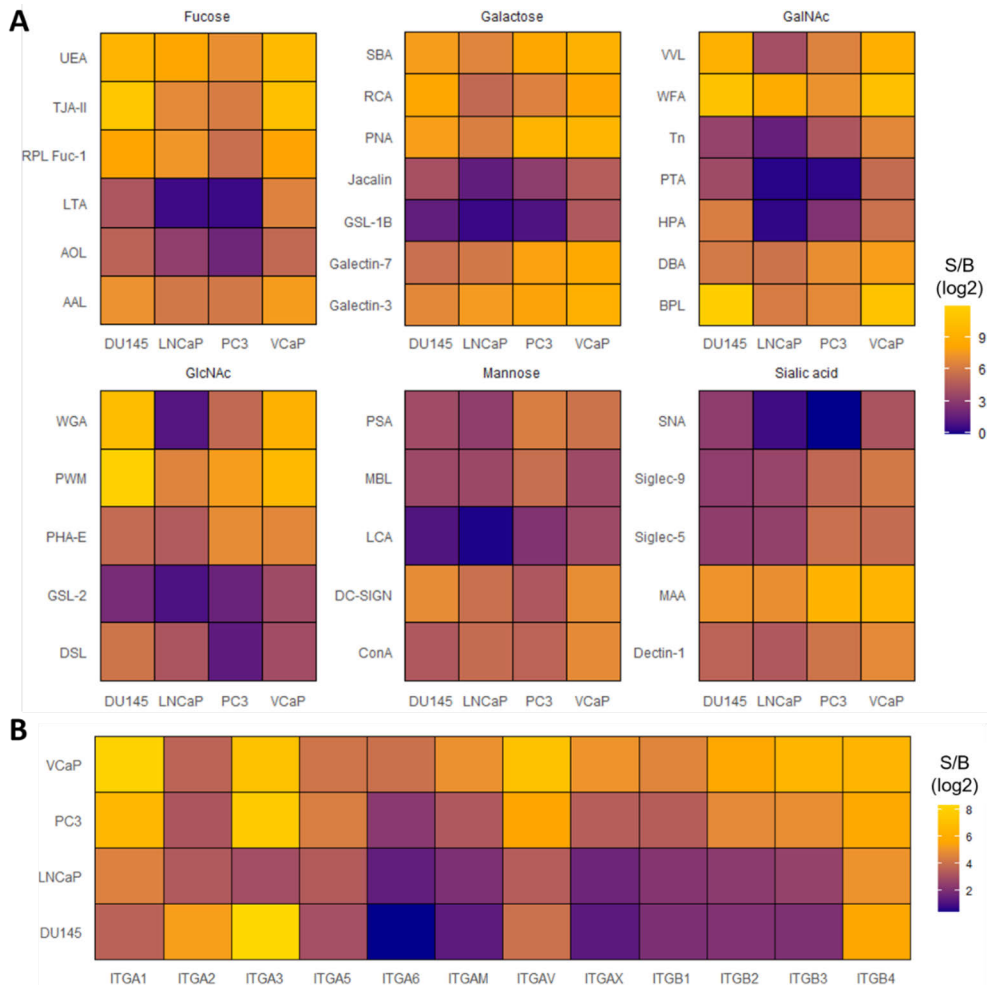

Figure 16: Characterization of cancer cell line derived EVs using direct assay. A) Six different glycans (fucose, galactose, GalNAc, GlcNAc, mannose, and sialic acid) specific lectin tracers were used to measure the S/B ratios obtained from EVs-derived from DU145, LNCaP, PC3, and VCaP cell lines in a direct immunoassay. B) 12 anti-integrin antibodies coated on NPs were used to measure the S/B ratios obtained from EVs-derived from four PCa cell lines in a direct immunoassay. The heatmap shows S/B (signal/background) ratios on a log2 scale.

