Acknowledgement

II

Abstract

The plasminogen activator system is assumed to play a key role in the degradation of extracellular matrix and basement membranes. During cancer invasion and metastasis, the activated plasminogen activator system cleaves plasminogen to the active plasmin. Plasmin is able to degrade a wide range of extracellular matrix proteins either directly or indirectly by activation of additional proteolytic enzymes.

Accumulating evidence has shown that the urokinase plasminogen activator system might be upregulated in many types of malignancies, including prostate cancer. Previous studies have found an association between the soluble form of the urokinase plasminogen receptor, abbreviated as suPAR, and the development and progression of prostate cancer. Furthermore, it has been proposed that the quantification of suPAR in serum might serve as a useful adjunct to current conventional diagnostic tools.

Published literature is limited regarding both the suPAR levels in patients diagnosed with benign prostate hyperplasia (BPH) and the differences in suPAR concentration between BPH- and prostate cancer patients. In the present thesis, the serum levels of suPAR in BPH- and prostate cancer patients are therefore quantified to determine whether there is a statistically significant difference between the two groups.

The present thesis concludes that there is no statistically significant difference in the suPAR concentration between BPH patients (median 0.22 ng/mL) and prostate cancer patients (median 0.17 ng/mL). However, methodological limitations of the immunoassay utilized for suPAR quantification was encountered, and further studies are warranted to give an exact estimation of the suPAR concentration in BPH- and prostate cancer patients. In addition, due to limited published literature, it was difficult to define the normal or abnormal levels of suPAR.

The serum samples obtained in the present thesis are stored at the Regional Research Biobank of Central Norway and are accessible for future studies. By including samples from the HUNT research biobank, it might be achievable to obtain a larger cohort of serum samples and further investigate the role of suPAR as a predictor of prostate cancer progression.

IV

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1. Introduction

1.1. The prostate gland

The prostate is an androgen-regulated gland of the male reproductive system. The gland produces a fundamental part of the seminal fluid, providing optimal environment and conditions for the activity and survival of the sperm cells. The components of the prostatic secretions include citric acid, acid phosphatase and several protein digesting enzymes such as the prostate specific antigen (PSA). Citric acid can be used by sperm for ATP production via the Krebs cycle, whereas PSA serves to liquefy the semen by cleavage of the gel proteins seminogelin I and II of the seminal fluid (1, 2).

The current consensual model of prostatic anatomy is based on the work of John E. McNeal. In a number of autopsy-based studies, McNeal redefined the anatomic perspective of, and terminology for, the prostate gland. The work of McNeal demonstrates that the human prostate gland is a composite organ that can be histologically divided into three glandular zones and a fourth nonglandular region termed the anterior fibromuscular stroma (AFMS). The AFMS is a wedge-shaped stromal barrier, occupying much of the anteromedial prostatic tissue that shields the prostatic urethra and glandular zones from overlying structures (3). Figure 1.1 further explains and illustrates the zonal organization of the prostate.

The histological architecture of the prostate includes two major cell types: Epithelial and stromal cells. Epithelial cells can be divided into different groups of urothelial (transitional), secretory (luminal), basal and neuroendocrine cells, and they are arranged in glands consisting of ducts which branch out from the urethra and terminate in acini. The stroma, surrounding the prostatic glands, contains smooth muscle cells and fibroblast. Blood vessels, tissue infiltrating white blood cells, peripheral nerves and ganglia are additional constituent cell elements of the normal human prostate (4, 5).

1.2. Pathology of the prostate

There are several disorders related to the prostate: Prostatitis, benign prostate hyperplasia (BPH) and prostate cancer (PCa).

1.2.1. Prostatitis

The syndrome of prostatitis is one of the most common diseases encountered in urologic practice, representing a heterogeneous mix of conditions. According to the National Institute of Health (NIH), these conditions can be divided into different subtypes including acute bacterial prostatitis, chronic bacterial prostatitis, chronic pelvic pain syndrome (CPPS) and asymptomatic prostatitis (6).

Acute bacterial prostatitis is an acute bacterial infection of the prostate. It is assumed that the most common route of infection is the intraprostatic reflux of infected urine with organisms such as *Escherichia coli* and *Klebsiella-*, *Proteus-*, *Pseudomonas-* and *Enterococcus species*. Left untreated, acute bacterial prostatitis can lead to overwhelming sepsis or development of prostatic abscess. In addition, it can lead to chronic bacterial prostatitis which is a persistent bacterial infection of the prostate lasting more than three months (7, 8).

The majority of patients with prostatitis syndrome suffer from CPPS. Unlike bacterial prostatitis where the causal organism can be identified, the etiology of CPPS is poorly understood and described; both inflammatory and infectious mechanisms have been postulated. The hallmark symptom of CPPS is pain and tenderness attributed to the prostate, or less common, the pelvis. In contrast, patients affected by asymptomatic prostatitis do not have these symptoms. These patients are commonly diagnosed for asymptomatic prostatitis by prostatic resection or biopsy specimens when being examined for BPH or PCa (8-10).

Studies have suggested that chronic inflammation of the prostate might play an important role in the development of BPH and PCa: The release of reactive oxygen species during chronic inflammation can induce DNA damage in prostatic epithelial cells and can contribute to the development of PCa. Early detection and treatment of asymptomatic prostatitis might therefore, in theory, participate in the prevention of inflammation associated PCa (10-12).
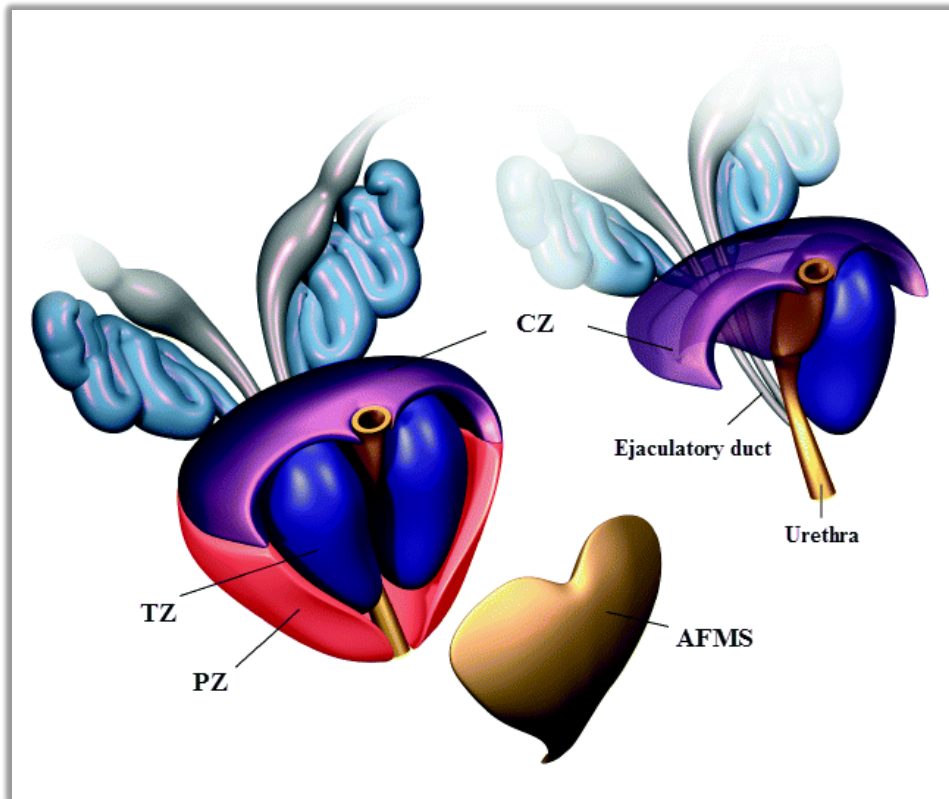
Figure 1.1: Zonal organization of the prostate. Modified from Shah *et al* (4) . The prostate gland is localized directly below the urinary bladder and in front of the rectum where it surrounds the urethra. The transitional zone (TZ) of the prostate comprises about 5 % of the glandular tissue and consists of two bulges of glandular tissue on either side of the urethra. This region grows throughout life and is the zone of the prostate that is most affected by benign hyperplastic nodules of the prostate. The central zone (CZ) comprises about 25 % of the prostate volume, forming an inverted cone surrounding the ejaculatory ducts. This region is seldom affected by disease. The peripheral zone (PZ), about 70 % of the prostate volume, extends posterolaterally around the central zone and distal prostatic urethra. The peripheral zone is the region of the prostate that is most susceptible to inflammation and prostate cancer (3, 4).

1.2.2. Benign prostate hyperplasia

BPH is defined as the nonmalignant enlargement of the prostate gland. Histologically, it is characterized as hyperplasia of epithelial- and stromal cells, with excessive nodular growth in the transitional zone of the prostate (figure 1.1). The proliferation rate increases about 9- and 37-fold in prostate epithelium and stroma respectively, suggesting that the prostate enlargement in BPH is primarily caused by the hyperproliferation of stromal cells (13-15).

An estimated 25% of men between the age of 50 to 60 years have histological evidence of BPH, increasing to more than 50% for men older than 60 years. With the prolonged average life span, increasing elderly population and increasing incidence, BPH has become a major disease of significant interest (16, 17).

The clinical relevance of BPH is highlighted by the fact that up to 50% of elderly men develop lower urinary tract symptoms (LUTS), which include urinary hesitancy, frequent urination, reduced urine flow and urinary retention. This explains the fact that transurethral resection of the prostate (TURP) remains one of the most common interventions in elderly men. Delayed treatment can cause severe complications, for example bleeding from the prostate, recurrent infections, renal stones and even kidney failure (18, 19).

Although the pathogenesis of BPH is complex and still unclear, several mechanisms appear to be involved in the development and progression of the disease. The role of androgens, including testosterone and dihydrotestosterone (DHT), in the progression of BPH is well established (20). Testosterone and DHT exert their function by binding to nuclear androgen receptors located in stromal- and epithelial cells, which in turn promotes the transcription of growth factors, including epidermal growth factor (EGF). The EGF is an important mitosis- and proliferation promoting factor that has been shown to play a role in the development of the prostate after binding to its specific receptor, EGFR. Upon interaction with EGF, EGFR induces the phosphorylation and activation of STAT3 (signal transducer and activator of transcription 3), a transcription factor essential for cell survival and proliferation. The phosphorylation of STAT3 in the cytoplasm induces its homodimerization, nuclear translocation and DNA binding, resulting in the expression of genes that mediate proliferation and prevent apoptosis. Abnormal activation of this EGF/STAT3 pathway causes an increase in the total number of epithelial and stromal cells, which appears to be associated with the development of BPH (21).

Furthermore, several studies have indicated a significant association between inflammation and BPH severity and progression (22-25). Inflammatory infiltrates are frequently observed in resections and biopsies from patients with enlarged prostates, and correlation between urinary symptom severity and intraprostatic inflammation has been proposed (24, 25). Although the exact mechanisms of inflammation to prostate growth are not yet fully understood, activation of macrophages or lymphocytes and secretion of cytokines to trigger abnormal growth appears to be important. Additionally, recent clinical studies have suggested a relationship between prostatic inflammation and LUTS associated with BPH (26, 27).

1.2.3. Cancer of the prostate

Human PCa is a significant cause of cancer morbidity and mortality worldwide. In the period 2006-2010, an average of 4264 new cases were diagnosed per year in Norway. This corresponds to around 30% of all new cancer cases in men, making it the most commonly diagnosed cancer in men (28).

PCa is a heterogeneous and multifactorial disease that proceeds through multiple pathological and cytological stages. In general, the etiopathogenesis occurs through the formation of pre-cancerous lesions designated as prostatic intraepithelial neoplasms (PINs). Progression of PIN to locally invasive adenocarcinoma and metastatic disease stages follows, often accompanied by the acquisition of androgen-independent disease states (29-31). Although the etiopathological causes of the PCa development are not well established, the risk factors associated with PCa often include intense oxidative stress, chronic inflammation and hormonal changes. In addition, advancing age also appears to be particularly important (10, 32, 33).

Multiple studies have revealed that alterations in key gene products and molecular pathways typically occur along PCa etiopathogenesis and progression (34-37). The genetic and epigenetic disruption and/or decreased expression of diverse tumor suppressor genes controlling cell cycle progression might provide critical roles for PCa development. In addition, the sustained activation of diverse oncogenic signaling cascades appears to provide fundamental functions for PCa progression to locally invasive, metastatic and androgen-independent PCa. For example, the downregulation of the tumor suppressor gene p27 appears to promote cell cycle progression and growth of PCa cells, while the enhanced expression of anti-apoptotic factors (Bcl-2) might provide their survival (34-37). A selection of the cellular signaling pathways and genes that appears to be involved in the etiopathogenesis and progression of PCa is schematically illustrated in figure 1.2.
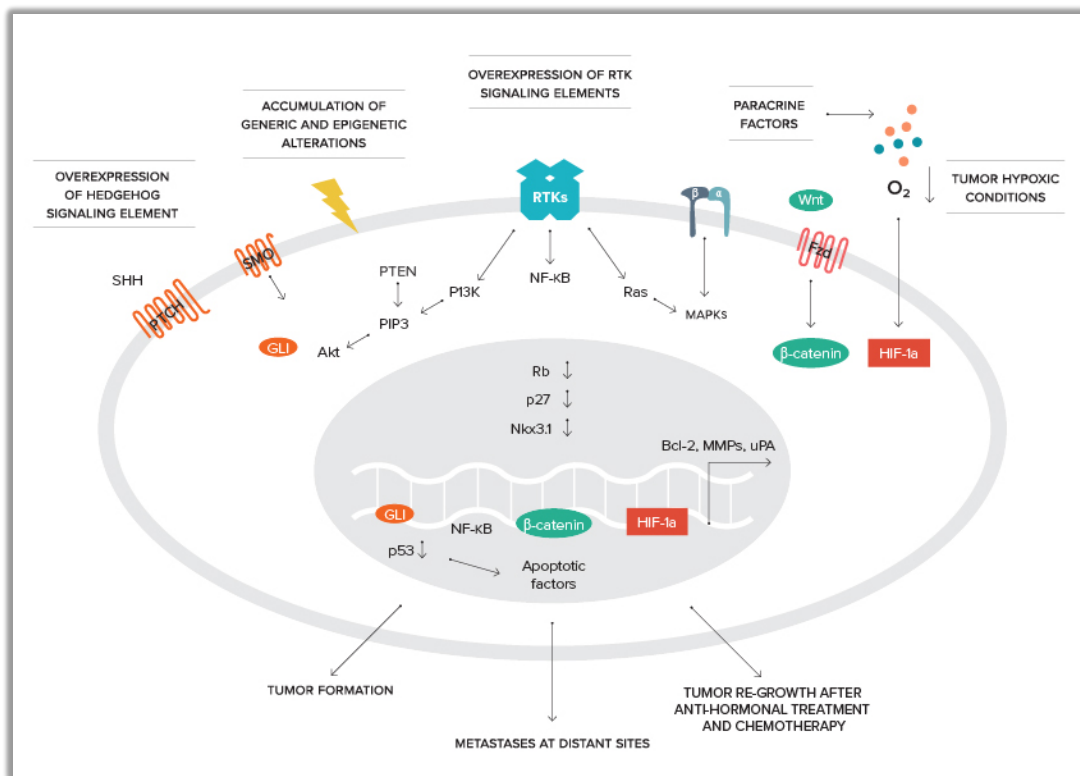
Figure 1.2: Cellular signaling pathways and genes involved in sustained growth, survival, invasion, metastasis and treatment resistance of PCa. Based on Mimeault *et al* (35). The accumulation of different genetic and epigenetic alterations can lead to a downregulation of diverse tumor suppressor genes such as phosphatases tensin homolog (PTEN), retinoblastoma (Rb), cyclin-dependent kinase inhibitor p27, homeodomain-containing transcription factor NKX3.1 and p53. The downregulations are combined with a persistent activation of oncogenic signaling pathways, including the receptor tyrosine kinases (RTKs), sonic hedgehog SHH/PTCH/GLI, Wnt/β-catenin and the downstream effectors PI3K/Akt, nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs). The result is an upregulation of the expression of different target genes that are involved in the malignant transformation (34-37).

Regions of focal atrophic prostate epithelium have been identified in aging men, frequently in association with a chronic inflammatory response. These regions usually exhibit increased epithelial proliferation and have been termed proliferative inflammatory atrophy (PIA) (38). Studies suggest that regions of PIA might give rise to carcinoma directly or indirectly via development into PIN, and PIA has therefore been proposed as a potential precursor for PCa (38-40). Given the possible influence of inflammation, it has been hypothesized that the use of anti-inflammatory drugs could reduce the risk of PCa. In fact, a recent study demonstrates that the use of certain nonsteroidal anti-inflammatory drugs might be associated with a small reduction in PCa risk. However, further studies are warranted to confirm the observed associations (41).

Locally invasive PCa can be well treated by radical prostatectomy with good prognosis. By contrast, advanced PCa is mainly treated with androgen-deprivation therapy, but androgen-independent lesions may eventually develop and result in the lethal disease termed castration resistant PCa. The androgen-deprivation can cause cancer regression since without androgen the rate of cell proliferation is lower than the rate of apoptosis, causing an extinction of the cancer cells (31).

Both local and castration resistant PCa can metastasize to different organs, including bone, lung and liver. Metastasis is defined as the formation of progressively growing secondary tumors at sites discontinuous from the primary lesion (42). At date, there is no curable treatment for metastatic PCa and the median survival is about one year (43). The early detection of PCa, before it develops into an incurable stage, is therefore essential for the effective treatment and positive clinical outcome of patients. The routine use of serum PSA testing has improved PCa detection and early diagnosis, but the role of PSA as a biological marker (biomarker) for PCa remains controversial (44).

1.3.   Prostate specific antigen

PSA is a serine protease produced by the luminal cells of the prostate gland. In healthy individuals, the retrograde release of PSA into the bloodstream is a rare event. This occurs with a frequency less than one PSA molecule per million secreted PSA molecules, leading to a concentration of <4.0 ng/mL in serum. Hence, the concentration is approximately one millionth part of the PSA concentration in seminal plasma (0.5-5.0 mg/mL). This significant difference in concentration is one of the reasons why PSA is argued to be a useful biomarker for prostatic disease (2, 45).

However, PSA has significant limitations as a biomarker for PCa. The PSA level of 4.0 ng/mL has been proposed as the upper limit of the normal range in serum, but this threshold is not well documented and defined (46). In fact, a study by Thompson *et al.* demonstrates that PSA levels ≤ 4 ng/mL are commonly found in men with biopsy-detected cancer, including men with advanced cancer. This might indicate that the threshold of 4.0 ng/mL can cause underdiagnosis and undertreatment of clinically significant disease. Lowering the threshold could on the other hand cause an increased risk of overdiagnosis and overtreatment of clinically unimportant disease (47).

