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Md. Ferdhos Liton Khan

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University of Turku

Faculty of Technology
Department of Life Technologies
Molecular Biotechnology and Diagnostics
Doctoral Programme in Technology (DPT)

Supervised by

Professor Emer., Kim Pettersson, Ph.D.
Department of Life Technologies
Molecular Biotechnology and Diagnostics
University of Turku
Turku, Finland

Eeva-Christine Brockmann, Ph.D.
Department of Life Technologies
Molecular Biotechnology and Diagnostics
University of Turku
Turku, Finland

Reviewed by

Docent Hannu Koistinen, D.Sc.
Department of Clinical Chemistry and Haematology
University of Helsinki
Helsinki, Finland

Professor Johan Malm, M.D., Ph.D.
Department of Translational Medicine, Clinical Chemistry
Lund University
Skåne University Hospital
Malmö, Sweden

Opponent

Docent Tobias Nordström, M.D., Ph.D.
Department of Clinical Sciences
Danderyd Hospital
Department of Medical Epidemiology and Biostatistics, Karolinska Institutet
Stockholm, Sweden

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To my parents

UNIVERSITY OF TURKU
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Molecular Biotechnology and Diagnostics
MD. FERDHOS LITON KHAN: Phage Display Modified Antibody to Free PSA Subform for Improved Prostate Cancer Detection
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ABSTRACT

Prostate specific antigen (PSA) is a key marker for early prostate cancer (PCa) detection. Multi-kallikrein immunoassays are commonly utilized to overcome the limitations of PSA in diagnosing aggressive PCa. PSA subforms have been extensively studied, and their usage has been demonstrated to improve PCa detection in the early stages. Early immunoassays for intact free PSA (iPSA) have demonstrated their clinical utility in multiple large PCa screening cohorts. Adding iPSA to a multi-kallikrein panel (total PSA, free PSA, and hK2) to evaluate the risk of clinically significant PCa in apparently healthy men substantially improves diagnostic specificity. From a technical perspective, the iPSA assay uses a unique monoclonal antibody (Mab) 4D4. The performance characteristics of this antibody are however less than ideal when considering the construction of a robust routine assay. The objectives of the thesis were to improve the binding affinity of 4D4 antibody utilizing phage display technology and to develop different assay formats for the sensitive and robust detection of iPSA and nicked PSA (nPSA).

The 4D4 Mab was cloned as recombinant Fab fragment and three mutant Fab libraries were constructed. Phage display technology was used to enrich iPSA-specific antibody Fab fragments from the libraries. Wild-type 4D4 Mab and an affinity-improved mutant 4D4-L3-2 Fab antibody were used as a tracer or capture in iPSA assay. Immunoassays for total PSA, free PSA, iPSA, nPSA, and total and free hK2 were used to assess perioperative plasma samples. The individual PSA and hK2 parameters alone, in different ratios or in the four-kallikrein combinations were specifically analyzed in patients with prostate gland volumes below or equal and above the median.

Several 4D4 mutants showing improved affinity and slower dissociation rate while maintaining the original iPSA specificity were identified from the Fab libraries. Using the mutant 4D4-L3-2 Fab in optimized versions of the iPSA assay yielded several-fold improvements in assay sensitivity compared with wild-type 4D4 Fab. The iPSA assay using 4D4-L3-2 Fab as a capture antibody offered an improvement in separating cancer from non-cancer, benign and low-grade cancers from clinically significant cancers especially in patients with lower prostate gland volumes.

KEYWORDS: prostate cancer, benign prostatic hyperplasia, multi-kallikrein, prostate specific antigen, phage display technology

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TIIVISTELMÄ

Prostataspesifinen antigeeni (PSA) on keskeinen merkkiaine eturauhassyövän varhaisessa toteamisessa. Monikallikreiini-immunomäärityksiä käytetään yleisesti vähentämään PSA:han liittyviä rajoitteita aggressiivisen eturauhassyövän diagnosoinnissa. PSA:n eri muotoja on tutkittu laajasti, ja niiden käytön on osoitettu parantavan eturauhassyövän havaitsemista varhaisvaiheessa. Varhaiset intaktin vapaan PSA:n (iPSA) immunomääritykset ovat osoittaneet kliinisen hyödyllisyytensä useissa laajoissa eturauhassyövän seulonnan kohorttitutkimuksissa. iPSA:n lisääminen monikallikreiinipaneeliin (kokonais-PSA, vapaa PSA ja hK2), jota käytetäänkliinisesti merkittävän eturauhassyövän kohonneen riskin arviointiin näennäisesti terveillä miehillä, parantaa huomattavasti määrityksen diagnostista spesifisyyttä. Teknisesti iPSA-määrityksessä käytetään ainutlaatuista monoklonaalista vasta-ainetta 4D4. Tämän vasta-aineen ominaisuudet eivät kuitenkaan ole ihanteelliset rutiinimäärityksen pystyttämiseen. Tämän väitöskirjatyön tavoitteena oli parantaa 4D4-vasta-aineen sitoutumisaffiniteettia faaginäyttötekniikan avulla ja kehittää erilaisia määrityksiä iPSA:n ja pilkkoutuneen PSA:n (nPSA) herkkään ja vakaaseen osoittamiseen.

4D4 Mab kloonattiin rekombinantti-Fab-fragmentiksi ja rakennettiin kolme mutatoitua Fab-vasta-ainekirjastoa. Kirjastoista rikastettiin faaginäyttötekniikalla iPSA-spesifisiä Fab-vasta-ainefragmentteja. Villityyppistä 4D4 Mab ja affiniteetiltaan parantanutta 4D4-L3-2 Fab-fragmenttia käytettiin leima- ja sitojavasta-aineena iPSA-määrityksessä. Kokonais-PSA:n, vapaan PSA:n, iPSA:n nPSA:n sekä kokonais- ja vapaan hK2:n immunomäärityksiä käytettiin perioperatiivisten plasmanäytteiden arviointiin. Erillisiä PSA- ja hK2-parametreja sellaisenaan, niiden eri suhteita tai neljän kallikreiinin yhdistelmää analysoitiin erityisesti potilailla, joiden eturauhasen tilavuus oli pienempi tai yhtä suuri tai suurempi kuin mediaani.

Fab-kirjastoista löydettiin useita affiniteetiltaan parantuneita 4D4-mutantteja, joilla oli hitaampi dissosiaatio samalla kun alkuperäinen iPSA-spesifisyys oli säilynyt. 4D4-L3-2 Fab mutantin käyttö optimoiduissa iPSA-määrityksessä paransi määrityksen herkkyyttä moninkertaisesti verrattuna alkuperäiseen 4D4 Fabiin. iPSA-määritys, jossa käytetään 4D4-L3-2 Fabia sitojavasta-aineena paransi syövän erottamista muista kuin syövistä sekä hyvänlaatuisten ja matala-asteisten syöpien erottamista kliinisesti merkittävistä syövistä erityisesti potilailla, joiden eturauhasen tilavuus on pienempi.

AVAINSANAT: eturauhassyöpä, hyvänlaatuinen eturauhasen liikakasvu, monikallikreiini, prostataspesifinen antigeeni, faaginäyttötekniikka

Table of Contents

Abbreviations

AMACR	alpha-methylacyl-CoA racemase
AUC	area under the curve
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
Ca	cancer
CDR	complementarity determining region
CN	calculated nPSA
CN(I-W)	calculated nPSA: fPSA – I-W
CN(I-M)	calculated nPSA: fPSA – I-M
CN(I-MC)	calculated nPSA: fPSA – I-MC
DRE	digital rectal examination
DTPA	diethylene triamine pentaacetic acid
Eu	europium
Fab	fragment antigen binding
F-hK2	free human kallikrein-related peptidase 2
fPSA or F	free prostate-specific antigen
fPSA(2C1)	immunoassay for measuring fPSA, Mab 2C1 as the tracer
GG	grade group
GS	Gleason score
KLK3	kallikrein-related peptidase 3
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-6	interleukin-6
I-M	assay of iPSA using mutant 4D4 (L3-2 Fab) as the tracer
I-MC	assay of iPSA using mutant 4D4 (L3-2 Fab) as the capture antibody
iPSA or I	intact free prostate-specific antigen
I-W	assay of iPSA using wt-4D4 Mab as the tracer
KG	Kaivogen assay buffer
Mab	monoclonal antibody

non-Ca	non-cancer
N-M	assay of nPSA using purified mutant 4D4 (L3-2 Fab) as the blocker
nPSA or N	internally cleaved “nicked” free prostate-specific antigen
N-W	assay of nPSA using purified wt-4D4 Mab as the blocker
PAP	prostatic acid phosphatase
PCa	prostate cancer
PCA3	PC antigen 3
phi	prostate health index
proPSA	pro-form (zymogen) of prostate-specific antigen
PSA	prostate-specific antigen
PSA-ACT	PSA complex with α1-antichymotrypsin
PSA-A2M	PSA complex with α2-macroglobulin
PV	prostate gland volume
RAM	rabbit anti-mouse IgG
ROC	receiver-operating characteristic
RP	radical prostatectomy
scFv	single chain variable fragment
T-hK2	total human kallikrein-related peptidase 2
TMPRSS2	transmembrane protease, serine 2
TMPRSS2:ERG	TMPRSS2 and etv-related gene fusion
TNM	tumor, nodes, metastasis
tPSA or T	total prostate-specific antigen
TRUS	transrectal ultrasound
wt	wild-type

List of Original Publications

This dissertation is based on the following original publications/manuscript, which are referred to in the text by the Roman numerals:

I. Liton M.F, Peltola M.T, Vehniäinen M, Kuusela E, Pettersson T, Lamminmäki U, Pettersson K, and Brockmann E.C. Phage display aided improvement of a unique prostate-specific antigen (PSA) antibody unreactive with Lys(145)-Lys(146) internally cleaved forms. *Journal of Immunological Methods.* 2015, 422: 72–79.

II. Khan M.F.L, Soikkeli M, Routila E, Karnnila S, Terävä J, Taimen P, Boström P.J, and Pettersson K. Clinical utility of mutant antibody-based assays for determination of internally cleaved and intact forms of free prostate-specific antigen. *The Journal of Applied Laboratory Medicine.* 2019, 3: 1014–1021.

III. Khan M.F.L, Perez I.M, Kekki H, Taimen P, Boström P.J, Jambor I, Ettala O, Pahikkala T, and Pettersson K. Improved diagnostic performance of a phage display antibody-assisted intact free PSA assay as used in the four kallikrein concept applied to the IMPROD/multi-IMPROD study. *Manuscript.*

In addition, some unpublished data is presented.

1 Introduction

Prostate cancer (PCa) is a major public health concern, being the second most common cancer in men worldwide. It is also the fifth leading cause of cancer-related death in men globally, accounting for an estimated 1.5 million new cases and 397,000 deaths in 2022 (Bray et al., 2024). In Finland in 2020, PCa was the most prevalent new cancer diagnosed in males (incidence 182.7, 5,035 new cases) (Finnish Cancer Registry, 2020). PCa is usually slow-growing cancer and rarely causes clinical symptoms at the early stage. Aggressive PCa often metastasizes to lymph nodes or other organs (Arya et al., 2006). Prostate specific antigen (PSA) is a key marker for early PCa detection. However, the lack of specificity is a major limitation of the PSA test. The PSA test is effective in detecting men with PCa, but it is also often elevated in men with benign prostatic hyperplasia (BPH) (Brooks et al., 2018). PSA-based PCa screening may reduce a small amount of PCa specific death but has no impact on overall mortality (Ilic et al., 2018). PSA-based PCa screening reduces PCa mortality, however, overdiagnosis and overtreatment may have adverse effects (Vickers, 2017). The discovery of various molecular forms of PSA (free PSA and complex PSA) led to the development of advanced immunoassays to enhance the specificity of PSA test to detect PCa (Catalona et al., 1995; Christensson et al., 1990; De Angelis et al., 2007; Gaudreau et al., 2016; Stenman et al., 1991). Measuring sub-forms of PSA or combining those with prostate-specific human kallikrein-related peptidase 2 (KLK2, hK2) is a major way to improve the diagnostic performance of PSA test. The Backman Coulter Prostate Health Index (phi) and the 4Kscore (four-kallikrein panel) test from OPKO Health are currently widely utilized (Agnello et al., 2022; Mi et al., 2021; Voigt et al., 2017). The internally cleaved or nicked form of free PSA (nPSA) concentrations are calculated by subtracting the concentration of intact free PSA (iPSA) from the concentration of free PSA (fPSA) (Peltola, Niemela, Vaisanen, et al., 2011), which has a positive correlation with the volume of prostate gland (Nurmikko et al., 2001).

iPSA, a subform of fPSA, has become an essential part of both four kallikrein concepts and commercial 4Kscore (Stattin et al., 2015; Vickers et al., 2018). The iPSA assay, which uses 4D4 monoclonal antibody (Mab), measures any fPSA isoforms in the circulation that do not have an internal cleavage at Lys145 and

Lys146 (Nurmikko et al., 2000). The performance characteristics of the 4D4 Mab are however less than ideal when considering the construction of a robust routine assay. In this thesis, the binding affinity of the 4D4 Mab was improved by phage display technology. The affinity-improved mutant 4D4 provides improved assay performance and offers new technical possibilities for including iPSA or nPSA assays in a multiplexed panel of kallikrein assays.

2 Review of the Literature

2.1 Prostate and Diseases

The prostate is the biggest male accessory gland, situated at the base of the urinary bladder. The gland looks circular, elliptical, or triangular in the axial view (Kumar & Majumder, 1995). During the ejaculation process, fluids are secreted from the seminal vesicles, prostate gland, and bulbourethral glands. These fluids help dilute the concentration of sperm and make an environment that is suitable for their survival. Fructose is the primary source of nutrition for sperm cells, and semen contains sulfates, which have a role in preventing the swelling of sperm cells (Britannica, 2022).

The prostate gland consists of four regions: peripheral zone, central zone, preprostatic region, and the anterior fibromuscular stroma (McNeal, 1968, 1980, 1981). The peripheral zone makes up more than 70% of the prostate gland. Most of the carcinomas develop in this location. The central zone makes up 25% of the prostate gland and it is the location of ejaculatory ducts. The preprostatic region constitutes about 5% of the prostate gland and the transition zone is located in this region. This is the area where BPH mainly occurs. The anterior fibromuscular stroma is a non-glandular region of the prostate that makes up approximately one-third of the prostate gland.

2.1.1 Prostatitis

Following PCa and BPH, prostatitis is the third most prevalent urinary tract disease in males. It accounts for 25% of all office visits to urological clinics across the globe regarding the genital and urinary systems issues (Khan et al., 2017). There are a few different types of prostatitis, including acute bacterial prostatitis, asymptomatic prostatitis, and bacterial or chronic pelvic pain syndrome. A considerably increased incidence of acute urine retention is linked to the presence of prostatic inflammation (Nickel, 2008). An acute infection of the prostate caused by bacteria may cause pelvic pain, and urinary tract symptoms. Diagnosis is primarily determined by evaluating the patient's medical history and conducting physical examination, however, urinalysis may be helpful (Coker & Dierfeldt, 2016). Prostatitis may

increase the risk of PCa (Zhang et al., 2020). According to the findings of a meta-analysis studies, having a history of clinical chronic prostatitis might considerably raise the risk of developing PCa in the general population (Perletti et al., 2017).