5.10 Characterization of cancer cell derived EVs by sandwich assay

Our previous studies (5.5 – 5.7) suggested that the overexpression of ITGA3 has potential in cancer detection. On the other hand, various other subunits of integrins (alpha unit α2, α3, α4, α5, α6, αM, αL, αV and VLA-4 and beta unit β1, β2, β4, and β5) have, previously, been identified on EVs-derived from cancer cells and urine

(Bijnsdorp et al., 2013; Rieu et al., 2000; Welton et al., 2010; Wubbolts et al., 2003). Moreover, integrins associated to EVs have been found to be overexpressed in BlCa and PCa (Bijnsdorp et al., 2013; Grossman et al., 2000; Krishn et al., 2019; Subbaram and Dipersio, 2011). Additionally, it is well-established that glycosylation of integrins have a vital role in cancer glyco-microenvironment and cancer progression (Marsico et al., 2018). This prompted us to further assess the expression of integrins and their glycosylations on EVs derived from cancer cell-lines.

However, to further assess the performance of the lectins, we set up a sandwich assay where ITGA3 was used as a capture for the immobilization of EVs-derived from DU145, LNCaP, PC3, and VCaP cell lines. The captured EVs were then detected by 12 lectins that previously showed highest binding intensities in the Figure 16 A. Among selected 12 lectin, UEA showed highest S/B ratios with EVs originating from four PCa cell lines (Figure 17 A).

Next, we explored the UEA-NPs to recognize several integrin sub-populations on EVs from cancer and non-cancer sources. The tested cell culture medium samples were taken from PCa (PC3 and DU145) and BlCa cell lines (T24 and J82), as well as non-cancerous control cells (MCF10A and HEK293). Among several anti-integrin antibodies, clearly the most intensive signals were obtained with use of ITGA3 on EVs from the prostate and bladder cancer cell lines compared to the control cell lines (Figure 17 B). Some other integrins, for example ITGAV, also showed elevated expression through the detection of EVs from cancer cell lines in comparison to control cell lines. However, detailed exploration of ITGAV was out of the scope of this study. In the future, we have plans to further explore other promising integrin and lectin candidates in clinical samples.

In this present study, we have systemically explored the glycovariant of ITGs on EVs to determine the most promising combination of integrins and lectins for the assay development. We have observed that UEA-NPs combining with integrin ITGA3 (ITGA3-UEA) could detect EVs from four aggressive cancer cell-lines (PC3, DU145, T24, and J82) showing very high signal-to-background ratios (S/B>10) in comparison to control cell-lines (HEK293 and MCF10A) (Figure 17 B).

