Early detection of prostate cancer using extracellular vesicles and nanoparticle-aided time-resolved fluorescence immunoassay

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Prostate cancer (PCa) is a type of cancer that affects the prostate, an organ in the male reproductive system. It is mostly diagnosed and deadliest cancers among men. In 2020, approximately 1.4 million new cases were diagnosed, causing 370,000 deaths. Although there are available biomarker-based techniques for detecting PCa, for example Prostate Specific Antigen (PSA), but they have a set of drawbacks. Therefore, extracellular vesicles (EVs) could be an option to solve this problem. In this study, we investigated whether utilizing a high-throughput method (FastEV^{TM}) for enriching EVs from clinical serum samples, in combination with nanoparticle-aided time-resolved fluorescence immunoassay (TRFIA) could enhance the accessibility of biomarkers for the early detection of PCa.

EVs- and soluble protein (SP)-fractions were separated from both PCa and benign prostate hyperplasia (BPH) patients using the FastEV technology. Biotinylated capture antibody was immobilized on streptavidin coated microtiter wells for capturing EV and SP-fractions of PCa. Then captured analyte was detected using glycan- binding lectin coated on nanoparticles (NPs).

We have observed that assay consisting with cancer antigen (Ca15.3) and (Ca19.9) in combination with WGA lectin, such as Ca15.3^{WGA} and Ca19.9^{WGA} assays were able to significantly separate the PCa patients from the BPH sources (p-value = 0.007 and 0.00001), respectively.

In this study we have demonstrated that high-throughput method (FastEV^{TM}) along with simple nanoparticle-aided time-resolved fluorescence immunoassay (TRFIA) could be used to detect PCa patients from clinically challenged BPH conditions.

Keywords: prostate cancer, prostate specific antigen, extracellular vesicles, immunoassay, microtiter, capture, lectin.

Contents

Abbreviations

ASR- age standardized rate

BITC- biotin-isothiocyanate

BPH- benign prostatic hyperplasia

BSA-bovine serum albumin

DRE-digital rectal examination

EDC- n-(3-dimethylaminopropyl)-n'-ethylcarbodiimide

EVs- extracellular vesicles

ILVs- intraluminal vesicles

lncRNAs- long non-coding RNAs

mAb- monoclonal antibody

miRNA- micro RNA

mpMRI- multiparametric magnetic resonance imaging

MVB- multivesicular body

MWCO- molecular weight cut off

NPs- nanoparticles

PCa- prostate cancer

PSA- prostate specific antigen

RALP- robotic-assisted laparoscopic prostatectomy

SEC- size-exclusion chromatography

SP- soluble protein

TPCC- turku prostate cancer consortium

TRFIA- time-resolved fluorescence immunoassay

tRNA- transfer RNA

TRUS- transrectal ultrasonography

TURP- transurethral resection of prostate

UC- ultracentrifugation

WGA-wheat germ agglutinin

1.0 Introduction

1.1 Prostate Cancer:

Prostate cancer (PCa) is characterized by the uncontrolled growth of cells in the prostate gland, leading to an abnormal accumulation of cells. Prostate is a gland in the male reproductive system that is located under the bladder and in front of the rectum. The prostate is approximately the size of a walnut and it surrounds the urethra. Prostate cancer is slow growing but in advanced conditions it can metastasize swiftly to the lymph nodes and in severe cases, it can spread to the bones and the central nervous system. Prostate cancer is the most common cancer in men all over the world. The European Randomized Study of Screening for Prostate Cancer (ERSPC) demonstrated that early detection of PCa can reduce PCa mortality (Schröder et al., 2009). Several methods are available for the detection of PCa but they have their own limitations. Thereby, there is an urgent need to develop a simple, sensitive, specific and non-invasive technique to detect PCa in its early stages.

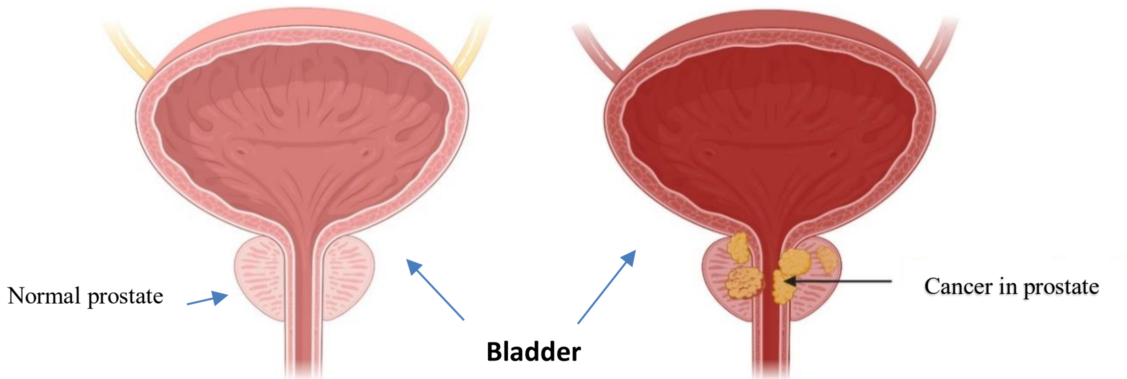

Figure 1: Normal vs cancerous prostate. Prostate is located just under the bladder. (*Picture is created in Biorender 2023*).

1.2 Epidemiology of prostate cancer:

According to the statistics of the World Health Organization (WHO), cancer is one of the deadliest diseases, accounting for nearly one-sixth of global deaths, with approximately 10 million deaths in 2020 alone. Among the various types of cancers, breast, lung, colon and prostate cancer (PCa) are the most prevalent types. Among them, PCa is the most commonly diagnosed cancer in men and is responsible for the significant number of cancer-related deaths. In 2020, approximately 1.41 million men were diagnosed with PCa, leading to 375,304 deaths worldwide (Wang et al., 2022). According to the estimation from the International Agency for Research on Cancer (IARC), PCa is projected to cause 740,000 deaths by the year 2040.

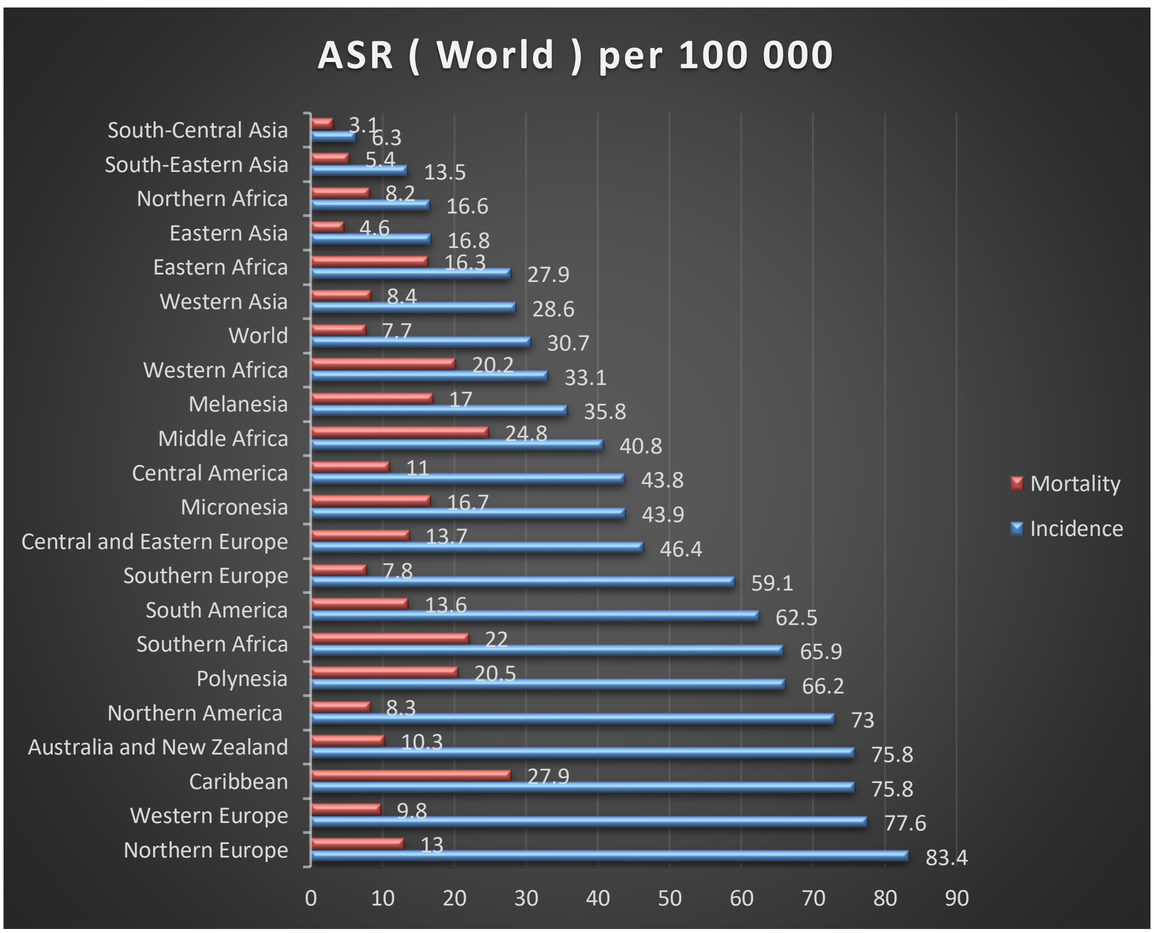

Figure 2: Region specific incidence and mortality rates by age group for PCa in 2020. The statistics shows that PCa is mostly common in European area followed by Australia and New Zealand. But the death rate is higher in Caribbean zone. ASR= Age Standardized Rate**.** *Graph is drawn in Microsoft Excel 2016. Concept is taken from Sung et al., 2020.*

However, the mortality rate was highest in the Caribbean region compared to others. In contrast, the overall incidence and mortality rates were lower in the Asian region during the same year. The Finnish Cancer Registry presented a somber picture in 2020 with 928 men losing their lives to PCa, while 5035 men faced the daunting diagnosis of this lethal cancer (Sung et al., 2020).

1.3 Risk factors associated with PCa:

A number of factors have identified as the risk factors for PCa, including advanced age, ethnicity (especially Black race individuals), gene mutations, growth factors like insulin growth factor (IGF) and family history of this malignancy.