2.1.2 Benign prostatic hyperplasia (BPH)

BPH is a prevalent disorder seen in older men that often causes symptoms related to the lower urinary tract (Ng & Baradhi, 2023). BPH-related lower urinary tract symptoms cause a significantly more human burden compared to other urological disorders (Launer et al., 2021). BPH is a condition where the prostate gland enlarges due to the proliferation of stromal and epithelial cells in the transitional zone. Both inflammation and sex hormones influence this enlargement (Chughtai et al., 2016). The progression and severity of BPH is associated with chronic inflammation (Nickel et al., 2016). Since BPH is a chronic disorder, patient age is the most important risk factor. The prostate continues to enlarge throughout the course of a man's lifetime. In males aged 21 to 30, the usual prostate weight is 20 gm plus or minus 6 gm. 50% of the male population between the ages of 51 and 60 have pathological BPH (Berry et al., 1984). BPH may induce renal failure, and renal failure can affect BPH management (Vikram & Mouli, 2021). Men with BPH may have symptoms like insufficient urine flow, dribbling after urinating, and nocturia (Devlin et al., 2021).

The incidence of BPH is very high in the same age group as men diagnosed with PCa. However, BPH and PCa are considered two distinct disorders, and the development of BPH is not related to an increased risk of PCa. Nevertheless, they often develop together, and it is critical to identify those BPH patients who also have PCa to treat both conditions effectively.

2.1.3 Prostate cancer (PCa)

PCa is a significant health concern affecting a large number of men worldwide (Cai et al., 2022). It is the most prevalent cancer in men and the second most common cancer globally (Bray et al., 2024; Gandaglia et al., 2021; Sung et al., 2021). In 2020, PCa was the most diagnosed cancer in men in 12 regions around the world (Ferlay et al., 2021). Early-stage PCa is usually asymptomatic and indolent. Most complaints include difficulty urinating, increased frequency, and nocturia. Urinary retention and back pain may occur in advanced stages (Rawla, 2019). PCa is often a disease that progresses slowly. PCa is most common in older men, typically occurring after the age of 40-50 years (Arcangeli et al., 2012; Bray et al., 2024; Rawla, 2019). Following a diagnosis, the typical amount of remaining life expectancy is more than 15 years. Although a low mortality rate is linked to slow disease, many men with PCa die

without knowing it, and the actual cause of their death is often something other than the cancer (Silberstein et al., 2013). PCa can lead to death through metastasis, where cancer cells spread to other parts of the body (Schatten, 2018).

PCa is a multifactorial disease, and the origin of PCa is unknown. However, both genetic and non-genetic risk factors are involved in the development of PCa (Gandaglia et al., 2021; Leitzmann & Rohrmann, 2012; Perdana et al., 2016; Schaid, 2004; Wilson & Mucci, 2019). According to World Cancer Research Fund International findings in 2018, being overweight and tall can increase PCa risk (Taitt, 2018). PCa occurrence differs depending on ethnic origin. The prevalence rate varies by region from 6.3 to 83.4 per 100,000 males, with the highest rates occurring in Northern Europe and the lowest rates occurring in South central Asia (Sung et al., 2021). This disparity is due to dietary and environmental factors that may influence the development and progression of the disease (Marks et al., 2004; Rawla, 2019; Shimizu et al., 1991).

2.2 Diagnosis of PCa

Early PCa detection is crucial, as it can greatly improve the chances of successful treatment and increase lifespan for patients (Carlsson & Vickers, 2020). However, there are currently no completely reliable tests for the early detection (Saghaeian Jazi, 2020; Schatten, 2018). The detection of PCa can be conducted by several approaches, such as serum PSA test, Digital rectal examination (DRE), Trans-rectal ultrasound (TRUS) and biopsy (Rebello et al., 2021). The primary component of PCa detection is the measurement of the PSA. The introduction of the PSA test has revolutionized the detection and management of PCa. However, the major issues that arise from using PSA include a high rate of overdiagnosis as well as an increased number of false positive biopsies. Both the phi and the 4Kscore are superior to PSA in terms of their ability to reduce the number of unnecessary biopsies (Bryant et al., 2015; Filella & Foj, 2018; Loeb & Catalona, 2014). Catalona et al. investigated the usefulness of PSA in the detection and staging of PCa using serum PSA measurements, rectal examination, and ultrasonography. The study involved 1653 healthy men 50 or more years old. The authors found that using a combination of serum PSA concentration measurement, rectal examination, and ultrasonography in men with abnormal findings on rectal examination, ultrasonography, or both provides a better approach for PCa detection than using rectal examination alone (Catalona et al., 1991). PSA is a better cancer predictor than DRE or TRUS (Mottet et al., 2017). DRE diagnosed most PCa patients when a clinician noticed a nodule in the rectum during a regular checkup. However, DRE by itself is not a very effective method for diagnosing cancers that are local and asymptomatic. PSA and DRE were shown to have an effect on PCa mortality in the randomized Prostate, Lung,

Colorectal and Ovarian (PLCO) screening trial. The results of the study showed that the rate of metastatic progression increased with an increase in PSA levels (Pinsky et al., 2019). A 150-patient retrospective cross-sectional study found that the diagnostic accuracy of DRE is less than PSA (Timilsina et al., 2020). TRUS is the method that is used most often to image the prostate. Histopathological examination of a sample of prostate biopsy tissue is commonly used to diagnose PCa. Prostate biopsies are obtained via the rectum using a needle under TRUS guidance (EAU Guidelines, 2024; Parkin et al., 2022). Multiparametric magnetic resonance imaging (mpMRI) is a highly reliable method for predicting the outcomes of TRUS biopsy and Radical prostatectomy (RP), potentially influencing PCa management, especially in screening population, where a larger number of patients may avoid TURS biopsy (Gaunay et al., 2017; Kasivisvanathan et al., 2018). mpMRI can identify significant PCa ranging from 44% to 87% in biopsy-naive men and those who have had previous negative biopsies (Fütterer et al., 2015). The detection and characterization of PCa are not improved by increasing the number of prostate biopsy cores taken (Andriole, 2009). In addition, the issue of missing cancers, under staging, and under grading of tumors based on biopsies is a concern that might induce inappropriate treatment selections (Andriole, 2009). So, new biomarkers are needed to replace or combine with PSA to improve the current diagnostic methods for early PCa detection, or better prognostic methods are needed.

2.2.1 Staging and grading

Accurate cancer staging is crucial for determining the most suitable treatment option for the patient. PCa staging and grading involves assessing the extent of the cancer's growth and spread, as well as examining the specific histology and cellular alterations in the tumor. Staging of PCa involves clinical and pathological stage. DRE, bone scans, x-rays, biopsy, and CT or MRI scans are used to determine the clinical stage, while the information acquired during surgery and test results from pathology laboratory are used to determine the pathologic stage (Schatten, 2018). The primary tumor (T) size and location, tumor spreading to lymph nodes (N), and metastasis (M) of cancer to other areas of the body are the three components that make up the TNM classification system (Buyyounouski et al., 2017; Edge et al., 2010; Madu & Lu, 2010). This system is used to determine the extent or spread of PCa. The T, N, and M variables are utilized to provide information on the size and extent of the tumor and its involvement (T1 to T4), the number of lymph nodes implicated (N0 or N1), and any further metastases (M0 or M1). The combined findings are used to determine the cancer stage of each patient. The extent of cancer is assessed using five stages, ranging from 0 (no cancer) to 4 (very advanced cancer). Organ-confined PCa, also known as stage 1 and stage 2 does not spread beyond the

prostate capsule and often has a favorable prognosis. Stage 3 indicates that the cancer has spread just beyond the prostate's outer layer and can be found in nearby tissue as well as the seminal vesicles. A tumor has reached stage 4 when it has metastasized to distant organs (Schatten, 2018). The PCa treatment is difficult when the cancer has spread beyond the prostatic capsule (stage 3 and T4).

Grading is a method used to determine the progression of the PCa by examining the histological tissue sample. It involves classifying the tissue patterns based on the morphology of the cells or structure of the tissue. Generally, if the cells and structures are well differentiated, the cancer is less aggressive. On the other hand, the presence of deformed cells and loss of glandular structure indicates of possibly aggressive cancer. Gleason grading method, developed by Dr. Donald Gleason in 1966, is a widely used method for assessing PCa tissue (Bailar et al., 1966). This system is used to evaluate the appearance of tissue in PCa. The system assigns the number from 1 to 5 to grade the cancer. Grade 1: the cells have an appearance that is practically identical to that of normal prostate cells. Grade 2-4: lower-scoring cells seem normal and are less aggressive. Higher scores seem more abnormal and will likely grow faster. Grade 5: most cells have an appearance that is substantially different from normal. The Gleason score (GS) is a grading system used to assess PCa. It utilizes a scale of 1 to 5 to assess the cell growth pattern of the tumor. The evaluation of cancer cell growth regions is done using a scale ranging from 2 to 10 and then adjusted to a scale ranging from 1 to 5. According to the ISUP system, PCa is categorized into five distinct Grade Groups (GG) based on the modified GS groups. GG 1 is similar to GS $\leq$6, GG 2 is similar to GS 3 + 4 = 7, GG 3 is similar to GS 4 + 3 = 7, GG 4 is similar to GS (4 + 4 = 8, 3 + 5 = 8 and 5 + 3 = 8) = 8, and GG 5 is similar to GS 9-10. A GS of 6 indicates low-grade cancer, and a GS of 8-10 indicates high-grade cancer (Buyyounouski et al., 2017; Epstein et al., 2016; Giannico & Hameed, 2018; Schatten, 2018).

2.3 Treatment

The treatment options for a patient depend on factors such as severity of the disease, recurrence risk, in addition to patient characteristics including age, comorbidities, and individual preferences (Miller et al., 2019). Since PCa frequently responds to treatment, life expectancy may be increased, and symptoms can be reduced. While early-stage PCa often has a favorable prognosis, the outlook for metastatic disease remains poor, with a 5-year survival rate of only 34% (Survival Rates for Prostate Cancer, American Cancer Society). Treating advanced PCa is difficult because regular chemotherapy is often not effective. Androgen deprivation therapy (ADT) is the treatment of advanced PCa, but their effectiveness typically lasts for only a few years. After this castration-resistant PCa often develops due to the re-activation of

androgen receptor signaling (Katzenwadel & Wolf, 2015; Knudsen & Kelly, 2011). PCa treatment works well for localized cancer, especially cancer that is confined to an organ. Surgery, radiation therapy, or expectant management are the three primary options for men diagnosed with localized disease (Litwin & Tan, 2017). Many men with localized PCa benefit from expectant management. It might include active surveillance with a purpose of cure or watchful waiting. Watchful waiting is a strategy utilized for older people or those with several comorbidities who will not benefit from curative treatment. On the other hand, active surveillance protocols use organized monitoring methods like PSA testing and repeat biopsies to detect changes in disease risk that need treatment (Filson et al., 2015). The active surveillance treatment option is possible for favorable risk PCa and safe in the 15-years period (Klotz et al., 2015). RP is a surgical procedure used to remove the whole prostate gland and potentially nearby tissues. It is mainly recommended for patients with localized and low-stage disease.

High-energy radiation kills cancer cells and shrinks tumors (NCCN Guidelines, 2024). After surgery, it may prevent cancer recurrence. Radiation therapy may be suggested to eliminate any cancer cells remaining if PSA levels rise after surgery. This therapy includes both internal form of radiation therapy known as brachytherapy and external beam radiation therapy (NCCN Guidelines, 2024). When treating PCa, RP and radiation therapy both have potential physical side effects. The side effects are urine incontinence, erectile dysfunction, and complications with the bowel (Miller et al., 2019; Mottet et al., 2017; NCCN Guidelines, 2024; Resnick et al., 2013).

Hormone therapy is often used in combination with radiation therapy for the treatment of patients with PCa in its early stages. It is typically given to patients with higher risk and can be administered before, during, or after radiation therapy to enhance its effectiveness. ADT is a hormone therapy that can inhibit the production of testosterone in the body or prevent cancer cells from utilizing testosterone. This treatment can lead to the reduction the tumor size or slowing of tumor growth temporarily (NCCN Guidelines, 2024). Hormonal therapy can cause various side effects such as libido loss, hot flashes, nocturnal sweats, irritability, and gynecomastia (Miller et al., 2019).

Chemotherapy is a drug-based therapy that kills cancer cells but may also harm healthy cells. PCa cannot be cured with this treatment, but it may reduce the size of the primary tumor and prevent the progression of cancer. It may extend people's lives and relieve their pain and other problems. Docetaxel is the chemotherapy drug used to treat advanced PCa (Thomas & Pachynski, 2018).

2.4 Prostate-specific antigen (PSA)

PSA is a member of the family of human kallikrein-related peptidases (KLKs). PSA, also known as KLK3, is highly expressed in the prostate and its gene has been mapped within a 300-kb region on chromosome 19q13.4 (Riegman et al., 1992; Schedlich et al., 1987; Yousef & Diamandis, 2001). PSA is released into the prostatic ducts in the form of an inactive proenzyme called proPSA, which consists of 244-amino acids (Balk et al., 2003; Mikolajczyk et al., 2002). Activation of proPSA occurs by the cleavage of seven amino acids from the N-terminus. The propeptide is cleaved by hK2 (KLK2) in the lumen of the prostatic ducts, resulting in the production of active mature PSA (Balk et al., 2003; Lovgren et al., 1997). PSA primarily involves the breakdown of gel in freshly ejaculated semen by proteolysis of the three key proteins (semenogelin I, II, and fibronectin) that are responsible for the formation of gel (Lilja, 1993; Malm & Lilja, 1995). PSA consists of 237 amino acids and has a molecular mass of 26,079 Daltons for the peptide moiety of the molecule (Bélanger et al., 1995). Recent research has revealed the complex nature of PSA, especially its post-translational modifications, such as glycosylation, which plays a crucial role in its biological function and diagnostic potential (Drake et al., 2015; Wang et al., 2023). The glycosylation of PSA has been the subject of extensive research, as it has been observed that the glycosylation profiles of PSA can differ between healthy individuals and those with PCa (Hsiao et al., 2016; Kammeijer et al., 2018; Tkac et al., 2019). PSA is mostly organ-specific; however, it has been found in various human body fluids and tissues in low quantities. The median concentrations of total PSA (tPSA) in seminal fluid (0.65 mg/mL) are significantly higher than in serum (0.50 ng/mL) (Sävblom et al., 2005). The risk of PCa increases with elevated PSA levels, but there is no definitive cutoff point. PSA levels in men without PCa are typically under 4 ng/mL. A PSA level between 4 and 10 ng/mL indicates a 1 in 4 chance of having PCa, while a PSA level above 10 ng/mL indicates a greater than 50% chance of having PCa (Caplan & Kratz, 2002; Screening Tests for Prostate Cancer, American Cancer Society).