The ability of PSA to accurately detect the presence of PCa is limited. In the circulation, elevated levels of PSA can be driven by conditions other than PCa such as BPH, prostatitis,

ethnicity, age and body mass index. As a consequence, about 60-80% of positive prostate biopsies are due to false-positive PSA values (45).

An attempt to improve the power of PSA as a biomarker includes the use of age-referenced PSA levels and identification of additional PSA-related biomarkers present in the circulation (48-50). Clinical studies of free and complexed PSA have significantly enhanced cancer specificity compared to the PSA-test alone. The serum levels of human kallikrein-related peptidase 2 (hk2) and various free PSA subfractions have been reported to differ significantly in benign versus malignant disease of the prostate (51-54).

A report by Mikolajczyk *et al.* proposes that multiple biomarkers are needed for the early detection and prognostication of PCa. As prostatic disease is multidimensional, Mikolajczyk *et al.* claims that indolent cancer, malignant cancer, metastatic cancer and BPH all must be differentiated as each of these have distinct treatments and medical interventions (55). However, the prognostic value of the abovementioned additional biomarkers appears to be limited, and traditional methods for grading and staging are used.

1.4.   Grading and staging of PCa

Grading refers to the microscopic histological characteristics of tumor tissue and is based on the Gleason scoring system. This is a scalar measurement that ranges the tumor biopsy from 1 to 5, with Gleason grade 1 being the most and Gleason 5 being the least differentiated (figure 1.3). A primary and secondary grade is classified from the two most abundant patterns of the tumor, and the sum of the two grades is the Gleason score. A high Gleason score does not necessary mean that the cancer has spread, but predicts the likelihood of the cancer to spread if left untreated (56, 57).

Pathological staging is another determinant of PCa prognosis and relies on the TNM system. The T is the extent of tumor, N is the presence or absence of disease in the regional lymph nodes, and M is the extent of metastatic disease. Stage T1 encompasses prostate tumor diagnoses on transurethral resection or needle biopsy. Unless metastases are present, the definitive stage can be determined only at prostatectomy. The extent of the tumor is divided into T1-T4, with higher T values indicating more involvement of the prostate and surrounding structures. For example, stage T2 refers to a tumor confined to the prostate. Stage T3 refers to tumor spread outside the prostate, with T3a indicating extensions into extraprostatic fat, T3b indicating unilateral seminal vesicle spread, and T3c indicating bilateral seminal vesicle spread. Spread to the urinary bladder or rectum is stage T4. The involvement of pelvic or

inguinal lymph nodes is denoted by N1, with N0 meaning no positive nodes. Metastatic disease is categorized as M1, with M0 meaning no metastasis (58).
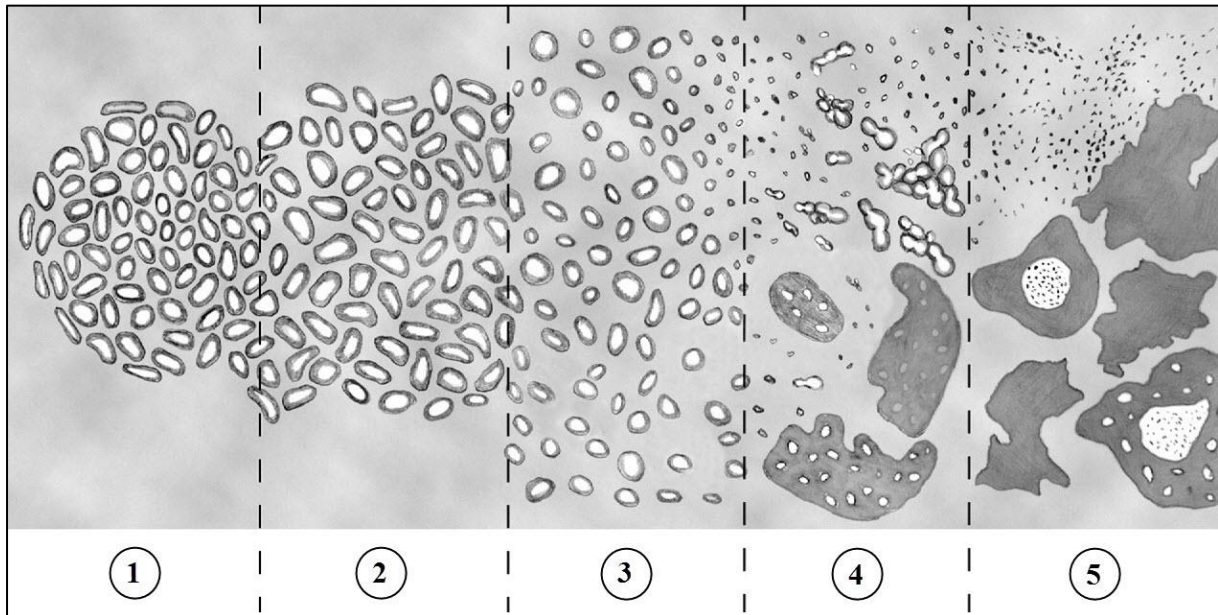
Figure 1.3: Overview of the Gleason system. Modified from Epstein, J.I. (56). The different Gleason scores are marked with number (1-5). Gleason score 1 is composed of circumscribed nodules of uniform, single, separate and closely packed glands. The glands of Gleason score 2 are not as uniform as glands of Gleason score 1, but are more loosely arranged. Gleason score 3 is composed of single glands, and the tumor infiltrates in and amongst non-neoplastic prostate acini. The neoplastic glands show marked variation in shape and size. Gleason score 4 consist of cribriform glands or fused, ill-defined glands with poorly formed glandular lumina. In this pattern the glands are no longer single and separate as in pattern 1-3. Finally, Gleason pattern 5 shows no glandular differentiation composed of solid sheets, cords or single cells (56, 57).

1.5.   The controversial relationship between BPH and PCa

A link between BPH and PCa has been recognized since the first autopsy studies of prostate gland in the 1950s. The original hypotheses were based on studies documenting the presence of both BPH and PCa at autopsy. In more recent studies, it has been found that approximately 20% of men with PCa also have BPH, and cancer has been found incidentally in 10-20% of surgically removed BPH specimens (59). However, it remains unclear whether the association indicates a causal link, shared risk factors, shared pathophysiological mechanisms or simply an increased surveillance and examination of patients with BPH leading to the diagnosis of PCa (16).

Although the two pathological conditions commonly coexist, it is widely thought that BPH is not a premalignant lesion (33). The differences in histology and anatomic location in the prostate gland support this hypothesis. BPH is histologically characterized by hyperplasia stromal and epithelial cells, whereas PCa involves complex histopathological changes in the glandular epithelium. Furthermore, BPH is known to develop from the transitional zone, while PCa usually originate in the peripheral zone. The conditions coexist in the same zone in only about 20% of the cases (3, 60, 61).

Schenk *et al.* recently examined the association between symptomatic BPH and PCa risk. Among the total 5,068 participating men, 30.5% had symptomatic BPH at baseline and 24.2% were diagnosed with PCa. However, no significant association was detected between symptomatic BPH and PCa (62).

Despite the differences, BPH and PCa also share some important characteristics. Both display an increasing prevalence with age, both require androgens for growth and development and both respond to androgen-deprivation treatments (59, 63, 64). In addition, a number of studies have indicated that chronic inflammation might be a common risk factor for both BPH and PCa (22, 39). If BPH really is a causal factor for PCa development, knowledge about this link could enable physicians to intervene at an earlier stage, thereby reducing the development of PCa and the number of metastatic PCa (16).

The similarities and differences of BPH and PCa are summarized in table 1.1.

Table 1.1: Similarities and differences between BPH and PCa

	BPH	PCa
Prevalence with age	Increases	Increases
Androgens	Required for growth	Required for growth
Androgen deprivation	Can decrease growth	Can decrease growth
Common anatomic location	Transition zone	Peripheral zone
Histology	Stromal and epithelial cells	Glandular epithelium
Premalignant potential	None	-

Although BPH can cause morbidity and reduce the quality of life, it is not a lethal disease *per se*. In PCa one of the major causes of morbidity and mortality is metastatic disease (65). As illustrated in figure 1.4, the metastatic process is a cascade encompassing multiple steps. In the initial step, the tumor cells disseminate from the primary tumor to locally invade the surrounding stroma. Once present in the stromal compartment, tumor cells can gain access to the blood and lymphatic vessels and subsequently intravasate. The circulatory system then provides transport of tumor cells to distant sites were they may extravasate and establish metastatic lesions (42, 66, 67).
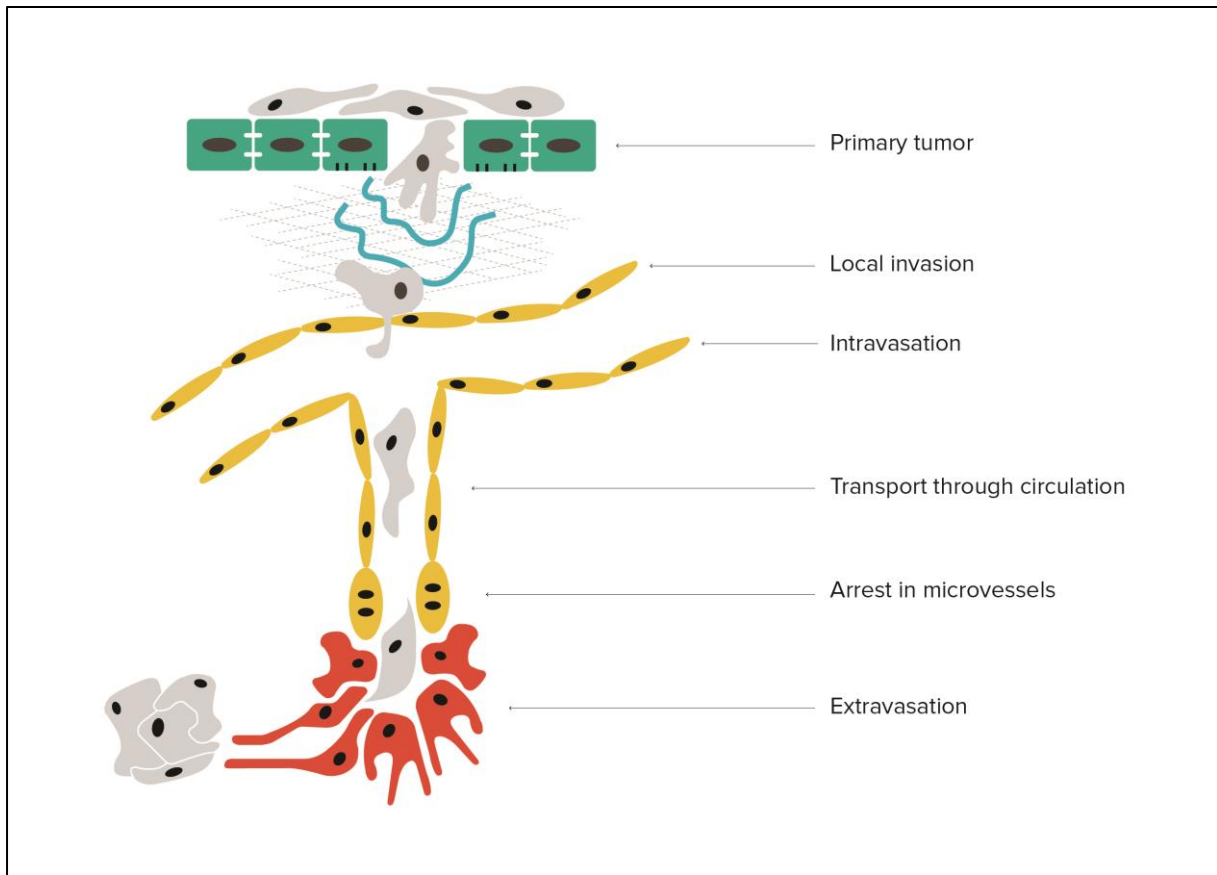
Figure 1.4: The metastatic cascade. Dissemination of cancer cells from the primary tumor and local invasion constitute the initial step of the metastatic cascade. Proteolytic enzymes degrade basal membranes and extracellular matrix so that tumor cells can pass through local stroma and subsequently penetrate the vessel wall. The intravasation into the circulatory system makes transport to distant sides possible. The tumor cells might then be arrested in microvessels and extravasate to establish metastatic lesions (42, 66).

As illustrated in figure 1.4, the local proteolysis of the extracellular matrix (ECM) and basal membranes (BMs) is at the core of the metastatic process (68). A complex array of proteolytic enzymes appears to be fundamental in local proteolysis, and among these, the plasminogen activator (PA) system is assumed to play a vital role (67, 69). In accordance to this, recent studies have demonstrated that the components of the PA-system could emerge as candidate markers to distinguish between BPH and PCa (70, 71).

1.6. The Plasminogen activator system

As described above, extracellular proteolysis of BM and ECM is a prerequisite for cancer invasion and metastasis, enabling tumor cells to pave their way through the surrounding tissues. The PA-system is a cascade reaction leading to the generation of the serine protease plasmin, which is able to degrade a wide range of extracellular proteins, either directly or through activation of other matrix degrading proteases (72-74).

Besides the role in ECM degradation, accumulating evidence has documented a role of the PA-system in several aspects of tumor development. This includes tumor cell proliferation, adhesion and migration, intravasation and extravasation, growth at the metastatic sites and tumor neo-angiogenesis (75-77). However, under normal conditions the PA-system is strictly controlled, and most physiological processes associated with tissue remodeling, comprising fibrinolysis and wound healing, depend on components of the PA-system (78-81).

The components of the PA-system include activators (uPA, tPA), inhibitors (PAI-1, PAI-2), and a receptor (uPAR). These components are presented in the following sections.

1.6.1. Plasminogen and plasmin

Plasminogen is the single-chain zymogen of plasmin and is predominantly synthesized in the liver. At relatively high concentrations, plasminogen circulates in plasma and interstitial fluids, but it is also localized at the cell surface bound to anexin II and plasminogen-RKT. The co-localization of plasminogen with plasminogen activators at the cell surface provides a mechanism for accelerated plasminogen activation (82-87).

Plasminogen is converted to active two-chain plasmin through proteolytic cleavage catalyzed by the plasminogen activators, uPA and tPA. The generated plasmin is an active trypsin-like serine protease with relatively wide substrate specificity (87, 88).

Although the primarily function of activated plasmin is fibrin clot lysis, it can also degrade several components of the ECM, including laminin, fibronectin, vitronectin and collagen (89, 90). In addition, plasmin can activate certain proteolytic enzymes of the matrix metalloproteinase (MMPs) family, including the MMP-3, MMP-9, MMP-12 and MMP-13 (91-93). The MMPs have been implicated as key proteases in removal of ECM barriers. Combined activities of plasmin and MMPs display an adequate proteolytic repertoire, enabling degradation of most protein constitutes in the ECM barriers (93).

Table 1.2: Components of the PA-system (79, 94, 95)

Component	Mw (kDa)[a]	Cellular source	Main function
Plasminogen	92	Mainly liver	Proenzyme of plasmin Converted to plasmin by uPA and tPA
uPA	54	Leukocytes, macrophages, endothelial cells, smooth muscle cells, epithelial cells, fibroblasts and tumor cells	Plasminogen activation during cell migration and invasion
tPA	70	Endothelial cells, keratinocytes, and leukocytes	Plasminogen activation during fibrinolysis
PAI-1	52	Endothelial cells, platelets, megakaryocytes, fibroblasts, smooth muscle cells, adipocytes, hepatocytes and tumor cells	Inhibition of uPA and tPA. Regulator of cell migration and invasion
PAI-2	47/60[b]	Monocytes, macrophages, keratinocytes and tumor cells	Inhibitor of mainly uPA.
uPAR	55	Leukocytes, macrophages, endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes and tumor cells	Localizes cell-associated proteolysis through binding of uPA. Also plays a role in signal transduction, cell adhesion and migration, chemotaxis, and proliferation.

[a] Mw, molecular weight in kilo-Dalton (kDa).

[b] Depends on degree of glycosylation.

1.6.2. Plasminogen activators: uPA and tPA

There are two types of plasminogen activators, tPA and uPA, both capable of catalyzing the conversion of plasminogen to plasmin.

The uPA molecule is secreted as an inactive single-chain urokinase (scuPA). Before uPA can exert its major biological function, to convert plasminogen to the active plasmin, scuPA must be proteolytically converted into an active enzyme (96). Activation of two-chain uPA occurs through cleavage of the Lys_{158}-Ile_{159} peptide bond after scuPA has bound to its receptor, uPAR (described in chapter 1.6.4). This activation is brought about by plasmin. Since uPA, in turn, generates plasmin from plasminogen, the activation of uPA by plasmin completes the loop for a feed- back-type activation (97, 98). In addition to plasmin also matriptase, hepsin, serase 1B and certain MMPs are capable of generating active uPA (99-101).

Like uPA, tPA is secreted as a single-chain precursor that is converted into active two-chain tPA through proteolytic cleavage by plasmin (102). Despite structural homogeneity, tPA is primarily involved in fibrinolysis, whereas uPA is primarily involved in tissue remodeling and cell migration (78, 79). At present, multiple studies have related uPA and plasmin to cancer progression and several aggressive cancers, including PCa (103-105).

In a recent study, Bekes *et al.* demonstrated that uPA-mediated plasmin generation might facilitate the early stages of metastasis, specifically during local invasion and intravasation. In several *in vitro* and *in vivo* studies, Bekers *et al* utilized highly disseminating variants of the human PCa-3 cell line, PC-hi/diss. In one of these studies the PC-hi/diss cells were implanted into the prostates of NOD/SCID mice. The tumor bearing mice were subsequently separated into two groups. The first group was treated with the specific pro-uPA activation blocking antibody, mAb-112, while the second group did not receive treatment. The result showed that the treatment significantly inhibited local invasion and tumor distant metastases of the PC-hi/diss cells. Immunostaining showed that tumors from mAB-112-treated mice appeared confined, whereas tumors from the untreated mice showed invasive fronts and were detected both in close association with blood vessels and intravascularly (77). Another study by Madsen et al. shows similar results and also suggest that uPA plays a key role in the process of early stage metastasis (106).

In normal tissues, the PA-system is however strictly controlled. Both uPA and tPA is tightly regulated at the level of pro-enzyme activation and can be inhibited by either two of the plasminogen activator inhibitors: PAI-1 and PAI-2 (107, 108).

1.6.3. Plasminogen activator inhibitors: PAI-1 and PAI-2

At date, the crystal structures and inhibitory mechanisms of PAI-1 and PAI-2 are well defined. Both PAI-1 and PAI-2 belong to the family of serine protease inhibitors (serpins) and form stable complexes with the catalytic site on their target protease. PAI-1 exists as a single chain glycoprotein, whereas PAI-2 exists in two forms; a cytosolic form and an extracellular glycosylated form (107, 108).