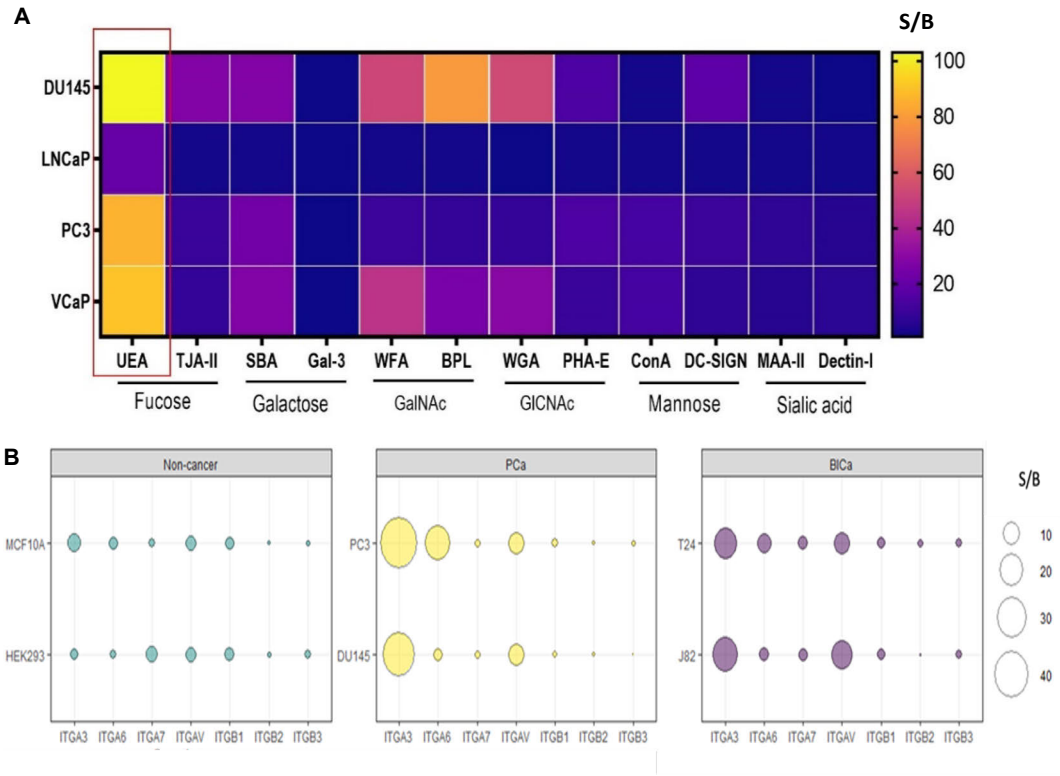

Figure 17. Characterization of cancer cell line derived EVs using sandwich assay. A) The S/B ratios obtained from sandwich assay where EVs-derived from PCa-cell lines were captured by ITGA3 and detected using 12-lectin tracers. B) Different integrin specific antibodies were used to capture EVs from PCa (PC3 and DU145) and BlCa (T24, and J82) cell lines compared to non-cancer (HEK293 and MCF10A) cell-lines in sandwich assay, where lectin UEA-NP was used to detect the captured EVs.

As expected, ITGA3-based capture showed exceptionally strong binding with fucose-binding lectins, particularly UEA lectin. UEA, a plant lectin isolated from *Ulex europaeus* seeds, has a strong binding affinity towards various fucosylated glycan structures, notably for Fucα1-2Galβ structure (Du et al., 1994). Noteworthy, it has been previously reported that fucose binding lectins such as AAL, LTA, and UEA have potential for the detection of PCa and BlCa (Haselhorst et al., 2001; Islam et al., 2021; Kekki et al., 2017; Li et al., 2015; Ohyama et al., 2004).

5.11 Characterization of biomarkers on urine derived EVs

Next, to find out where our biomarkers are localized, we separated the EV-enriched and protein-enriched fractions from urine of BPH, PCa, and BlCa patients through

the collaboration with Ghent University, Belgium. The EV-enriched and protein-enriched fractions were isolated from urine following the previously published articles (Dhondt et al., 2020a; Dhondt et al., 2020b).

Herein, we set out to evaluate to which extent the ITGA3-associated aberrant glycosylation is localized on EVs. To explore this, the EV-enriched and protein-enriched fractions from individual urine of BPH, PCa, and BlCa patients were passively coated on microtiter plates and then detected with NPs-coated with tetraspanin (CD9-NP &CD63-NP), integrin (ITGA3-NP), and lectin (UEA-NP). In BPH urine samples, the NPs-coated tetraspanin (CD63 &CD9), integrin (ITGA3), and lectin (UEA) assays showed 2-, 2-, 4-, and 66-fold higher S/B ratios in EV-enriched factions compared to protein-enriched fractions, respectively (Figure 18 A). However, in PCa urine samples, the tetraspanin (CD63 &CD9), integrin (ITGA3), and lectin (UEA) assays showed 46-, 3-, 3-, 139-fold higher S/B ratios in EV-enriched fractions compared to protein-enriched fractions, respectively (Figure 18 B). Furthermore, in BlCa urine samples, the tetraspanin (CD63 &CD9), integrin (ITGA3), and lectin (UEA) assays showed 9-, 4-, 3-, 48-fold higher S/B ratios in EV-isolates compared to protein-enriched isolates, respectively (Figure 18 C). Remarkably, the UEA-NP assay showed the highest S/B ratios in EVs enriched fractions compared to protein-enriched fractions from urine of BPH, PCa, and BlCa patients. This experiment demonstrated that the investigated biomarkers are highly enriched on EV-fractions as compared to protein-enriched fractions of urine.