PSA is primarily produced by the epithelial cells of the prostate gland. Only a small amount of PSA normally enters the bloodstream. Disruptions to the microstructure of the prostate gland allow more PSA to enter the extracellular space. Extracellular PSA is drained by the lymphatic system, which results in its entry into the bloodstream and subsequent increase in serum (Jain et al., 2024). The serum PSA levels showed a positive correlation with the progression of clinical stage and a direct relationship with the estimated tumor volume (Stamey et al., 1987). PSA has also been found to be present in the different glands of both females and males (Sokoll & Chan, 1997). PSA is found in circulation in two forms: fPSA (non-complexed forms), having a range of 5 to 40% of tPSA, and PSA complexes with the protease inhibitors α 1-antichymotripsin (ACT), α 2-macroglobulin (A2M) and protein c

inhibitor (Christensson et al., 1990; Espana et al., 1991; Finne et al., 2000; Lilja et al., 1991; Lilja et al., 2008; Stenman et al., 1991; Stephan et al., 2002). These inhibitors form complexes with PSA, which are mostly in circulation but also form complexes in a small fraction in the prostate (Bjartell et al., 1993; Igawa et al., 1996; Lin et al., 2005; van Sande & Van Camp, 1983). The proportion of the PSA-ACT complex was shown to be lower in patients with BPH compared to those with PCa. However, the PSA-A2M complex exhibited low levels in patients with PCa, as well as in healthy men and women (Stenman et al., 1991). The fPSA fraction in the circulation comprises various forms, categorized as intact and nicked, based on cleavage of the peptide backbone. iPSA includes enzymatically inactive proenzyme (proPSA) and mature PSA. iPSA lacks an internal cleavage at Lys145 and Lys146, while nPSA has an internal cleavage at Lys145 and Lys146 (more details are in chapter 2.4.2) (Nurmikko et al., 2000; Peltola, Niemela, Vaisanen, et al., 2011).

2.4.1 PSA as marker for PCa

The measurement of PSA in serum was introduced by Catalona et al. in 1991 as a screening method for PCa. This method is considered a valuable addition to rectal examination and ultrasonography in identifying PCa (Catalona et al., 1991). The mortality rate of PCa was shown to be reduced with PSA screening in population-based studies (Hugosson et al., 2010; Schröder et al., 2012). Blood PSA levels are a powerful predictive indicator of long-term aggressive PCa (Vickers & Lilja, 2012). A significant issue with routine PSA screening is overdiagnosis. In a European screening study, PSA screening reduced PCa deaths by 20% but increased the risk of overdiagnosis and overtreatment (Schröder et al., 2009). In this study, the PCa detection rate using PSA alone was 4.6%, which was higher than the rate using DRE alone (3.2%). However, combining PSA with DRE improved the detection rate to (5.8%) (Catalona et al., 2017). PSA screening can detect PCa patients with higher proportion of organ-confined tumors than DRE (Catalona et al., 1993). The prevalence of PCa in men increased with higher levels of PSA (Thompson et al., 2004). PSA levels above 4.0 ng/mL are often considered elevated, but only 25% to 30% of men with this levels have cancer on biopsy (Parekh et al., 2007). Operating characteristics of PSA for PCa detection were evaluated in a randomized study at 221 US centers, including 18882 healthy men without PCa and with PSA levels less than or equal to 3.0 ng/mL. The results showed that there is no PSA cutoff with a high level of specificity and sensitivity that can effectively monitor healthy men for PCa (Thompson et al., 2005). Parameters such as PSA density, PSA velocity, and age or race-specific reference ranges have been developed to enhance PSA specificity (Polascik et al., 1999; Stephan et al., 2014). The percentage of fPSA (fPSA%) was shown to have the most effect on PCa probability in a population-

based PCa screening trial conducted in Finland (Finne et al., 2002). %fPSA significantly enhances cancer detection sensitivity compared to reference ranges of age-specific PSA (Catalona et al., 2000). %fPSA may assist in discriminating between PCa and BPH with tPSA levels ranging from 4.0 to10.0 ng/mL (Partin et al., 1996). %fPSA improves PCa screening specificity in men with higher total serum PSA levels and decrease unnecessary biopsy (Catalona et al., 1995). The ratio of fPSA to tPSA (f/tPSA) in PCa patients is impacted by the volume of the prostate, aiding in distinguishing PCa from BPH patients with a small volume of prostate (Stephan et al., 1997). The ratio of f/tPSA test may reduce unnecessary biopsies and increase the detection rate of PCa (Roddam et al., 2005). fPSA, complexed PSA, and subforms of fPSA (proPSA, iPSA and nPSA) can be measured to enhance the sensitivity and specificity of the PSA test (Ayyıldız & Ayyıldız, 2014; Lilja et al., 2008; Nurmikko et al., 2001; Peltola, Niemela, Alanen, et al., 2011; Stenman et al., 1991). The different forms of PSA are shown in **Figure 1**.

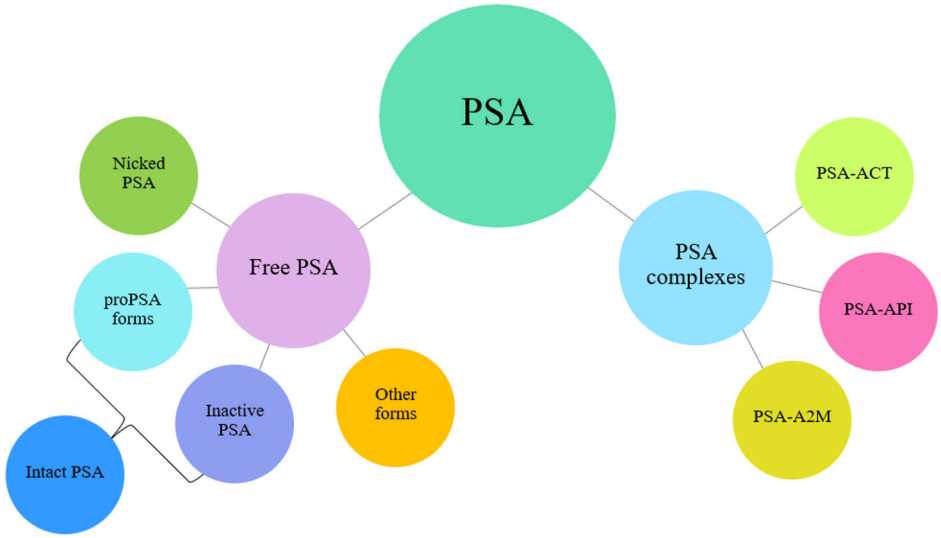

Figure 1: Different forms of PSA. The figure is drawn by PowerPoint following the original idea described by Stephan et al. (Stephan et al., 2002).

The phi is a test that combines tPSA, fPSA, and [-2]proPSA into a single score and this test may reduce unnecessary biopsies and increase specificity for detection of PCa in males 50 years or older with PSA range from 2.0 to 10.0 ng/mL, and with negative DRE results, and as well as phi is useful for the detection of PCa that is clinically significant (Catalona et al., 2011; Loeb et al., 2015). The use of phi in a multivariable risk assessment can greatly enhance the detection of aggressive PCa and may reduce unnecessary biopsies (Loeb et al., 2017). Moreover, phi is a reliable

biomarker for detecting PCa accurately and distinguishing aggressive and non-aggressive PCa (Agnello et al., 2022).

The 4Kscore is a blood test that was commercialized by OPKO Diagnostics. The test includes tPSA, fPSA, iPSA, and hK2 and has shown diagnostic efficacy in identifying significant PCa (Parekh et al., 2015). A large prospective study was conducted to validate a statistical model that utilizes the four kallikrein markers. The study included 6129 men with increased PSA (3.0 ng/mL). The inclusion of the four kallikreins in the model enhanced the detection of PCa compared to relying only on PSA levels. Additionally, using the model led to a reduction of unnecessary biopsies (Bryant et al., 2015). A panel of four kallikreins measured in the blood can predict the outcome of biopsy in men who have not been previously screened with a high PSA levels (Vickers et al., 2008).

2.4.2 proPSA, iPSA and nPSA

There are three different types of fPSA in serum, proPSA, iPSA and nPSA. The proenzyme (proPSA) form is commonly associated with PCa, whereas internally cleaved (BPSA or nPSA) form of PSA is predominantly associated with BPH. The third PSA form is mainly composed of intact, similar to native, active PSA (iPSA) but enzymatically inactive due to structural or conformational changes (Ayyıldız & Ayyıldız, 2014; Mikolajczyk et al., 2002; Mikolajczyk et al., 2000).

ProPSA, a unique molecular form of fPSA that can be measured in circulation by immunoassays, is produced when pre-proPSA is cleaved, yielding an inactive precursor protein with 244 amino acids. Active PSA is produced by cleaving the N-terminal 7 amino acids of proPSA (Peyromaure et al., 2005). proPSA has been investigated to distinguish BPH from PCa more precisely. Sokoll et al. investigated proPSA for PCa detection at 2.5–4.0 ng/mL tPSA. tPSA, fPSA, and proPSA were measured in serum samples from 119 men (88 non-cancer, 31 cancer) before biopsy. The authors found that the %proPSA (proPSA/fPSA) can possibly identify 75% of cancer while reducing 59% of unnecessary biopsies (Sokoll et al., 2003). Catalona et al. showed that the %proPSA improved substantially the specificity of PCa detection and reduced a significant number of unnecessary biopsies (Catalona et al., 2003). [-2]proPSA is a truncated isoform of proPSA that may distinguish PCa from BPH years before diagnosis and aid in preventing overtreatment in non-aggressive and consequently nonsignificant PCa individuals (Heidegger et al., 2014). According to research by Park et al. % [-2]proPSA is more effective than tPSA and %fPSA in predicting the prevalence and aggressiveness of PCa in Korean males (Park et al., 2018).

iPSA is a subform of fPSA that does not have an internal cleavage at Lys145 and Lys146. The iPSA assay uses a monoclonal antibody 4D4 that does not recognize

the PSA that is internally cleaved at Lys145 and Lys146 (Nurmikko et al., 2000). The iPSA alone or its ratio to fPSA (iPSA/fPSA) showed a substantial difference between the two groups. The iPSA/fPSA ratio was considerably higher in PCa samples (median 59%) than in BPH samples (median 47%) (Nurmikko et al., 2001). The ratio of iPSA/fPSA showed a statistically significant increase in patients with PCa (median 48.5%) compared to those with BPH (median 41.8%). This finding suggests that iPSA/fPSA ratio can potentially enhance the specificity of PCa detection (Steuber et al., 2002). A lower ratio of iPSA to tPSA is associated with advanced PCa pathological stage and grade (Peltola, Niemela, Vaisanen, et al., 2011). Christensson et al. assessed the variation of iPSA measurement at different time points and found that the intra-individual variability of iPSA seems to be quite low over a short period of time (Christensson et al., 2011). iPSA, as part of a four kallikrein panel, was able to contribute to the reduction of unnecessary biopsies in the screening populations of 740 men in Göteborg, Sweden (Vickers et al., 2008). A recent study by Vickers et al. indicates that iPSA and hK2 contribute significantly to the discriminatory power of the kallikrein panel (Vickers et al., 2018).

nPSA is a subform of fPSA that consists of an internal cleavage at Lys145 and Lys146 (Nurmikko et al., 2000). Despite being cleaved, the molecule stays structurally intact in vivo due to many disulfide bonds (Sokoll & Chan, 1997). Since there has not been direct immunoassay, nPSA concentrations were calculated in several studies by subtracting iPSA from fPSA. Pauliina Niemelä developed a model of a direct nPSA assay in her PhD thesis (Niemelä, 2002) and the assay has been developed by Peltola et al. (Peltola, Niemela, Alanen, et al., 2011) and Khan et al., 2019. The calculated nPSA and the ratio to tPSA separate the PCa groups from the BPH groups significantly (Nurmikko et al., 2001; Steuber et al., 2002). A lower ratio of calculated nPSA to tPSA is associated with advanced PCa pathological stage and grade (Peltola, Niemela, Vaisanen, et al., 2011). The concentrations of measured nPSA were found to be higher in BPH than PCa patients and the concentrations of measured nPSA were found to be 23% lower than the calculated nPSA (Peltola, Niemela, Alanen, et al., 2011). Chen et al. reported that nPSA is more common in BPH nodules (Chen et al., 1997). nPSA accounts for benign volume dependency and provides strong indications of nodular hyperplastic changes in the prostate (Steuber et al., 2005). Univariate regression analysis showed a strong correlation between the nPSA/tPSA ratio and PCa on biopsy (Steuber et al., 2007).

2.5 Human kallikrein-related peptidase 2 (hK2)

hK2 (KLK2) and PSA (KLK3) belong to the same human family of kallikrein-related peptidases and have an 80% amino acid sequence similarity (Finlay et al., 1998; Yousef & Diamandis, 2001). hK2 is mostly found in the prostate epithelium,

and it has trypsin-like substrate specificity. It can convert proPSA to mature, enzymatically active PSA (Kumar et al., 1997; Lovgren et al., 1997). Part of serum hK2, like PSA, is free form and 4-19% complexed with ACT or protein C inhibitor (Becker et al., 2000) and in contrast to PSA, hK2 is expressed at higher levels in PCa tissue than BPH tissue (Finlay et al., 1998). hK2 concentrations are 100-fold lower than PSA, and the high variation of the ratio of free hK2 (F-hK2) to total hK2 (T-hK2) suggested that hK2 in plasma is not consistently free form in the PCa patients (Vaisanen et al., 2004). It has been shown that the serum hK2/fPSA ratio outperformed the fPSA/tPSA ratio in identifying PCa in the diagnostic "gray zone" of 4 to 10 ng/mL tPSA (Kwiatkowski et al., 1998). hK2 has been shown to help discriminate PCa from non-cancer patients and predict cancer stage or grade pre-operatively (Becker et al., 2001; Becker et al., 2003; Haese et al., 2000; Haese et al., 2001). Including hK2 in a multikallikrein panel can predict the biopsy outcome in males with a high PSA level and help reduce of unnecessary biopsies (Vickers et al., 2008). An individual patient data meta-analysis showed that adding hK2 to the kallikrein panel provides statistically significant predictive discrimination (Vickers et al., 2018).

2.6 Other biomarkers for PCa detection and prognosis

In addition to PSA, other PCa biomarkers have been studied for PCa detection and prognosis based on serum/blood, urine and tissue samples.