Both PAI-1 and extracellular PAI-2 are effective inhibitors of both soluble and receptor-bound uPA. The formation of covalent complexes with active uPA results in a substantial increase of serpin stability, making it able to effectively trap uPA in a stable serpin-protease complex. As illustrated in figure 1.5, the complex is associated with endocytic receptors of the low density lipoprotein (LDL) family which are subsequently internalized into clathrin-coated vesicles and degraded in lysosomes (109-111).

Despite the shared serpin function of PAI-1 and PAI-2, high tumor levels of PAI-1 promote tumor progression, whereas high levels of PAI-2 appear to decrease tumor growth and metastasis. This divergence in function may be related to the complex array of molecular interactions between PAI-1 and uPA-uPAR and various ECM components and co-receptors, which are not apparent with PAI-2 (95).

As an example, uPAR is a receptor with high affinity for the ECM protein vitronectin as well as the adhesion receptors of the integrin family (112-114). On vitronectin matrixes, cells are attached to the ECM through the binding of uPAR and integrins to vitronectin. The affinity of these interactions is supported by binding of uPA to uPAR. PAI-1 binding and inactivation of uPA cause impaired uPAR- and integrin affinity for vitronectin and thereby detachment of cells that relay on uPAR for adhesion to the ECM. The uPA-uPAR-integrin-PAI-1 complexes are later removed from the surface by LDL-receptor-mediated endocytic clearance. However, the receptors are recycled to the cell surface and can reengage to ECM molecules and then promote cell attachment to vitronectin. PAI-1 is unable to promote binding to vitronectin by competitive displacement of pre-engaged integrins form vitronectin. These events illustrate the cycle of cellular attachment and de-attachment which are fundamental for efficient cell migration and stromal invasion (113-116).

The protective effect of PAI-2 facilitates cell surface inhibition and clearance of uPA, but it might also counteract PAI-1 stimulatory actions on tumor invasion and metastasis. High levels of PAI-2 might prevent the removal of vitronectin-bound PAI-1 for uPA binding and thereafter decrease vitronectin-dependent cell migration (95).

The presence of the cytosolic form of PAI-2 suggests that the inhibitor might have accessory functions in addition to the serpin activity, but these exact functions of PAI-2 remain unclear. However, the observation of non-glycosylated extracellular PAI-2 might indicate that cytosolic PAI-2 is released during inflammation or other conditions resulting in cell damage. This release could enhance the local secretion of glycosylated PAI-2 in tissue, thereby limiting the pericellular and extracellular proteolysis during tissue remodeling processes (95).
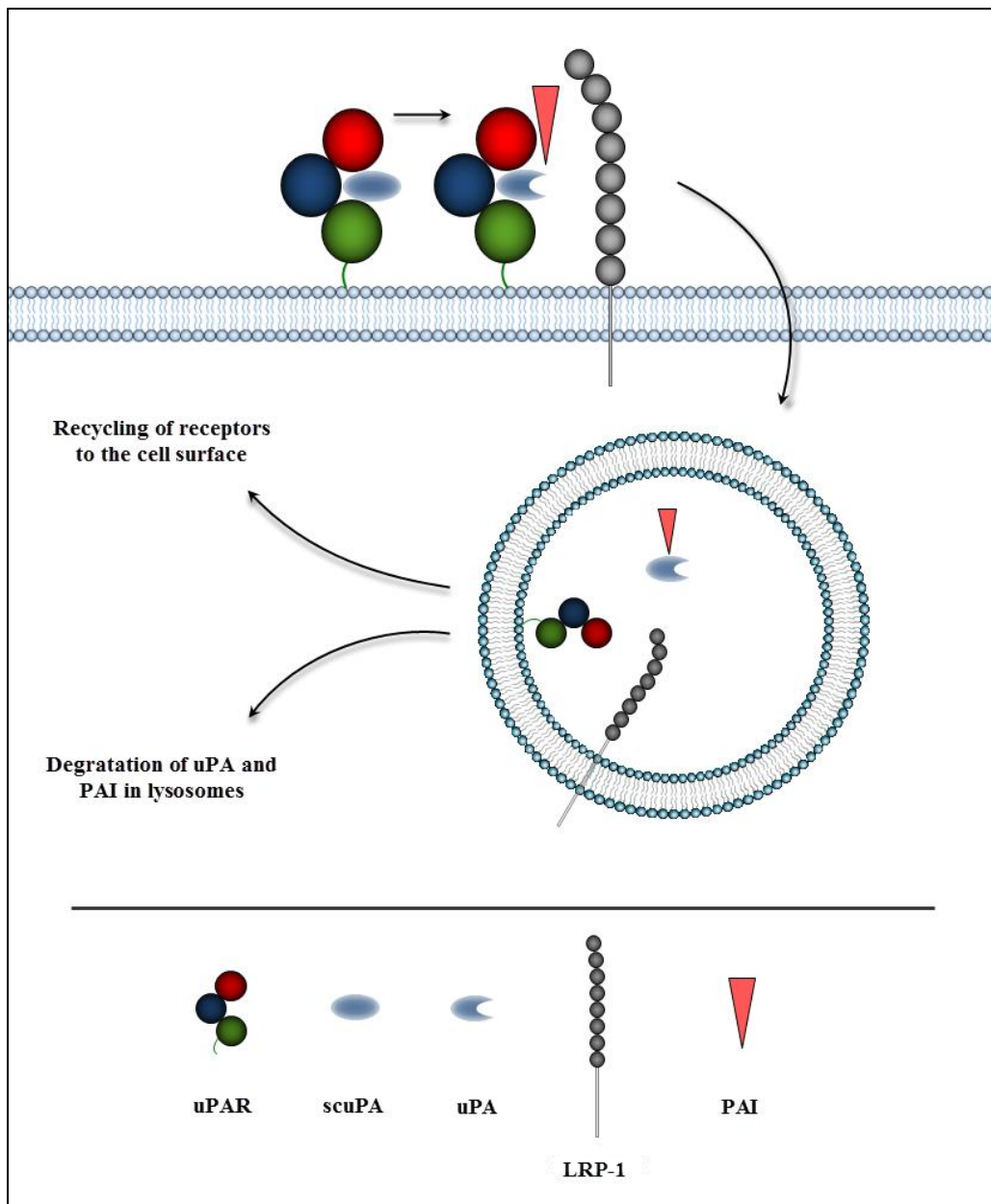
Figure 1.5: Regulation of uPA-uPAR complexes. Based on Binder *et al.* (117). Following uPA inhibition, the uPA-uPAR-PAI-1 complex interacts with certain members of the LDL receptor family, in this case the LDL related protein (LRP)-1 receptor. This leads to internalization of the complex through formation of clathrin-coated vesicles. Subsequently, uPA and PAI-1 are degraded in lysosomes while the receptors are recycled to the cell surface (109, 110). Moreover, studies have demonstrated that the direct interaction of uPA-PAI-1 with LRP-1 receptor can induce the activation of the Jak-Stat pathway, subsequently leading to enhanced cell migration (not shown) (118). In addition, the interaction of uPA-PAI-1 with very-low-density lipoprotein (VLDL) receptor can increase cell proliferation by stimulating sustained ERK activation (not shown) (119). Although uPA-PAI-2 is cleared from the cell surface through interactions with LRP-1 and VLDL-receptors, these are of lower affinity than those of uPA-PAI-1 and are not able to induce cell migration or proliferation (120, 121).

1.6.4. Plasminogen activator receptor: Urokinase receptor, uPAR

The structure of uPAR consists of three homologous domains anchored to the cell membrane through a glycosyl-phosphatidylinositol (GPI) moiety (figure 1.6). The three domains, designated D1, D2 and D3, have an apparent mutual sequence homology and belong to the Ly-6/uPAR (LU) family of domains (81, 122, 123).
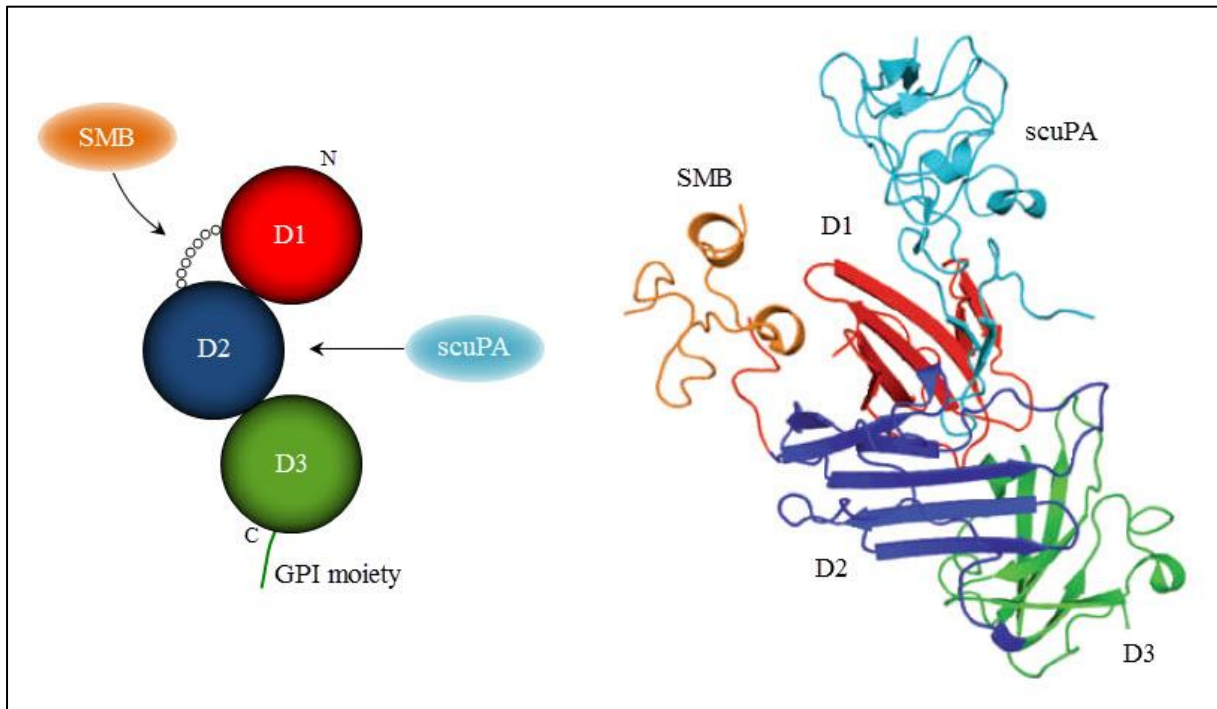
Figure 1.6: The structure of uPAR and the location of the ligand-binding sites of scuPA and SMB region of vitronectin. Modified from Huai *et al.* **(124).** D1 (colored red) is the N-terminal domain, D2 (colored blue) connects D1 to D3, and D3 (colored green) is the C-terminal domain that anchors the receptor to the plasma membrane through the GPI moiety (96). The three domains have a typical three-finger fold with three adjacent loops rich in β-pleated sheets and a small C-terminal loop (123-125). The scuPA binding site is localized in the central cleft of the receptor which is generated by an interdomain assembly of D1, D2 and D3. In contrast, the binding site for the somatomedin B (SMB) domain of vitronectin is found at the outer side of the receptor; at the two loops connecting the central four β-sheets in D1 as well as the linker region connecting D1 and D2 (112, 124).

Under normal physiological conditions, the level of uPAR expression on cell surfaces is relatively low. This has been demonstrated in homeostatic mouse tissues that have been investigated (126). The expression is however consistently upregulated during certain tissue remodeling processes. This is illustrated during skin wound healing, where uPAR expression

is selectively induced at the very tip of the leading edge of the keratinocytes migrating to close the wound (126). In cancer tissue, uPAR is frequently overexpressed and linked to local invasion and metastasis. The actual contribution of the tumor and stroma compartments to this expression varies among the different types of cancer (127, 128).

According to Usher *et al.*, uPAR is expressed on the surface of macrophage-like cells and neutrophils in both malignant and benign prostatic tissue. In most of the carcinomas (~90%) the macrophage-like cells were found in the interstitial tissue between the tumor cell islands. Contrary, in most of benign tissues they were located in the lumen of the glands and found in the interstitial tissue in fewer cases (~30 %). This indicates that macrophages are recruited to the stroma of malignant tissue (103). Furthermore, Usher et al. found that uPAR expression of malignant tissue appeared to be upregulated in response to the presence of cancer cells (103). Previously it has been demonstrated that upregulation of uPAR can be induced by the activation of monocytes to macrophages and by inflammatory factors produced by PCa cells such as tumor necrosis factor (TNF)-α and monocyte chemotactic proteins (MCP) (129, 130). The overexpression of uPAR participates in tumor proteolysis by increasing the ability of cancer cells to overcome barriers of BMs and ECM. Additionally, signaling through uPAR in stromal cells might also contribute to the tumor-promoting functions of the stroma (103).

Other studies have demonstrated that uPAR is predominantly expressed on the surface of the cancer cells, with little or no expression in the stromal cells (131, 132). Gavrilov *et al.* exemplify that there is no expression of uPAR on neither macrophages nor neutrophils, despite the well documented expression in these cell types (130).

The uPAR interactome

A relatively large number of potential biological ligands and interactors have been identified for uPAR. Based on the current level of evidence, these interactors may be divided into two groups (133). The first group is formed by uPA and the ECM protein vitronectin, which is considered to be the main uPAR ligands. Their physical and functional interaction with uPAR is well characterized as illustrated in in figure 1.6 (113, 114, 134, 135).

The binding site for scuPA on uPAR is localized in the central cleft of the receptor, making the entire external structure of uPAR free for interactions with other proteins. In fact, the somatomedin B (SMB) domain of vitronectin binds uPAR at the outer side of the receptor. Studies have found that uPAR can accommodate both uPA and the SMB domain simultaneously and that uPAR binds more readily to vitronectin when it is pre-bound to uPA

(112, 123-125). The binding of uPA stimulates the receptor to cluster in cholesterol- and sphingolipid- enriched domains, referred to as lipid rafts, in the plasma membrane. This action also increases the ability of uPAR to bind vitronectin (135).

In contrast to uPA and vitronectin, the structural basis and binding sites of the second group of interactors are less understood. This group encompasses a long series of proteins including a variety of receptor tyrosine kinases, integrins, G-protein coupled receptors (GPCRs), EGFRs and receptors of the LDL family (114, 135-140). Through these interactions, uPAR activates intracellular signaling molecules including the tyrosine kinase Src, the serine kinase Raf, focal adhesion kinase (FAK) and extracellular-signal-regulated kinase (ERK). Activation of these proteins subsequently results in profound changes in cell migration, adhesion and proliferation (141-143).

Proteolytic and non-proteolytic function of uPAR

Coherent with uPA being a serine protease, uPAR is involved in the regulation of extracellular proteolysis by promoting cell surface activation of plasminogen, generating plasmin. The proteolytic function of uPAR, together with its signaling functions, enable it to facilitate tumor cell adhesion and invasive migration through the ECM and to determine whether tumor cells will, or will not, proliferate *in vivo* (72, 73).

Cell migration across the blood barrier and into tissues is an essential process during the immune response against infections, as well as during inflammation and metastasis. The process is tightly linked to adhesion and chemotaxis, and the role of uPAR in these mechanisms is supported by several studies (144-146). Rijneveld *et al.* examined the migration pattern of immune cells in uPAR-deficient (uPAR$^{-/-}$) and wild-type mice after inoculation with *Streptococcus pneumoniae*. The results demonstrated that recruitment of neutrophils into inflamed tissues is significantly reduced in the absence uPAR. Whereas the wild-type mice recovered completely from the *Streptococcus pneumoniae* infection, the uPAR-deficient mice eventually died from the infection, indicating a fundamental role of uPAR in the inflammatory response (144).

The capacity of uPAR to influence cell migration depends on the functional linkage and physical association with integrins (136, 140, 146, 147). Integrins are α/β heterodimeric cell adhesion receptors that mediate bidirectional interactions between cells and the ECM. To date, 18 α- and 8 β-subunits have been mapped on the human genome, together forming 24 known heterodimers with substantially distinct ligand specificities and affinities (148).

The vitronectin receptor, $\alpha_v\beta_3$ integrin, is frequently found co-expressed with uPAR in aggressive tumors, and studies have demonstrated that uPAR-$\alpha_v\beta_3$ interactions can play an essential role in tumor cell migration and invasion (149). An initial binding between uPAR and vitronectin increases the contact between the cell and ECM, bringing all matrix receptors present in the plasma membrane in closer contact with their extracellular ligands. Specifically, $\alpha_v\beta_3$ integrin can bind vitronectin and thereafter trigger changes in cell morphology, migration and signal transduction (113). The activation of Rac, a small GTPase of the Rho family, stimulates cytoskeleton reorganization and morphological changes at the leading edge of the cell by activating actin polymerization and membrane protrusion. The final result is tumor cell motility and invasion, onto the bound vitronectin (150-152).

In addition to the $\alpha_v\beta_3$-uPAR interaction, uPAR can also interact with $\alpha_M\beta_2$, $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins (149). Several studies have demonstrated a physical association between uPAR and the fibronectin receptor, $\alpha_5\beta_1$ integrin. This association increases the activity state of $\alpha_5\beta_1$ integrin, and consequently enhances fibronectin binding. Aguirre Ghiso *et al.* found that adhesion to fibronectin resulted in a robust and persistent ERK activation, which appears to be required for the cancer cells to sustain proliferation *in vivo* (figure 1.7) (153). Interestingly, *in vivo* studies have demonstrated that blockage of uPAR leads to abatement of uPAR-$\alpha_5\beta_1$ dependent signal transduction thereby reducing the ERK pathway activation and resulting in tumor cell dormancy (141, 153-157). In its dormant state, the tumor cells are present in a quiescent mode and are not biologically or clinically apparent. Both local and metastatic cancers undergo a period of dormancy before entering a stage of progressive growth.