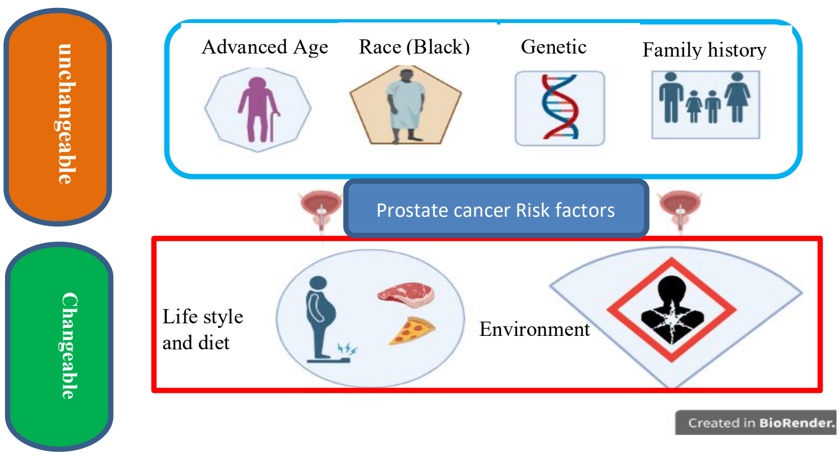

Figure 3: Prostate Cancer Risk factors: Changeable and unchangeable. Some risk factors cannot be controlled by men like age, race, family and genes associate with it. But there are some risk factors that depend on life style and diet. Physical inactivity, red meat and high fatty food can lead to obesity. Exposer to occupational and environmental carcinogens can also cause PCa. *Picture created in Biorender 2023.*

In addition, certain lifestyle choices such as dietary patterns, tobacco and alcohol use, obesity, physical inactivity and exposure to environmental elements like chemicals or ionizing radiation have been associated with an increased risk of developing advanced PCa (Berenguer et al., 2023).

Some risk factors and their roles in PCa given in Table 1. (Berenguer et al., 2023)

Table 1: Some risk factors associated with PCa

Risk factors	Role in PCa
Ethnicity	African and Caribbean men have a higher tendency of progression in PCa compared to other populations and the studies have identified that the risk associated loci is found in chromosome 8q24.
Family history and gene	Around 5-15% PCa cases have a suspected hereditary component. Research has shown that 170 loci is believed to be susceptible to PCa progression.
Obesity and physical inactivity	Obesity is responsible for imbalances in hormonal pathways that can result in higher levels of insulin and IGF, testosterone, inflammatory cytokines, and lower levels of adiponectin, and sex hormone-binding globulin.
Tobacco Use	Tobacco use escalates the likelihood of developing PCa and can cause some biochemical changes that may lead to metastasis.
Calcium, Vitamin D and dairy products	Daily intake of dairy products above the recommended dose can pose a risk to develop PCa while the intake of Vitamin D has been associated to reduce the risk of PCa.
Cruciferous vegetables, soy, and green tea	Cruciferous vegetables, soy, and green tea can reduce the risk of progressing PCa and hence it is thought that Asian men, who consume these foods frequently, exhibit lower rates of PCa.

1.4 Current methods for the detection of PCa:

Several methods are available for the detection of PCa such as Prostate Specific Antigen (PSA), Digital Rectal Examination (DRE), Multiparametric Magnetic Resonance Imaging (mpMRI) and Transrectal Ultrasonography (TRUS) (Descotes 2019, and Penzkofer and Tempany-Afdhal 2013). However, all of these methods have their own limitations. Benign prostatic hyperplasia (BPH) and inflammation are the two non-cancerous diseases of the prostate gland. PSA testing, for example, can result in false positive that can cause over diagnosis of PCa. Conversely, it may also result in false negatives, failing to detect PCa, leading to missed diagnoses (Chou et al., 2011). DRE has limited sensitivity and specificity, and its accuracy depends on the experience and skill of the examiner. DRE may also cause anxiety and discomfort in patients and may be less reliable in detecting early-stage prostate cancer (Egawa et al., 1994). The mpMRI may have variable accuracy depending on the experience and expertise of the radiologist in interpreting the images. mpMRI can result in false positives, leading to unnecessary biopsies or overdiagnosis of clinically insignificant prostate cancer (Sosnowski et al., 2016).

One drawback of TRUS-guided prostate biopsy is that there is no reliable visual guide to ensure the even distribution of biopsy cores. Up to 72% of hospitalizations due to complications from prostate cancer can be attributed to the significant infection risk associated with TRUS (estimated to be as high as 7%) (Panzone et al., 2022).

Therefore, developing a non-invasive, specific and sensitive method to improve the detection of prostate cancer is still a critical research goal. In this perspective, extracellular vesicles (EVs) have emerged as a promising candidate and a potential future tool for PCa diagnosis.

1.5 Extracellular Vesicles:

EVs are 40 nm to 1000 nm sized lipid-bound particles secreted by various cell types. Cells utilize extracellular vesicles as channels for intercellular communication. Moreover, EVs contain a diverse cargo, such as mRNA, miRNA, circRNA, proteins, metabolites and DNA.

EVs can promote cancer development by the cargo they carry. One interesting characteristics of EVs is their ability to mimic the cell surface. The biomolecules present on the surface of cells, such as proteins, lipids, and carbohydrates, can also be found on the surface of EVs.

For example, glycans which are carbohydrate moieties, are present on both the cell surface as well as on the EV surface. In terms of cancer, altered glycosylation patters are observed on the cancer cell surface and the same alterations are also known to be present on the EV surface (Islam et al., 2019; Tao and Guo 2020; Zhang et al., 2021). Thus, the detection of these altered glycans on the surface of EVs could be a useful tool for the detection of prostate cancer.

1.6 Types of Extracellular Vesicles:

EVs are classified into three main types based on their size and the route of origin: exosomes, microvesicles and apoptotic bodies.

Exosomes are the smallest type of EVs with a size range of 40-150 nm. Exosomes are surrounded by a single outer membrane and are released by almost all types of cells. They originate from the endosomal system by the formation of multivesicular bodies (MVBs). Exosomes are released from the cells when the MVBs fuse with the plasma membrane. These particles can be found in all body fluids like urine, saliva, bronchial fluid, breast milk, serum, semen, tears, plasma, amniotic fluid, lymph, synovial fluid, bile, cerebral spinal fluid (CSF) and gastric acid. Exosomes also known as Intraluminal Vesicles (ILVs). (Doyle and Wang 2019).

Microvesicles are larger than exosomes with a size ranging from 100-1000nm and they originate from budding and fission at special “lipid raft” domains of the plasma membrane (Cocuuci and Meldolesi 2015). These are also referred to as ectosomes, shedding vesicles or microparticles. (Nederveen et al., 2021; Ramirez-Garrastacho et al., 2021.)

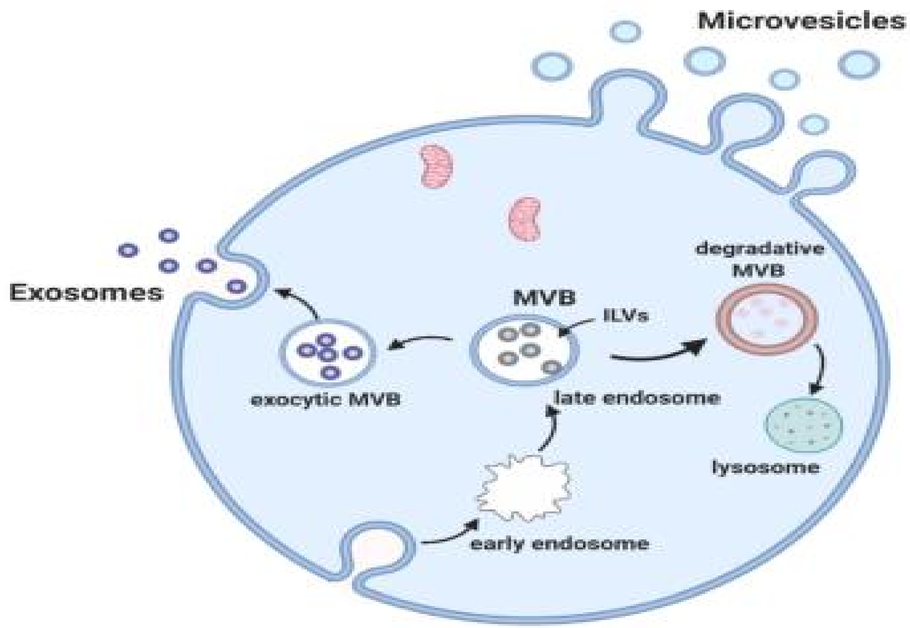

Figure 4: A diagram illustrating the release of EVs is presented. Proteins within early endosomes are enclosed within intraluminal vesicles (ILVs) of larger multivesicular bodies (MVBs). ILVs of MVBs are formed when they bud inward into the endosome's lumen. During biogenesis, MVBs can follow two pathways: degradative, where they are directed towards lysosomes, or exocytic, where they fuse with the plasma membrane, releasing their contents known as exosomes. Microvesicles are vesicles that form directly through fusion of the plasma membrane. *Picture is taken from the PhD booklet of Islam (2022) upon permission.*

Apoptotic bodies are the third category of EVs and sizes range from 1000-5000 nm. Apoptotic bodies are mainly composed of nucleus, cytosol and endoplasmic reticulum. (Cline et al., 2004; Dieker and Muller, 2009). Apoptotic bodies are formed when the cell undergoes programmed cell death. (Nederveen et al., 2021; Ramirez-Garrastacho et al., 2021) (Figure-4).

1.7 Functions of EVs:

The EVs were initially regarded as cellular waste disposed into the extracellular matrix. However, over the past decade, our understanding of EVs has significantly evolved. It is now clear that EVs play an important role in cell-to-cell communication. EVs function as cargo ships in the body fluids, carrying DNA, RNA, proteins or drugs and delivering the generated signals or biological molecules to the corresponding recipient cells (Islam et al., 2019 and Zhang et al., 2021).

Over the last few decades, researchers have identified miRNA and mRNA as significant component of EVs. The advancement of techniques for the detection of EVs has enabled the observation of a wide array of RNA species, such as transfer RNA (tRNA), long non-coding RNAs (lncRNAs) and viral RNAs. Emerging evidence indicates that various types of RNA, including lncRNAs, plays a pivotal role in influencing the growth and progression of cancer cells (Zhang et al., 2021).

The functional impact of EVs on tumorigenesis is evident as breast, pancreatic, and colon cell lines secrete abundant EVs that carry proteins like MHC I, MHC II, FasL, TRAIL, NKG2D ligand and TGF- β (Simona et al., 2013; Mincheva-Nilsson and Baranov, 2014; Zhang et al., 2015).

The presence of FasL in EVs released by tumor cells has been found to impact the apoptotic process of $CD8^{+}$ T cells, resulting in immune suppression (Wieckowski et al., 2009). EVs originating from tumor cells have demonstrated their ability to create an immunosuppressive environment within the tumor microenvironment through the transmission of TGF- β, modulation of tumor-associated fibroblasts, and promotion of angiogenesis (Webber et al., 2010).