Prostatic acid phosphatase (PAP), prostate secretory protein 94, and interleukin-6 (IL-6) are serum/blood-based detection markers (Gaudreau et al., 2016; Sardana et al., 2008; Xu et al., 2019).

PAP, transmembrane protease, serine 2 and etv-related gene fusion (TMPRSS2:ERG), IL-6, c-reactive protein, urokinase plasminogen activator, transforming growth factor β1, circulating tumor cells, and cell free DNA are serum/blood-based prognosis markers (Gaudreau et al., 2016; Liu et al., 2021; Sardana et al., 2008; Xu et al., 2019).

PC antigen 3 (PCA3 or DD3) is a PCa-specific gene and a potential urinary biomarker for the early identification of PCa (Bussemakers et al., 1999). PCA3 has a key advantage in its specificity for PCa cells. Unlike PSA, which can be elevated in benign conditions, PCA3 is highly overexpressed in PCa cells, with virtually no expression in normal or non-cancerous prostatic tissues (Bourdoumis et al., 2010). The PCA3 score is useful indicator for predicting the outcome of a repeat prostate biopsy. Higher PCA3 scores are associated with a higher probability of a positive biopsy result (Haese et al., 2008). Progensa test is used to determine if a repeat biopsy is needed after a prior negative result. Progensa test measures the concentrations of

urine PCA3 and PSA RNA molecules following DRE (Gaudreau et al., 2016; Groskopf et al., 2006). Urinary PCA3 testing with PSA can reduce unnecessary prostate biopsies (Crawford et al., 2012). Furthermore, TMPRSS2:ERG is also a urine-based detection marker (Laxman et al., 2006).

TMPRSS2:ERG, prostarix, and mi-prostate score urine test are urine-based prognosis markers (Gaudreau et al., 2016).

Alpha-methylacyl-CoA Racemase (AMACR) is an enzyme and a tissue biomarker that has been extensively studied in the context of PCa. It is increased in PCa cells (Lloyd et al., 2013). AMACR not only upregulates at the protein level, but also localizes the enzyme to the peroxisomal compartment of PCa cells (Kong et al., 2020). In clinical PCa samples, AMACR mRNA levels are 9-fold higher than normal (Luo et al., 2002). It has been shown that the levels of AMACR mRNA in PCa are 55-fold higher than in benign prostate tissue (Jiang et al., 2004). Furthermore, early prostate cancer antigen is also a tissue-based detection marker (Sardana et al., 2008).

AMACR, TMPRSS2:ERG, hepsin, cysteine-rich secretary protein 3, proMark, and prolaris are tissue-based prognosis markers (Gaudreau et al., 2016; Sardana et al., 2008).

2.7 Antibody phage display technology

2.7.1 Antibody structure

Antibodies are immune-related glycoproteins that are produced in reaction to the presence of foreign substances known as antigens. Foreign substances including bacteria, viruses, fungi, and parasites are recognized as foreign by the immune system because their surface molecules are different from those in the body. The interactions between immunoglobulins and antigens usually happen in the paratope and epitope sites. The paratope is the site of the immunoglobulin (Ig) where the antigen binds, and epitope is the site of the antigen to which the Ig binds (Schroeder & Cavacini, 2010).

IgM, IgD, IgG, IgA, and IgE are the five distinct classes of Ig, which show differences in terms of their structure of amino acid, contents of carbohydrates, charge, and size (Altshuler et al., 2010; Charles A Janeway et al., 2001; Paul, 2012; Schroeder & Cavacini, 2010). IgG antibodies are 150 kDa molecules with four polypeptide chains, two of which are identical light (L) and two of which are identical heavy (H). An IgG consists of three structural domains: two fragment antigen binding (Fabs) and one fragment crystallizable (Fc), all connected by a flexible “hinge” region (Altshuler et al., 2010; Schroeder & Cavacini, 2010). As a heavy chain dimer, the Fc fragment consists of constant H2 (CH2) and constant H3 (CH3) domains, whereas the Fab fragment contains L-H chain dimer with variable

L (VL) – constant L (CL) linked with variable H (VH) – constant H1 (CH1) domains. The structure of polypeptide chains differs on the isotype. The amino acid sequence of the antibody's variable region varies significantly for different antibodies. Usually, it contains 110-130 amino acids, giving the antibody its specificity for binding to the target antigen. The light chain contains a VL and CL domain. There are two kinds of light chains: lambda and kappa. The heavy chain contains a VH domain and three CH domains (**Figure 2**). However, not all antibodies have structures like Ig; camelid antibodies lack the light chain, consisting solely of heavy chains (Arbabi-Ghahroudi, 2022).

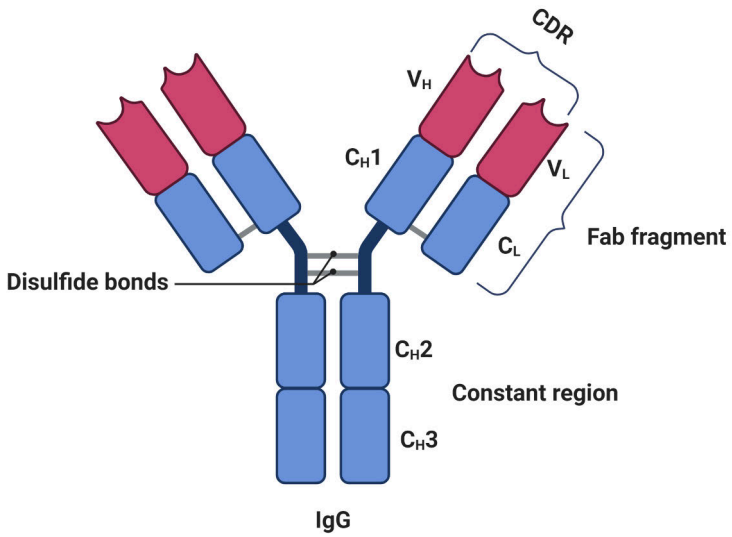

Figure 2: Schematic representation of antibody structure. *This figure is created with BioRender.com (2022).*

The variable domains of the H and L chains are generated by a genetic recombination process termed V, D, J (VH) or VJ (VL) recombination, which may potentially generate an enormous range of antibody sequence combinations. Each VL and VH domain has three complementarity-determining regions (CDR) loops that directly interact with the antigen. These loops extend from the framework region, which remains the same structurally and makes most interactions with the antigen. The length, sequence, and structure of the CDR H3 is the most variable of the six CDRs (H1, H2, H3, L1, L2, and L3). The V-D-J junction forms this loop, which is highly variable due to additional nucleotides added or deleted between V-D and D-J (Stanfield & Wilson, 2014). Arnaout et al. used deep sequencing to sequence heavy chain VDJ-rearranged genes from two healthy donors and found several million heavy chain CDR3s sequence in one adult human blood (Arnaout et al., 2011).

2.7.2 Antibody production

Immunization is the simplest method for producing antigen-specific antibodies, which can recognize the target antigen with high affinity and specificity. Blood from an animal that has been immunized can be used to isolate polyclonal antibodies. Antibodies are produced by immune cells called lymphocytes. To create antibodies with identical structure and specificity, lymphocytes are isolated and immortalized by fusing them with tumor cells. This fusion creates hybridoma cells, which can produce antibodies that are identical in their properties. These antibodies, known as monoclonal antibodies (Mab) (Altshuler et al., 2010; Nelson et al., 2000; Saeed et al., 2017; Zaroff & Tan, 2019). In 1975 Hybridoma technology was invented by Kohler et al. (de Almeida et al., 2018; Kohler & Milstein, 1975; Zaroff & Tan, 2019). Hybridoma technology has shown to be an impressive and important platform for producing high-quality Mabs, and Mabs derived from hybridomas have become the fastest-growing category of therapeutic biologics (Castelli et al., 2019; Moraes et al., 2021; Zhang, 2012). Mabs are valuable in research and diagnostic tests since they are specific for their target proteins and can be utilized as vehicles to transport therapeutic agents to malignant cells in the body (Nelson et al., 2000). Antibodies have several applications in research in the biochemistry, cytology, and clinical chemistry, as well as in diagnosis, and disease therapy (Altshuler et al., 2010; Modjtahedi et al., 2012; Saeed et al., 2017; Steplewski et al., 2015).

2.7.3 Recombinant antibodies

Recombinant antibodies are generated by gene engineering technique, allowing for expression of L and H chains of Igs as separate proteins. This method facilitates the generation of diverse antibody fragments and modification of numerous antibody characteristics, such as affinity, specificity, and immunogenicity. Two distinct methods are used to generate recombinant antibodies. The first approach is the generation of recombinant analogs of Mabs by the utilization of genetic components from hybridoma cells. The second approach involves developing libraries of various recombinant antibody sequences and then selecting antibodies with the desired specificity and affinity (Altshuler et al., 2010).

Fab and single-chain Fv (scFv) antibody fragments are the most common recombinant antibody fragments. ScFv molecule consists of just the antigen-binding VL and VH domains of the whole Ig antibody, joined by polypeptide linker. The Fab of recombinant antibodies is usually more stable and functional than the scFv. The Fab fragments contain the VL and VH chain joined the CL and CH1 to improve the stability of antibody constructs. Recombinant antibodies have been found to be highly effective in immunoassays, with detection limits as low as 3 ng/mL (Dillon et al., 2003). The development of recombinant antibody production technology to

produce antibodies with better affinity than natural antibodies seems promising (Altshuler et al., 2010; Altshuler et al., 2012). Recombinant antibodies offer significant potential for improving the accuracy and efficiency of diagnostic tests across a wide range of medical applications (Basu et al., 2019; Jin et al., 2022; Roth et al., 2021). Recombinant antibody fragments are becoming increasingly popular due to their advantages, such as permeability, small size, ability to maintain antigen identification, and ease of production. Recombinant antibodies have been used to develop robust diagnostic reagents (Holliger & Hudson, 2005).

2.7.4 Phage display technology

Phage display is a method used to isolate binding peptides or proteins from various mutagenic libraries. It involves linking the phenotype (displayed protein) to the genotype (encoded DNA), allowing for the targeted enrichment of specific library pools and efficient screening of resultant clones (Frei & Lai, 2016). A combinatorial library comprising protein or peptide coding DNA that are fused to the gene encoding coat protein of a filamentous phage is created using phage display technology. Proteins or peptides that are fused to the phage coat protein are then displayed on the phage surface. The library can be screened to identify phages that display proteins or peptides that bind to a specific target. The screening is done by several rounds of panning and amplification (Jain et al., 2007).

In 1985, Smith et al. first introduced the phage display technology, enabling cloned antigens to be expressed on the surface of virus (Smith, 1985), and since then, this technology has been substantially and thoroughly utilized in numerous disciplines of study, including antibody production. McCafferty et al. demonstrated that entire antibody variable domains can be displayed on fd bacteriophile surfaces (McCafferty et al., 1990). Phage display is one of the most used techniques in the laboratory for expressing antibody libraries on bacteriophages (Schmitz et al., 2000). Phage display has shown to be a powerful technique for selecting antigen specific antibodies from antibody libraries (Bradbury & Marks, 2004). Phage display technique is one of the most effective molecular diversity techniques, which have been used in the discovery and development of a number of therapeutic tools (Bazan et al., 2012).

For in vitro selection of human antibodies, phage display is considered to be the gold standard method (Lim et al., 2014). Phage display method has become an effective technique for the expression of target genes and the selection of ligands that are specific to targets (Tan et al., 2016). Phage display technology is becoming more important in the medical and health sciences because a large number of different recombinant antibodies that target different antigens have been produced in a short period of time (Kumar et al., 2019). Phage display is a powerful approach

for improving antibody binding qualities by seperating antibody fragments with required characteristics from enormous libraries of mutants formed by mutagenesis from the parental antibody (Korpimaki et al., 2004; Saviranta et al., 1998).

Phage display technology uses a filamentous bacteriophage (Marvin et al., 2014). The filamentous bacteriophage consists of a circular single-stranded DNA genome enclosed by surface coat proteins. The most commonly used are the Ff class of filamentous phage, which includes M13 (**Figure 3**), fl, and fd (Specthrie et al., 1992). The phage infects *Escherichia coli (E.coli)* male strains by attaching the phage's end (p3) to the F pilus on the bacterial surface (Stassen et al., 1994). The M13 phage infects in its host continuously and releases new phages (Ledsgaard et al., 2018).

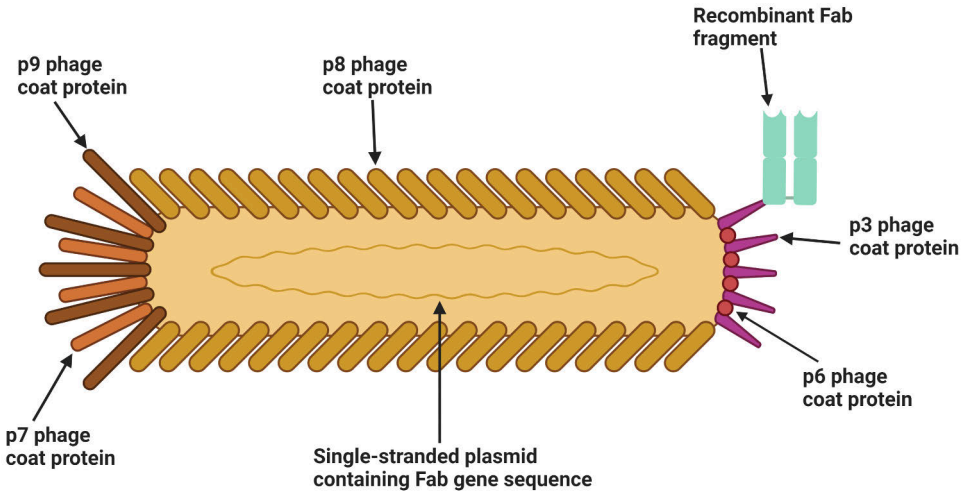

Figure 3: A schematic representation of the filamentous M13 phage. The expression of the recombinant Fab fragment occurs on the surface of phage and gene that codes for fusion protein is inside the phage genome. The phage is about 900 nm long and 6.5 nm wide (Ledsgaard et al., 2018). It has a single-stranded DNA genome and contains 6407 nucleotides (Ledsgaard et al., 2018; van Wezenbeek et al., 1980). The genome consists nine genes that encode a total of 11 proteins. Among these 11 proteins, five are coat proteins, while remaining six proteins are involved in the process such as genome replication, phage assembly, and phage extrusion. Among five coat proteins, the phage capsid is composed of approximately 2700 protein units of the major coat protein p8, while about 5 copies of minor coat proteins p7 and p9 are present at one end, and p3 and p6 are present at other ends of the phage particle (Ledsgaard et al., 2018; Sidhu, 2001). *This figure is created with BioRender.com (2022).*

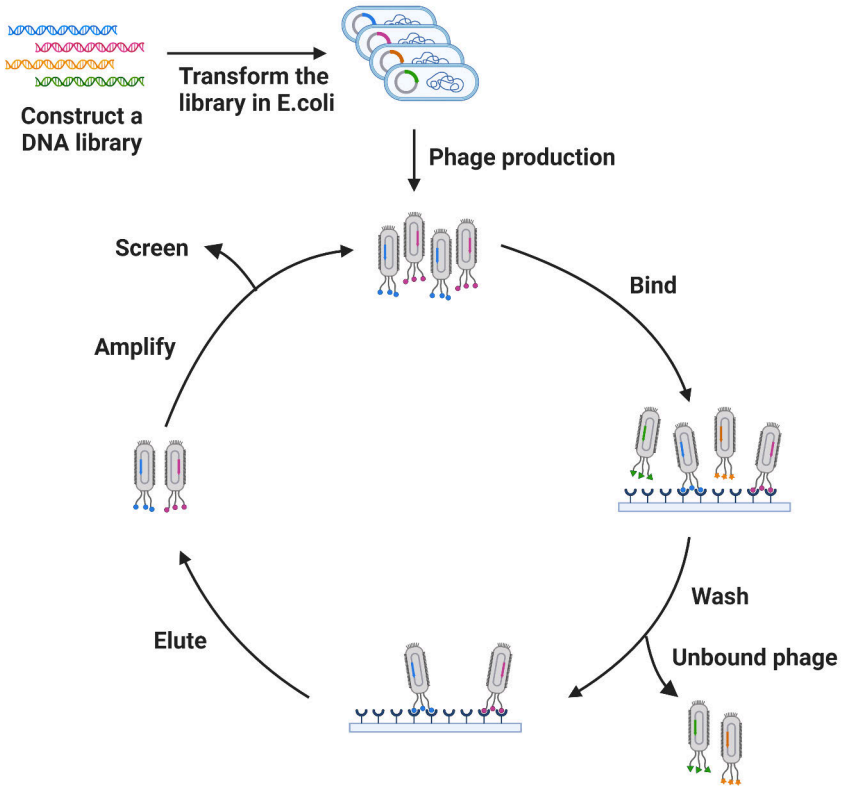

Figure 4: The phage display panning cycle. This figure is created with BioRender.com (2022).