The results from the abovementioned studies implicate a fundamental role of uPAR in determining whether tumor cells will proliferate or become dormant (42, 153).
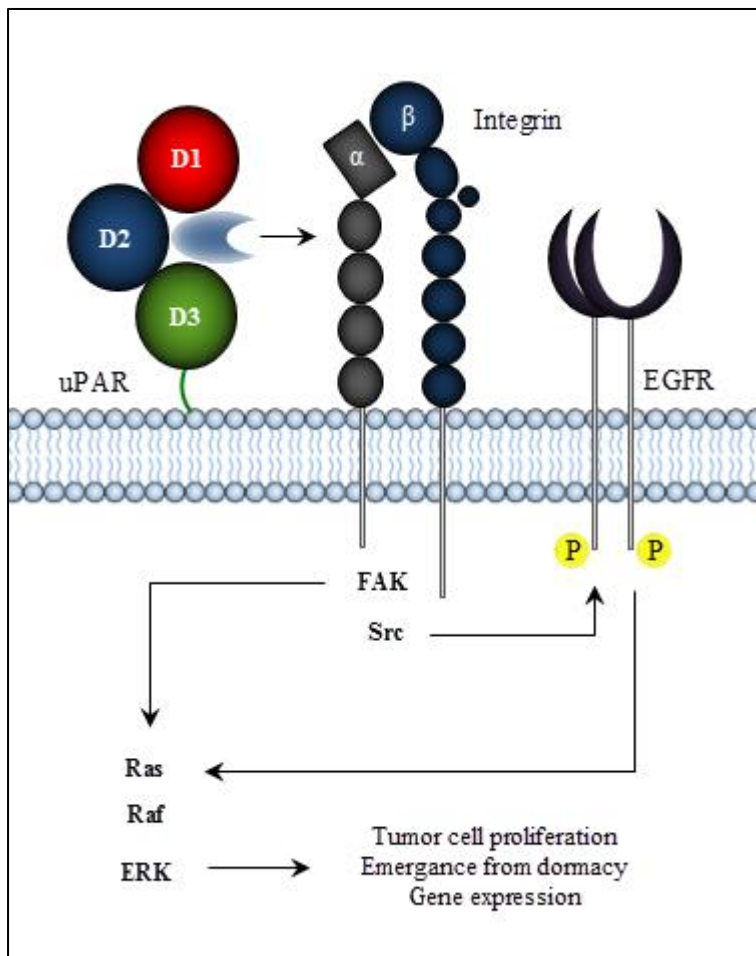
Figure 1.7: uPAR and $\alpha_5\beta_1$ integrin signaling pathway. Based on Smith and Paez *et al.* (158, 159). Rapid growth of metastatic carcinoma *in vivo* is regulated by high expression of uPAR that, by interacting and activating $\alpha_5\beta_1$ integrins, initiates a signaling cascade that culminates ERK activation. Initially, the uPAR-$\alpha_5\beta_1$ interaction promotes auto-phosphorylation of FAK. The tyrosine kinase Src is also activated and can further phosphorylate and activate EGFR to enhance the ERK pathway activation. Studies have demonstrated that activated ERK is involved in tumor cell proliferation and emergence from tumor dormancy. Furthermore, ERK targets the transcription of uPAR suggesting a positive feedback loop for uPAR expression. The uPA-uPAR binding is required for uPAR-$\alpha_5\beta_1$ integrin-EGRF signaling, whereas in $\alpha_v\beta_3$ signaling uPA does not appear to be required (113, 135, 153, 156, 160-162).

The role of uPAR in chemotaxis depends on the proteolytic cleavage of the receptor. The cleaved uPAR fragments provide new functionalities to uPAR, as the conformational changes facilitate the appearance of previously hidden ligand binding sites and further broadening the array of uPAR interactions (117).

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As illustrated in figure 1.8, the proteolytic cleavage of the linker region, connecting D1 and D2-D3, creates a soluble D1 fragment and a cell-anchored D2-D3 fragment (uPAR$_{2-3}$). After cleavage, the uPAR$_{2-3}$ expose a chemotactic epitope termed the SRSRY sequence. Previous studies have demonstrated that the SRSRY sequence is associated with the recruitment of cells expressing GPCRs of the formyl peptide receptor (FPR) family (117, 163). Furthermore, the new uPAR$_{2-3}$ form is unable to bind uPA or vitronectin, making the cleavage a regulatory mechanism to attenuate the biological function of uPAR (164).

As described in the next section, the soluble form of uPAR (suPAR) also appears to interact with receptors of the FPR family and thereby attracting and regulating cell migration (138).
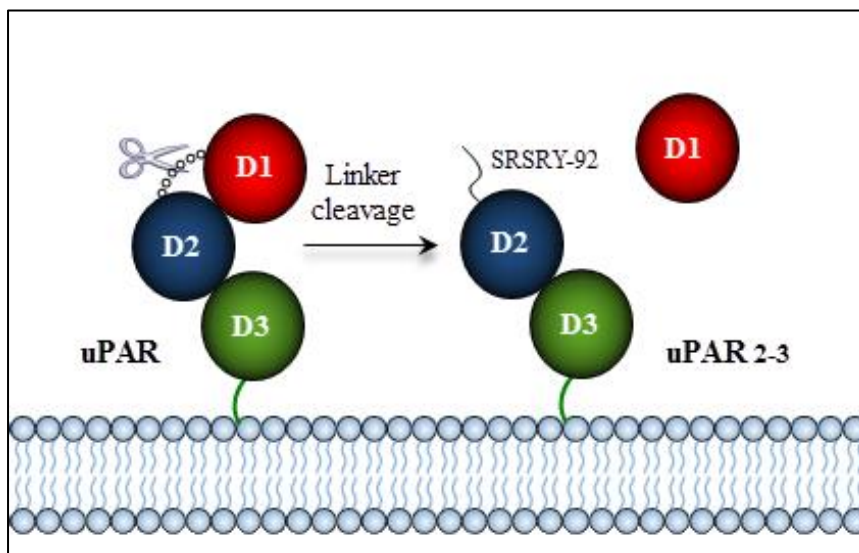
Figure 1.8: A schematic illustration of uPAR cleavage. Based on Thuno *et al*. (165). The linker region is susceptible to cleavage by different proteases, including uPA, plasmin, MMP-12, tissue kallikrein 4 and cathepsin G (166-168). The cleavage creates a soluble D1 fragment and a D2-D3 fragment (uPAR$_{2-3}$) anchored to the cell surface. The uPAR$_{2-3}$ expresses the SRSRY-sequence. (169, 170).

1.6.5. Soluble plasminogen activator receptor: suPAR

The proteolytic cleavage of the GPI moiety leads to the total release of uPAR from the cell-surface, generating suPAR. Proteases including phospholipase D, phospholipase C, cathepsin G and PI-PLC are known to be GPI-specific, and a mixture of these appears to be required to regulate the generation of suPAR (171).

In addition, full length suPAR can be cleaved in the linker region within the receptor. Full length suPAR shares the overall structure as uPAR and can be cleaved in the linker region by the same proteases as uPAR (figure 1.8). One exception is the uPA-catalyzed cleavage which appears to be limited to only GPI-anchored uPAR (172). The cleavage of the linker region creates a soluble D1 fragment, and a soluble D2-D3 fragment (suPAR$_{2-3}$), as illustrated in figure 1.10.
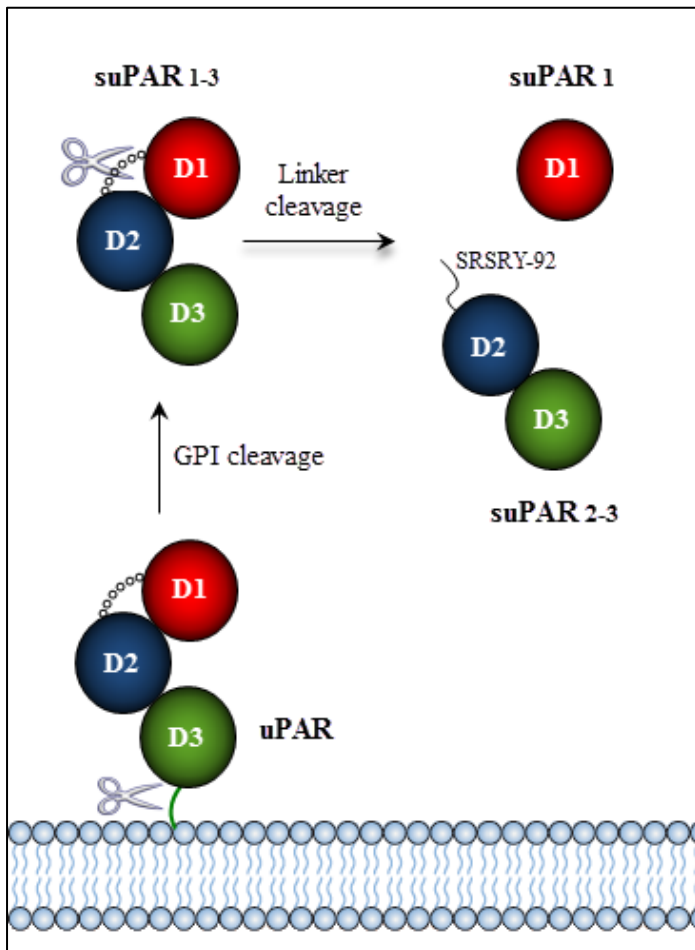
Figure 1.9: A schematic illustration of the generation and cleavage of suPAR. Based on Thuno *et al* (165). The intact uPAR can be released from the cell-surface by proteolytic cleavage of the GPI moiety. The linker region of full length suPAR (suPAR$_{1-3}$) can also be proteolytically cleaved be the same proteases as uPAR, generating a soluble D1 (suPAR$_1$) and a soluble D2-D3 fragment (suPAR$_{2-3}$).

Cleavage of the GPI moiety result in a reduced number of uPAR on the cell-surface, and the activity of the receptor is impaired. However, the released suPAR may provide new biological activities and the suPAR fragments have distinct properties related to their structural differences (165, 173, 174).

Function of suPAR and suPAR fragments

The full length suPAR, suPAR$_{1-3}$, undergo only a slight conformational change when shed form the cell surface, and *in vitro* studies have demonstrated that suPAR$_{1-3}$ is capable of specifically binding uPA as well as vitronectin (175-179). By competing with cell-bound uPAR, suPAR$_{1-3}$ may function as a scavenger and inhibit cell-associated plasminogen activation and cell adhesion to vitronectin (175-178).

In an *in vivo* study of nude mouse models, the effect of recombinant suPAR$_{1-3}$ on ovarian cancer cells was found to inhibit cell proliferation and reduce cell-associated matrix degradation. As a consequence, tumor cell growth and metastasis were reduced (175). A similar study by Piccotella *et al.* demonstrated that the mechanism of action of suPAR$_{1-3}$ appeared to be associated to a decreased level of ERK and FAK activation in PCa cells. The role of suPAR$_{1-3}$ as an uPA scavenger prevents binding of ligands to uPAR, thereby producing a lower level of ERK and FAK activation in the cells (180). These findings indicate that suPAR$_{1-3}$ might have possible implications of inhibiting cancer-promoting actions (165). Furthermore, the study showed that cleavage of suPAR by chymotrypsin reversed these effects (180).

The linker region of suPAR$_{1-3}$ is, similar to the cell anchored receptor, sensitive to proteases. Cleavage in this region leads to the generation of suPAR$_{2-3}$. Published work indicates that suPAR$_{2-3}$ is capable of inducing chemotaxis (138, 163, 166, 181). The exposed SRSRY sequence of suPAR$_{2-3}$ can chemoattract hematopoietic stem cells and monocytes by activating the high-affinity FPR and the low-affinity FPR-like 1 (FPRL1), respectively. Further it can chemoattract basophils by activating both FPRL1 and the low-affinity FPRL2 (138, 181). Activation of FPRs leads to the heterologous desensitization of chemokine receptors, such as CXCR2, which are strongly involved in the mobilization of hematopoietic stem cells (182, 183). This means that chemotactically active suPAR$_{2-3}$ may act as a classical chemokine being able to attract and regulate cell migration. Furthermore, a rapid release of suPAR$_{2-3}$ by *in vitro* activated neutrophils has been identified. The actual biological function of this is still unclear, but it is hypothesized that the production of suPAR$_{2-3}$ on sites of acute inflammation can

contribute to the recruitment of monocytes to these sites during an inflammatory response (184, 185).

Origin of suPAR

Under conditions of inflammation, uPAR is cleaved from the cell surface and converted to suPAR which is subsequently distributed in blood, urine and cerebrospinal fluid. Immune activation caused by infectious disease, autoimmune disease and a wide variety of solid tumors results in the cleavage of uPAR from the cell surface, thereby creating an easily detectable signal in the form of increased levels of suPAR in body fluids (186-189).

In PCa, the exact source and biological role of suPAR is not clearly defined. In breast cancer, Holst-Hansen *et al.* reported that uPAR was directly released by the cancer cells in a cell-dependent manner with a constant and direct correlation between cell number and amount of uPAR releases. In addition, Holst-Hansen *et al.* showed that tumor cells in breast cancer xenograft models could release uPAR into blood, and that the concentration of plasma suPAR is highly correlated with tumor volume (165, 190, 191).

Shariat *et al.* propose that high levels of suPAR are, at least in part, prostatic in origin. This suggestion is based on the observation that suPAR levels tend to decrease after prostatectomy. Additional to the findings of Holst-Hansen *et al.* on breast cancer cells, these results suggest that direct local production by cancer cells significantly contributes to the increased circulating levels of suPAR in patients with PCa (190, 191). The increased shedding of uPAR could be caused by the release from the surface of tumor cells and/or stromal cells in the cancer tissue, as a result of increased intra-tumoral proteolysis. However, the release of suPAR from the tumor and into the blood may not be proportional to the amount of uPAR present in the tumor tissue. Riisbro *et al.* did not find any correlation between suPAR levels in serum and tumor cytosols of breast cancer patients, suggesting that the increased levels found in breast cancer patients could not have been solely the result of an increased amount of uPAR being shed from the primary tumor tissue (192).

In accordance to these findings, a growing body of evidence support the theory or suggests that some of the increase in blood suPAR levels could occur because of a systematic reaction to the cancer cells, including activation of monocytes and neutrophils (190-192).

1.7. Soluble uPAR levels in blood reflect underlying pathology

In healthy individuals the median concentration of suPAR is, according to Stephens *et al.*, relatively low and has been cited as 1.2 ng/mL in both serum and plasma (186, 193).

The suPAR levels are present in various concentrations depending on the activation level of the immune system, and several studies have focused on the suPAR levels during infectious disease and among intensive care-treated patients. In a study by Koch *et al.*, the prognostic impact of suPAR was demonstrated by measurement of serum suPAR concentration in critically ill patients at a medical intensive care unit. Samples were obtained by the time of admission, prior to intensive care treatment, as well as during the first week of clinical course. Compared to healthy volunteers, critical care patients showed significantly elevated suPAR levels in serum. In addition, critically ill patients with low suPAR levels upon admission, day 3 and on day 7 of the clinical course, had a significantly better outcome during the one year follow up. Based on these observations, Koch *et al.* suggest that suPAR in serum might be utilized as a marker to assess disease severity in critically ill patients (194). Further, they note that suPAR is a stable and robust marker exhibiting favorable properties due to its high stability in serum samples and limited circadian changes in plasma concentrations. Other studies have demonstrated that suPAR levels in serum and plasma also remain constant through several freezing and thawing cycles and are more stable in room temperature compared to other markers (195, 196).

Similar to high-sensitive C-reactive protein (hsCRP), suPAR is believed to be a marker of low grade inflammation. Eugene-Olsen *et al.* have indicated that elevated suPAR levels in plasma are associated with the increased risk of developing cancer, cardiovascular disease (CVD), diabetes and mortality in the general population. The suPAR levels were measured in 2,602 individuals over a 12.5 years period, and the presence of an elevated baseline suPAR level was associated with an increased risk of disease and mortality during the course of the study. These findings indicate that suPAR might be used as an early warning, inflammatory biomarker that potentially may improve the current ability to predict the development of these major diseases, and also to predicting mortality. Further studies are however warranted to confirm whether the associations between suPAR and diseases are present in other populations, as the study by Eugene-Olsen *et al.* only included Caucasian participants (197).

Moreover, enhanced levels of suPAR in blood have been found in several pathological conditions including human immunodeficiency virus (HIV), malaria, active tuberculosis,

sepsis, pneumococcal- and streptococcal pneumonia bacteraemia. Various forms of solid tumors such as lung-, breast-, colorectal-, ovarian- and prostate cancer have also shown elevated levels of suPAR. Enhanced suPAR levels in all these conditions have been found to be associated with an unfavorable prognosis (186, 193, 194, 198-209).

1.8. Elevated suPAR levels appears to be associated with aggressive PCa

In PCa, high levels of suPAR have been found in more aggressive tumors. Shariat *et al.* measured the plasma levels of suPAR in patients who underwent radical prostatectomy for clinically localized PCa, patients with PCa metastases to regional lymph nodes, patients with newly diagnosed PCa metastases to bone and healthy individuals. The results showed that the levels of suPAR were elevated in patients with PCa compared to healthy individuals. In patients with PCa, levels of suPAR were significantly higher in patients with bone metastasis than patients with lymph node metastasis, which in turn had higher levels than patients with non-metastatic PCa (191).

Similar findings have been reported by Miyake *et al*. Measurements of serum suPAR in 54 healthy controls, 62 BPH patients and 72 PCa patients showed that the suPAR concentration where significantly higher in PCa patients with metastases than in those without metastases. In addition, the mean serum levels of suPAR in patients with PCa appeared to be enhanced compared to those in BPH patients and healthy individuals (205).

Further, Shariat *et al.* demonstrated that among the patients with clinically localized PCa who underwent radical prostatectomy, higher preoperative levels of suPAR were associated with patients with features of biologically aggressive PCa compared to patient with features of non-aggressive progression. However, the authors of the study were not able to demonstrate that the collective amount of suPAR was a predictor for biochemical progression during the follow-up time, and a possible value of suPAR as a prognostic marker in PCa must be evaluated in a larger cohort of patients (191).

Based on the results from these two studies, an association between suPAR levels and PCa progression has been suggested. Both plasma and serum levels of suPAR indicate the degree of PCa progression and patients' prognosis, and measurements of suPAR might therefore serve as a useful adjunct to current conventional diagnostic tools.

1.9.   The role of suPAR in early PCa detection

Specific immunoassays have been developed to enable quantification of the individual fragments of suPAR (71, 210). In a clinical study, a cohort of men referred for prostate biopsy were used to assess whether measurements of intact and cleaved suPAR might discriminate patients found with PCa from men with no evidence of malignancy. A significant difference in levels of $suPAR_1$ and $suPAR_{2-3}$ was found in men with PCa compared to men with no evidence of cancer. The selective detection of suPAR fragments suggested enhanced discrimination compared to the PSA test, indicating that measurement of the individual suPAR forms in serum might improve the specificity of PCa detection and be complementary to PSA for PCa detection (71, 211).

To further investigate this, Steuber *et al.* measured several PCa-related biomarkers in serum to evaluate if a combination of these could improve PCa detection. The results showed a significant association of PSA, free PSA and suPAR forms with the presence of PCa on biopsy. This indicates that free PSA isoforms and different suPAR forms might be used to improve the selection of patients for PCa biopsy, thereby enhancing the diagnostic accuracy of early cancer detection when combined with total PSA and age (212).

Further research of larger cohorts of men is, however, warranted to confirm these results. The use of combined biomarkers, including suPAR, might allow clinicians to estimate the probability of a positive biopsy of an individual patient and thereby selecting appropriate patients for prostate biopsy. Clearly, no single analyte is likely to achieve the desired level of diagnostic accuracy for early PCa detection (55).