The contents of EVs remain intact and protected from outer proteases and RNases. This is facilitated due to their encapsulation within membranes and close interaction with RNA or DNA binding proteins or lipoprotein complexes. Additionally, EVs exhibit stability even in challenging circumstances like extreme pH level, multiple freeze thaw cycles and prolonged storage, which enhances their potential as possible biomarker candidates (Nawaz et al., 2014).

1.8 Isolation of Extracellular Vesicles:

Several techniques are available for the isolation of EVs from biological fluids and cell culture supernatants. However, a single method alone is ineffective for the separation of EVs. During isolation of EVs, several considerations have to be taken into account such as, EVs types, sample number, volume of sample and sources, percentage of EVs purity, downstream analysis application, cost and processing time (Witwer et al., 2013). The following methods can be employed for the isolation of EVs:

- Ultracentrifugation
- Ultrafiltration
- Size exclusion chromatography
- Kit Based precipitation
- FastEV Technology

1.8.1 Ultracentrifugation:

Ultracentrifugation (UC) is the most common method used for the isolation of EVs. Approximately half of the researchers use this technique to isolate EVs from cell culture supernatants and biological fluids (Zarovni et al., 2015). The underlying concept of this approach involves utilizing centrifugal force to separate the constituents of the cellular compartments, based on their size, density and shape (Li et al., 2017). A centrifugal force exceeding 1000,000 x g is generally used during the process (Théry et al., 2006; Fernández-Llama et al., 2010). For the isolation of EVs from urine, a centrifugal force of 200,000 x g is generally used for 2.5 hours whereas EVs isolation from cell culture media needs 100,000 x g for 1.5 minutes (Zhou et al., 2006; Chen et al., 2013; Hogan et al., 2014). For isolating exosomes, the sample is first purified to remove contaminants and then mixed with protease inhibitors to preserve the integrity of proteins. Through repeated centrifugations, the collected supernatant or pellet is re-suspended in PBS and subjected to further centrifugation steps until the final exosomal fraction is obtained. This fraction can then be used for subsequent analysis (Li et al., 2017). Unfortunately, this method of EV isolation has several drawbacks. It is expensive, time consuming and this method is not suitable for handling a large amount of samples. (Hogan et al., 2014, Van et al., 2014: Wang and Sun 2014).

1.8.2 Size exclusion chromatography:

Size-exclusion chromatography (SEC) is another method employed for the isolation of EVs. One of the benefits of using SEC is its ability to separate lipoproteins and proteins from EVs. This technique yields isolation results comparable to ultracentrifugation from cell culture supernatant, as demonstrated in studies conducted by Lobb et al., 2015; Nordin et al., 2015. However, the initial concentration step and collection of EV fraction can be laborious and troublesome. Consequently, this isolation method is not extensively considered by researchers.

1.8.3 Ultrafiltration:

Ultrafiltration is a widely used technique for the isolation of EVs. The utilization of commercially available centrifugal devices with molecular cut-offs (MWCO) ranging from 3K Da to 100K Da has been observed in various studies. The ultrafiltration method offers several advantages, including a shorter isolation period, cost-effectiveness, user-friendly operation, and suitability for handling large sample volumes (Cheruvanky et al., 2007). However, certain limitations are associated with ultrafiltration, such as EV aggregation in pressure-driven cells, loss of smaller EVs, and decreased sample quality (Lobb et al., 2015; Cheruvanky et al., 2007; Gerlach et al., 2013; Rood et al., 2010; Merchant et al., 2010).

1.8.4 Kit-based Precipitation:

In recent times, various techniques have been developed for the isolation of EVs, each with its own advantages and disadvantages. Some commercially available kits, such as the miRUCRY^{TM} exosome isolation kits, ExoQuick and Invitrogen Total Exosome isolation reagent, rely on the precipitation of membrane particles. However, one significant drawback of this method is its inapplicability when dealing with a substantial quantity of samples (Karttunen J 2019).

1.8.5 FastEV Technology:

FastEV is a novel, high throughput technology that has been developed as a platform for biomarker discovery. FastEV technology begins by subjecting test samples e.g. pooled plasmas from cases vs controls, to isolate with an array of FastEV conditions. The obtained fractions are then analyzed using array technologies. FastEV enables efficient identification of the optimal condition for separating cases from controls.

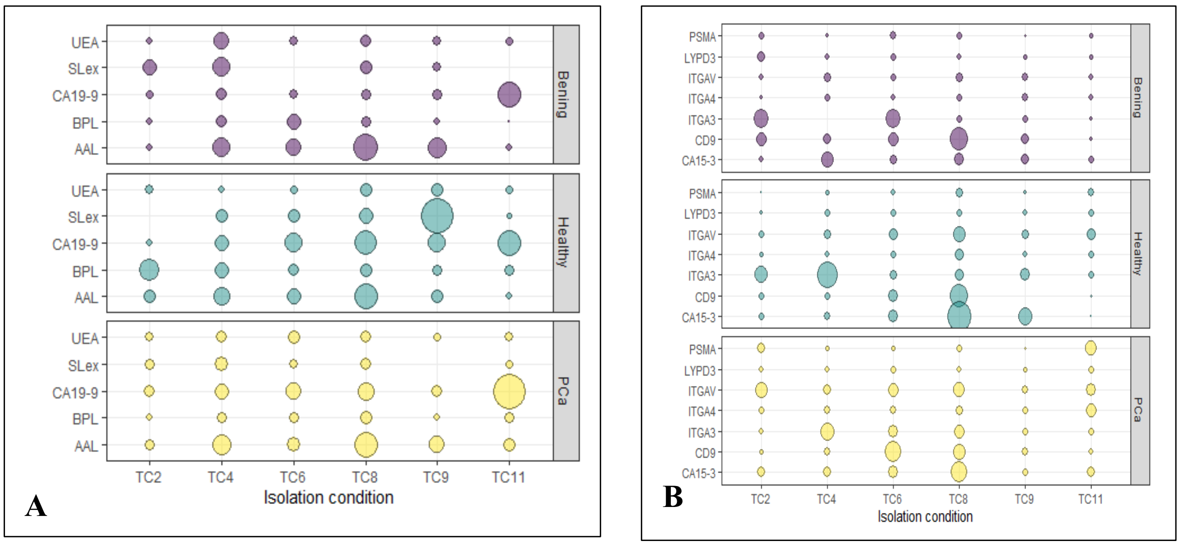

Figure 5: The bubble chart in Figure 5-A and B shows how many folds the biomarker is enriched to the EV fraction vs SP fraction. Pooled benign, healthy and PCa sample are treated in different chemical conditions by the different antibody and anti-antibody or lectins to discover respective biomarkers. *Picture is made in Biorender 2022.*

The selected optimal FastEV conditions can easily be applied to a large number of samples, facilitating significant breakthroughs in biomarker discovery research. This technology greatly cuts down the cost, time, and resources required to find the optimal method for each sample type.

This promising technique developed by Pia Siljander and Maija Puhka from the EV core group at the University of Helsinki. In our research project, this novel technique is used to isolate the EVs from clinical serum samples.

1.9 Glycosylation:

Glycosylation is a post-translational modification that involves the attachment of carbohydrate moieties, known as glycans, on the proteins and lipids present on the cell surface. The cell surface is composed of a diverse array of proteins and lipids. Glycosylation occurs in almost all human proteins and approximately half of the proteins in eukaryotes. This modification thus enhances the functional activity of proteins and lipids for example, protein folding, cell adhesion, immune response, trafficking. This process is mediated by two main types of enzymes: glucosidases and glycosyltransferases (Reyes et al., 2021; Marth and Grewal, 2008; Reily et al., 2019)

Glycosylation can be categorized into two main types: N-linked glycosylation and O-linked glycosylation, which are distinguished by the specific attachment site of the glycan to a protein. N linked glycosylation occurs when glycans are linked to the amide group of asparagine side chains, whereas O-linked glycosylation involves the attachment of glycans to the hydroxyl groups of serine and threonine side chains (Syed et al., 2015).

N-linked glycosylation can be classified into the following categories:

- Complex oligosaccharides, which comprise multiple sugar types such as fucose, galactose and N-acetylneuraminic acid
- High-mannose oligosaccharides, that contain mannose and GlcNAc residues
- Hybrid branch, that exhibit a combination of both complex oligosaccharides and high- mannose structures.

Among the various types of O-linked glycosylation, O-GlcNAc is a prominent type that plays an important role in the biosynthesis of mucins. In cancer cells, O-glycosylation is commonly observed and these alterations hold promise as a potential tool for the diagnosis of cancer (Steen et al., 1998; Varki, 2017).

1.10 Glycosylation in cancer and EVs-A promising candidate in cancer diagnostics:

A study conducted by Pinoh and Reis, 2015 demonstrated that glycosylation is prevalent in cancer. Several factors are involved in stimulating altered glycosylation, such as evading the immune system, attaching to the endothelium and creating new adhesions for metastasis (Stowell et al., 2015 and Varki, 2017)

Sialylation, a specific type of glycosylation, plays a vital role in cellular recognition, cell adhesion, and cell signaling. Sialylated carbohydrates contribute significantly to these processes. The development of cancer has been strongly linked to the dysregulation in glycosyltransferase expression, leading to a notable increase in global sialylation, particularly in α2,6-and α2,3-linked sialylation (Pinoh and Reis, 2015).

One of the prominent features of EVs is that they reflect the glycosylation patterns of their cell of origin. As N-glycans and O-glycans are present on the cell surface, similar glycosylation patterns are also prevalent in EVs (Batista et al., 2011; Stowell et al., 2015). Moreover, many cancers associated altered glycans are commonly found in EVs. Therefore, the identification of these altered glycans in EVs has the potential to serve as diagnostic tool for the identification cancer. For example, O-GlcNAc glycosylation is present on the proteins found on EVs, thus, GlcNAc identification could be a potential biomarker for cancer diagnostics (Badr et al., 2014; Chaiyawat et al., 2016).

1.11 Lectins:

Previous discussions have highlighted the presence of altered glycans in both cancer cells and EVs. Therefore, the detection of these altered glycans hold great potential as a marker for cancer detection. Studies have demonstrated that various types of glycan-binding proteins known as lectins are present in nature. Lectins have the ability to bind to specific glycans. Thus, the interaction between lectin and glycan can act as a lock and key model, illustrated in Figure 5. (Varki A et al., 2015; Krautter F and Iqbal AJ 2018).