Phage display technique has enabled the generation of antibody libraries comprising a large number of phage particles, each of which encodes and displays a distinct molecule. Biopanning is an important method for selecting specific binders to enrich the levels of desired molecule through multiple cycles of incubation, washing, amplification, and reselection of phage that are bound to target antigen (Bazan et al., 2012). Biopanning process is used to select individual phage particles that display the Fab-p3 fusion proteins. In this process (**Figure 4**), the phage-Fab and antigen are incubated together to capture the specific antibody phage. The non-specific phages are then washed away, and the antigen-specific phages are eluted. For further enrichment, antigen-specific phages are recovered, re-infected into bacterial (*E. coli*) cells for amplification and analysis. The phage pools from every selection round are assessed by immunoassay to assure the enrichment of antigen-specific antibody fragments. Single clones are screened, sequenced, and their affinities are determined (Bazan et al., 2012; Koivunen et al., 1999; Smith & Scott, 1993; Wu et al., 2016).

2.7.5 Antibody affinity maturation in vitro

Affinity maturation is a process to improve affinity and binding interactions in vitro. In vitro affinity maturation involves diversifying antibody fragment sequences and then selecting antibody fragments with higher affinity to the target antigen by phage display selections (Ledsgaard et al., 2018). The affinity of an antibody for its antigen is dependent on the identity and structure of the amino acid sidechains present in the CDRs of both the H and L chains (Bhupal & Diane, 2012). In vitro affinity maturation approaches such as random mutagenesis, site-directed mutagenesis, and chain shuffling are used to improve the antibody fragments' affinity (Altshuler et al., 2010; Bhupal & Diane, 2012; Bradbury & Marks, 2004).

Random mutagenesis is a method that involves the random alteration of any amino acid residues within the sequence of an antibody. The advantage of random mutagenesis is that it does not require any prior information of the structure of antibodies or the amino acid residues involved in their interactions (Altshuler et al., 2010). Error-prone polymerase chain reaction (PCR) is one of the approaches used to perform random mutagenesis (Altshuler et al., 2010; Hawkins et al., 1992; Labrou, 2010). Error-prone PCR utilizes the natural error rate of Taq polymerase to introduce mutations into DNA segment that has been amplified using PCR (Bradbury & Marks, 2004). Error-prone PCR introduces a small number of point mutations randomly over a large area of a target gene by using low-fidelity polymerization conditions (Bhupal & Diane, 2012). Error-prone PCR was used to generate libraries of mutated scFv antibodies and results indicated that libraries with higher mutation rate produced more active clones with improved affinity (Daugherty et al., 2000). Miyazaki et al. found three clones with improved cortisol-binding activity using error-prone PCR (Miyazaki et al., 1999). Error-prone PCR can improve the interaction and strengthen the stability of the antibody's structure by introducing logically inconceivable mutations throughout the sequence (Nagano & Tsutsumi, 2021).

Site-directed mutagenesis is a highly effective method utilized to insert specific and targeted alterations to the DNA sequence at a specific site (Bhatia et al., 2023). Site-directed mutagenesis can improve affinity by mutating selected residues, resulting in a library of high affinity mutants (Sheedy et al., 2007). Site-directed mutagenesis is a method used to introduce specific mutations at desired positions or residues within wild-type gene of an antibody (Sheedy et al., 2007). Site-directed mutagenesis involves altering amino acid residues and is commonly utilized in combination with silico modeling, crystallographic data, and ligand docking programs, to investigate the functions of specific binding site amino acid residues (Bhupal & Diane, 2012; Sheedy et al., 2007; Sivasubramanian et al., 2009). X-ray crystallography analysis was used to conduct alanine mutagenesis scanning (each amino acid, one at a time, was changed to an alanine) of the interfacial residues of

an antibody. This led to a 30-fold increase in binding affinity for the antigen (Yamashita et al., 2019). The advantage of site-specific mutagenesis is that it allows for the efficient production of antibodies with higher affinity without affecting their structural integrity (Nagano & Tsutsumi, 2021).

Chain shuffling is a technique that involves fixing one of the two chains and combining it with a repertoire of partner chains to produce a secondary library that can be searched for better pairings against antigens (Bhupal & Diane, 2012; Ledsgaard et al., 2022). Chain shuffling is an efficient method for accessing to combinatorial libraries, as it allows the expansion of antigen binding clones (Kang et al., 1991). Chain shuffling is an appropriate mutagenesis method when immune libraries have VH and VL sequences, but not useful with naïve libraries because the heavy and light chains have not been exposed to the target antigen (Bhupal & Diane, 2012; Sheedy et al., 2007). Furthermore, chain shuffling is an important technique to increase the binding affinity of antibodies to a specific antigen (Lai et al., 2018).

3 Aims of the study

This study's main goals were to improve the binding affinity of 4D4 antibody utilizing phage display technology and to develop different assay formats for the sensitive and robust detection of iPSA and nPSA.

The following were the main objectives outlined in the original publications or manuscript:

I. To improve the binding affinity of the antibody 4D4 to iPSA using phage display technology.

II. To evaluate the new mutant L3-2 Fab-assisted assay constructs against wt-4D4 Mab-based assays for iPSA and nPSA, along with the reference assays for fPSA and tPSA in plasma samples from a cohort of 105 patients.

III. To expand the clinical cohort for evaluation of iPSA assay using mutant L3-2 Fab as the capture antibody relative to the original iPSA reference assays.

4 Summary of Materials and Methods

In the original publications or manuscript (I-III), the materials and methods used are described in detail. A summary of materials and methods is provided here.

4.1 Bacterial strains and helper phage (I)

E. coli strain XL-1-Blue (Stratagene, USA) was used for the production of new phage stock, *E. coli* strain SS320 was used for the production of phage display libraries (Sidhu et al., 2000), and *E. coli* strain BL21 (New England Biolabs, USA) was used for the production of Fab. VCSM13 helper phage (Stratagene, USA) was used for the production of phage.

4.2 Clinical samples (II-III)

In order to assess the accuracy of biomarkers in the diagnosis of PCa, plasma samples from a cohort of 105 male patients were prospectively collected for study II between 2013 and 2015 (ClinicalTrials.gov identifier NCT01864135) (Jambor et al., 2017). In study III, a total of 310 plasma samples were collected from two cohorts of male patients: 61 from the IMPROD and 249 from the multi-IMPROD trial (ClinicalTrials.gov identifier NCT02241122). The purpose of the study was to assess the accuracy of MRI and biomarkers in the diagnosis of PCa (Jambor et al., 2019). Studies II and III included men with clinical suspicion of PCa due to serum PSA levels higher than 2.5 ng/mL or an abnormal DRE. All patients had systematic biopsies (6 + 6), and if a suspected lesion in MRI, two targeted biopsies were obtained from the lesions for biomarker research.

4.3 Antibodies and PSA standard

4.3.1 Antibodies (Mab or Fab)

Table 1 contains a list of the antibodies that were used.

Table 1: The antibodies that were used for the study.

Antibodies	Specificity	Immunoassay	Study	References
4D4 Mab	iPSA	iPSA tracer	I-III	(Nurmikko et al., 2000)
		iPSA capture	I	
		nPSA blocker	II	
L3-2 4D4 Fab	iPSA	iPSA tracer	I-II	(Liton et al., 2015)
		iPSA capture	I-III	
		nPSA blocker	II	
2C1 Mab	tPSA, T-hK2	nPSA (tracer)	II	(Pettersson et al., 1995)
		fPSA (2C1)[a] tracer	II	
H117 Mab	tPSA	tPSA capture	II-III	Abbot, USA. (Eerola et al., 1997; Piironen et al., 1998)
		fPSA capture	II-III	
5A10 Mab	fPSA	fPSA tracer	II-III	(Lilja et al., 1991)
		iPSA tracer	I-III	
5A10 Fab	fPSA	iPSA capture	I-III	(Eriksson et al., 2000)
		nPSA capture	II	
		fPSA (2C1)[a] capture	II	
H50 Mab	tPSA, T-hK2	tPSA tracer	II-III	Abbot, USA. (Eerola et al., 1997; Piironen et al., 1998)
6H10 Mab	T-hK2	T-hK2 capture, F-hK2 tracer	III	(Becker et al., 2000)
11B6 Mab	F-hK2	F-hK2 capture	III	(Vaisanen et al., 2004)
7G1	tPSA, T-hK2	T-hK2 tracer	III	(Nurmikko et al., 2000)
5F7	tPSA	T-hK2 blocker	III	(Nurmikko et al., 2000)
5H6	tPSA	T-hK2 blocker	III	(Nurmikko et al., 2000)
2E9	tPSA	T-hK2 blocker	III	(Lilja et al., 1991)

[a] fPSA (2C1) assay was performed in parallel with the nPSA assay.

4.3.2 PSA standard

The recombinant proPSA was produced utilizing a baculovirus expression system Trichoplusia ni (Lovgren et al., 1997). The purification of proPSA was carried out using affinity chromatography. The Mab 5A10 was coupled with the AffiGel 10 support matrix. The equilibration of the columns was carried out using 50 mM Tris, pH 7.2, and 0.5 M NaCl. Subsequently, the elution process was performed using

0.2 M glycine, pH 2.5. The eluted proPSA fractions were neutralized by adding Tris-HCl, pH 9 to 100 nM concentration. The standard material was diluted in TSA (pH 7.75) containing 1 g/L protease-free BSA. The concentration range for proPSA in iPSA assay was 0.027-236 ng/mL (I), while the concentration range for iPSA and nPSA assays was 0.019-238 ng/mL, and for tPSA and fPSA assays it was 0.027-236 ng/mL (II). The proPSA concentration range for the iPSA assay (III) was 0.007-167 ng/mL.

4.4 Production of recombinant antibody fragments (I)

4.4.1 Cloning of 4D4 Fab fragment

Reverse transcription PCR was used to clone the recombinant 4D4 Fab fragment from the hybridoma cell line 4D4 Mab (Nurmikko et al., 2000). Subsequently, the Fab fragment was cloned to the bacterial expression vector pAK400 (Krebber et al., 1997).

4.4.2 Mutant 4D4 Fab library construction

Oligonucleotide-directed mutagenesis method was used to construct the three Fab libraries (CDR-L3, CDR-H1, or CDR-H2). The cloned 4D4 Fab was then amplified by PCR. The *BspQI* enzyme was used to digest the PCR products and then ligated to complete Fab gene. The Fab cassette was amplified by PCR, followed by digestion with *SfiI* and ligation with *SfiI*-digested vector pEB32x, according to the method described by Huovinen et al., 2013. The ligation product was inserted into *E. coli* SS320 cells by electroporation, followed by infection with VCS M13 helper phage to produce phage display libraries according to a previously reported protocol by Brockmann et al., 2011.

4.4.3 Panning mutant libraries for affinity-improved binders

Phage display selection against wt-PSA was done three rounds on the libraries. The phage libraries were subjected to negative selection against streptavidin and Mab H117. The libraries were incubated for one hour on microtiter plate coated with streptavidin and then on a microtiter plate coated with streptavidin and bound with biotinylated H117 (bio-H117). Subsequently, native mouse IgG and streptavidin that had been blocked with biotin were added. Next, the wt-PSA was subjected to incubation with the preselected libraries for one hour. Following this, the phages that were bound to PSA were captured for one hour by streptavidin beads immobilized

with bio-H117 Mab. Beads were collected using a magnet and washed two or three times with TBT-0.1 and subsequently one wash with TBS. The phages were eluted using hydrochloric acid, and subsequently neutralized with Tris buffer. The eluted phages were used to infect XL1-Blue cells, and fresh phage stocks were produced (Brockmann et al., 2011). The calculation of the panning output was done by plating aliquots of infected cells. To evaluate the background, a similar panning was done without PSA.

4.4.4 Antibody fragment production, purification, biotinylation and labeling

The Fab was produced in BL21 cells in one-liter flask cultures. The cells were sonicated to release the Fab. The purification of Fab was done using DEAE matrix anion-exchange column, immobilized metal affinity column, and HiTrap Protein G HP chromatography column. Site-specific biotinylation was performed on recombinant 5A10 Fab and 4D4 Fab fragments with maleimide-PEG2-biotin (Eriksson et al., 2000). The Mabs were biotinylated with biotin isothiocyanates (Eriksson et al., 2003). Europium N1 chelates were used to label the Mab, Fab fragments, and PSA (Vaisanen et al., 2006).

4.5 Immunoassays

4.5.1 General immunoassay protocol (I-III)

The immunoassays were preformed manually using 96-well microtiter plates coated with either streptavidin or polyclonal rabbit anti-mouse IgG antibody (RAM). The basic protocol was followed with three steps: one-hour incubation at room temperature (RT), washing two times after capture antibody and sample incubation, and washing four times after label antibody incubation. Time-resolved fluorescence (TRF) was measured with the Victor 420 Multilabel Counter (Perkin-Elmer Life Sciences) after 5 to 10 minutes of enhancement solution incubation. MultiCalc software (Perkin-Elmer Life Sciences) was used to calculate the concentrations of the unknown samples.