1.10.   The aim of the thesis

As described in the preceding chapters, previous studies provide arguments and findings to substantiate that serum levels of suPAR may be linked to PCa. The PA-system has been reported to participate in the degradation of ECM and BMs during cancer invasion and metastasis. The levels of suPAR are argued to reflect the activity of the PA-system and are therefore considered to be informative for the identification and prognostication of PCa.

However, previous studies utilizing serum measurements of suPAR for early detection of PCa, have reported conflicting results. Miyake *et al.* found a statistically significant difference in the suPAR values between BPH- and PCa patients, with higher levels in those with PCa (205). In contrast, McCabe *et al.* measured increased levels of suPAR in both BPH and PCa

patients compared to healthy individuals, but they were not able to establish a statistically significant relationship between suPAR levels and the presence of BPH and PCa (211).

With limited literature giving contradictory conclusions as basis, this present thesis aim to quantify the serum concentration of suPAR in BPH- and PCa patients and to determine whether there is a statistically significant difference between the two groups. Elevated levels of suPAR in PCa- compared to BPH patients might implicate that quantification of suPAR in serum could improve the discrimination of cancer from benign patients, which in turn is important as these diseases have distinct treatments and medical interventions.

2. Materials and methods

2.1 Selection of cohort

Blood samples were obtained with informed consent (see appendix 7.1) from 41 patients at Orkdal Hospital and St. Olavs Hospital between September and December 2012.

21 samples were collected from BPH patients hospitalized for surgery at Orkdal Hospital. The blood samples were obtained at the same day as surgery, and the mean age of the patients were 70.8 years (range: 59-82 years).

The remaining 20 samples were collected from PCa patients with small volume cancer, characterized by a Gleason score of 6 (3+3) and a PSA $\leq$ 10 ng/mL. Blood samples were obtained during a routine visit to the cancer policlinic at St. Olavs Hospital, and the mean age of these patients were 63.7 years (range: 57-75 years)

All samples were drawn before any prostatic manipulation. Patients receiving treatment for their prostatic disorder were excluded from the study. A previous study demonstrates that the suPAR concentration in blood decrease after radical prostatectomy, indicating that removal of the prostate, or part of the prostate, might eliminate the cells from which suPAR originate (191).

2.2 The material of choice

All samples were collected to the Regional Research Biobank of Central Norway and registered in their database. The remaining material after completion of the present thesis will represent a valuable resource for future studies, and for this purpose the biobank requested a collection of serum samples.

The Nord-Trøndelag health study (HUNT) research biobank store large quantities of serum samples and additional information about participant's health status. The collection of serum samples in this project opens for collaborative studies with HUNT in the future.

Previous studies quantifying suPAR concentration in BPH- and PCa patients have not reported any disadvantages of the use of serum samples as the material of choice (71, 205, 211). A quantitative study of healthy donors demonstrated that all forms of blood preparations (serum, citrate plasma, EDTA-plasma and heparin-plasma) are suitable for determination of suPAR concentrations (186).

Based on this information, serum was decided to be the material of choice.

2.3 Blood collection and preparation

Peripheral venous blood was collected on serum tubes (Vacuette®l) with gel and centrifuged at 2500 gravy (g) in 15 minutes in room temperature. Serum was apportioned into six aliquots (500 µL each) and immediately stored at -80 ºC.

After blood collection, each sample was fully anonymized and the only personal information obtained was age and gender. Consequently, the possibility to trace the sample back to the identity of the patient emanates, and the scientist is not able to obtain further information about the sample. Furthermore, the participant's right to demand destruction, deletion or surrender of biological material does not apply to anonymized samples as stated by the Health Research Act (§16).

The BPH samples were collected and prepared by the laboratory staff at Orkdal Hospital, and thereafter they were sent directly to St. Olavs Hospital to be apportioned into aliquots and stored. Procedures for blood sampling, sample preparation and transport were distributed to May-Britt Sætre and Eva Gundersen at the laboratory (see appendix 7.2 and 7.4).

The PCa samples were collected at the Cancer Polyclinic of St. Olavs Hospital. The samples were centrifuged, apportioned into aliquots and stored at the laboratory. Procedures for blood sampling were distributed to the urotherapist Inger Stokkan at the polyclinic (see appendix 7.3 and 7.4).

2.4 Collection of samples to the Regional Research Biobank of Central Norway

The Regional Research Biobank of Central Norway is a research facility where collections of human biological material, mostly from patients, are stored. A flexible database store relevant sample information, medical information regarding the patients from whom the samples originate and information obtained from analyzing the samples (213).

2.4.1. Establishing a new project in the biobank

All serum samples obtained in the present thesis were entered into the biobank as a new project. In general, every research project must have an approval from the Regional Ethical Committee (REC) before start. The REC evaluates the ethical values concerning the objective and method of the project, the consent and confidentiality, as well as the risk and security for the participating individuals.

Approval from REC was obtained in a previous study organized by the biobank, where a reference range for suPAR in serum and urine was established. The same approval was used in the present thesis with some amendments related to end date, number of participants and changes to the recruitment procedure. According to the Health Research Act (§11), an approval from REC must be submitted for amendments to the project's purpose, methodology, duration or organization. The REC had no ethical objections to the change of the project, allowing the project to continue under the new conditions (see appendix 7.5).

Before the present thesis were granted an account in the biobank database system, all users had to sign a safety- and declaration of confidentiality form, stating to not link samples to the personal identity of the donor due to private lists (see appendix 7.6) . A declaration of confidentially to Helse Midt-Norge iT (HEMIT), the operator of the database system, was also endorsed (see appendix7.7).

2.4.2. Registration of samples in the database

The database of the biobank is constructed to automatically encrypt the personal identification number by using a strong encryption algorithm, making the samples and the associated information completely anonymous to the scientists. The link between the patient identity and the encrypted number are kept under strict access control, in key files separated from the project data (213).

In the present thesis, the samples were fully anonymized directly after blood sampling and no personal identification number was available. To be able to register samples in the database, dummy numbers were generated to replace the personal identification numbers normally registered on every donor.

Further, the database generates a unique identification number for each sample. This number is a combination of the biobank identification number, a project number and a sample identifier. This unique number can be converted to a machine readable barcode. The barcode is printed on a label sticker and attached to the sample container.

The following figures (figure 2.1 to 2.5) illustrate the steps when entering a sample to the database of the biobank.

Figure 2.1: Generation a dummy number using an Excel script. The present thesis obtained anonymized samples, and the age and gender was used to generate dummy numbers. Note that the figure is only to illustrate the procedure and the generated number was not used in the study.

Figure 2.2: Adding a new sample to the database of the biobank. When a new sample is added, the date of sampling (1) as well as the dummy number (2) is entered into the database. The consent form and the time of signature is also added (3).

Figure 2.3: Characteristics of the sample. Data concerning sample date (1), age (2), sex (3), blood fraction (4), diagnosis (5) and storage (6) is added to the database. Only men were participating in the study and serum samples were obtained. For the diagnosis, either BPH or PCa were selected. The samples were stored in a freezer at - 80 ° C.

Figure 2.4: Location of the samples in the biobank. The exact location and position of each sample in the biobank is registered in the database. Samples are placed in a boxes in positions ranging from 1-91 (1). The box is placed in specific racks and positioned in particular shelfs in the freezers of the biobank (2).

Figure 2.5: Bar codes is generated and attached to the sample tube. The bar code number is then converted form of the unique identification number for each sample unit. The number is a combination of the biobank identification number, a project number (i.e. the identification number of the project in which the original specimen was acquired) and a sample identifier (i.e. a serial number).

Id	Klasse	Oppbrukt	Donor	Opprettelsesdato	Prøveenhet	Lokasjon	Beholder	Prøve-Id	Prøvedato_dato	Alder_heltall	Kjønn_flerval
50186	Blod	false	2	2012.10.08	50186	Norge / St. Olavs Hospital / Laboratoriesenteret / 231.04.045 / Fryser 3 / Hylle 1 / Rack 02 / Boks 01 /	Rør	0010002400001638	2012.10.08	56	Mann
50187	Blod	false	2	2012.10.08	50187	Norge / St. Olavs Hospital / Laboratoriesenteret / 231.04.045 / Fryser 3 / Hylle 1 / Rack 02 / Boks 01 /	Rør	0010002400001752	2012.10.08	56	Mann
50188	Blod	false	3	2012.10.08	50188	Norge / St. Olavs Hospital / Laboratoriesenteret / 231.04.045 / Fryser 3 / Hylle 1 / Rack 02 / Boks 01 /	Rør	0010002400001866	2012.10.08	31	Mann
50189	Blod	false	3	2012.10.08	50189	Norge / St. Olavs Hospital / Laboratoriesenteret / 231.04.045 / Fryser 3 / Hylle 1 / Rack 02 / Boks 01 /	Rør	0010002400001980	2012.10.08	31	Mann
50190	Blod	false	3	2012.10.08	50190	Norge / St. Olavs Hospital / Laboratoriesenteret / 231.04.045 / Fryser 3 / Hylle 1 / Rack 02 / Boks 01 /	Rør	0010002400002066	2012.10.08	31	Mann

Figure 2.6: Overview of saved samples. A donor number is generated from the dummy number.

2.5   Measurement of suPAR concentration in serum

The IMUBIND® uPAR ELISA Kit (American Diagnostica, see appendix 7.8) was used for the quantitative determination of suPAR in serum. This ELISA kit was made available for the project and alternative kits were not evaluated.

ELISA is an abbreviation for "enzyme-linked immune-sorbent assay" and the principle of the assay is illustrated in figure 2.7. In the assay, the uPAR standards of 0.00, 0.25, 0.75, 1.5, 2.0 and 3.0 ng/mL and patient samples (100μL) were added in duplicates to the plate of 96-microwells. The microwells are precoated with a solid phase of mouse monoclonal uPAR antibodies (figure 2.7, step 1). When standards and samples were added, the suPAR antigen binds the monoclonal uPAR antibodies (figure 2.7, step 2).

Following overnight incubation, a washing procedure was performed to remove unbound material. Washing buffer (300 μL) was added to the microwells by using a multi canal pipette (Eppendorf Xplorer plus, range: 50-1200 μL). Subsequently, biotinyl-labeled secondary anti-human uPAR (100 μL) were added to the microwells. These antibodies recognize the bound suPAR molecules and create a "sandwich complex" (figure 2.7, step 3).
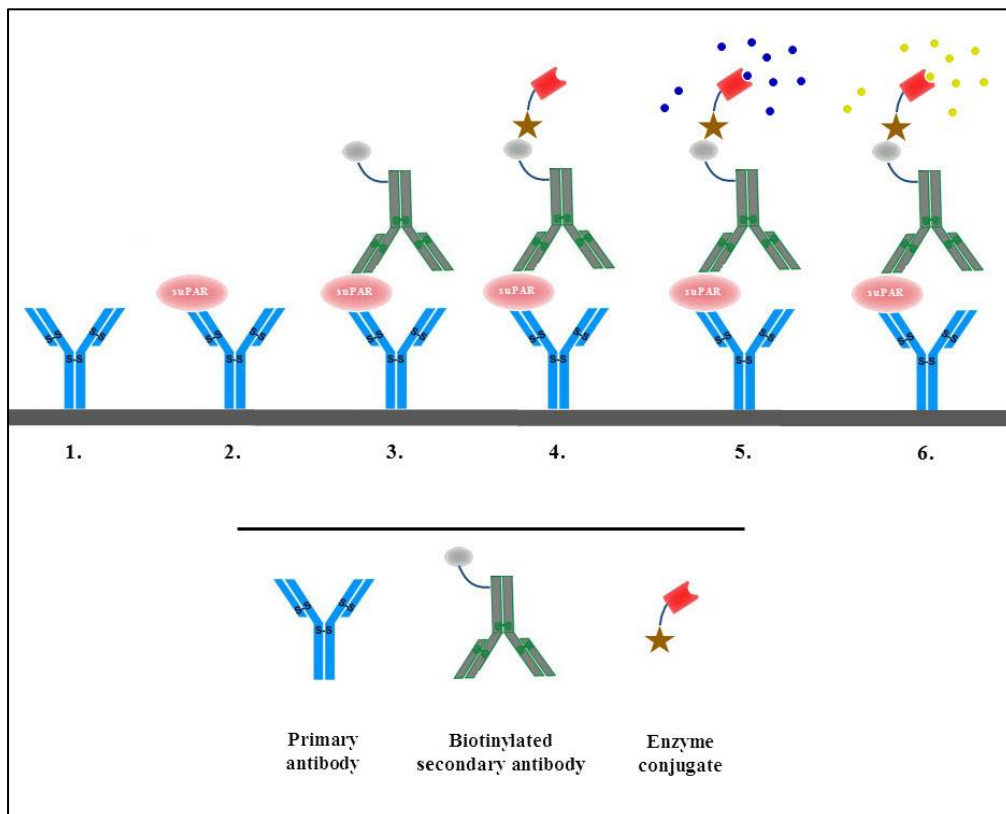
Figure 2.7: The principle of IMUBIND® uPAR ELISA (appendix 7.8).

Following a new washing step, the enzyme conjugate (100 μL) was added. The conjugate consists of streptavidin conjugated horseradish peroxidase (HRP). During the one hour incubation, streptavidin binds biotin of the secondary antibody to complete the formation of the antibody-enzyme detection complex (figure 2.7, step 4).

Following another washing step, perborate/3,3',5,5'-tetramethylbenzidine (TMB) substrate (100 μL) was added to the microwells. As depicted in figure 2.8, the reaction between the enzyme and the substrate creates a blue colored solution in the microwells (figure 2.7, step 5). The more suPAR a sample contains, the more intense is the blue color which develops.

After 20 minutes of incubation, the color development was stopped by the addition of sulphuric acid (H_2SO_4) (50 μL). The color of the solution changes to yellow (figure 2.7, step 6). A plate of microwells after addition of H_2SO_4 is depicted in figure 2.9.

The absorbance at 450 nm was measured using a microtiter plate reader (depicted in figure 2.10). To calculate the suPAR concentration, the corrected absorbance of all wells must be calculated by subtracting the absorbance of the blank standard (0 ng/mL).

The standard curve was constructed in a linear system by plotting the corrected absorbance of the standard (ordinate, y-axis) against the corresponding suPAR concentration (abscissa, x-axis), and draw the best fitting line. The determination of the suPAR concentration of the patient samples was performed by interpolation on the curve. The mean suPAR concentration of the duplicate samples was calculated and used for further analysis.
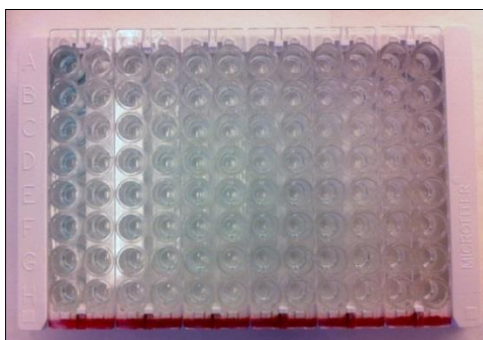
Figure 2.8: The 96-microwells after TMB substrate is added. The more suPAR a sample contains, the more intense is the blue color which develops.
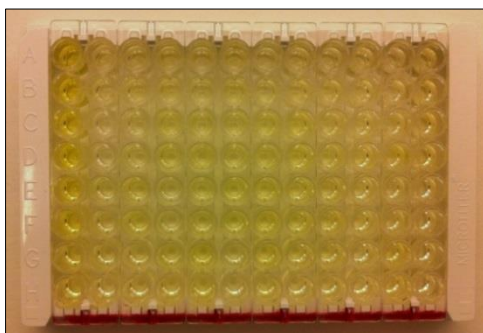
Figure 2.9: The 96-microwells after addition of the stop solution, H_2SO_4. The blue color development in the microwells is stopped, and the color changes from blue to yellow.
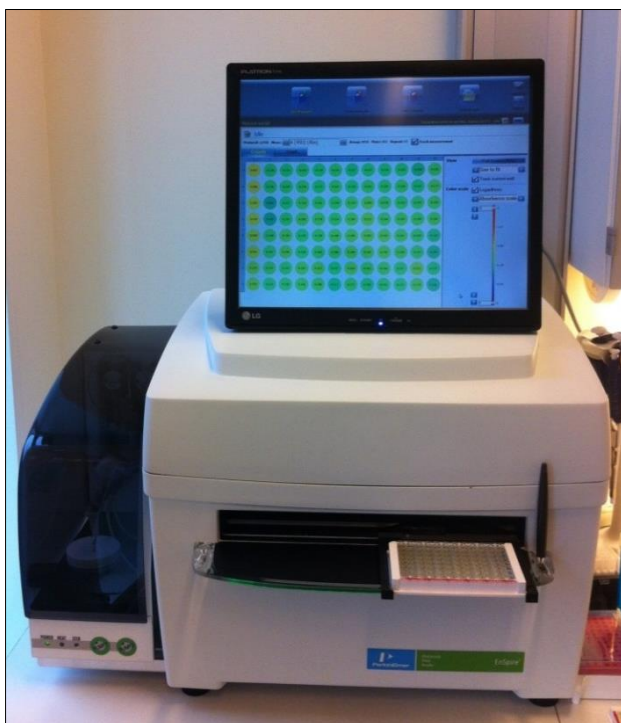
Figure 2.10: The absorbance (450 nm) is measured by Perkin Elmer microtiter plate reader delivered by EnSpire.

2.5.1. Optimization of the IMUBIND® uPAR ELISA

The IMUBIND® uPAR ELISA kit is validated on citrate collected platelet poor plasma. The material of choice, serum, was decided without this knowledge. However, according to the kit supplier, the ELISA kit had been successfully used with all blood preparations, including serum. Since the assay was not validated on serum samples, it had to be optimized for this type of sample material in order to achieve optimal detection of suPAR.

Several initial test runs were performed by using serum samples with different dilutions (undiluted, 1:2, 1:4). This was performed to determine which, if any, dilution of the sample would give absorbance values within the limits of the standard curve. A sample with high levels of suPAR must be diluted to ensure that its value fall within the standard curve range and a low-level sample must be assayed without dilution.

2.5.2. Precision measurements IMUBIND® uPAR ELISA

To describe the precision of the measurements, the coefficient of variation (CV) of the duplicates was calculated in Excel.

The CV is defined as the standard deviation (SD) divided by the mean and is a function of how much an individual result (x_i) differs from the mean (x_{mean}). (214, 215). The acceptance criteria for the CV in the present study was 10%, based on information from the IMUBIND® uPAR ELISA supplier.

The formulas for calculating the mean, SD and finally the CV are:

$$X_{mean} = \frac{\sum_{n-1}^{N} x_i}{N} \qquad [2.1]$$

$$SD = \sqrt{\frac{\sum_{i=1}^{N}(x_i - x_{mean})^2}{N-1}} = \sqrt{\frac{\sum_{i=1}^{N} d_i^2}{N-1}} \qquad [2.2]$$

$$CV = \frac{SD}{x_{mean}} \qquad [2.3]$$

2.6   Statistical analysis

All statistical analyses were performed by using Excel 2010 (Microsoft) and SPSS version 21(IBM). Prism version 6 (GraphPad) was used to plot some of the graphs used in the thesis.