Lectins are a diverse group of proteins found in animals, plants, viruses and bacteria (Loris 2002). One widely studied lectin is Wheat Germ Agglutinin (WGA), which is commonly used as a tool for studying glycans. Studies have shown that WGA has a binding affinity with GlcNAc glycans (Wu et al., 1998). However, a challenge in lectin-glycan interactions is that the binding affinity is generally weaker compared to antigen-antibody interactions, as summarized in a review by Syed et al., 2016.

To address this problem, researchers have explored the use of nanoparticles (NP) coated with lectins, such as Europium-doped NPs. These NPs are embedded with approximately 30,000 chelates, allowing for multivalent interaction between lectins and glycans (Islam et al., 2019; Harma et al., 2001). This interaction provides signal amplification which can be measured by using a specialized method called NPs-aided time resolved fluorescence immunoassay (NP-TRFIA) (Gidwani et al., 2016).

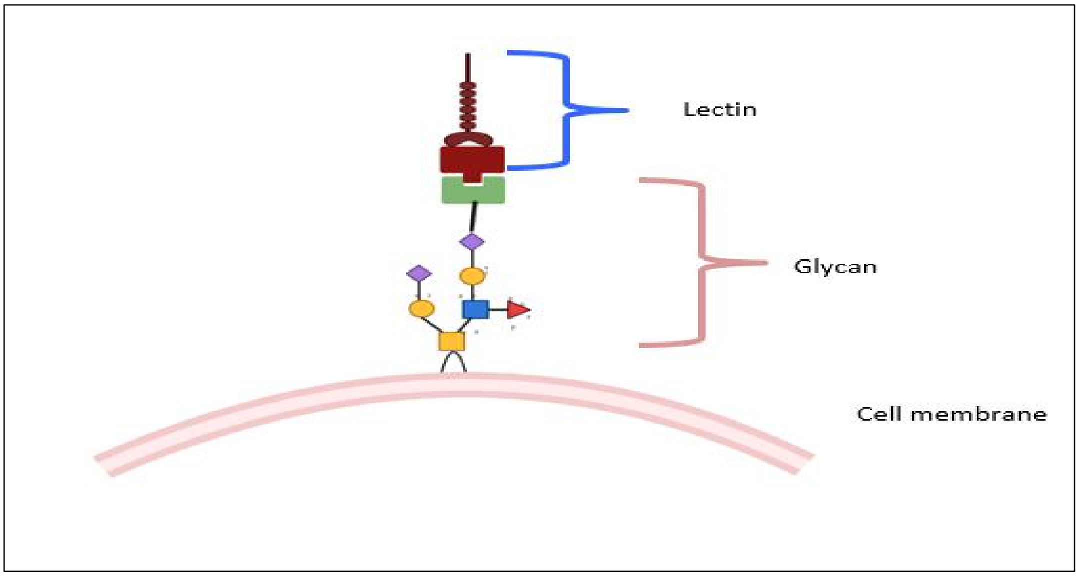

Figure 6: The interaction between lectin and glycan on cell surface that acts as a lock and key model. Cell surface has different types of proteins. By the process glycosylation, these proteins are attached with glycan. Glycan has affinity with lectin that acts as a lock and key model. *Illustration is made by Biorender 2023*.

1.12 TRFIA (Time-resolved fluorescence immunoassay):

In 1959, two scientists Rosalyn Yalow and Solomon Berson introduced the concept of immunoassay, marking a significant milestone in the field of medical diagnostics. Since then, the immunoassays have been modified and improved. One such advancement is Time-Resolved Fluorescence Immunoassay (TRFIA), which utilizes time-resolved fluorescence to measure the close proximity between antibodies and antigens (Hagan and Zuchner, 2011; Sy et al., 2016).

TRFIA offers several advantages over the conventional immunoassays. Firstly, it provides a larger detection range, allowing the measurement of a broad spectrum of analytes. It is less time consuming. Another advantage is its reduced susceptibility to sample interference. It also exhibits high sensitivity, enabling the detection of low analyte concentrations.

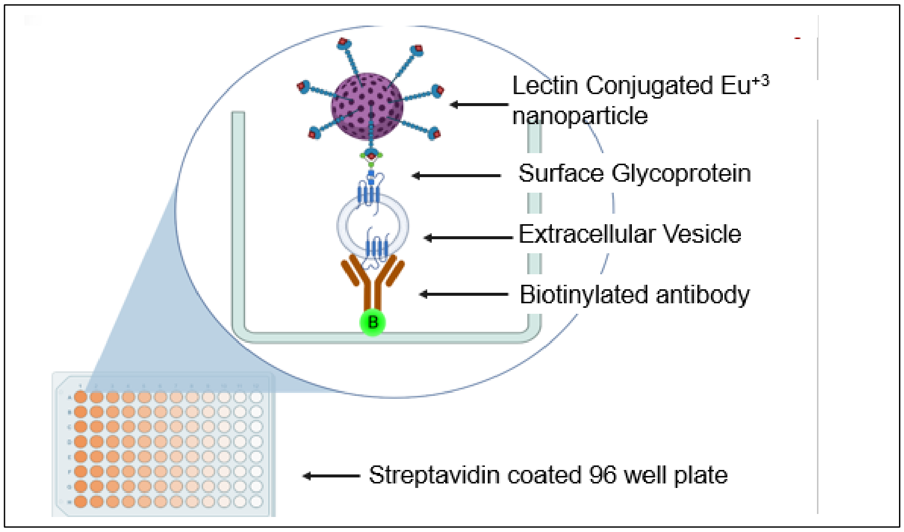

Figure 7: A schematic representation of mechanisms of TRF assay. A biotinylated antibody is immobilized in the streptavidin-coated plate followed by the recognition of antigen (in this case-EVs). Glycan on EVs are detected by lectin coated on NPs**.** *Figure drawn in Biorender 2023.*

At the biotechnology division of the University of Turku, Finland, some scientists used lanthanide ions and their chelating agents as tracers in TRFIA. They demonstrated that lanthanide ions (Eu^{3+}, Tb^{3+}, Dy^{3+}, Sm^{3+}) possessed higher capability to detect analytes with increased specificity.

Lanthanide nanoparticles have diverse applications in bioanalysis and biomedicine, including bio-labeling, bio-detection, cancer therapy, fluorescence imaging, and drug delivery. The unique properties, such as long fluorescence lifetimes and resistance to self-quenching, make them superior to traditional labels. These nanoparticles are produced by encapsulating thousands of lanthanides chelates in a protective shell. In this study, commercially available polystyrene nanoparticles doped with europium (Eu^{3+}) were employed for the TRFIA technique.

2.0 Aim

The aim of our research was to investigate the detection of glycosylation alterations in EVs derived from the serum of patients with prostate cancer (PCa) using a cutting-edge technique called NP-TRFIA in combination with a high-throughput EV isolation approach called FastEV. By utilizing different combinations of lectins and glycans, we were able to differentiate between prostate cancer and benign conditions.

3.0 Materials and Methods

This study was based on the nanoparticles-assisted technique, utilizing biotinylated anti-antibody to capture the epitope on the surface of EVs that enabled highly sensitive detection of PCa applying sandwich immunoassay. In immunoassay set-up, a monoclonal antibody was used as a capture and lectin was used as a tracer. In this project, different assay combinations were evaluated in large cohort of PCa clinical samples collected from Turku Prostate Cancer Consortium (TPCC) at Turku University Hospital and ethical approval was taken from the University of Turku ethics committee (ClinicalTrial.org: NCT01864135). Prior to their participation in the study, informed consent was obtained from all individual participations, ensuring their voluntary agreement to be involved. All methods employed in the study were conducted following local ethical guidelines and regulations governing research practices.

3.1 Samples:

The synopsis of sample cohort used in this study is described in the following tables (table 1). All the samples were collected following the specific guidelines, rules and regulations following the Helsinki declaration. All samples were centrifuged at 2000 x g for five minutes to remove all cell debris or cell associated waste products. Samples were usually stored at -80 ^{0}C and the immunoassay was performed in the room temperature.

3.2 Reagents:

3.2.1 Antibodies:

In this study, mainly two antibodies Ca19-9 and Ca15-3 were used which specifically binds with the epitopes presented on EVs. These two antibodies were either biotinylated or conjugated on nanoparticles to detect the EVs. Some related information is given in table 3. Regarding these two antibodies.

Table 2: Clinical information of the samples used in this study. Gleason Score (GS), T-category, n (%), Nodal stage, n (%)

Patient characteristics	Prostate cancer (n=87)	Benign Prostatic Hyperplasia (BPH) (n=53)
	Age (years) Medium	
	66 (44.5-75.5)	70 (52-76)
	Gleason Score (GS)	
GS 6	n=39 (45%)	
GS 7	n=21 (24%)	
GS 8	n=6 (7%)	n=1
GS 9	n=20 (23%)	n=1
	Pre-PSA	
	8.6 (2.3-6.2)	4 (0.2-770)
	Prostate Size (Cm3)	
	35(11.6-144)	NA
	T-category, n (%)	
Tx	1 (1%)	1 (2%)
T1c	40 (46%)	
T2	33 (38%)	
T3	13 (15%)	1 (2%)
	Nodal stage, n (%)	
Nx	26 (30%)	
N0	55 (63%)	1 (2%)
N1	4 (5%)	
NA	2 (2%)	
		Tumor Grade, n (%)

MX	27 (31	
M0	57 (66%)	1 (2%)
M1a	1 (1%)	
NA	2 (2%)	

Table 3: Name along with its specificity, clone and manufacturer of the antibodies that we used in our study.

Antibody name	Specificity	Clone	Manufacturer
Ca19-9 Antibody	Ca19-9 antigen	C192	Fujirebio
Ca15-3 Antibody	Ca15-3 antigen	Ma552	Fujirebio

3.2.2 Lectins:

This project was mainly based on a plant lectin named WGA. WGA stands for Wheat germ agglutinin. To know the nature of glycan-present on EVs surface- the lectin WGA was coated on nanoparticles to increase the floor of interaction between glycoprotein and WGA. This lectin has specific binding site on glycan namely N-Acetylglucosamine.

3.3 Nanoparticles coatings with antibody and lectin:

To create the reporter molecules, we utilized Fluoro-Max Carboxylate-modified Eu^{3+}-doped nanoparticles (Seradyn Indianapolis IN, USA). These nanoparticles were conjugated with anti-Ca19-9, anti-Ca15-3 antibodies and WGA by the method described by Islam et al., 2019. The conjugation process involved covalently linking the antibodies to the carboxyl groups present on the nanoparticle surface. First, the nanoparticle surface was activated using 0.75 mmol/L EDC (N-(3-dimethylaminopropyl)-N`-ethylcarbodiimine; Sigma Aldrich) to include crosslinking, followed by the stabilization with 10mmol/L sulfo-NHS (N-hydeoxysulfosuccinimide; Sigma Aldrich). The activation of the nanoparticle surface took place in a phosphate buffer (10mM, pH 8), and the subsequent coupling of antibodies to the activated nanoparticles was achieved by shaking the mixture for 2 hours at room temperature.