4.5.2 Assay buffer

The buffers utilized in the immunoassays consisted of Kaivogen assay buffer (KG buffer) and Kaivogen iPSA assay buffer (KG iPSA buffer) were purchased from Kaivogen, Turku, Finland. The MES assay buffer was prepared with either 50 or 200 mM of 2-(N-morpholino) ethanesulfonic acid with pH values of 6.0 or 6.75 with

the addition of 0.9% NaCl, 0.01% Tween-40, and 0.05 or 0.5% BSA. A blocking component consisting of denatured mouse IgG at a concentration of 25 µg/mL was included in the KG and MES assay buffers.

4.5.3 Soluble Fabs screening (I)

The process involved the inoculation of individual clones into a 96-well culture plate containing 200 µL Super Broth medium. The cellular membranes were disrupted through a freeze-thaw process, which was subsequently followed by the addition of a lysis buffer to facilitate the release of the periplasmic Fab. The diluted Fab containing supernatant was added to a plate coated with RAM. The plate was incubated for one hour and washed four times. The PSA binding activity of Fab was measured by adding 10 ng per well of Eu-labeled PSA (Eu-PSA), while the total amount of Fab per well was quantified by adding 20 ng per well of Eu-RAM. The plate was incubated for one hour and washed four times. TRF was measured as previously. Purified wt-4D4 Fab was used as a calibrator to measure the Fab concentrations. In order to determine the affinity, the ratio of the Eu-PSA signal to the ng Fab was calculated.

4.5.4 Binding affinity and kinetics determination (I)

Fabs were grown in 20 mL cultures to compare the rates of association (on-rates) and dissociation (off-rates) and the corresponding dissociation constants (Kds). Sonication was used to lyse the cells, and the supernatants were utilized for analysis. Immunoassay utilizing Eu-RAM was used to determine Fab concentrations. To initially measure the binding characteristics of the isolated clones, mutated and wt-4D4 Fabs (20 ng) were added in 100 µl of KG assay buffer on RAM well, incubated for one hour at RT, and washed four times. Eu-PSA was added 10 ng per well in 100 µl MES buffer (pH 6.5) and incubated for five hours at 12 °C. The fluorescence was enhanced and measured after aspiration and a single wash. The effect of pH was investigated by repeating the same procedure using MES buffer with pH ranging from 6.0 to 6.75 and Tris buffer with pH ranging from 7.0 to 8.5. The off-rates of each mutant were measured by binding 10 ng per well of Eu-PSA to RAM immobilized Fabs at 12 °C for five hours in MES assay buffer, pH 6.5. Cold MES assay buffer, pH 6.5 (100 µl) was added and aspirated after incubation at 12 °C for a range of periods (0-60 minutes) to observe the dissociation of the PSA from the Fab. To determine the on-rates, RAM immobilized Fabs were incubated with Eu-PSA (5 ng per well) in 100 µl of MES assay buffer, pH 6.75 at RT for a range of periods (0–60 minutes). To determine the Kd values, Eu-PSA at concentrations ranging from 0.015 to 600 ng per well was added in 100 µl of MES assay buffer, pH

6.75 and incubated for five hours at 12 °C. The Scatchard method (Scatchard, 1949) was used to calculate the Kd values.

4.5.5 Analytical sensitivity and linearity determination (I)

The standard (proPSA) curve was used to determine the analytical sensitivity of the iPSA assays. To calculate the analytical sensitivity, the measurement data were fitted with a power model and reading the analyte concentration at the cut-off value. The equation that was used to calculate the cut-off level was "average background of three replicates + 3x standard deviation (SD) of the background".

The standard (proPSA) curve of the iPSA assay with MES 6.75 or KG assay buffer was used to determine the linearity. The binding efficiencies of Mab and Fabs were directly compared by dividing the corresponding signals by the labelling degree. Furthermore, labelled 4D4 antibody signals were divided by concentrations of PSA standard to determine the linearity of the signal.

4.5.6 tPSA and fPSA immunoassays (II-III)

The concentrations of tPSA and fPSA were measured as reported previously (Vaisanen et al., 2004). The bio-H117 was used as the capture antibody in both assays at a concentration of 300 ng per well in 100 µL of assay buffer. In each well, 25 µL of standard (proPSA) and samples were added to 100 µL of assay buffer. The Europium labelled (Eu) tracer Eu-H50 and Eu-5A10 Mab were used for tPSA and fPSA assays, respectively, at a concentration of 100 ng per well in 200 µL of assay buffer.

4.5.7 iPSA immunoassay (I-III)

The iPSA assays using wild-type 4D4 (I-W) or mutant 4D4 L3-2 Fab as a tracer (I-M) and capture (I-MC) were performed as previously described (Khan et al., 2019; Liton et al., 2015). For study III, the capture antibody bio-5A10 Fab was used for the I-W assay at a concentration of 150 ng per well in 100 µL of KG assay buffer and bio-L3-2 Fab was used for the I-MC assay at a concentration of 100 ng per well in 100 µL of 200 mM MES assay buffer. In each well, 50 µL of standard (proPSA) and samples were added to 100 µL of assay buffer (KG iPSA buffer for I-W and 200 mM MES assay buffer for I-MC). The Eu-4D4 Mab was used for I-W assay at a concentration of 200 ng per well in 200 µL of KG iPSA buffer, and Eu-5A10 Mab was used for I-MC assay at a concentration of 100 ng per well in 200 µL of 200 mM MES assay buffer.

4.5.8 nPSA immunoassay (II)

The nPSA assay was performed as previously described (Khan et al., 2019; Peltola, Niemela, Alanen, et al., 2011). The capture antibody bio-5A10 Fab was used for nPSA assay at a concentration of 150 ng in 100 µl of 50 mM of MES assay buffer, pH 6.75. In each well, 50 µL of standard (proPSA) and samples were added to 100 µL of 50 mM of MES assay buffer. To block all iPSA, 2000 ng of 4D4 Mab or mutant L3-2 Fab were added in 100 µL of 50 mM of MES assay buffer in each well. The Eu-2C1 Mab was added at a concentration of 100 ng in 50 µL of 50 mM of MES assay buffer after two hours incubation at RT and without a washing step.

A similar procedure was used for measuring fPSA concentrations in parallel with the nPSA immunoassay. In the blocking step, 50 mM of MES assay buffer was used instead of the iPSA blocking antibody. Since the intact proPSA standard material was blocked in the nPSA assay, the nPSA concentrations were calculated using the fPSA (2C1) immunoassay standard curve. The efficiency of the 4D4 Mab or mutant L3-2 Fab blocking was calculated using the nPSA assay standard curve.

Calculated nPSA (CN) concentrations (II-III)

The CN concentrations were used in publication II and publication/manuscript III. The concentration of CN was obtained by subtracting the concentration of iPSA from the concentration of total fPSA as previously described (Peltola, Niemela, Vaisanen, et al., 2011).

4.5.9 hK2 immunoassay (III)

The T-hK2 and F-hK2 assays were done as previously reported (Vaisanen et al., 2004). The capture antibodies bio-6H10 for T-hK2 and bio-11B6 Mab for F-hK2 were used at a concentration of 300 ng per well in 100 µL of assay buffer. For T-hK2, the blocking antibodies 2E9, 5H6, and 5F7 in assay buffer were added. In each well, 100 µL of standard (recombinant hK2) and samples were added to 100 µL of assay buffer immediately after the blocking antibodies. Eu-7G1 Mab for T-hK2 and Eu-6H10 Mab for F-hK2 were added at a concentration of 100 ng per well in 200 µL of assay buffer. TRF was measured as described previously.

4.6 Statistical analyses (II-III)

In study II, statistical analysis [receiver operating characteristics (ROC) and estimating areas under the curve (AUC)] was performed on each assay results and the corresponding ratios in relation to the clinical parameters, namely the status of

biopsy positive (cancer) or biopsy negative (non-cancer), the grading of the biopsy (Gleason 6-9), and the prostate gland volume (PV).

In study III, the ISUP Gleason Grade Group (Epstein et al., 2016) is provided in terms of frequency and proportion using the whole cohort. For the analysis, the GS of the patient was used as the "ground truth" and dichotomized into benign/GS = 6 and GS ≥ 7. The DeLong approach (DeLong et al., 1988; Hanley & Hajian-Tilaki, 1997) was used to calculate the AUC with the corresponding 95% confidence intervals (CI). The statistical significance of the difference between benign/GS = 6 and GS ≥ 7 was assessed using a Mann-Whitney U-test. Logistic regression models were fitted in order to analyze the combination of variables. The model's predictive performance was assessed by calculating the mean AUC and SD over hold-out cross-validation repeated 10000 times.

5 Summary of Results

5.1 Oligonucleotide-directed mutagenesis libraries: construction and enrichment (I)

To enhance affinity of the 4D4 antibody, recombinant 4D4 Fab was cloned and three different Fab libraries were constructed. Each library was designed to contain mutations in one CDR loop. Table 2 in the original publication I show the planned mutation in the libraries. The Phage display approach was used to select the libraries against wt-PSA to enrich the affinity-improved binders. The number of phages recaptured during each cycle of panning was counted to determine the enrichment of specific binders. Low specific binders and a high background proportion were seen in the first round of panning. In the subsequent rounds, the output of specific binders increased while the proportion of background decreased. The ratio of background to output for CDR-H1, CDR-L3, and CDR-H2 libraries decreased from 30, ~100, and 45% in the first round to 1, 2 and 28%, respectively, in the third round.

5.2 Mutant clones selection and characterization (I)

A total of 930 individual clones were isolated from the panned libraries and screened to find the highest affinity clones. In the screening assay, the signal of 31 clones was two to five times greater than the wt-4D4 Fab, whereas the remainder had lower signals (data not shown). When the clones were sequenced, 14 of the 31 were unique. In these 14 clones, 1 to 5 amino acids were mutated either in CDR-H1, -H2 or -L3 loop. To remove the effect of expression level on the result, these clones were evaluated for affinity by dividing the signal from bound PSA with the amount of Fab in the assay. The PSA/ng Fab signal of the selected five best clones was two to four times higher than the wt-4D4 Fab (**Figure 5**).

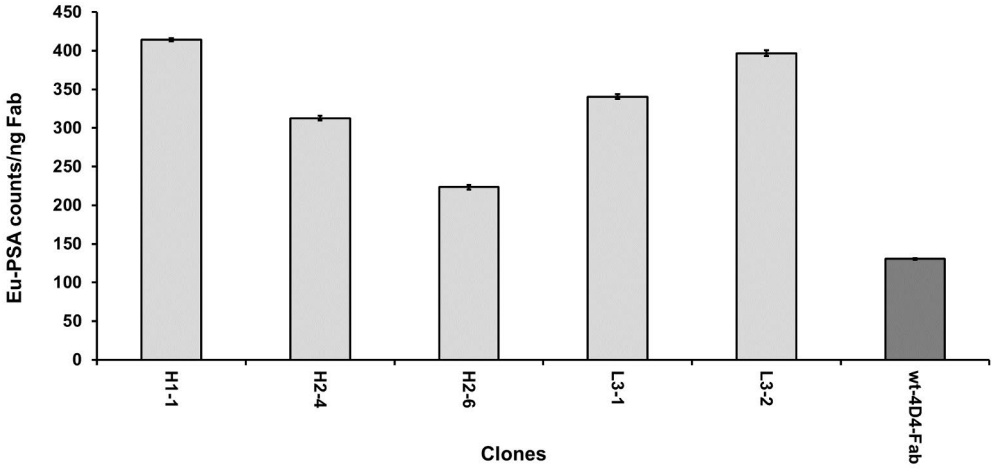

Figure 5: Affinity screening of the best five unique clones. The SD of the three replicates are shown as error bars in the graph.

The off-rates of both wt-4D4 and the mutated clones were determined by observing the dissociation of a complex of antigen and antibody in the immunoassay. In the off-rate assay, it was observed that when KG buffer was added for off-rate incubation, the signal dropped immediately down to ~10% (results not shown). The low signal was a problem in the immunoassay. DTPA and pH were found to be a problem. Therefore, assay buffer was optimized without DTPA with pH 6.0–8.5 at +12 ºC with wt-4D4 Fab and five mutated clones. The wt-4D4 Fab and mutated clones gave the highest signal at pH 6.5–7.0 (Figure 2 in original publication I).

The off-rate of the mutated clones was measured at pH 6.5. After 60 minutes of incubation at +12 ºC in the MES assay buffer, pH 6.5, the mutated clones selected from the libraries had a slower off-rate than the wt-4D4 Fab (**Figure 6**). The on-rate was similar with all clones, and both the mutated clones and the wt-4D4 Fab achieved maximum binding (100%) at 25 minutes (results not shown).

At pH 6.75 in the optimized assay buffer, the Kd value of all the five selected mutated clones was lower (0.3–0.9 nM) than the wt-4D4 Fab (1.8 nM). Among the mutated Fabs, L3-2 had the lowest Kd value (0.3 nM), showing six times better affinity that was than the wt-4D4 Fab.

The specificity of the five mutated clones for iPSA was characterized by measuring their cross-reactivity against seminal plasma containing ≈ 95% nPSA (Zhang et al., 1995). The cross-reactivity of Fabs L3-2, H2-6, and H1-1 (6, 4, and 3%, respectively) is somewhat similar, and the cross-reactivity of Fabs H2-4 and L3-1 (18 and 12%, respectively) is higher than that of the wt-4D4 Fab (5%).

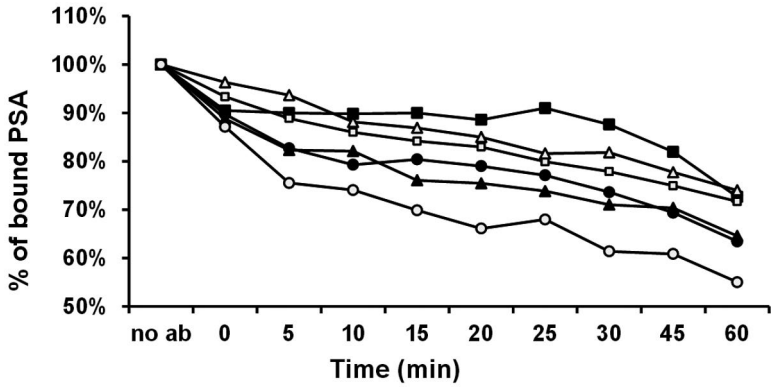

Figure 6: Dissociation of wt-PSA from the Fab clones as a function of time. Eu-labeled PSA was bound to Fab on anti-mouse IgG coated microtiter plate, and unbound PSA was then washed away. MES assay buffer pH 6.5 was added and the off-rate was followed by measuring the percentage of bound PSA after 0–60 minute incubation. No ab refers to the original signal. Zero (0) minute refers to the initial signals after adding and immediately removing the assay buffer. 100% is the maximum binding. Symbols refer to clones H1-1(■), H2-4 (▲), H2-6 (●), L3-1 (□), L3-2 (Δ), wt-4D4 Fab (○) (Figure 3 in original publication I).