2.6.1. Parametric versus nonparametric analysis

Parametric tests (e.g. independent t-test, ANOVA) are based on the assumption that the data are sampled from a normal (Gaussian) distribution. The nonparametric tests do not assume that data follow a normal distribution. Rather than using normal distribution, the non-parametric test rank values from high to low and analyses the distribution of ranks.

Nonparametric statistical procedures are appealing because they make fewer assumptions about the distribution of the data, but they are less powerful as they use less information in their calculation.

2.6.2. Testing for normality

When analyzing differences between groups using parametric tests, a common requirement is that the dependent variable is approximately normally distributed for each category of the independent variable. This means that the suPAR concentration must be approximately normally distributed for the BPH- and PCa patient group. Normality testing was therefore performed prior to the statistical analysis to determine whether a parametric test could be used.

There are three approaches for assessing normal distribution: Graphical methods (histograms, normal probability plot, box plots), numerical methods (skewness and kurtosis) and formal normality tests (216). The Shapiro-Wilk test is a formal test of normality, and it is, according to SPSS, appropriate for small sample sizes (< 50 samples). The statistical test might however be over- or under sensitive, and the visual inspection of histograms and normal probability plots are advantageous to assess normality.

Shapiro-Wilk test

The Shapiro-Wilk test is a statistical method for assessing normal distribution. The test analyzes the null hypothesis that all values are sampled from a population that follows normal distribution. The null hypothesis is tested at $\alpha=0.05$ (95%) confidence level in SPSS.

By calculating the correlation between the given sample values and ideal normal scores, the Shapiro-Wilk test can determine a W-value by the following formula:

$$W = \frac{(\sum_{i-1}^{N} a_i x_{(i)})^2}{\sum_{i-1}^{N}(x_i - x_{mean})^2}$$

[2.4]

The x_i represent the given sample values and a_i are constants generated from the means, variances and covariances of the ordered statistics of a sample size (N) from a normal distribution. The W-value is then used to compute the p-value (216).

If the p value is greater than α=0.05, the given values are not inconsistent with a normal distribution and pass the test. The normality test cannot, however, prove that the values are samples from normal distribution; it only demonstrate that the deviation from the ideal normal distribution is not more than what is expected with chance alone.

If the p-value is less than α=0.05, the null hypothesis is rejected and the alternative hypothesis, that the data is not sampled from normal population, is accepted.

Normal probability plot and histograms

The normal probability plot is a plot of the ordered suPAR concentrations, y_i, $i = 1, 2, \ldots, N$, versus the theoretical normal distribution z-scores. If the sample is actually drawn from a normal distribution, the plot of y_i versus the z-score will be approximately a straight line. Deviations from linearity correspond to various types of non-normality (217, 218).

Constructing a normal probability plot requires calculating probabilities and corresponding z-scores for each observation. The probability is corresponding to the normal contribution and is calculated by the following formula:

$$prob = \frac{i - \frac{3}{8}}{n + \frac{1}{4}}$$

[2.5]

The z-score is a standard normal random variable and represents the number of standard deviations (σ) the data value is from the mean (μ). The z-score is important because if the variable x is normally distributed, z is as well. The z-score is calculated by using the function = NORMSINV (probability) in Excel or by the following formula:

$$z = \frac{x - \mu}{\sigma}$$ [2.6]

The correlation coefficient of the points of the normal probability plot is used to measure and evaluate the linearity of the probability plot. The correlation coefficient is compared to a table of critical values for the given N and significance level to provide a formal test of the null hypothesis that the observations arise from normal distribution. If the correlation coefficient is greater than the tabulated value, the hypothesis of normal distribution cannot be rejected. Table 2.1 lists the critical values of the normal probability plot correlation coefficient for the present thesis. (219, 220).

Table 2.1: Critical values of the correlation coefficient

N[a]	$\alpha = 0.05$[b]
20	0.9498
21	0.9515

[a] The given N is 20 (PCa samples) and 21 (BPH samples).

[b] The significance level is set as $\alpha=0.05$.

The inspection of histograms is a graphical method for assessing normal distribution. The histogram shows the frequency distribution of the suPAR concentrations within specified suPAR ranges. If the data samples are from a normal distributed population, the histogram will have a shape similar to a symmetrical and bell-shaped curve (217).

If the data set in one or both groups is non-normally distributed, an appropriate transformation can often yield a data set that does follow a normal distribution. This might increase the applicability and usefulness of statistical tests which is based on the normal distribution of the variables. The Box Cox transformation is a particular useful family of power transformations (221).

Box Cox transformation

The Box Cox transformation is given by:

$$y_i^{(\lambda)} = \frac{x_i^{\lambda} - 1}{\lambda} \quad if \ \lambda \neq 0$$
$$y_i^{(\lambda)} = \log(x_i) \quad if \ \lambda = 0$$

[2.7]

where x_i is the suPAR concentration, and λ is the transformation parameter to be determined such that the values of $y_i^{(\lambda)}$ are approximately normally distributed. The transformation given by [2.7] is defined only for positive values of x_i (218).

There are several methods of finding the appropriate value of the Box Cox transformation parameter, λ. One method involves the use of the normal probability plot and the correlation coefficients for normality. In essence, the method is to find the λ that will make the normal probability plot most linear. Various values of λ are tested until the maximum value of the correlation coefficient is obtained, and the value of λ corresponding to the maximum correlation of the plot is then the optimal choice for λ. Furthermore, the range of λ-values where the corresponding correlation coefficient is greater than the critical value for the given N and significance level would be acceptable as a suitable transformation (218).

Transformations are not always successful and must be verified with a normal probability plot of the transformed data, called a normal Box Cox probability plot. Nonparametric tests are generally an appropriate alternative when the normality assumption does not hold (217).

2.6.3. Mann-Whitney test

The Mann-Whitney test is a nonparametric test used to compare the suPAR concentrations in the BPH- and PCa patients and to determine if there is a statistically significant difference between these two groups.

The test is based on converting the test variables into rank scores. First, the Mann-Whitney test ranks all values from low to high, paying no attention to which group each value belongs to. The smallest number gets a rank of 1 and the largest number gets the rank of N, where N is the total number of values in both groups. Then, the Mann-Whitney test averages the ranks in each group and evaluates whether the mean ranks of the two groups differ significantly from each other.

The null hypothesis of the Mann-Whitney test is that the mean of ranks for the BPH- and PCa group are equal. If the mean ranks of the two groups are very different, the p-value will be very small. If the p-value is large, the data do not give any reason to reject the null hypothesis, but it is, however, not the same as saying that the two groups are similar. The null hypothesis is tested at $\alpha=0.05$ (95%) confidence level in SPSS.

The Mann-Whitney test calculates a U- and a z-value. The sum of ranks of the groups (R), together with the lowest N_1 ($N_{PCa}=20$) and greatest N_2 ($N_{BPH}=21$) is substituted in the following formula for U_1 and U_2:

$$U_1 = N_1N_2 + \frac{N_1(N_1+1)}{2} - R_1 \qquad [2.8]$$

$$U_2 = N_1N_2 + \frac{N_2(N_2+1)}{2} - R_2 \qquad [2.9]$$

The smallest of U_1 and U_2 is called the U-value. For cases where both N_1 and N_2 do not exceed 20, the U-value is used to determine whether the null hypothesis is rejected or not. Critical U-values for the Mann-Whitney test are given in statistical tables. When both N_1 and N_2 exceed 20, as in the present study, the sampling distribution of U approaches the normal distribution (217).

The U value is therefore used to calculate the z-value by using the following formula:

$$z = \frac{U - N_1N_2/2}{\sqrt{\dfrac{N_1N_2(N_1+N_2+1)}{12}}} \qquad [2.10]$$

The z-value is then used to find the corresponding p-value in the statistical table for normal distribution (217).

3. Results

3.1 Optimization of IMUBIND® uPAR ELISA

The results from the test run are summarized in table 3.1, and the raw data from the assay are found in appendix 7.10.

Table 3.1: Test run of IMUBIND® uPAR ELISA

Sample	DF*	Absorbance	suPAR ng/mL
1	1	0.288	1.74
	2	0.227	2.68
	4	0.134	2.92
2	1	0.233	1,38
	2	0.187	2.16
	4	0.110	2.29
3	1	0.278	1.68
	2	0.154	1.72
	4	0.093	1.85

* Dilution factor (DF)

The absorbance values of the tested serum samples decreased less than expected with increasing dilution factor, in such a way that the calculated suPAR concentration increased with increased dilution (figure 3.1 and 3.2, respectively). This indicates that some dilution dependent factor interferes with the suPAR measurement in serum samples.

In addition, the results of the test run revealed low absorbance values in general, corresponding to low suPAR concentrations. To ensure that the absorbance values were within the limit set of the standard curve in subsequent assays, the serum samples were not pre-diluted with sample buffer.
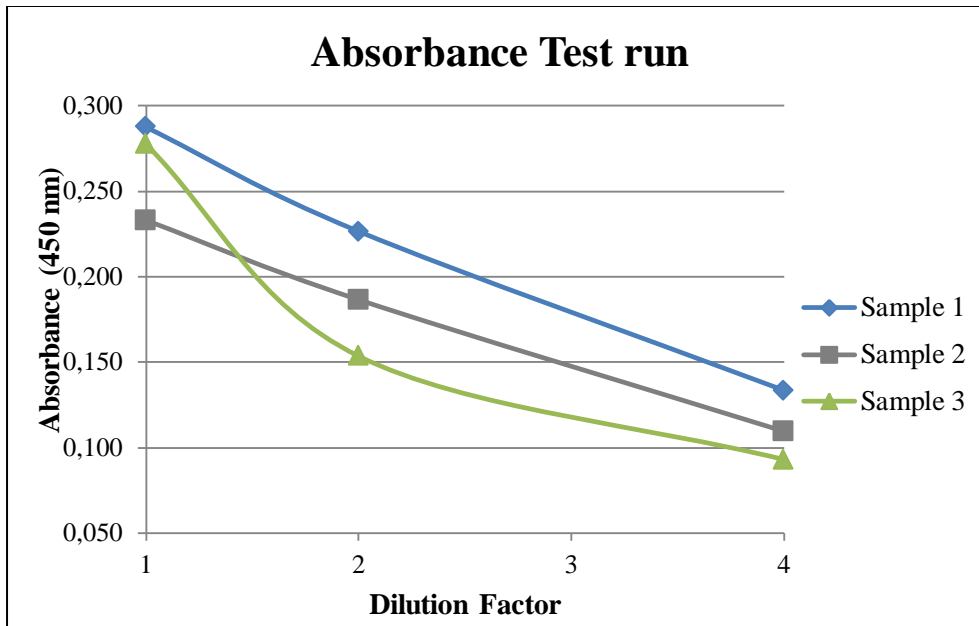
Figure 3.1: Scatter plot illustrating absorbance values (450 nm) against dilution factor. The absorbance decreases in correlation with increased dilution factor in all three samples.
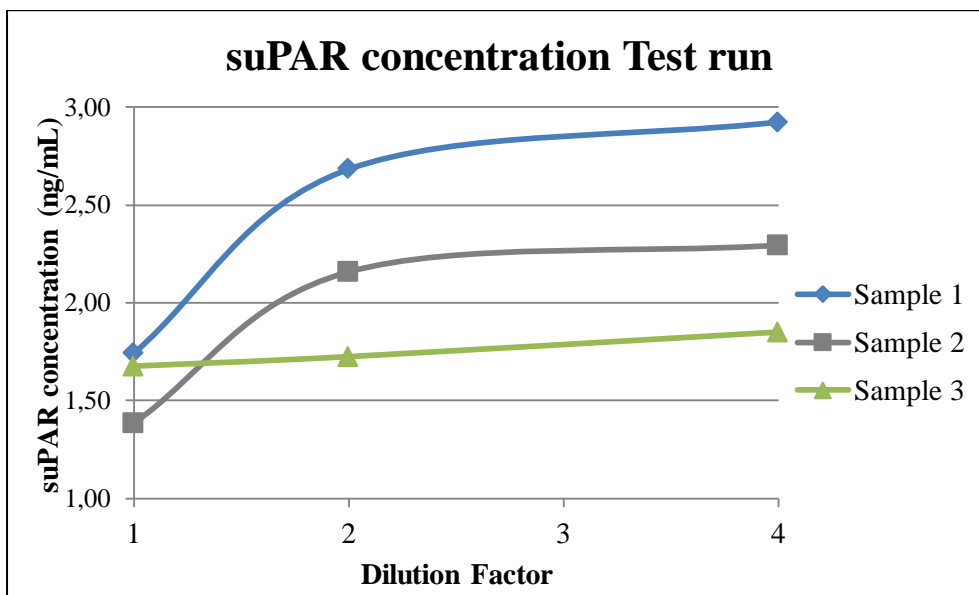
Figure 3.2: Scatter plot illustrating suPAR concentrations (ng/mL) against dilution factor. The calculated suPAR concentrations increase with increased dilution factor for all three samples. The different dilutions of a sample should always derive the same final analyte concentration. The lines of the scatter plot should be horizontal as the diluted samples are multiplied by the additional dilution factor.

3.2   Serum suPAR concentration of the BPH group

Table 3.2 summarizes all patient data and measurements of the BPH group. The raw data of the assay are found in appendix 7.11.

The suPAR concentrations ranged from 0.00 ng/mL to 0.47 ng/mL, with a median value of 0.22 ng/mL. The median value is calculated for the purpose of comparing the results with previous studies.

Table 3.2: Patient data and suPAR concentration

Patient	Age	suPAR ng/mL	CV(%)
1	82	0.19	0.0
2	60	0.27	7.6
3	67	0.03	90.7
4	65	0.37	4.1
5	77	0.04	101.0
6	59	0.00	-835.7
7	69	0.35	34.4
8	75	0.46	13.2
9	63	0.36	12.5
10	80	0.40	35.1
11	72	0.37	15.0
12	69	0.47	9.7
13	79	0.22	2.3
14	69	0.19	10.7
15	70	0.20	24.9
16	65	0.09	16.4
17	69	0.23	24.4
18	76	0.17	12.1
19	65	0.09	49.3
20	82	0.36	2.8
21	75	0.20	5.0
Range		0.00-0.47	
Median		0.22	

The calculated CV of 14 samples was over the acceptance criteria of 10%. This indicates poor precision and repeatability of the sample duplicates. Calculated CVs are also sensitive to small absolute divergences between duplicates in samples due to low suPAR concentrations.

3.3   Serum suPAR concentration in PCa patients

Table 3.3 summarizes all patient data and measurements of the PCa group. The raw data of the assay are found in appendix 7.11.

The suPAR concentrations range from 0.00 ng/mL to 0.56 ng/mL, with a median value of 0.17 ng/mL. The median value is calculated for the purpose of comparing the results of the present thesis with previous studies.

Table 3.3: Patient data and suPAR concentration

Patient	Age	suPAR ng/mL	CV (%)
22	57	0.26	21.7
23	64	0.52	2.9
24	67	0.26	19.4
25	71	0.24	6.4
26	68	0.00	-97.1
27	64	0.12	65.0
28	55	0.10	39.3
29	66	0.23	0.0
30	62	0.10	45.8
31	61	0.22	11.4
32	65	0.00	-217.6
33	67	0.23	9.0
34	62	0.49	6.2
35	61	0.41	37.0
36	62	0.11	22.2
37	63	0.04	72.2
38	55	0.00	0.0
39	65	0.08	6.5
40	63	0.00	-471.4
41	75	0.56	19.1
Range		0.00-0.56	
Median		0.17	

The calculated CV of 13 samples was over the acceptance criteria of 10%. This indicates poor precision and repeatability of the sample duplicates. Calculated CVs are also sensitive to small absolute divergences between duplicates in samples due to low suPAR concentrations.

3.4   Test of normal distribution

The p-values for the Shapiro-Wilk test are 0.315 (BPH) and 0.027 (PCa). This implies that the PCa sample set is not normally distributed because the p-value was smaller than α=0.05. The BPH sample set is greater than α=0.05 and the deviation from the ideal normal distribution is not more than what is expected with chance alone.

Table 3.4: Shapiro-Wilk test of normality

		Shapiro-Wilk test [a]		
		W	df [b]	p
suPAR	BPH	0.948	21	0.315
	PCa	0.890	20	0.027

[a] The null hypothesis is tested at α=0.05, [b] Degree of freedom (df).

The normal probability plots of the BPH and PCa group are shown in figure 3.3 and 3.4. The correlation coefficient (R^2) of the linear line of the BPH plot (0.9620) is over the critical value of the normal probability plot correlation coefficient (table 2.1), while the correlation coefficient of the linear line of the PCa plot (0.9043) is under the critical value. These results correspond to the Shapiro-Wilk test.
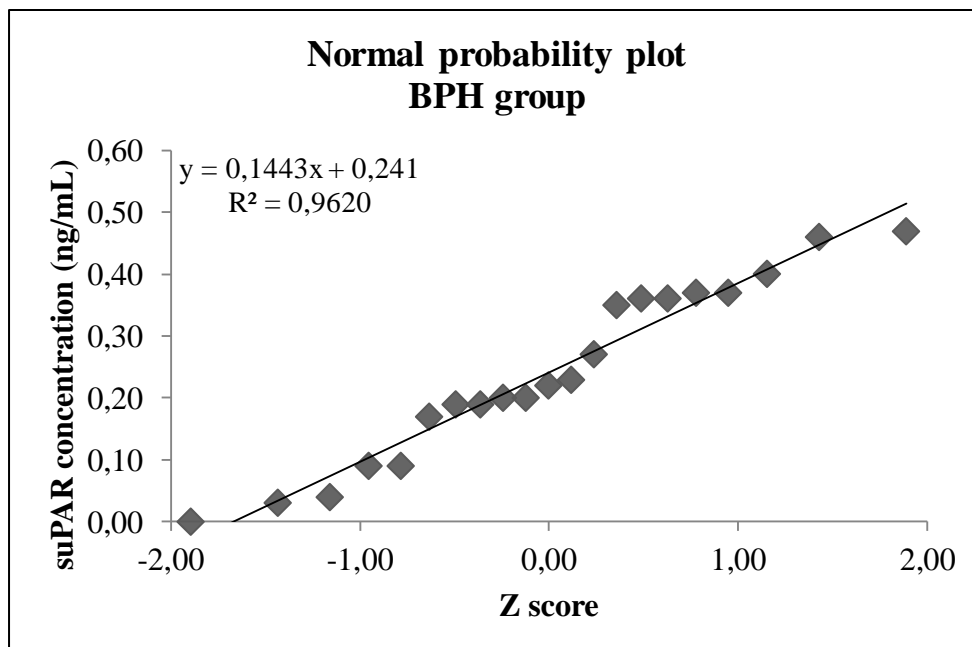
Figure 3.3: Normal probability plot of the BPH group. The z-score is plotted on the x-axis and the ranked suPAR concentration is plotted on the y-axis. The plot illustrates that the suPAR concentrations are plotted close to the linear line indicating normality.
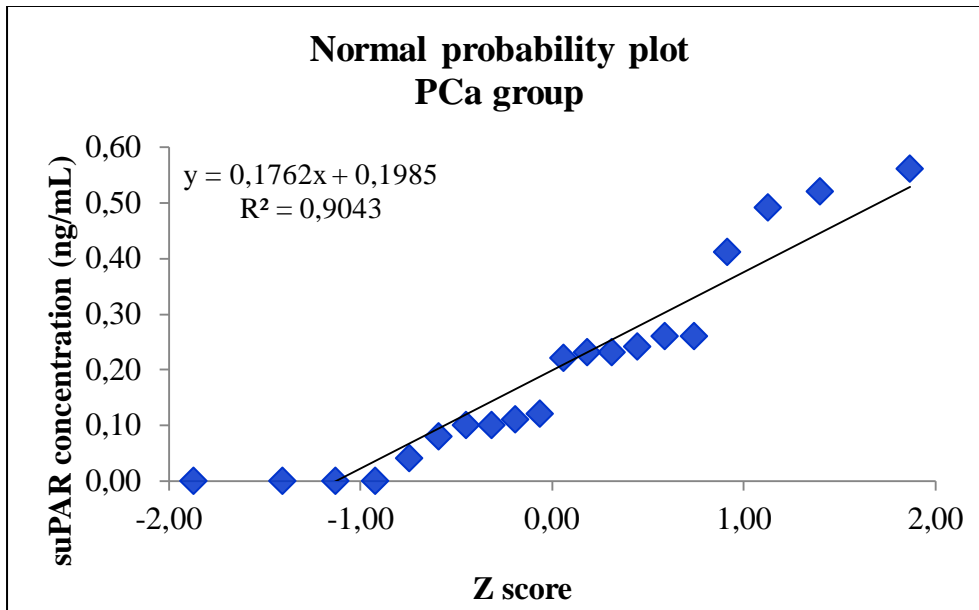
Figure 3.4: Normal probability plot of the PCa group. The z-score is plotted on the x-axis and the ranked suPAR concentration is plotted on the y-axis. The suPAR values do not reflect the straight-line appearance and appears to deviate from normal distribution.