As expected, EV-associated tetraspanin and integrin biomarkers were significantly elevated in the EV isolates compared to other protein enriched isolates ($p<0.05$). Interestingly, fucose-binding lectin UEA showed greater differences in EV fractions compared to protein enriched fractions. The UEA lectin is known to bind the carbohydrate structure Fucα1-2Galβ which is evidently enriched on EVs (Molin et al., 1986; Saito et al., 2018). Moreover, several studies claimed that fucose-binding lectins such as UEA, AAL, AOL, PSA, LTA can be used as a diagnostic marker in different cancers (Blonski et al., 2007; Dwek et al., 2010; Haselhorst et al., 2001; Kekki et al., 2017; Li et al., 2015; Ohyama et al., 2004). It was also predictable that biomarkers alone were unable to discriminate cancer cases from benign control. Thus, next we have introduced a sandwich assay combining integrins and lectins in clinical samples.

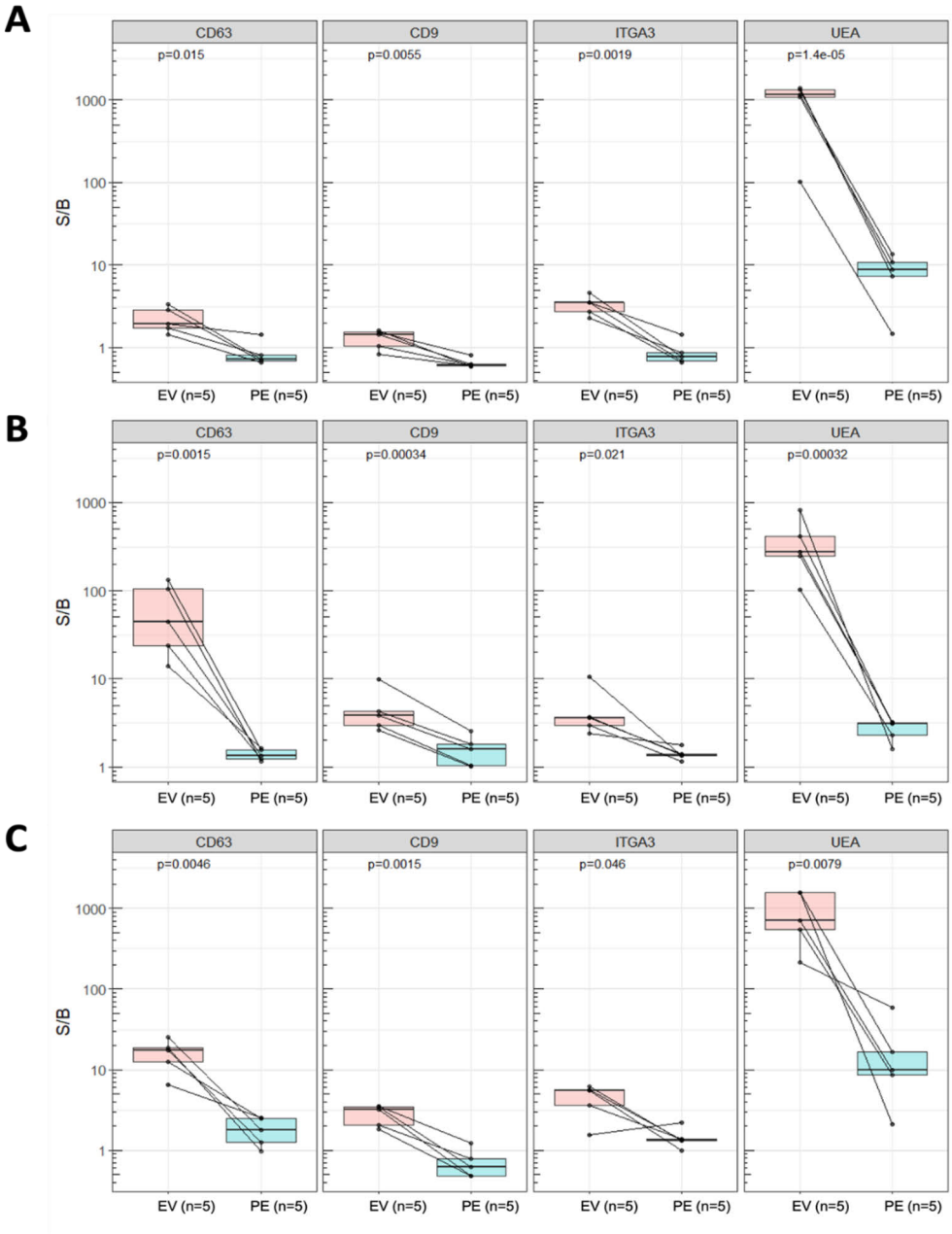

Figure 18. Enrichment of biomarkers: Biomarkers are localized on urine derived EVs. CD63, CD9, ITGA3, and UEA associated assays were characterized on EV- and protein-enriched fractions of A) BPH, B) PCa, and C) BlCa patients. The x-axis shows EVs and protein fraction of individual urine samples used while the y-axis shows the S/B ratios. Herein, EV=extracellular vesicles, PE= protein enriched fraction.

5.12 Specificity of ITGA3-ITGA3 and ITGA3-UEA assay