To remove any unbound antibodies, the bio-conjugated mixture of antibodies and nanoparticles were washed with a tris-based buffer (0.05% NaN_3, 10mM tris pH 7.8). To block any remaining active sites on the nanoparticles, the mixture was then stored at +4°C overnight in the presence of 2g/L BSA buffer. Following this, the mixture of nanoparticles was allowed to settle for two days at +4°C to eliminate any aggregates through centrifugation (350 x g and 5 minutes). The concentration of NPs was measured with standard known concentration and stored at +4°C. The calculations were performed with 1420 Victor™ Multilabel Counter (Perkin Elmer, Finland).

3.4 Biotinylation of antibody:

Mainly two antibodies (Ca19-9 and Ca15-3) were biotinylated in this study. To begin, a solution of biotin isothiocyanate (BITC) in ethanol was prepared at a concentration of 10mM. The pH of the antibody solution was adjusted to 9.8 using a carbonate buffer with a concentration of 50mM. Next, a 40-fold excess of biotin compared to the monoclonal antibody (mAb) was added to achieve a final reaction volume of 500µl, aiming for a mAb concentration of 2mg/ml. the reaction mixture was incubated at room temperature (RT) for 4hours. Gel filtration was employed to eliminate any unreacted BITC using NAP-5 and NAP-10 columns (GE- healthcare, Belgium), and a solution containing 50 mmol/L Tris-HCL (pH 7.5), 150 mmol/L NaCl, and 0.5 g/L NaN_3. Finally, the biotinylated antibodies were stored at +4°C in the presence of 1g/L BSA for preservation.

3.5 Isolation of Extracellular Vesicles:

In this experiment, we utilized a novel platform called FastEV for high-throughput EVs isolation. To isolate EVs, we sent our PCa serum sample to the research group named "Extracellular Vesicles Core" in the University of Helsinki, Finland. As this is going to be established in the market so, this technique is now kind of a trade secrets that's why we were known a little about this technique. But in short, PCa (n=87) and benign (n=53) serum samples were treated with different chemical conditions to yield Extracellular vesicles (EVs)- and other soluble proteins (SP)-fractions. Thesis EVs and SP-fractions were studied with several biomarkers in 96 microtiter well plate for one hour using direct assay. And then the best biomarker combination was selected from the biomarker characterization assay.

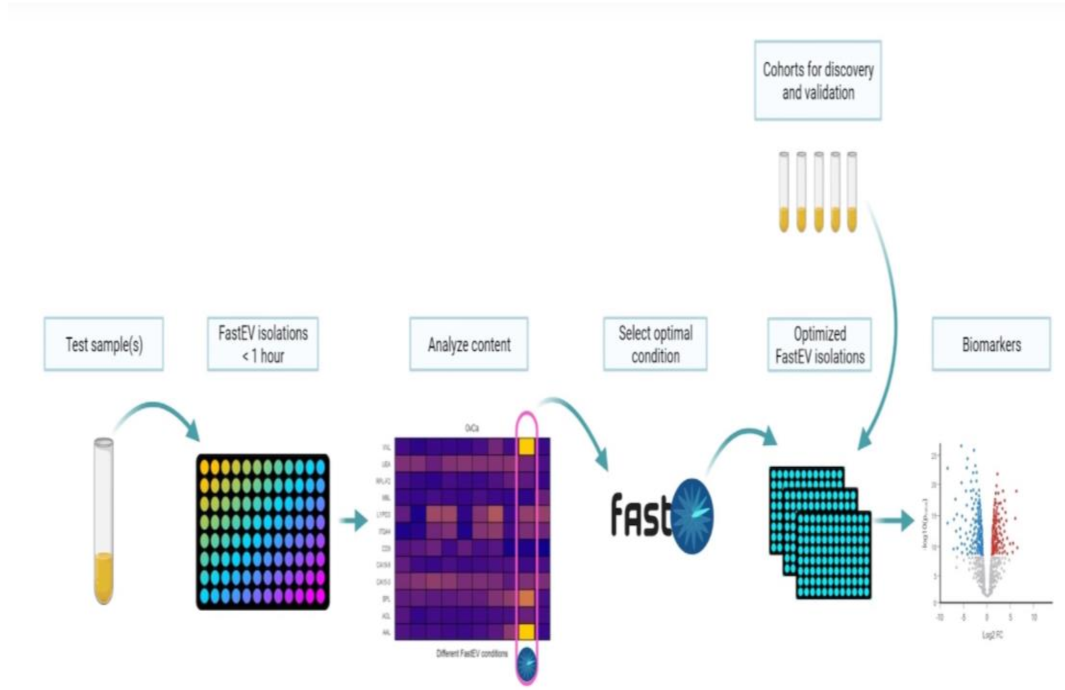

Figure 8: FastEV workflow for optimal biomarker discovery. Samples are immobilized to 96 well plate by 1 hour shaking. EVs are isolated from test samples using different chemical conditions and analyzed to select the optimal FastEV condition. The selected condition is used to isolate EVs from a larger chohort of samples and used for biomarker discovery**.** *Picture is made in Biorender 2022.* (Siljander and Puhka, 2022, University of Helsinki)

3. 6 Immunoassay Protocol:

In this section, we used sandwich immunoassay method (Figure 9), and the procedure are given below:

- Biotinylated capture-mAb (bC192, bMa552) from Fujirebio were added at a concentration of 100 ng/well.

❖ Yellow streptavidin-coated 96-well plate wells (Kaivogen Oy in Turku, Finland) were prewashed using DELFIA Paltewash By Wallac Oy. Each well received 30 µl of the capture-mAbs.

❖ The binding reaction was allowed to incubate 1 hour at room temperature, with shaking at 750 rpm.

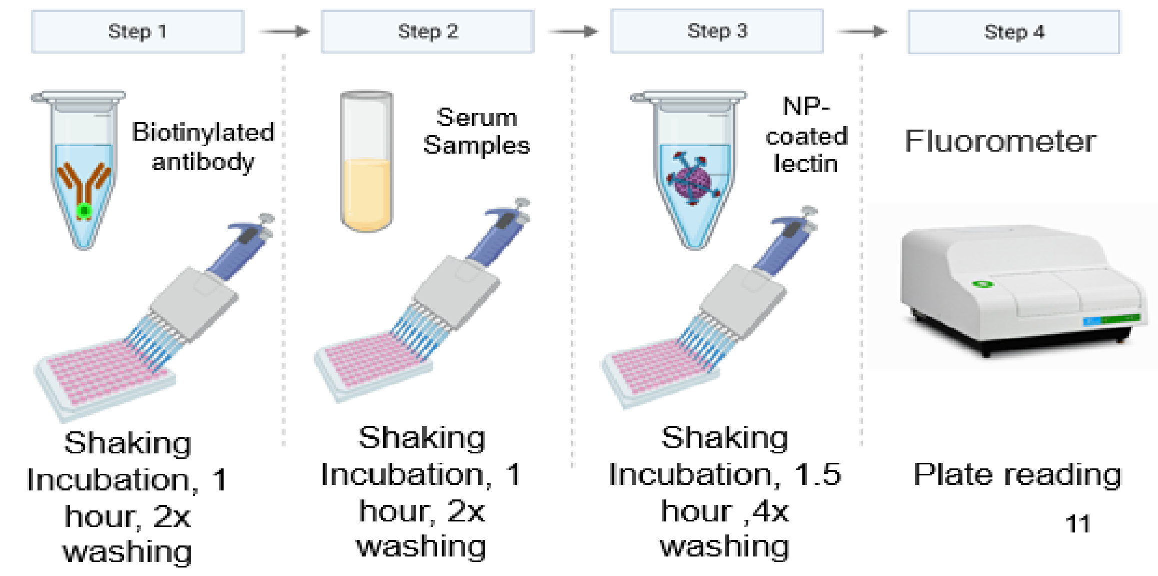

Figure 9: **Sandwich Immunoassay protocol:** Biotinylated antibody was immobilized in the 96 well plate followed by the addition of serum samples with 1 hour shaking incubation at RT. To increase the multivalency, NP coated lectin was allowed to the reaction mixture. Lastly, fluorimeter was used to measure the interactions among the antibody, analyte and NP coated lectin**.** *Picture created in Biorender 2023.*

❖ After the incubation, unbound samples washed twice.

❖ To create 1/10 sample dilution, benign and PCa serum samples were mixed with commercially available Red assay Buffer from Kaivogen Oy. The dilutions consisted of 1-part sample and 9 parts buffer.

❖ As a blank, red assay buffer used.

❖ The experiment involved the addition of samples in triplicates (100 µl per well), followed by an incubation period of 1 hour at room temperature with gentle shaking at 750 rpm.

- ❖ Subsequently, the unbound sample washed twice to remove any excess material. To each well, lectin and antibody nanoparticles (NPs) were introduced at a concentration of 10,000,000 nanoparticles per well (30 µl per well).
- ❖ The reaction mixture allowed to incubating for 1.5 hours at room temperature with continuous shaking at 750 rpm. To ensure optimal results, any unbound capture antibodies were thoroughly washed 4 times. The signal emitted by the nanoparticles was quantified using time-resolved fluorometer (Victor, Wallac Oy, Turku, Finland), employing the surface (normal) protocol to measure Europium fluorescence. The excitation wavelength was set at 340 nm and the emission wavelength was 615 nm.

3.7 Statistical analysis:

Data generated during the sandwich assay was analyzed using MS excel, GraphPad and Origin Lab 2016. To separate the significance of PCa sample result from Benign conditions, Mann-Whitney U test was used to obtain the *p-value,* where <0.05 indicated the statistical significance. Standard deviation, standard error, co-efficient of variance and specific signal was calculated using excel. Standard error was calculated as the standard deviation divided by the square root of the sample size. Co-efficient of variance was determined as the standard deviation divided by the average signal. To calculate specific signals, average signals of the blank was subtracted from the average signals of the sample. The sample data is available in the appendix.