The visual inspection of the normal probability plots shows that the suPAR concentrations of the BPH group are plotted against the z-score in an approximate linear line. This supports the statistical evaluation above. Divergences from a straight line are revealed for the PCa group, indicating a deviation from normal distribution.

Similar results are found by visual inspection of the histograms for the BPH- and PCa group (figure 3.5). The histograms show that the BPH group has an approximate classical bell shape with an approximate symmetrical distribution of the suPAR concentrations. The histogram of the PCa group does not have the classical bell shape, and the suPAR concentrations appear to be non-normally distributed among the PCa group.
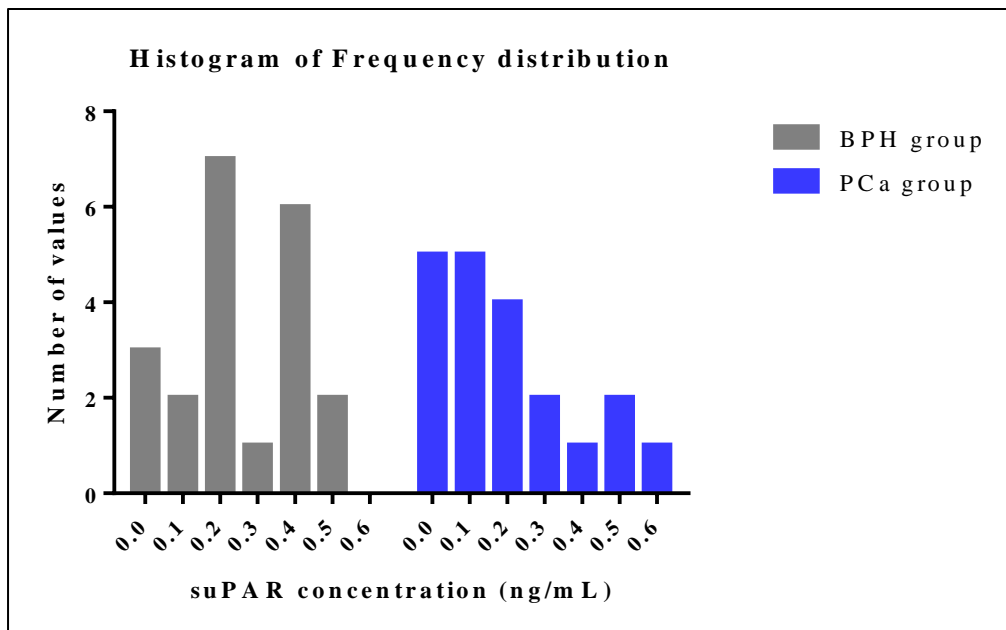
Figure 3.5: Histogram representation. The histogram shows the frequency distribution of suPAR concentrations in the BPH group (grey) and the PCa group (blue).

Evaluation of the data showed that the PCa samples were not sampled from a population following normal distribution. To analyze the differences between groups using parametric tests, normal distributed variables are required for both the BPH- and PCa patient groups. An appropriate transformation of the data could yield a data set that follow normal distribution.

Box Cox transformation ($\lambda=0.65$) of the suPAR concentrations was not successful. This is assessed by the normal Box Cox probability plots and the correlation coefficients (figure 3.6 and 3.7). The transformed suPAR values are not plotted close to the straight line appearance, and the deviation from normal distribution might be explained by the several suPAR concentrations of 0.00 ng/mL obtained.
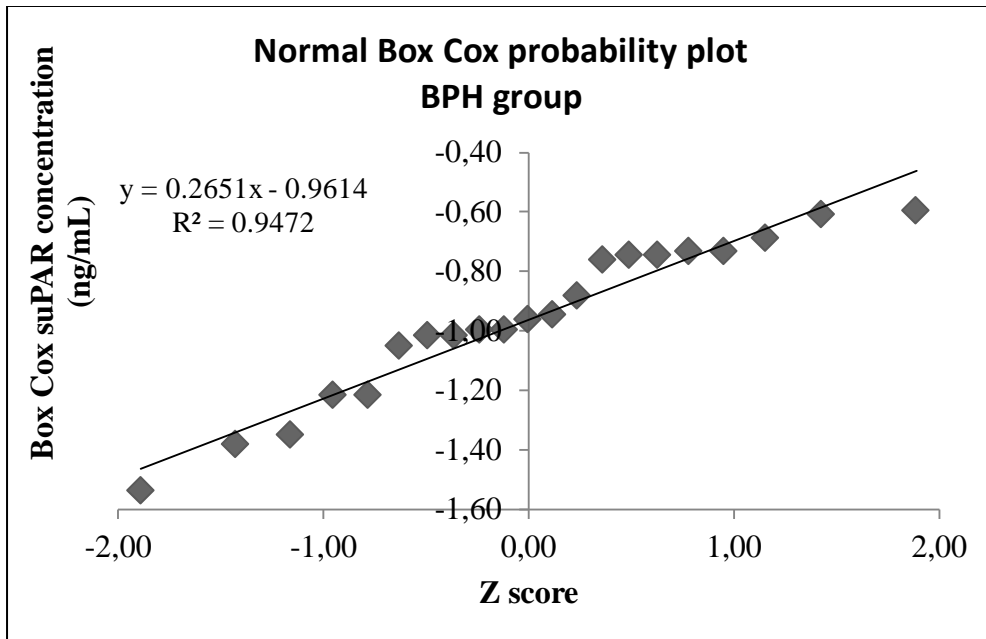
Figure 3.6: Normal Box Cox probability plot of the BPH group, λ=0.65. The z-score is plotted in the x-axis and the Box Cox transformed suPAR concentrations are plotted on the y-axis.
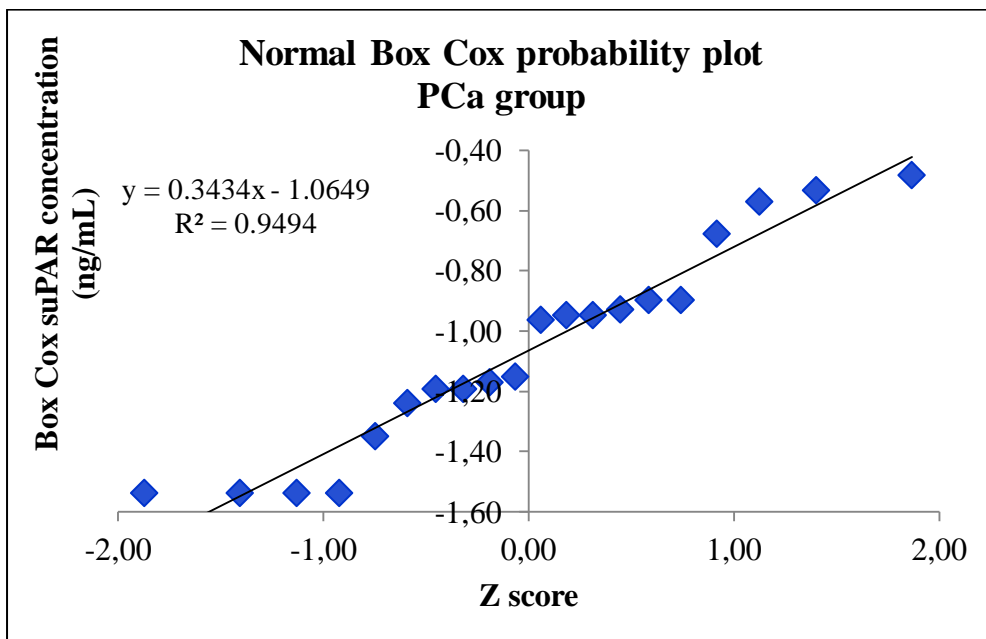
Figure 3.7: Normal Box Cox probability plot of the PCa group, λ=0.65. The z-score is plotted in the x-axis and the Box Cox transformed suPAR concentrations are plotted on the y-axis.

Based on these findings, it was concluded that the requirement for normal distribution was not fulfilled. A nonparametric test was therefore used for further statistical analysis.

3.5   Difference in suPAR concentration between the BPH and PCa group

The distributions of the suPAR values across the BPH- and the PCa groups appear to be approximately similar for the two groups, as illustrated in figure 3.8. The grey and blue dots represent the suPAR values of the 41 serum samples, and the horizontal lines show the median suPAR concentration in each group.
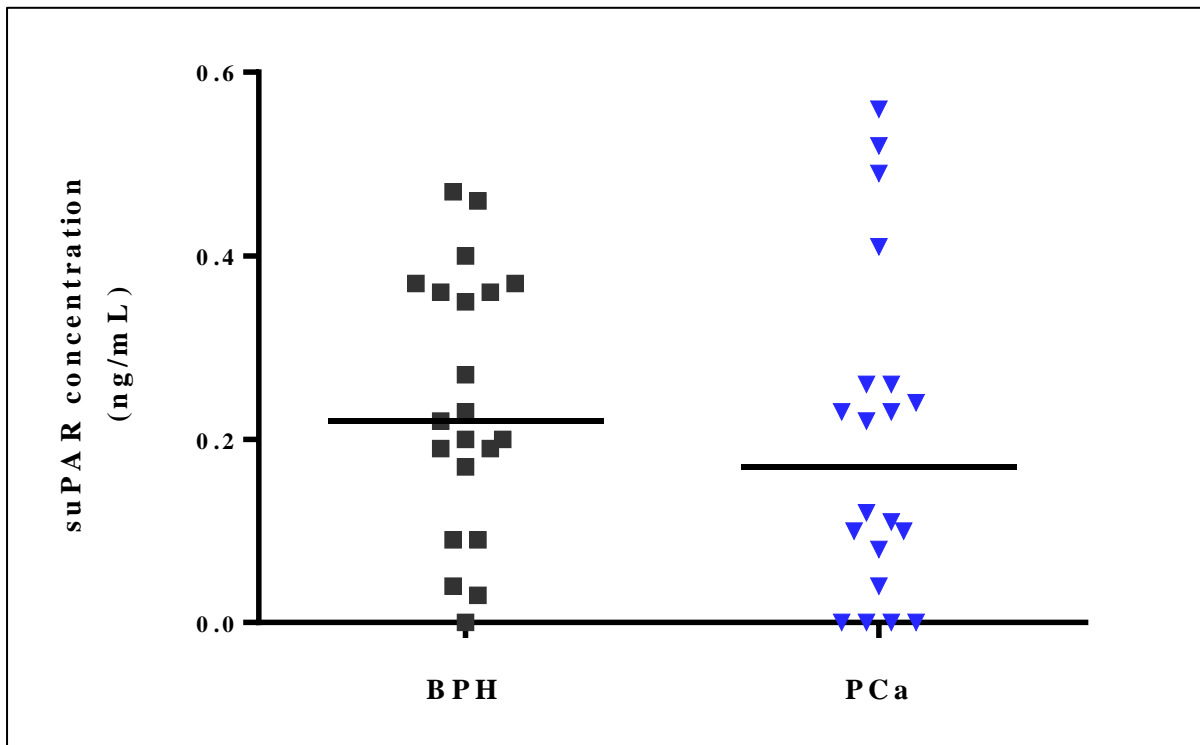
Figure 3.8: Serum suPAR concentration (ng/mL) in the BPH- and PCa patient groups.

Due to the deviations from normal distribution, a nonparametric Mann-Whitney test was run to determine whether there was a statistically significant difference in the serum suPAR levels between the BPH- and PCa patients. All values were ranked irrespectively of the group which they pertained, and the mean rank and sum of ranks of each group were computed.

In the PCa group, a mean rank of 19.45 and a sum of rank of 389 were calculated. In the BPH group, higher levels were found with a mean rank of 22.48 and a sum of rank of 472. These results are summarized in table 3.5 and are the basis for the Mann-Whitney test. The output from the Mann-Whitney test is presented in table 3.6.

Table 3.5: Mean rank and sum of ranks

		N	Mean Rank*	Sum of Ranks*
suPAR	BPH	21	22.48	472
	PCa	20	19.45	389
	Total	41		

*The table shows the mean rank and the sum of ranks by which the Mann-Whitney test is based on.

Table 3.6: Results of the Mann Whitney test

Table analyzed	Test data*
p	0.418
U	179.00
z	- 0.810

*The null hypothesis is tested at α=0.05.

The null hypothesis of the Mann-Whitney test is that the mean of ranks for the BPH- and PCa group are equal. The two-tailed probability associated with z=-0.810 is p=0.418. As the p>α the data do not give any reason to reject the null hypothesis. This is not the same as saying that the two groups are similar, but there is no compelling evidence that they are different.

From the Mann-Whitney test, it can be concluded that there is no statistically significant difference in the serum suPAR levels between the BPH- and the PCa patients found in the present thesis.

4. Discussion

The aim of the present thesis was to quantify the serum concentration of suPAR in preoperative BPH- and PCa patients and to determine whether there was a statistically significant difference between the two groups.

The median serum suPAR concentration in BPH- and PCa patients was measured to 0.22 ng/mL (range: 0.00-0.47 ng/mL) and 0.17 ng/mL (range: 0.00-0.56 ng/mL), respectively. There was no statistically significant difference in serum suPAR concentration between BPH- and PCa patients when using the Mann-Whitney test.

Similar studies have reported considerably higher median concentrations for BPH- and PCa patients. McCabe *et al.* detected median suPAR concentrations of 3.15 ng/mL in BPH patients and 4.21 ng/mL in PCa patients, with overall suPAR concentrations ranging from 1.36 to 16.29ng/mL. Another similar study by Miyake *et al.* found suPAR concentrations ranging from 1.70 to 7.20 ng/mL and 1.60 to 11.40 g/mL in BPH- and PCa patients, respectively. Even studies of healthy individuals have reported higher suPAR concentrations than the results obtained in the present thesis, with values ranging from 1.40 to 5.50 ng/mL (205, 211).

As described in section 1.10, the abovementioned studies draw contrary conclusions regarding the differences of suPAR concentration between the two clinical manifestations. McCabe *et al.* were not able to find a statistically significant difference in suPAR levels between BPH- and PCa patients, whereas Miyake *et al.* found significantly higher serum levels of suPAR in PCa patients than in patients with BPH (205, 211). Since published literature is limited and it is only possible to compare the results to these two previous studies, it is difficult to evaluate the normal or abnormal levels of suPAR in the present thesis. The results appear to correspond with McCabe *et al.*, but there are some limitations of the present thesis that must be considered and discussed.

The low quantities of suPAR were detected in all serum samples. It is therefore suspected that a systematic error might have affected the accuracy of the IMUBIND® uPAR ELISA assay, causing overall low suPAR concentrations. The poor precision and repeatability of the IMUBIND® uPAR ELISA assay further emphasize that there might be limitations of the method for detecting suPAR in serum samples using this assay, despite successful experiences claimed by the supplier.

4.1 Potential sources of systematic errors

Systematic errors can arise from impaired chemical and physiological actions of the sample matrix and reactions on which an immunoassay is based on. The source of these actions might include the possible occurrence of side reactions that interfere with the measured process or the non-specific binding of components in the sample matrix (214). The next sections will discuss the potential effect of these actions on IMUBIND® uPAR ELISA determination of suPAR in serum.

4.1.1. Interference of IMUBIND® uPAR ELISA

In general terms, interference of immunoassays occurs when substances in the sample matrix modify the antigen-antibody interaction, preventing an assay from recognizing its designated analyte. Serum contains several potential interfering factors, for example albumins and immunoglobulins (214). These might affect the accuracy of IMUBIND® uPAR ELISA in the detection of suPAR and might be a possible reason for the systematically low suPAR concentrations in the present thesis.