Next, we explored the specificity of our capture and tracer of the conventional ITGA3-ITGA3 and glycovariant ITGA3-UEA assays. The total ITGA3-ITGA3 and glycovariant ITGA3-UEA assays use anti-ITGA3 antibody as a capture which is immobilized on streptavidin coated 96-well plate for capturing EVs from pooled urine of benign (BPH), PCa and BlCa patients. Then the captured EVs were detected using either the same ITGA3 antibody or the lectin UEA coated on NPs. Following the MISEV2018 (minimal information for studies extracellular vesicles 2018) guideline, one negative EV marker and one tetraspanin marker were used to rule out non-specific and specific detection of EVs. The non-specific detection in the assay was evaluated using antibody-NPs against molecules that are not usually present on the surface of EVs (monoclonal antibody 5E4, specific for kallikrein 2). The signal obtained from negative marker was barely countable. Whereas, for the specific detection of EVs, a tetraspanin based CD9-CD9 assay was performed with urine samples. The signal from CD9-CD9 assay was used as a normalization factor for ITGA3-ITGA3 and ITGA3-UEA assay. After normalization, the total ITGA3-ITGA3 and glycovariant ITGA3-UEA assay showed nearly 3-flod higher signal/background (S/B) ratio in terms of EV expression in BlCa urine samples compared to benign and PCa samples (Figure 19).

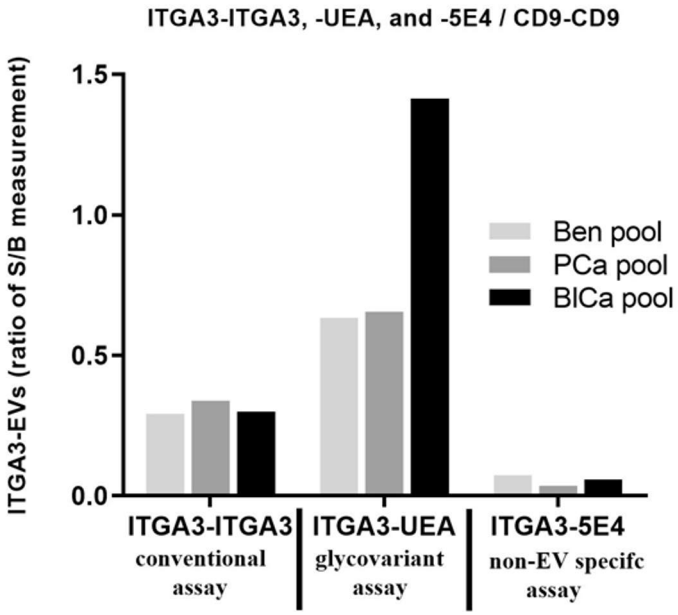

Figure 19: Capture and tracer specificity of conventional ITGA3-ITGA3 and glycovariant ITGA3-UEA assays in pooled urine of benign, BlCa and PCa patients.