4.0 Results

Back in 1981, EVs were treated as cellular waste with no apparent value. But fast and forward to today, it has captured a great deal of attention for the role in cell-cell communication. Not only that, but these tiny vesicles also carry biomolecules that can drive cancer progression and metastasis, making them an area of intense research and fascination (Morelli 2017). EVs can be detected in biological fluids such as serum, blood, urine, plasma and saliva, making them a promising source of biomarkers for the early detection and monitoring of diseases. Analyzing the bio-molecular contents of these EVs paves the way for more accurate and efficient diagnosis (Islam et al., 2022). But isolation of these EVs from complex body fluid is prerequisite for potential biomarker discovery. Isolation of EVs is still a major concern as the different isolation methods encounter with different limitations. To unravel the intricacies of EV biology, researchers rely heavily on obtaining highly purified EVs. Traditionally, the ultracentrifugation (UC) method has been the go-to technique for EV collection. While it remains a popular method, recent studies suggest that UC-purified EVs have lower purity, compromised structure due to high-gravity conditions, and the process can be quite time-consuming (Yamamoto et al., 2019). Therefore, we explored the novel FastEV technique to isolate the EVs from patients' serum. In this approach, using FastEV technology, we were able to successfully separate extracellular vesicles (EVs) and soluble protein fractions (SP) from the serum samples of both PCa and BPH patients. To capture the EV and SP fractions of PCa, we immobilized a biotinylated capture antibody on streptavidin-coated microtiter wells. Then, we used glycan-binding lectins coated on NPs to detect the capture analyte. Our results showed the effectiveness of this technique in accurately detecting and isolating EV and SP fractions, which could potentially lead to improved diagnosis and treatment options for PCa patients.

Our research work is based on the detection of PCa biomarker on EVs by the help of lectin. So, we focused on the available biomarker in the market approved by Food and Drug Administration (FDA). Studies showed that Ca19.9 and Ca15.3 could be the potential candidate for the detection of cancer (Ghosh et al., 2013). Therefore, we explored both Ca19.9 and Ca15.3 in combination with lectin WGA assay to investigate any significant difference between PCa patients and BPH. In our study, we used 53 benign samples named as TURP: Transurethral Resection of Prostate and 87 PCa samples named as RALP: Robotic-Assisted Laparoscopic Prostatectomy.

The following section comprises a summary of the results obtained from our thesis work.

4.1 Construction of conventional assay (Ca19.9-Ca19.9) to discriminate PCa from Benign conditions:

In this experiment, a conventional assay (Ca19.9-Ca19.9) was constructed to explore the performance of assay in the whole serum sample. In the assay set up, biotinylated anti-Ca19.9 antibodies were immobilized on the streptavidin coated 96 well microtiter plate. The serum sample was then applied into the plate where immobilized Ca19.9 antibody capture Ca19.9 antigen. Then, as a tracer we used the same Ca19.9 antibody coated on NPs. The conventional assay Ca19.9-Ca19.9 was utilized to observe the discrimination between PCa patients and benign conditions. Unfortunately, the conventional assay did not separate prostate cancer from benign conditions (*P value* = 0.21).

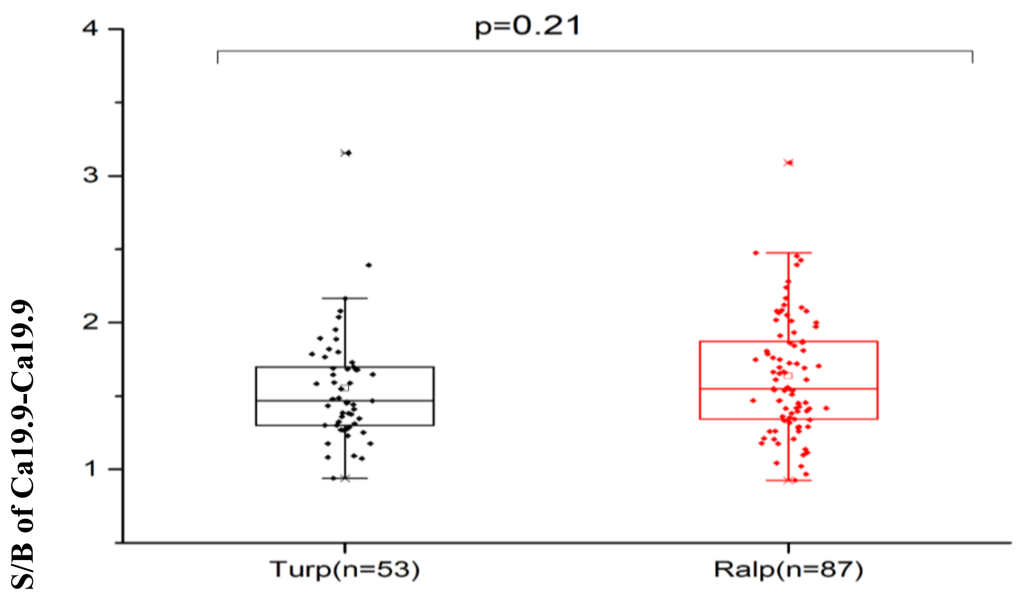

Figure 10: **The signal by background (S/B) ratio of PCa patients (n=87) and benign conditions (n=53).** The signal by background (S/B) ratio calculated by comparing with control and sample, and the *p-value* was measured using the Mann-Whitney test.

4.2 Construction of glycovariant assay (Ca 19.9-WGA) using Standards:

As the conventional assay did not separate PCa from benign conditions mentioned above in section 4.1, thus, we have tried to explore the glycovariant standard assay constructing with the lectin WGA.

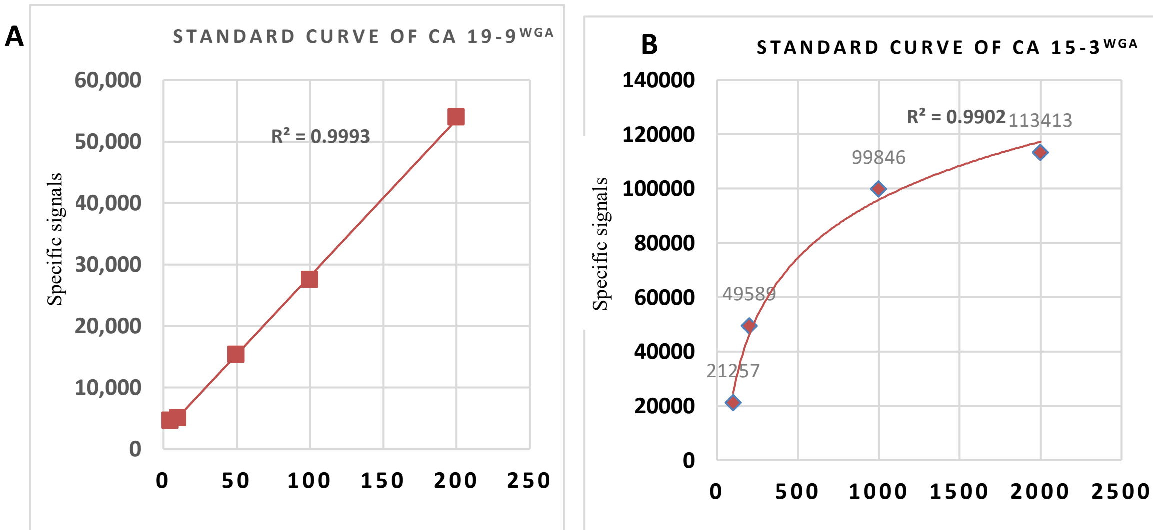

Figure 11: Glycovariant assay of A) Ca19.9-WGA and B) Ca15.3-WGA with standard Ca19.9 antigen and DU145-EVs (prostate cancer cell line) respectively. Both assays showed excellent linearity.

To develop any assay, analytical standards should be performed using standard materials. As a reference stated by Islam (2022), in his PhD booklet "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 have used DU145-EVs derived from prostate cancer cell line as a standard for Ca15.3-WGA assay. Similarly, Ca19.9-WGA assay was validated using Ca19.9 standard antigen. The graphs of Ca19.9-WGA and Ca15.3-WGA assays (Figure 11: A &B) represent excellent linearity (R^2 = 0.9993 and 0.9902, respectively).

4.3 Construction of glycovariant assay (Ca19.9-WGA) using serum samples:

The aim of this study was to investigate the efficacy of glycovariant assay using whole serum sample to separate prostate cancer (PCa) from benign prostatic hyperplasia (BPH) conditions. Previous results mentioned in section 4.2 suggested that combining the standard Ca19.9 and Ca15.3 assay with lectin WGA may show promise in this regard. However, our attempts to employ the Glycovariant assay (Ca19.9 and WGA) with the whole serum sample did not yield significant discrimination between PCa and BPH conditions. The data generated from this glycovariant assay (Ca19.9-WGA) was subjected to statistical analysis, and an insignificant p-value of 0.35 was obtained (Figure 12). These findings suggest that further investigation is needed to develop a more reliable combination for distinguishing between PCa and BPH using the glycovariant assay with our serum samples.

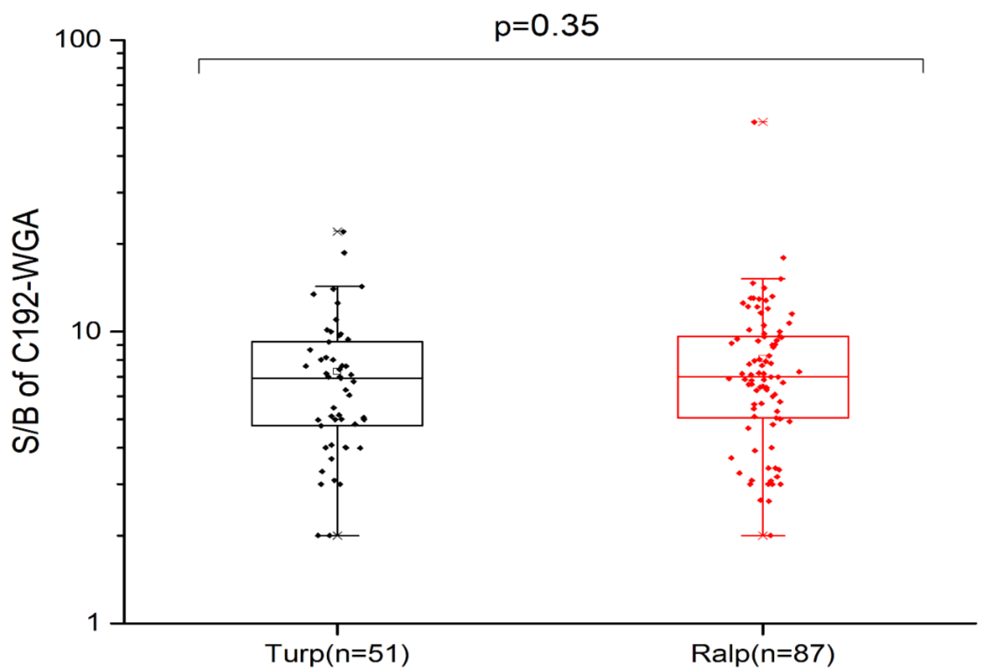

Figure 12: Performance of Ca19.9-WGA glycovariant assay with serum sample was observed with its signal by background (S/B) ratio. In this study, 51 benign patients were distinguished from 87 PCa patients by using nanoparticles coated with WGA as a tracer. This assay combination did not separate the PCa from BPH.