5.3 Analytical sensitivity and linearity of iPSA assay (I)

In the iPSA assay, the mutant L3-2 Fab was compared to the wt-4D4 Fab and the wt-4D4 Mab that was used in the reference iPSA assay as a tracer (Nurmikko et al., 2000). When wt-4D4 Mab, wt-4D4 Fab and mutant Fab were used as tracer in iPSA assay, the assay signal with L3-2 Fab was 26 times higher than with the wt-4D4 Fab and two times higher than with the wt-4D4 Mab. The relative analytical sensitivity with L3-2 Fab, wt-4D4 Fab, and wt-4D4 Mab was 0.12, 4.46, and 0.26 µg/L, respectively (**Figure 7**).

The linearity of the signal was also calculated to compare how well the wt-4D4 Mab, Fab and mutant Fab worked in the iPSA assays as a tracer when different assay buffers (KG pH 7.75 or the MES pH 6.75) were used. When utilizing the MES assay buffer with a pH of 6.75, both the wt-4D4 Mab and mutant L3-2 Fab demonstrated similar and highly linear performance (Figure 5A in original publication I). However, it was observed that wt-4D4 Mab deviated from the expected signal linearity when the KG buffer pH 7.75 was used (Figure 5B in original publication I). The wt-4D4-Fab's performance as tracer lags behind significantly in both buffer options, showing a 25 to 50-fold lower signal than the wt-4D4 Mab or L3-2 Fab.

When an equal molar amount of bio-wt-4D4 Mab, wt-4D4 Fab and mutant L3-2 Fab were used as capture antibody in the iPSA assay, the assay signal with L3-2 Fab in MES assay buffer was three and five times higher than with the wt-4D4 Fab and wt-4D4 Mab, respectively. The relative analytical sensitivity with wt-4D4 Mab,

wt-4D4 Fab and mutant L3-2 Fab was 0.25, 0.18 and 0.07 µg/L, respectively. Mutant L3-2 Fab showed optimal performance in the MES assay buffer with almost perfect linearity. However, the signals of wt-4D4 Mab and Fab showed deviations from linearity, particularly at lower concentrations of PSA (Figure 7A in original publication I).

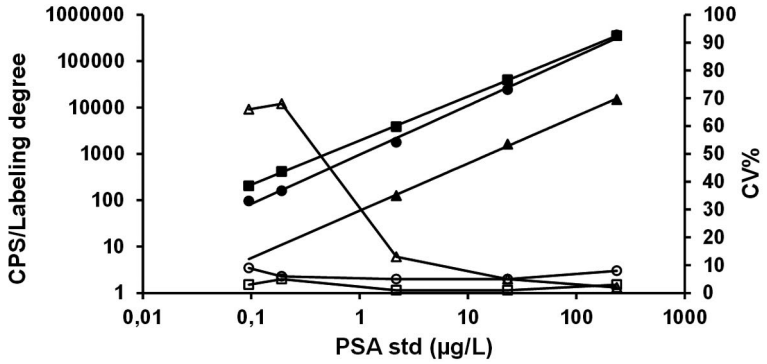

Figure 7: iPSA assay using 4D4 binders as tracers options. iPSA assay standard curves (filled symbols) and CVs of standard concentrations (open symbols). The assay used bio-5A10 Fab as a capture antibody and wt-4D4 Mab (● and ○) (200 ng/well/200 µl in KG assay buffer), wt-4D4 Fab (▲ and ∆) (50 ng/well/200 µl in MES assay buffer) and L3-2 Fab (■ and □) (50 ng/well/200 µl in MES assay buffer) as a tracer antibody. The labeling degrees of Eu3+-labeled wt-4D4 Mab, wt-4D4-Fab and L3-2 Fab were 6.6 Eu3+/Mab, 1.7 and 1.5 Eu3+/Fab, respectively. The counts per second (CPS) from iPSA assay were divided by the labeling degree of Mab and Fab (Figure 4 in original publication I).

5.4 Evaluation of mutant L3-2 Fab-assisted assay using IMPROD plasma samples (II)

A total of 105 plasma samples were collected from male patient with or without cancer. The objective was to compare the performance of the mutant L3-2 Fab-assisted assay constructs with the reference wt-4D4 Mab-based assays to determine iPSA and nPSA. Central features and abbreviated versions of the assay designs and calculations that were used to obtain iPSA or nPSA measurements are shown in **Table 2.** Antibody 5A10, which is specific to fPSA, was used in all the assays either for capturing or as a tracer; the two assays for nicked PSA used Mab 2C1 as a tracer (**Table 2)** (Table 1 in original publication II).

Table 2: Use of 4D4 in 5 various assays and calculations for the determination of iPSA and nPSA.

Assays and calculations	Short form
1. Assay of iPSA using wt-4D4 Mab as the tracer (reference assay)	I-W
2. Assay of iPSA using mutant 4D4 (L3-2 Fab) as the tracer	I-M
3. Assay of iPSA using mutant 4D4 (L3-2 Fab) for capturing	I-MC
4. Assay of nPSA using wt-4D4 Mab for blocking of iPSA	N-W
5. Assay of nPSA using mutant 4D4 (L3-2 Fab) for blocking of iPSA	N-M
6. Calculated nPSA: fPSA − I-W	CN(I-W)
7. Calculated nPSA: fPSA − I-M	CN(I-M)
8. Calculated nPSA: fPSA − I-MC	CN(I-MC)

The patient population consisting of 73 men with cancer and 32 without cancer (non-cancer) is outlined in Table 2 in the original publication II. Statistically significant difference of cancer vs. non-cancer groups was not achieved by any of the three versions of the iPSA assays, namely I-M, I-MC, and I-W ($P > 0.05$). The calculated levels of nPSA (CN: I-W, I-M, I-MC) concentrations significantly distinguished the two groups, the I-MC assay demonstrated the best performance ($P = 0.008$).

Table 3: AUCs and *P* values of the ratios of different plasma PSA forms with Cancer vs non-Cancer, prostate volume (PV) ≤ 35 mL, prostate volume (PV) > 35 mL, and Cancer + Gleason Score (GS) ≥ 7 vs non-Cancer + GS6 groups. PSA values were measured with the different assay formats described in **Table 2**.

	Ca (n = 73) vs non-Ca (n = 32)		**Ca (n = 41) vs non-Ca (n = 11) PV ≤ 35 mL**		**Ca (n = 24) vs non-Ca (n = 20) PV > 35 mL**		**Ca + GS ≥ 7 (n = 56) vs non-Ca + GS6 (n = 49)**	
Parameter	AUC	*P* value	AUC	*P* value	AUC	*P* value	AUC	*P* value
F/T	0.857	≤0.0001	0.805	0.002	0.840	0.0001	0.795	≤0.0001
I-W/F	0.650	0.016	0.722	0.025	0.570	NS	0.515	NS
I-M/F	0.634	0.029	0.605	NS[a]	0.602	NS	0.538	NS
I-MC/F	0.753	≤0.0001	0.825	0.001	0.710	0.017	0.693	0.001
N-W/T	0.819	≤0.0001	0.776	0.005	0.834	0.0002	0.832	≤0.0001
N-M/T	0.789	≤0.0001	0.700	0.044	0.831	0.0002	0.809	≤0.0001
CN(I-W)/T	0.854	≤0.0001	0.818	0.001	0.824	0.0002	0.722	0.0001
CN(I-M)/T	0.828	≤0.0001	0.772	0.006	0.824	0.0002	0.741	≤0.0001
CN(I-MC)/T	0.870	≤0.0001	0.861	0.0003	0.865	≤0.0001	0.806	≤0.0001

[a] NS, not significant.

The AUC values corresponding to various calculated ratios of different PSA forms are shown in **Table 3** (Table 4 in original publication II). In the whole cohort, it was observed that the ratio of calculated nPSA: fPSA − I-MC [CN(I-MC)] over total PSA (T) showed the highest AUC value of 0.870 when compared to all other ratios over tPSA. The I-MC performed significantly better than the I-W and I-M assays when evaluated as ratios to fPSA (F) (**Figure 8**). The I-MC/F and CN(I-MC)/T ratios showed the best performance among the parameters in the PV ≤ 35 mL group. The AUC for I-MC/F and CN(I-MC)/T was 0.825 (P = 0.001) and 0.861 (P = 0.0003), respectively. The highest performance was seen in the PV > 35 mL group with the ratio of CN(I-MC) over tPSA (AUC 0.865; P ≤0.0001).

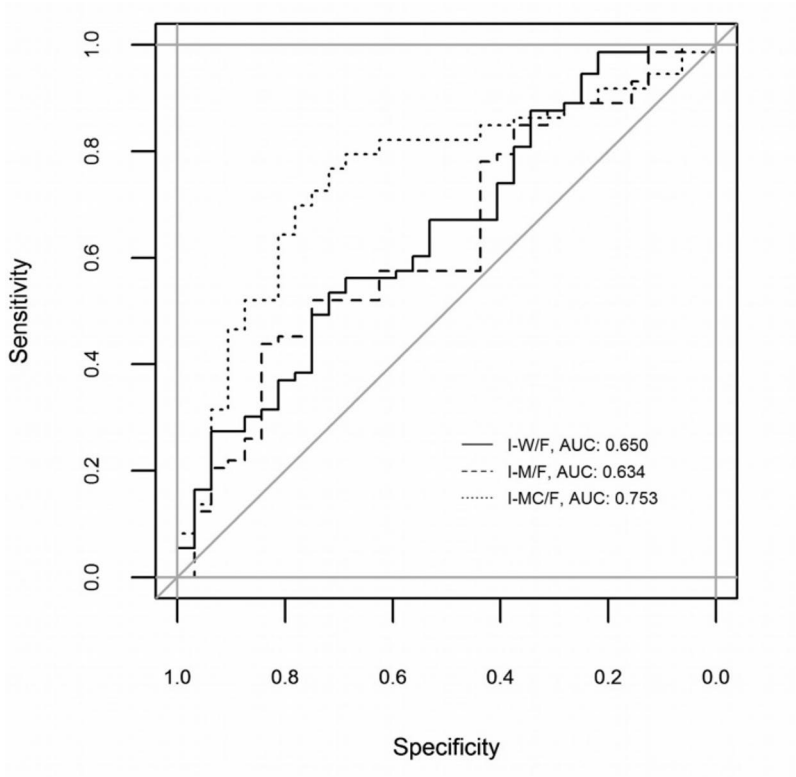

Figure 8: ROC curve for the differentiation of patients with Ca from those with non-Ca. Plot represents (specificity) at different sensitivities for percent I-W/F, I-M/F, and I-MC/F (Figure 1 in original publication II).

ROC analysis was further used to examine any combination of three assays (tPSA, fPSA, iPSA and nPSA). The highest partial AUCs (90 -100% specificity range) were seen for the combinations of tPSA + fPSA + I-MC and tPSA + fPSA + CN(I-MC) (0.784) when comparing cancer vs. non-cancer. The combination of tPSA + fPSA + measured nPSA proved most effective in distinguishing Ca + GS ≥7 from non-ca + GS6 (Figure 2 in original publication II).

5.5 Evaluation of mutant L3-2 Fab-assisted assay using IMPROD/multi-IMPROD plasma samples (III)

The clinical cohort (plasma samples, n = 310) was increased to evaluate the I-MC assay utilizing the mutant L3-2 Fab as the capture antibody in comparison to the original iPSA reference assays that are frequently used in clinical settings. In publication II, iPSA was compared to fPSA and tPSA alone. In the

manuscript/publication III, the diagnostic performance of two assay designs of hK2 (T-hK2 and F-hK2) was also compared.

The patient cohort includes 163 patients with benign and low-grade cancer (Benign/GS = 6), as well as 147 patients with clinically significant cancer (GS ≥ 7). The calculated nicked PSA based on the I-W and I-MC substantially and equally differentiated the two groups. All the iPSA over fPSA ratios and all the calculated nPSA over tPSA ratios (including the F/T ratio) significantly distinguished the two groups (P <0.0001) (Table 1 in manuscript/publication III).

Table 4: AUCs and *P* values of different plasma PSA forms and ratios with Benign/GS = 6 vs GS ≥ 7, PV ≤ 38 mL and PV > 38 mL groups.

	Benign/GS = 6 (n = 163) vs GS ≥ 7 (n = 147)		**Benign/GS = 6 (n = 67) vs GS ≥ 7 (n = 90) PV ≤ 38 mL**		**Benign/GS = 6 (n = 96) vs GS ≥ 7 (n = 57) PV > 38 mL**	
Parameter	AUC (95% CI)	*P* value	AUC (95% CI)	*P* value	AUC (95% CI)	*P* value
Age	0.65 (0.59–0.71)	<0.0001[b]	0.69 (0.61–0.77)	<0.0001[b]	0.67 (0.58–0.76)	0.001[b]
PV			0.54 (0.45–0.63)	0.359	0.59 (0.50–0.68)	0.061
T	0.62 (0.56–0.68)	0.0003[b]	0.70 (0.61–0.79)	<0.0001[b]	0.60 (0.51–0.69)	0.039[a]
F	0.54 (0.48–0.60)	0.2224	0.61 (0.52–0.70)	0.017[a]	0.56 (0.46–0.66)	0.230
I-W	0.52 (0.46–0.58)	0.6211	0.62 (0.53–0.71)	0.010[a]	0.50 (0.40–0.60)	0.991
I-MC	0.56 (0.50–0.62)	0.0667	0.67 (0.58–0.76)	0.0002[b]	0.55 (0.45–0.65)	0.293
T-hK2	0.56 (0.50–0.62)	0.0659	0.69 (0.61–0.77)	<0.0001[b]	0.53 (0.43–0.63)	0.603
F-hK2	0.55 (0.49–0.61)	0.1053	0.71 (0.63–0.79)	<0.0001[b]	0.51 (0.41–0.61)	0.768
CN(I-W)	0.58 (0.52–0.64)	0.0106[a]	0.56 (0.47–0.65)	0.169	0.60 (0.51–0.69)	0.032[a]
CN(I-MC)	0.58 (0.52–0.64)	0.0164[a]	0.56 (0.47–0.65)	0.239	0.60 (0.51–0.69)	0.047[a]
F/T	0.69 (0.63–0.75)	<0.0001[b]	0.61 (0.52–0.70)	0.019[a]	0.67 (0.58–0.76)	0.0004[b]
CN(I-W)/T	0.69 (0.63–0.75)	<0.0001[b]	0.57 (0.47–0.67)	0.156	0.71 (0.63–0.79)	<0.0001[b]
CN(I-MC)/T	0.71 (0.65–0.77)	<0.0001[b]	0.63 (0.54–0.72)	0.006[a]	0.71 (0.62–0.80)	<0.0001[b]
I-W/F	0.63 (0.57–0.69)	<0.0001[b]	0.53 (0.43–0.63)	0.508	0.65 (0.56–0.74)	0.002[b]
I-MC/F	0.68 (0.62–0.74)	<0.0001[b]	0.65 (0.56–0.74)	0.002[b]	0.66 (0.57–0.75)	0.001[b]

[a] Significance at the 0.05 level.