5.13 Performance of ITGA3-ITGA3 and ITGA3-UEA assay

The next objective was the assessment of analytical performance of the ITGA3-UEA and ITGA3-ITGA3 assays. Considering potential translation of EV-based biomarkers to the clinics, an analytical standard is one of the key prerequisites for the development of assays that are robust and reproducible enough. Therefore, we here used well characterized EVs samples derived from DU145-EVs as a standard analyte. As shown in the Figure 20 A, the measured limit of detection (LoD) of total ITGA3-ITGA3 assay was 2.56 $x10^8$ EVs/mL, whereas the LoD of glycovariant ITGA3-UEA assay was 1.38 $x10^8$ EVs/mL (Figure 20 A). The sensitivity of glycovariant ITGA3-UEA assay for the detection of EVs was about 2-fold higher compared to total ITGA3-ITGA3 assay. Both ITGA3-ITGA3 and ITGA3-UEA assays showed excellent linearity (R^2=0.991).

5.14 Clinical evaluation of ITGA3-UEA assay

To address the clinical performance of ITGA3-UEA assay, we applied the assay for the detection of EVs from crude urine of four different groups including healthy individuals as well as patients with BPH, PCa and BlCa. The DU145-derived EVs were used as a calibration standard. The assay demonstrated statistically significant discrimination of BlCa patients from benign (BPH) controls (5.5-fold; p=0.004) (Figure 20 B**)**. Similarly, this assay revealed highly significant discrimination of BlCa patients from healthy sources (23-fold: p=0.0001). Remarkably, the ITGA3-UEA assay showed significant discrimination of BlCa compared to PCa patients (9.2-fold; p=0.00038). On the other hand, this assay showed no separation between BPH and PCa patients (p=0.6) (Figure 20 B).

From the preclinical study, we have observed that our investigated biomarker combination was most prominently found on PCa cell line-derived EVs. Unfortunately, this finding did not translate in the clinical study, as we did not see any discrimination between PCa patient urine samples and benign control samples. The reason why ITGA3-UEA assay cannot discriminate PCa patients from benign sources might be due to the prognostic value of ITGA3 resulting in signal elevation only in metastatic PCa rather than early-stage cancer. Previously, we also observed that ITGA3 expression is relatively higher in EVs derived from metastasis prostate cancer PC3 cell-line compared to early-stage LNCaP (Islam et al., 2019). Urine samples from PCa used in this study were mostly from early-stage cancer patients. Similarly, Bijnsdorp et al., have previously reported that ITGA3 is highly expressed on urinary EVs of metastatic PCa patients compared to early-stage PCa (Bijnsdorp et al., 2013). Another reason of getting less signal from PCa urine samples may be due to the collection of samples through catheterization which may decrease the

possibility of the prostate fluid leakage into the urethra, thereby, did not become a part of the urine samples.

This platform could be utilized for the detection of other cancers by using different combination of integrins and lectins. However, the number of samples used in this project is relatively limited so further assessment is required with a large cohort of samples for the validation of our findings.