4.4 Construction of glycovariant assay (Ca19.9-WGA) using EV fractions isolated from FastEV technique:

This assay was developed by using EV fractions that were isolated from whole serum samples using novel FastEV technique. The EV fractions were immobilized in the microtiter plate using an anti-Ca19.9 biotinylated antibody, which was attached to the streptavidin-coated plate. Then the lectin WGA coated on NPs were utilized to interact with glycan on EV surface. This interaction was amplified by the TRFIA. The data generated by this assay was calculated and interestingly, Ca19.9-WGA assay was able to significantly discriminate prostate cancer from benign sources using isolated EV from serum samples (*p*-value=0.00001).

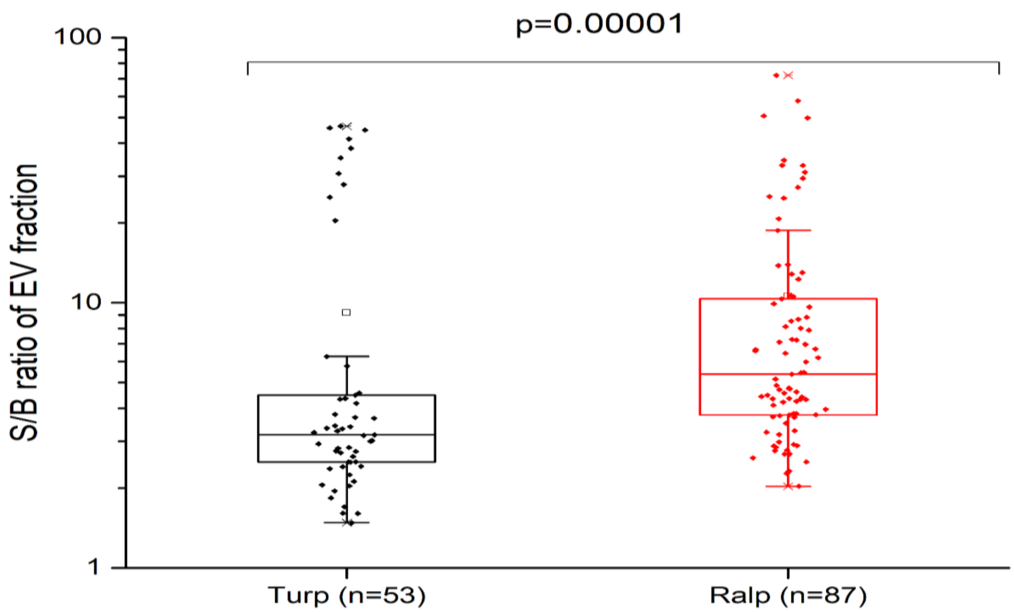

Figure 13: The assay's performance using Ca19.9 and WGA was evaluated based on its signal to background (S/B) ratio. It was found that, this assay can differentiate between patients with PCa and those with benign conditions. Nanoparticles coated with WGA were employed as a tracer, enabling the differentiation of 53 benign patients from 87 PCa patients in the study.

4.5 Construction of glycovariant assay (Ca 19.9-WGA) using SP fractions:

Similarly, we utilized SP fractions, the by-product of FastEV technique, isolated from serum samples. These SP fractions were immobilized into a microtiter plate using a biotinylated anti Ca19.9 antibody that was attached to a streptavidin coated plate. This biotinylated Ca19.9 antibody was subjected to capture analyte from SP fractions. Then the WGA was coated on NPs and utilized to observe if there is any glycan-lectin interaction present in the SP fractions. The resulting interaction was then amplified using time-resolved fluorescence immunoassay (TRFIA). The data we obtained from this assay was not significant where the *p-value* was 0.44. So, this assay demonstrated that SP fractions isolated from serum samples cannot differentiate PCa patients from benign conditions.

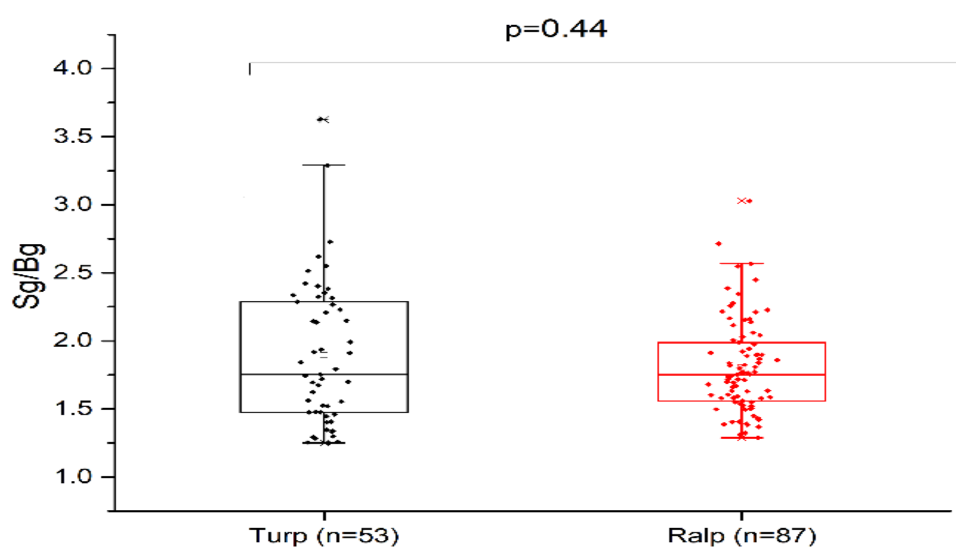

Figure 14: Here, SP fractions were tested to observe the interaction between glycan and lectin. But this assay does not separate the PCa patients from its benign conditions. The data was analyzed as signal to background ratio (S/B) from control to samples.

4.6 Construction of glycovariant assay (Ca15.3- WGA) using EV fractions.

As like as Ca19.9 WGA assay, we have also developed another prominent PCa biomarker Ca15.3 associated assay, Ca15.3-WGA. In this glycovariant assay (Ca15.3-WGA), biotinylated anti-Ca15.3 antibody was immobilized to a 96-well microtiter plate followed by the interaction of analyte (EVs). To explore the glycan alteration on the EV surface, WGA coated on NPs were allowed to interact with the glycan residues. Then the amplified interaction was measured with TRFIA. The data generated from this assay was analyzed. Surprisingly, this assay also significantly discriminated prostate cancer from benign sources using isolated EV from serum samples (*P*-value=0.007).

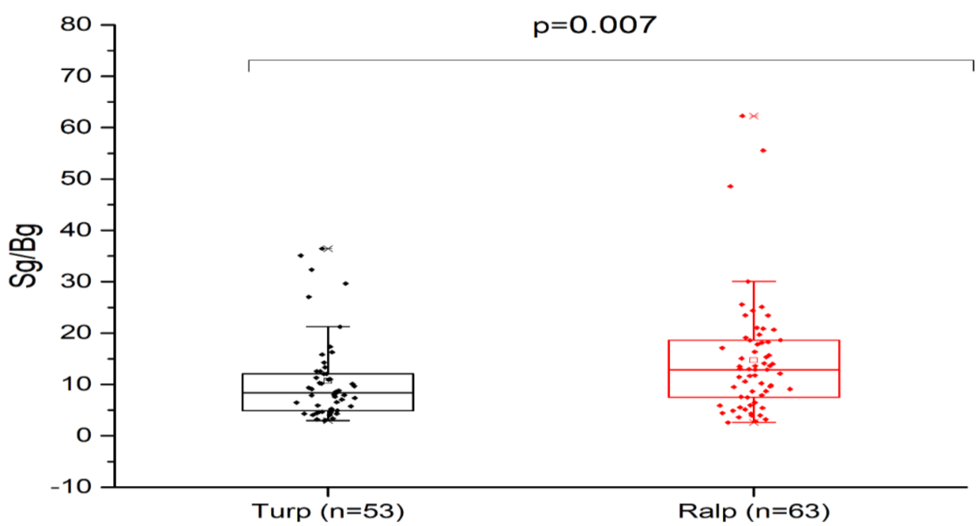

Figure 15: In this glycovariant assay, we have explored another biomarker named Ca15.3 in combination with lectin WGA using 53 benign and 63 PCa patients' samples. The generated data demonstrates that this assay, Ca15.3-WGA, can discriminate PCa patients from the benign conditions patients.

5.0 Discussion

Prostate cancer has recently gathered considerable attention due to its high mortality rate and has been the focus of significant efforts to lessen its devastating effects on men's health. There are some methods available for the detection of prostate cancer but they have some drawbacks which makes the detection approach questionable. Hence, we have focused to develop an approach to detect prostate cancer in a simple, effective and non-invasive manner. In this study, we have developed an easy technique with its high specificity and simplicity. In this regard, EVs played a significant role for the development of biomarker based non-invasive assay to diagnose prostate cancer.

Over the past two decades, there has been significant research on circulating EVs as potential biomarkers in liquid biopsy. The scientific community's fascination with EVs has surged dramatically, primarily due to its exceptional stability and the cargo they carry can represent their site of origin (Islam et al., 2019, Zohu et al., 2020). Thus, serum samples from prostate cancer patients were used in our study because of the abundance of EVs in the serum. However, one major problem is the isolation of EVs from serum samples as EV isolation is labor intensive, time consuming and a complex process (Sidhom et al., 2020). To overcome this complexity, we used a novel, high-throughput FastEV technique as it is quick and automatable.

In our study, isolated EVs from PCa serum sample were captured using biotinylated anti-Ca19-9 and anti-Ca15-3 antibodies immobilized on microtiter plate. Then the captured EVs were detected by lectins coated on nanoparticles. The signals were quantified utilizing a TRFIA based detection method. Subsequently, the developed assay was subjected to comprehensive characterization. This characterization step allowed us to evaluate the specificity and reliability of the assay in distinguishing PCa-derived EVs from EVs associated with non-malignant conditions.

To explore the presence of biomarkers on the surface of EVs, we performed glycol-profiling experiments. We utilized biotinylated anti-Ca19.9 and anti-Ca15.3 antibodies to capture the EVs and lectin WGA as a tracer. Prior studies have shown that Ca19.9 and Ca15.3 are glycoprotein antigens that carry GlcNAc and GalNAc residues in their structure and these residues have been shown to interact with lectins (Yan et al., 2013, Fukuda 1991).) Our study aligns with their previous findings, as we observed a similar interaction between GalNAc-GlcNAc residues on glycoproteins and the lectins. Their studies corroborate our observations regarding the interaction between glycoproteins and lectins.