[b] Significance at the Bonferroni-adjusted 0.004 level.

The outcome of ROC analysis of benign/GS = 6 vs GS ≥ 7 are presented in **Table 4** (Table 2 in original manuscript/publication III). The analysis includes both individual parameters and their corresponding ratios in the whole cohort and subgroups with PV ≤ 38 mL and PV > 38 mL. In the PV ≤ 38 mL group, the measured tPSA, T-hK2 and F-hK2 assays separated two groups significantly (AUC 0.69-0.71, P <0.0001). The I-MC assay provided the best separation (AUC 0.67, P = 0.0002)

among the fPSA forms, significantly outperforming fPSA (AUC 0.61). The ratio of CN(I-MC/T (AUC 0.63, $P = 0.006$) and I-MC/F (AUC 0.65, $P = 0.002$) provided the best separation. However, the ratios calculated using the I-W did not achieve a level of significance. In the PV > 38 mL group, the CN(I-W)/T and CN(I-MC)/T ratios provided the best and similar separation (AUC 0.71, $P < 0.0001$). The AUC of F/T ratio was 0.67 ($P = 0.0004$).

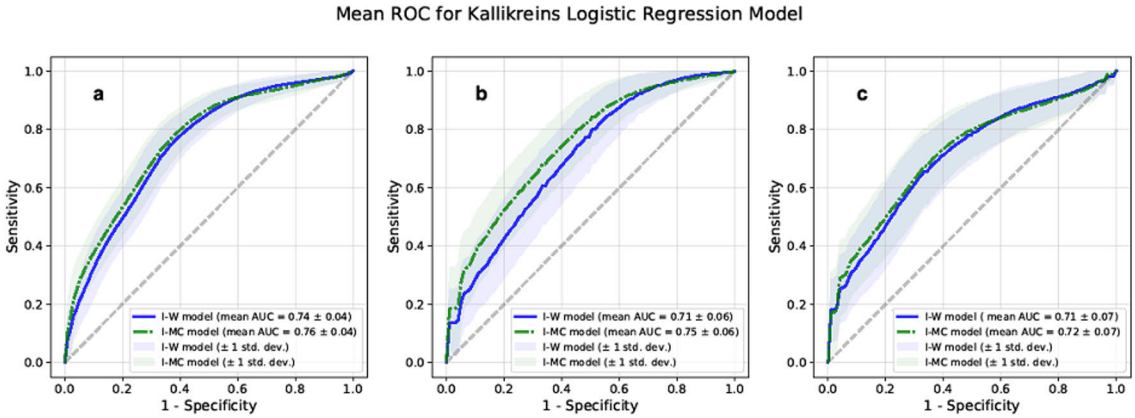

Figure 9: Mean ROC curves and mean AUC obtained by performing holdout cross-validation (30% test and 70% training) 10000 times to assess the two Kallikreins Logistic Regression models. a) whole cohort b) gland volume ≤ 38 mL and c) gland volume > 38 mL (Figure 1 in original manuscript/publication III).

The logistic regression model of tPSA, fPSA, and T-hK2 was subjected to further analysis in combination with I-W or I-MC with the whole cohort and the subgroups with PV ≤ 38 mL and PV > 38 mL, as shown in Table 3 in the original manuscript/publication III. In the whole cohort, when comparing Benign/GS = 6 with GS ≥ 7, the respective AUCs of I-W and I-MC were 0.74 and 0.76 **(Figure 9a)**. The contribution of I-MC to the model (OR, 33.254; $P < 0.001$) was significantly higher than that of I-W (OR, 4.663; $P = 0.009$). In the PV ≤ 38 mL group, the AUCs of I-W and I-MC were 0.71 and 0.75 **(Figure 9b)**. The contribution of I-W to the model was not statistically significant (OR, 2.301; $P = 0.334$). However, the odds ratio for I-MC was significantly increased, reaching statistical significance (OR, 83.650; $P = 0.011$). In the PV > 38 mL group, the AUCs of I-W and I-MC were 0.71 and 0.72, respectively **(Figure 9c)**. Both I-W (OR, 6.368; $P = 0.028$) and I-MC (OR, 19.062; $P = 0.008$) contributed substantially to the model. It is important to note that T-hK2 ($P = 0.110$ or 0.091) did not contribute to the model in the PV > 38 mL group.

The Logistic Regression model of tPSA and T-hK2 was also subjected to analysis in combination with CN(I-W or I-MC) and with I-W/F or I-MC/F.

In the whole cohort, when comparing Benign/GS = 6 with GS ≥ 7, the AUC of CN(I-W) (AUC 0.75) was almost identical to CN(I-MC) (AUC 0.76). In the PV ≤ 38 mL group, the AUC of the CN (I-MC) (AUC 0.74) was slightly higher than the CN (I-W) (AUC 0.72). However, the AUC of CN (I-MC) was identical to CN (I-W) (AUC 0.72) in the PV > 38 mL group (data not shown).

In comparing Benign/GS = 6 with GS ≥ 7, I-MC/F (AUC 0.72) performs better than the I-W/F (AUC 0.66) in the whole cohort as well as in the PV ≤ 38 mL group (AUC was 0.74 for I-MC/F and 0.69 for I-W/F). However, the AUC of I-MC/F (AUC 0.69) was almost identical to the I-W/F (AUC 0.68) in the PV > 38 mL group (data not shown).

6 Discussion

PSA is a widely used marker for PCa. The use of a panel of four kallikrein immunoassays has shown potential in improving the prediction of prostate biopsy outcomes in men with elevated PSA levels. The assay of iPSA, one of the kallikrein forms, relies on a unique antibody 4D4 Mab. Due to low binding affinity and fast dissociation rate (off-rate) of the 4D4 Mab (Nurmikko et al., 2000), it cannot capture the iPSA tightly or in the nPSA assay cannot block all the iPSA in the sample (Peltola, Niemela, Alanen, et al., 2011). The aims of this study were to improve the binding affinity of 4D4 antibody utilizing phage display technology to develop different assay formats for the sensitive and robust detection of iPSA and nPSA, and to compare the performance of the affinity improved mutant L3-2 Fab-assisted assay constructs with the reference wt-4D4 Mab-based assays to determine iPSA and nPSA in plasma samples.

In this section, the results of this study are discussed. Detailed information is available in the original publications or manuscript (I-III).

6.1 Improvement of antibody affinity (I)

The objective was to improve the binding capability of the 4D4 antibody to achieve more accurate and reliable measurements of iPSA. To improve the affinity, the recombinant form of Mab 4D4 was generated. Recombinant Fab fragments have an advantage over Mabs because they allow site-specific chemical changes, making site-specific biotinylation of Fab more effective than chemical biotinylation of Mab (Ylikotila et al., 2006). Antibody affinity significantly impacts immunoassay performance. The affinity of the mutant Fabs was improved by 2 to 6 times when compared to the wt-4D4 Fab. This improvement was achieved by using oligonucleotide-directed mutagenesis and phage display-based selection and screening. Previously, Muller et al. have utilized phage display technology in their study to improve the affinity (8.5 fold) of the 5D3D11 antibody targeting PSA (Muller et al., 2011). A reduction in off-rate is often the main cause of an improved affinity by phage display (Marks et al., 1992), as was seen with the mutants in this study. The cross-reactivity towards nPSA was measured against seminal plasma containing ≈ 95% nPSA (Zhang et al., 1995), and it was as low as that of wt-4D4

Fab. The L3-2 Fab, with its sub nanomolar affinity, was anticipated to be advantageous in the iPSA assay. The higher affinity of the L3-2 Fab led to increased signal per labeling degrees in both iPSA assay settings than the wt-4D4 Fab. Similarly, using the L3-2 Fab as a capture antibody in the iPSA assay yielded a higher signal than using 4D4 Mab. In the iPSA assay, streptavidin-coated microtiter wells were used to capture bio-Mab and bio-Fabs. The L3-2 Fab showed higher signal in comparison to the wt-4D4 Fab or Mab, even though having an equal number of binding sites. This indicates that L3-2 Fab has a higher affinity and slower off-rate, resulting in improved sensitivity in the iPSA assay. The wt-4D4 Mab signal was significantly weaker than wt-4D4 Fab, possibly because the chemical biotinylation of Mab is less optimal than site-specific biotinylation of Fabs (Ylikotila et al., 2006). The decreased off-rate of L3-2 Fab produced superior linear standard curve with MES assay buffer at low concentrations when compared to the wt-4D4 Mab. The detection limit of the new iPSA assay using mutant L3-2 Fab as a tracer and the MES assay buffer (pH 6.75) was lower than that of the reference iPSA assay using wt-4D4 Mab.

6.2 Performance of immunoassays in IMPROD plasma samples (II)

The mutant L3-2 Fab-assisted assay constructs were compared to previously reported wt-4D4 Mab-based assays (Nurmikko et al., 2001; Peltola, Niemela, Alanen, et al., 2011). According to previous studies of iPSA, it was observed that none of the three iPSA versions (I-W, I-M, and I-MC) were able to discriminate the two groups. Notably, it was observed that the ratio of I-MC to fPSA substantially improved the distinguishing of cancer and non-cancer groups, and this was seen in all four of the groups that were analyzed. The positive impact is notably apparent in the low PV group, where the two I-MC based ratios (I-MC/F and CN(I-MC)/T) performed better than other ratios. According to the results, the capture mutant L3-2 Fab offers a distinct separation of fPSA into subgroups that are more informative for discriminating cancer from non-cancer. The measured nPSA effectively differentiated cancer from non-cancer in the whole group, high PV group, and high GS grade group. The measured concentrations of nPSA were found to be 45–55 percent of the calculated nPSA concentration, consistent with the findings of the previous report (Peltola, Niemela, Alanen, et al., 2011). The study demonstrates that the phage displayed mutant L3-2 Fab can be used to construct a new assay. This mutant as a capture antibody shows technical feasibility and offers significant improvements in distinguishing between cancer and non-cancer, in the whole cohort as well as in the subgroups analysed. The study focused on comparing diagnostic potential with various ratios of measured parameters, with particular emphasis on

iPSA assays. When utilising the four parameters (I-W, tPSA, fPSA, hK2) of the 4Kscore, weighted statistical model (algorithm) were used instead of ratios. The predictive performance of the model was shown to be greatly improved by the inclusion of iPSA (I-W) and hK2, as indicated by a previous meta-analysis study (Vickers et al., 2018). The potential improvements provided by the mutant antibody based iPSA (I-MC) assay need to be thoroughly evaluated utilizing the algorithm. The small size of the cohort is a limitation of this study. However, the cohort was suitable for demonstration of cancer detection rate and tumor differentiation that are consistent with previous study (Ahmed et al., 2017). Further confirmation of the results is necessary by including a larger patient cohort.

6.3 Performance of immunoassays in IMPROD/multi-IMPROD plasma samples (III)

The technical improvement of the iPSA assay construct using mutant L3-2 Fab was evaluated in order to solve certain technical deficiencies (such as a high off-rate dissociation causing decreased assay sensitivity, linearity, and stability in the low standard range) in the original assay using 4D4 Mab. The size of the sample cohort was increased to include 310 patient samples, with 163 samples grouped as benign/GS = 6 and 147 samples grouped as GS ≥ 7. The newly developed I-MC assay was then validated using a logistic regression model that included tPSA, fPSA, hK2, and iPSA. The results from the whole cohort indicate a moderate performance improvement when using the mutant L3-2 Fab-based iPSA assay compared to the reference 4D4 Mab-based iPSA assay, as seen by the AUC values of 0.76 and 0.74, respectively. The two assays performed quite similarly in patients with gland volumes > 38 mL. Conversely, the mutant L3-2 Fab-based iPSA outperformed the reference iPSA (AUC 0.75 vs. 0.71) in the low PV group, as observed in both the univariate comparison and in the logistic regression model. The improved accuracy and precision shown in this study may be due to the better binding characteristics acquired by oligonucleotide-directed mutation efforts and the utilization of site-specific biotinylated Fab fragment for capturing iPSA. In contrast to study II (Khan et al., 2019), the performance of the F-hK2 assay concept (Vaisanen et al., 2004) in the model was also assessed instead of the T-hK2. T-hK2 and F-hK2 assays perform similarly. Both assays perform very well in the low PV group but poorly in the high PV group. However, the T-hK2 assay's contribution to the whole cohort remains quite evident.

7 Conclusions

Detection of PSA has provided significant advancements in the diagnosis and prognosis of PCa, although its sensitivity and specificity have limited its effectiveness. PSA-based PCa screening reduces PCa mortality, however, overdiagnosis and overtreatment may have adverse effects (Vickers, 2017). The discovery of various molecular forms of PSA led to the development of advanced immunoassays to enhance the specificity of PSA test to detect PCa (Christensson et al., 1990; De Angelis et al., 2007; Gaudreau et al., 2016). Inclusion of intact PSA (iPSA) to a multi-kallikrein panel to evaluate the risk of clinically significant PCa in apparently healthy men substantially improves diagnostic specificity (Vickers et al., 2008).

This study was carried out to improve the binding affinity of 4D4 Mab antibody using phage display technology to develop different assay formats for sensitive and robust detection of iPSA and nPSA.

The following are the main conclusions obtained from the original publication/manuscript:

I. The sensitivity and robustness of the reference iPSA assay using wt-4D4 Mab as a tracer antibody were poor. Initial efforts to capture iPSA using wt-4D4 Mab were unsuccessful. The mutant L3-2 Fab derived from phage display library provides improved assay performance in both formats and offers new technical possibilities for including iPSA or nPSA assays in a multiplexed panel of kallikrein assays.

II. The novel assay construct that utilizes the mutant L3-2 Fab as a capture (I-MC), significantly improves distinguishing cancer from non-cancer in all subgroups analyzed. This improvement is especially noticeable in patients with lower PV.

III. The use of mutant L3-2 Fab as a capture in the iPSA assay improves the ability to distinguish benign and low-grade cancers from clinically significant cancers, particularly in patients with lower PV. In the four-kallikrein logistic regression analysis, the mutant I-MC performs slightly better than the I-W in the whole cohort but is nearly identical in the higher

PV group. However, the mutant I-MC performs significantly better than the I-W in the lower PV group.

In conclusion, the iPSA assay construct using L3-2 mutant of the 4D4 antibody, discovered through phage display technology, is not only technically feasible but also improves the separation of the patient groups. This suggests that the new assay construct has the potential to improve PCa detection.

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Turku, September 2024

Md. Ferdhos Liton Khan

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