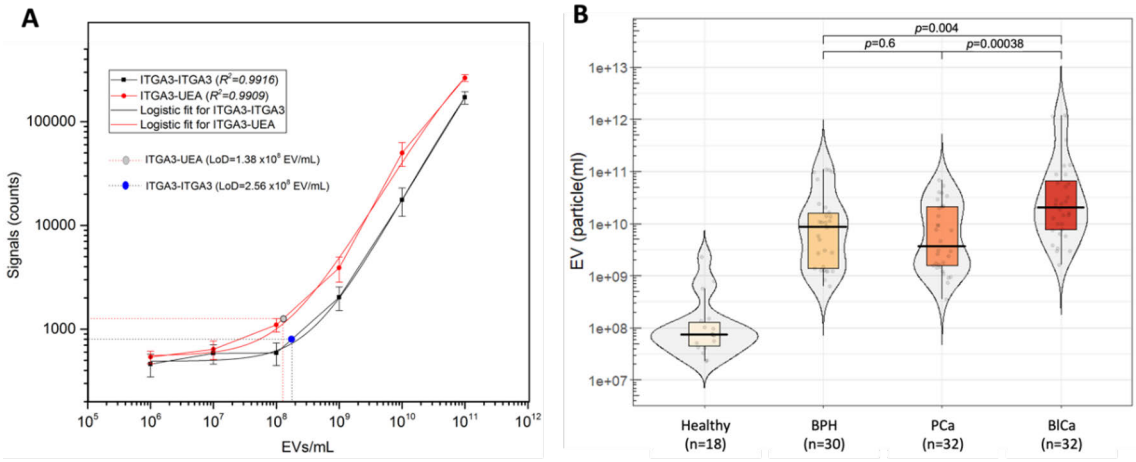

Figure 20: A) Standard curves of ITGA3-ITGA3 and glycovariant ITGA3-UEA assay for the detection of EVs-derived from DU145 cell line. B) The performance of ITGA3-UEA assay for the analysis of uEVs-derived from healthy, BPH, PCa, and BlCa patients. The ITGA3-UEA assay exhibited discrimination of BlCa from PCa, BPH, and healthy controls in term of signal counts, and *p* value.

6 Conclusion

This doctoral thesis was carried out for the development of a simple but highly sensitive assay platform to explore the potential of urine-EVs as biomarkers for prostate and bladder cancers. This assay was utilized for screening various combinations of capture and tracer including antibodies and lectins against tumor-associated proteins and glycans presented on the surface of EVs for biomarker discovery.

The main conclusions were drawn based on the original publications (I-III):

I. NP-based time-resolved fluorescence immunoassay was developed to detect EVs directly from unprocessed urine and cancer cell line supernatant without the need of any extensive preprocessing and isolation technique. Our lectin-NPs based assays exhibited 2-10-fold higher sensitivity over lectin-chelate based assay for the analysis of urinary EVs. Further, this approach opens the platform for the identification of disease-specific markers on the surface of EVs.

II. To find out the functional biomarker combinations, we constructed an assay combining tumor marker integrin and lectin for the discrimination of BlCa from non-cancer patients. A panel of lectin-NPs was screened to detect fucosylated glycan structure on integrin, ITGA3, from urine of BlCa patients. Among the lectins tested for the identification of BlCa-specific ITGA3 glycoisoform, lectin UEA showed potential value for the detection of BlCa from urine samples.

III. To explore to what extent our investigated biomarker combination is localized, several members of the integrin family and a rich lectin library were tested for the detection of EVs-derived from PCa and BlCa cell lines. After screening of integrin family and lectin library, we found that the ITGA3-UEA assay combination was abundant on EVs derived from cancer cells. We also applied this ITGA3-UEA assay for the detection of EVs from urine of PCa and BlCa patients. We found that the ITGA3-UEA assay could discriminate BlCa compared to benign control and PCa cases.

Our NPs-assisted immunoassay could be used as a simple tool for the analysis and characterization of urinary EVs for urological cancer detection. Till now, the analysis of EVs typically requires time-consuming and tedious isolation process. Our immuno-capture assay can detect EVs without the need for isolation and extensive preprocessing, which is a clear advantage over the existing isolation-based methods. Furthermore, for the first time, we have set an internal analytical standard with cancer cell derived EVs and compared it with clinical patients' samples. Additionally, the glycovariant assay ITGA3-UEA demonstrated better performance over assay targeting the mere ITGA3 protein.

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