In a study, Terava et al., (2019) demonstrated that the conventional assay utilizing Ca15.3 was not significant. Another study conducted by Matsuoka K (2014) demonstrated that there is a great affinity between lectin and GlcNAc residues of glycans. So, in our experiment we first constructed a conventional assay using Ca19.9 both as a capture and a tracer. However, it was unable to effectively discriminate PCa from benign conditions (*p-value*: 0.21, Figure 10). To further investigate, we followed the findings of Terava et al., (2019) and Matsuoka K (2014) and constructed Ca19-9-WGA and Ca15-3-WGA glycovariant assays for further study. When we used EVs isolated via FastEV technique, both Ca19.9-WGA and Ca15.3-WGA glycovariant assays showed promising results compared to the conventional assays. The *p-value* obtained were 0.00001, 0.007 and 0.21 respectively (Figure 13, Figure 15 and Figure 10). This noteworthy discrimination between PCa and benign conditions may be attributed to distinct features stated by Terava et al., 2019: first, the signal amplification provided by thousands of Eu^{+3} chelates doped in a single particle and second, the enhanced functional avidity of lectins towards their target glycan epitopes enabled by the high-density immobilization of lectin on the particles.

Prior to the glycovariant assay with isolated EVs, we also tested unprocessed clinical serum samples to explore the potential interaction between WGA lectin and the glycans present in Ca19.9. Unfortunately, this assay did not yield significant separations between the PCa conditions and benign ones (*p-value*: 0.35, Figure 12).

During the isolation of EVs by the FastEV technique we collected some by products from patient's serum sample as a soluble protein (SP) fractions. We also explored these SP fractions to investigate if there is any glycan present on these protein fractions. As expected, we did not find any considerable interaction between glycan and lectin (Figure 14).

From the above discussion, we can say that both glycovariant Ca19.9-WGA and Ca15.5-WGA assay are significant. Between these two assays Ca19.9-WGA stands out more prominent. Therefore, we calculated the average signals, standard deviation, standard error, co-efficient of variance and specific signals that has been shown in the appendix.

6.0 Conclusion

To conclude we can demonstrate that:

- ❖ EVs from patient serum sample might be a good source of new biomarker discovery.
- ❖ FastEV technology was used in combination with simple nanoparticle-aided time resolved fluorescence immunoassay (TRFIA) to improve the detection of prostate cancer patients from benign prostatic hyperplasia (BPH).
- ❖ Ca 19.9-WGA assay, demonstrated significant discrimination of PCa patients from BPH (p=0.00001).
- ❖ Ca 15.3-WGA assay, demonstrate significant discrimination of PCa patients from BPH (p=0.007).
- ❖ In the result section, 4.4 and 4.5 graphs showed some outliers that might be the cause of running the assay in different days. Now we have planned to run the big cohort of samples in a day to observe if there are any variations is encountered.

7.0 References

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9.0 Appendix

The Glycovariant assay Ca19.9-WGA was significant more than the others assay. Therefore, we highlighted the average signals, standard deviation, standard error, coefficient of variance and specific signals of our tasted samples. Here, TURP is the benign conditions and RALP is the cancerous patients. Every specific sample ID represents every individual patient.

Ca19.9-WGA assay with EV fractions

EV Ca19.9-WGA					
TRACER	NP				
Sample ID	Average Signals	Standard Deviation	Standard Error	Coefficient of Variance	Specific Signals
Blank	583	39	28	7%	
TURP_0005	24203	2175	1538	9%	23,620
TURP_0007	22289	1049	741	5%	21,706
TURP_0010	17898	1052	744	6%	17,315
TURP_0015	20509	1118	791	5%	19,926
TURP_0032	11904	508	359	4%	11,321
TURP_0034	16279	64	45	0%	15,696
TURP_0044	14581	1210	856	8%	13,998
TURP_0077	27039	4756	3363	18%	26,456
TURP_0097	26616	1148	812	4%	26,033
TURP_0119	1499	58	41	4%	617
TURP-0120	2217	431	304	19%	1,335
TURP-0122	2215	274	193	12%	1,333
TURP-0155	2787	327	231	12%	1,905
TURP-0163	2434	530	375	22%	1,552
TURP-0166	1814	56	39	3%	932
TURP-0169	2804	830	587	30%	1,922
TURP-0171	2212	82	58	4%	1,330
TURP-0178	2969	311	220	10%	2,087
TURP-0181	1417	127	90	9%	535
TURP-0184	2319	229	162	10%	1,437
TURP-0193	2952	215	152	7%	2,070
TURP-0203	5089	278	196	5%	4,207
TURP-0211	3006	786	556	26%	2,124

TURP-0217	3955	1222	864	31%	3,073
TURP-0223	2858	691	488	24%	1,976
TURP-0226	2087	110	77	5%	1,205
TURP-0231	2651	781	552	29%	1,769
TURP-0232	2588	27	19	1%	1,706
TURP-0242	2242	535	378	24%	1,559
TURP-0251	2523	32	22	1%	1,840
TURP_0252	3119	1098	776	35%	2,436
TURP_0257	1877	43	30	2%	1,194
TURP_0263	1644	352	249	21%	961
TURP_0269	1929	365	258	19%	1,246
TURP_0274	2504	341	241	14%	1,821
TURP_0278	1392	176	124	13%	709
TURP_0289	1095	113	80	10%	412
TURP_0290	1011	41	29	4%	328
TURP_0293	1332	280	198	21%	649
TURP_0309	1532	168	118	11%	849
TURP_0315	2592	739	522	28%	1,909
TURP-0336	2061	59	42	3%	1,378
TURP-0338	1447	233	165	16%	764
TURP-0340	2975	113	80	4%	2,292
TURP-0341	1943	575	406	30%	1,260
TURP-0343	4284	190	134	4%	3,601
TURP-0353	1651	615	435	37%	968
TURP-0388	1254	172	121	14%	571
TURP-0391	1623	274	193	17%	1,026
TURP-0392	2052	284	201	14%	1,455
TURP-0393	2493	361	255	14%	1,896
RALP_0026	17165	208	147	1%	16,582
RALP_0034	18117	496	350	3%	17,534
RALP_0045	20105	2127	1504	11%	19,522
RALP_0050	29049	307	217	1%	28,466
RALP_0051	33666	2411	1705	7%	33,083
RALP_0054	19211	482	341	3%	18,628
RALP_0057	42013	6812	4816	16%	41,430
RALP_0080	29533	2057	1454	7%	28,950
RALP_0161	19230	1956	1383	10%	18,647
RALP_0175	15886	1309	925	8%	15,303

RALP-0176	2633	54	38	2%	1,751
RALP-0178	2294	40	28	2%	1,412
RALP-0186	3951	1373	971	35%	3,069
RALP-0191	4024	577	408	14%	3,142
RALP-0192	3370	563	398	17%	2,488
RALP-0194	3720	352	249	9%	2,838
RALP-0198	2808	308	218	11%	1,926
RALP-0215	3276	358	253	11%	2,394
RALP-0217	2044	50	35	2%	1,162
RALP-0221	4743	652	461	14%	3,861
RALP-0230	6378	1715	1212	27%	5,496
RALP-0242	3621	527	373	15%	2,739
RALP-0246	4545	167	118	4%	3,663
RALP-0290	2370	80	56	3%	1,488
RALP-0307	3499	755	534	22%	2,617
RALP-0323	6267	133	94	2%	5,385
RALP-0333	4170	284	200	7%	3,288
RALP-0353	3806	68	48	2%	2,924
RALP-0355	5908	666	471	11%	5,026
RALP-0369	4069	636	450	16%	3,187
RALP_0371	2219	391	276	18%	1,536
RALP_0412	1388	102	72	7%	705
RALP_0420	1968	636	449	32%	1,285
RALP_0427	1890	104	74	6%	1,207
RALP_0436	2579	1131	799	44%	1,896
RALP_0450	1552	47	33	3%	869
RALP_0452	2904	7	5	0%	2,221
RALP_0456	2580	774	547	30%	1,897
RALP_0463	1896	840	594	44%	1,213
RALP_0467	4496	92	65	2%	3,813
RALP_0470	3729	990	700	27%	3,046
RALP_0475	3255	506	358	16%	2,572
RALP_0482	2562	367	260	14%	1,879
RALP_0490	1994	0	0	0%	1,311
RALP_0493	1947	601	425	31%	1,264
RALP_0502	2974	392	277	13%	2,291
RALP_0506	5378	580	410	11%	4,695
RALP_0519	5830	371	262	6%	5,147

RALP_0533	3010	420	297	14%	2,327
RALP_0546	3717	770	544	21%	3,034
RALP_0563	1605	265	187	17%	1,008
RALP_0565	2593	500	353	19%	1,996
RALP_0576	2098	292	206	14%	1,501
RALP_0596	4165	195	138	5%	3,568
RALP_0612	6179	479	339	8%	5,582
RALP_0616	3978	430	304	11%	3,381
RALP_0620	2580	73	52	3%	1,983
RALP_0624	5917	38	27	1%	5,320
RALP_0627	1966	335	237	17%	1,369
RALP-0637	1726	24	17	1%	1,129
RALP-0640	3851	620	438	16%	3,254
RALP-0647	2646	980	693	37%	2,049
RALP-0650	4786	681	482	14%	4,189
RALP-0661	5259	409	289	8%	4,662
RALP-0667	7328	182	129	2%	6,731
RALP-0671	2282	578	409	25%	1,685
RALP-0676	2809	256	181	9%	2,212
RALP-0679	5172	36	25	1%	4,575
RALP-0690	6304	1977	1398	31%	5,707
RALP-0692	3574	706	499	20%	2,977
RALP-0708	5760	226	159	4%	5,163
RALP-0740	1498	82	58	5%	901
RALP-0744	11182	804	568	7%	10,585
RALP-0746	8252	165	116	2%	7,655
RALP-0751	4158	285	202	7%	3,561
RALP-0752	15014	4170	2948	28%	14,417
RALP-0753	2911	596	421	20%	2,314
RALP-0758	2209	281	199	13%	1,612
RALP-0765	4342	1358	960	31%	3,745
RALP-0772	4851	233	164	5%	4,254
RALP-0818	7759	1394	985	18%	7,162
RALP-0829	3704	125	88	3%	3,107
RALP-0834	8316	3517	2487	42%	7,719
RALP-0863	6351	1637	1158	26%	5,754
RALP-0869	12380	886	626	7%	11,783
RALP-0873	14813	1942	1373	13%	14,216

RALP-0885	7660	1629	1152	21%	7,063