Currently, there has been no report of an association between interfering factors in serum and suPAR. However, the dilution dependent suPAR concentrations found in the test run might implicate that interfering factors are present. The undiluted samples showed lower concentrations of suPAR than diluted samples. This might be due to interfering factors that prevent the suPAR molecules from binding to the antibody in the solid-phase reaction. This leads to less sandwich complexes generated, and falsely low concentrations are detected. When the samples are diluted, the effects of the interfering factors are reduced, and the detected suPAR concentration becomes higher. This scenario is illustrated in figure 4.1(B). Since the samples used in the present thesis were not diluted, such mechanism might distort the accuracy of IMUBIND® uPAR ELISA assay, causing the systematically low levels of suPAR recorded.
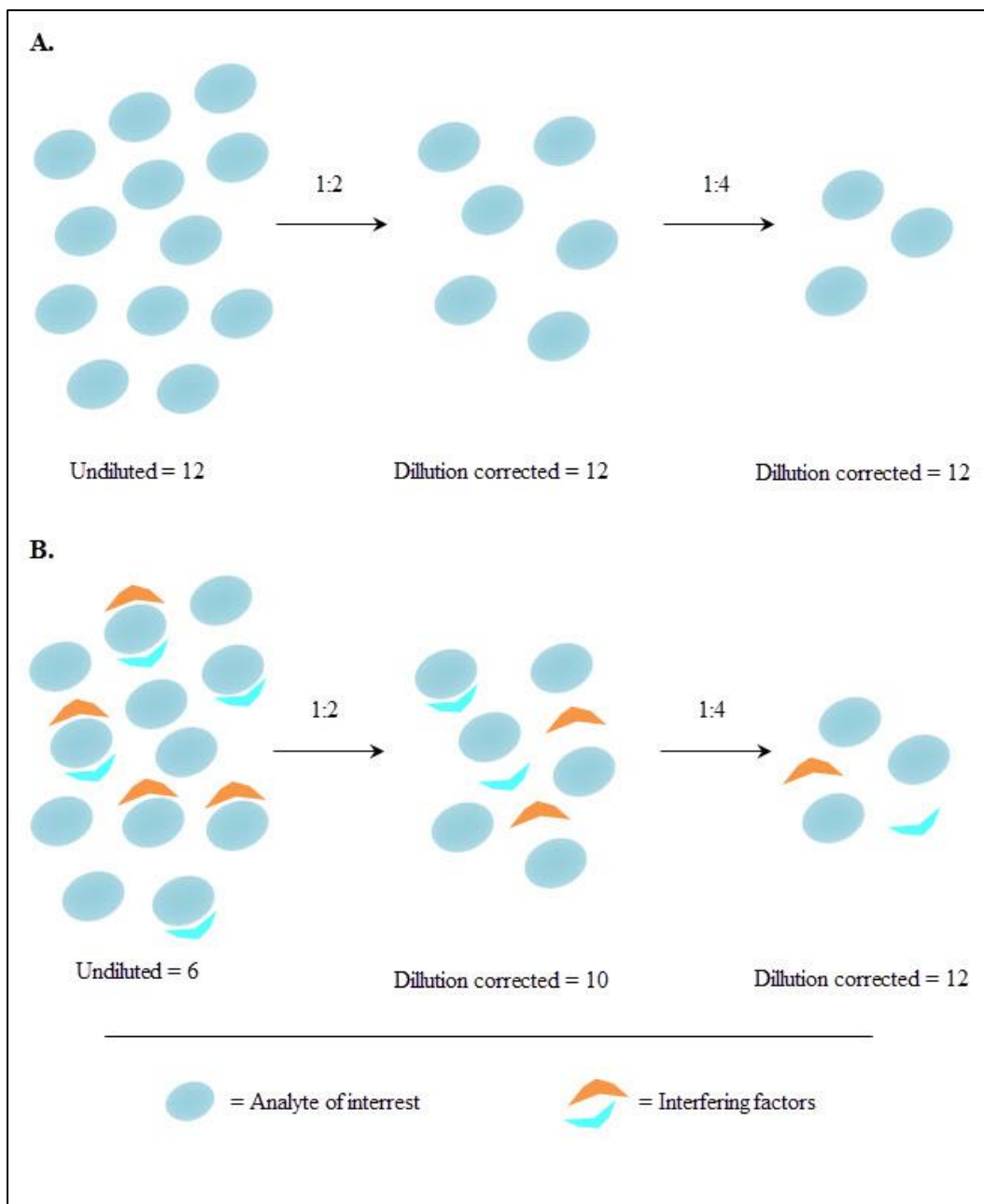
Figure 4.1: Schematic illustration of the effect of interference. Based on R&D systems (222). A: Expected results from a dilution experiment when no interfering factors are present in the sample matrix. The analyte concentration (=12) is the same in all serial dilutions. B: Potential results from the same experiment if interfering factors are present in the sample matrix. In the undiluted sample, these factors can interfere with the analyte of interest, causing low concentrations of the analyte (=6). The effect of the interfering factor is reduced when the sample is diluted. The appropriate dilution of the sample (1:4) might exclude the effect of the interfering factors and give a more correctly detection of the concentration (=12).

Under optimal conditions, different dilutions of a sample should always end up with the same value of the original analyte concentration, as demonstrated in figure 4.1 (A). Assays are generally designated to overcome the effect of interfering factors. However, IMUBIND® uPAR ELISA is only validated for use with citrate plasma and not serum, and it might therefore be suspected that serum is unsuitable for this assay. To further investigate this, suPAR levels in both serum and plasma from the same patient should be determined by IMUBIND® uPAR ELISA. The results could be used to evaluate whether the assay detects different suPAR levels in the two sample materials.

Another possible cause for the low quantities of suPAR can be the formation of aggregates. In a structural study of ligand-free uPAR, Xu *et al.* demonstrate that circulating suPAR is prone to aggregation in the absence of uPA. The aggregates might bind to the solid-phase antibodies differently than the single protein molecules and adversely affect the results of the assay (125). However, these findings are preliminary and little is known about the exact cause for the agglutination.

4.1.2. Non-specificity of IMUBIND® uPAR ELISA

The supplier of IMUBIND® uPAR ELISA recommended a 1:10 dilution of plasma samples with sample buffer (appendix). The sample buffer contains bovine serum albumin (BSA), a potent blocking reagent that minimizes nonspecific binding of antibodies or proteins to unoccupied spaces of the microwells.

In the present thesis, the absence of blocking reagent in the undiluted samples could potentially have had a negative effect on the specificity of the IMUBIND uPAR ELISA assay. Proper blocking is paramount to minimize IMUBIND® uPAR ELISA background signaling and attaining an accurate signal. The non-specific bindings might not explain the systematically low suPAR concentrations detected in the present thesis, but they might explain the poor precision between the duplicate measurements.

4.1.3. Hook effect

In addition to reducing the effect of interfering factors and background signaling, sample dilution might also prevent the occurrence of the Hook effect. When an ELISA system is overwhelmed with the target antigen, the high concentration of the analyte might exceed the concentration of the assay antibodies and simultaneously react with the primary and secondary antibodies. The result is a reduction of sandwich-complexes formed, and finally an

underestimated result (215). This might be a legitimate explanation to the systematically low suPAR concentrations detected in the present thesis, as it is likely that the samples might contain high levels of suPAR.

However, the Hook effect appears to occur in assays where all reactants are simultaneously mixed together. In the assay of IMUBIND® uPAR ELISA, the reagents are sequentially mixed. The primary antibody in the solid phase captures antigen from the sample, and after a washing step the secondary antibody is added to react with the bound antigen. This stepwise approach prevents simultaneously binding of both primary and secondary antigens causing the Hook effect. The Hook effect is therefore probably not the cause for the systematically low suPAR levels detected in the present thesis.

4.2 Preanalytical sources of error

Studies have demonstrated that suPAR is a highly stable molecule in serum (187, 194-196, 223). The effects of preanalytical factors on suPAR levels in serum, such as sample handling and storage, have previously been examined. Riisbro *et al.* found that there were no significant alterations in suPAR concentrations when whole blood was processed into serum after 1, 3, 8 or 24 hours at 20°C. Furthermore, it was found that repeated freeze and thaw cycles of the serum samples did not influence the determination of suPAR (196).

In the present study, differences in the sample handling procedure regarding transport and time before freezing occurred between the two patient groups. Samples obtained from BPH patients were collected in the morning, centrifuged and subsequently transported from Orkdal. The samples were received at St. Olavs Hospital at 2:00 pm. In accordance with the study of Riisbro et al., the suPAR levels in these samples seem to be stable and not affected by the prolonged storage at room temperature. This might be assumed because the quantified suPAR in the BPH samples did not significantly differ from the PCa samples which were collected, apportioned and stored in a freezer within 2 hours. If the present thesis had found differences of suPAR concentration between BPH- and PCa samples, the differences in sample handling must have been taken into consideration.

The protocol provided by IMUBIND® uPAR ELISA does not contain information about other preanalytical variables associated with the method (appendix 7.8). The effect of Bilirubin, Hemoglobin and Intralipids has been tested by suPARnostic®, another supplier of uPAR ELISA kits, and no significant interference was found from any of the substances tested (224).

4.3 Precision of IMUBIND® uPAR ELISA

Immunoassay precision is defined as the repeatability of independent results of measurements obtained under controlled conditions. The degree of precision was determined by calculating the coefficient of variation (CV) of the sample duplicates (214, 215).

According to the supplier of the IMUBIND® uPAR ELISA kit, the assay has a CV value less than 10 %. In the present study, all standards had an acceptable CV value under 10%. This indicates good precision and repeatability of the duplicate standard measurements. However, only 14 of the total 41 serum samples had an acceptable CV, indicating poor precision of the majority of samples. Given the low suPAR concentrations measured, the calculated CVs are very sensitive to small absolute divergences between duplicates. The CVs must therefore be mathematically expected to be high, and the precision and repeatability is linked to the low suPAR levels discussed above.

The poor precision obtained from the serum samples might also be related to random errors, some of which have already been discussed. Random errors might be due to lack of repeatability in pipetting and mixing of samples and reagents, lack of stability of time regulation at incubation steps or improper washing steps. However, the standards and the samples within one assay were handled under exactly the same conditions. The same pipettes, the same reagents, the same incubation times, the same washing procedure were used and performed by the same individual. The random errors are a major source of uncertainty in the assay and cannot be totally eliminated, but it is more likely that a combination of errors are the causative factor for the systematically low suPAR levels obtained from the IMUBIND® uPAR ELISA assay. These are difficult to detect and explicitly pinpoint, and further testing of the method is required.

4.4 Future perspectives

All serum samples collected in the present thesis were apportioned into aliquots, registered and stored in the Regional Research Biobank of Central Norway. Unused serum aliquots are stored in the biobank and are available for future scientific projects.

As a continuation of the present thesis, further optimization of the IMUBIND® uPAR ELISA assay for serum samples could be assessed. Experiments improving the precision and specificity of the method might increase the accuracy of detecting suPAR in serum by using this method. One suggestion is to run recovery experiments to determine whether the

IMUBIND® uPAR ELISA assay is affected by interfering factors in the sample matrix, which has been suspected in the present thesis. Spiked samples can be prepared by adding varying amounts of suPAR to serum matrix and to the standard dilution. If the recovery observed for the spike is identical to the recovery obtained for the analyte prepared in standard diluent, the sample matrix is considered valid for the assay procedure. If the values differ, then components in the sample matrix are causing the difference and adjustments of the method must be made to minimize the discrepancy.

The Quantikine Human uPAR ELISA kit, provided by R&D systems®, is validated on serum samples. As a continuation of the present thesis, a comparison study of the Quantikine Human uPAR ELISA- and IMUBIND® uPAR ELISA assays could be relevant. The same sample set as used in the present thesis could be reanalyzed by the Quantikine Human uPAR ELISA. This could determine if this assay can provide more valid results, with good precision and specificity, than the IMUBIND® uPAR ELISA assay. When reanalyzed, the statistical difference of suPAR between the BPH- and the PCa samples could also be recalculated.

Limited data is available regarding the suPAR concentrations of BPH patients. We cannot draw any new conclusions from the results of the present thesis due to the possible limitations of the IMUBIND® uPA ELISA. The serum suPAR concentrations in PCa patients have been more exclusively studied, and several reports describe enhanced levels of serum suPAR levels in these patients.

The serum suPAR levels in patients with metastatic disease have previously been found to be significantly higher compared to patients with localized disease. These findings indicate that levels of serum suPAR might be a useful marker for the detection of patients with metastatic disease, and it is of particular interest whether the elevations of serum suPAR concentrations are associated with poor prognosis in PCa patients. To further investigate this, additional samples could be collected to the Regional Research Biobank of Central Norway, from PCa patients with different Gleason Scores and with or without evidence of metastases. However, a great challenge is to find patients who have not received treatment, as the suPAR levels tend to decrease after prostatic manipulation (191). Alternatively, this could be solved by accessing serum samples from the HUNT 2 study. For example, samples from presumptively healthy men who later developed PCa and a control group of men who never developed PCa could be collected and suPAR concentrations quantified. The differences in suPAR concentration between the men who developed cancer and men that never did might implicate the role of

suPAR in PCa development and whether suPAR levels could be utilized to predict PCa progression.

Furthermore, Miyake *et al.* demonstrated that the overall survival rate of PCa patients with suPAR elevation was significantly lower than the survival rate of patients with normal levels (205). These findings suggest that measurement of serum suPAR may provide additional information in determining prognosis and patient treatment strategy: Intensive treatment might be focused to patients with elevated suPAR since patients with normal levels of suPAR may not need to undergo intensive, additional therapies if the initial treatment is successfully performed.

In spite of this seemingly alluring potential, suPAR has not yet been integrated into clinical practice. Hence, further studies are required to validate the role of suPAR in PCa progression and patient prognosis. The study of HUNT 2 samples would be a good supplement to today's published work.

5. Conclusion

The present thesis was not able to detect a statistically significant difference in the suPAR concentration between BPH- and PCa patients. The median serum suPAR concentration in BPH- and PCa patients was measured to 0.22 ng/mL (range: 0.00-0.47 ng/mL) and 0.17 ng/mL (range: 0.00-0.56 ng/mL), respectively.

The IMUBIND uPAR ELISA used for suPAR quantification in serum had methodological limitations. These involve low precision and systematically low levels of suPAR recorded.

Further studies are warranted to give an exact estimation of the suPAR concentration in BPH- and PCa patients. The samples obtained from the present study are stored at the Regional Research Biobank of Central Norway and are accessible for further studies regarding suPAR or other scientific work organized by the biobank. By including samples from the HUNT research biobank, it might be achievable to obtain a larger cohort of serum samples and further investigate the role of suPAR as a predictor of prostate cancer progression.

6. References

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Le risque élevé de mortalité chez les personnes assumées être TB-négatives peut être prédit à l'aide d'un simple test

El alto riesgo de mortalidad entre individuos que se asume son negativos para TB puede predecirse utilizando un test sencillo. Tropical Medicine & International Health. 2009;14(9):986-94.

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7. Appendix

7.1 Consent

Forespørsel om å gi biologisk materiale til Regional forskningsbiobank Midt-Norge

Det regionale helseforetaket Helse Midt-Norge og Norges teknisk-naturvitenskapelige universitet (NTNU) har sammen etablert Regional forskningsbiobank Midt-Norge (heretter kalt Forskningsbiobanken).

Formål
Forskningsbiobanken tar vare på biologisk materiale (for eksempel blod, vev og urin) som kan benyttes til medisinsk forskning når det ikke lenger er bruk for det til diagnostisering og behandling. Formålet er å gi økt kunnskap om årsaker til sykdom, for derved å bidra til bedre forebygging, diagnostikk og behandling av sykdom. Av og til trenger man også materiale fra friske personer som kan brukes som kontroll ved forskning på sykdomsgrupper eller undersøkelse av friske bærere av sykdomsgener. I denne forbindelse forespørres du om å gi materiale til Forskningsbiobanken. Dette innebærer at det tas av en liten mengde blod tilsvarende ett prøvetakingsglass (ca 5 ml) av deg. Dette materialet blir satt av til medisinsk forskning og forvaltet av Forskningsbiobanken. Materialet vil bli brukt til forskning på store sykdomsgrupper som kreft, hjerte- og karsykdom, diabetes m.fl. Forskningsbiobanken er offentlig eid, og den drives ikke kommersielt.

Biologisk materiale og helseopplysninger
All bruk av biologisk materiale og helseopplysninger i forskning vil skje i form av prosjekter, som først må godkjennes av Regional komité for medisinsk og helsefaglig forskningsetikk Midt-Norge (REK). Materialet vil bli oppbevart og brukt så lenge det er noe igjen, og oppbevaring og bruk blir utført i samsvar med Helseforskningsloven og annet relevant lovverk.

Opplysninger/sporbarhet

Blod avgitt fra blodgivere til Forskningsbiobanken vil være fullt anonymisert. De eneste opplysningene som er knyttet til ditt materiale, er kjønn og alder. Det vil derfor ikke være mulig å spore blodprøven til din identitet eller for forskere å innhente noen andre opplysninger om deg.

Databehandling/taushetsplikt

Alle som behandler biologisk materiale og opplysninger om dette, er underlagt taushetsplikt i henhold til Forvaltningsloven § 13 og Helsepersonelloven § 21. Det meste av forskningen vil foregå i Midt-Norge, men i enkelte tilfeller kan det være nødvendig å overføre deler av materialet til forskere andre steder i landet, eventuelt i utlandet.

Formelle godkjenninger

Opprettelsen av Forskningsbiobanken er godkjent av Regional komité for medisinsk og helsefaglig forskningsetikk Midt-Norge, Helse- og omsorgsdepartementet og Sosial- og helsedirektoratet. Datatilsynet har gitt Forskningsbiobanken konsesjon til å behandle

helseopplysninger. Dette er en generell godkjenning. I ditt tilfelle vil ikke helseopplysninger være knyttet til materialet.

Dine rettigheter

Det er frivillig om du vil tillate at biologisk materiale kan bli brukt til forskning eller ikke. Fordi ditt materiale ikke er identifiserbart og kun lagres under et referansenummer som ikke er sporbart til deg, vil det ikke være mulig å trekke samtykket etter at materialet er avlevert.
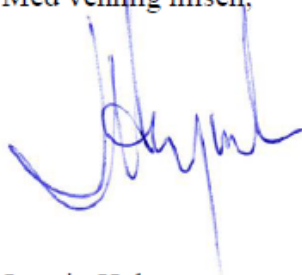
Har du noen spørsmål kan disse rettes til:
Regional forskningsbiobank Midt-Norge
St. Olavs Hospital HF, Laboratoriesenteret
Erling Skjalgssonsgt. 1
7006 Trondheim

Tlf: 72 57 13 44, telefax: 72 57 38 01
E-postadr: biobank@medisin.ntnu.no
www.ntnu.no/dmf/biobank

Dersom du ønsker å samtykke, ber vi deg signere vedlagte samtykkeerklæring.

Med vennlig hilsen,

Jostein Halgunset,
Ansvarlig for Regional forskningsbiobank Midt-Norge

INNSAMLING AV SERUM MATERIALE FRA BPH- PASIENTER VED ORKDAL SYKEHUS

Masteroppgave
Oda Moe Stokke
Veileder: Haakon Skogseth

Institutt for laboratoriemedisin,
kvinne – og barnesykdommer

Beskrivelse av prosjektet

Prosjektet omhandler proteinet urokinase plasminogen activator reseptor (uPAR) og den løselige formen for reseptoren, suPAR. SuPAR er påvist ved flere kreftformer og pågående forskning viser at suPAR kan ha diagnostisk og prognostisk verdi ved prostatakreft.

Den høye forekomsten av prostatakreft og begrensingene med PSA som kreftmarkør tilsier at det er et stort behov for alternativer og/eller supplementer til PSA. Mens PSA viser en tendens til å øke med alderen, ser suPAR ut til å holde seg mer stabil hos friske individer. I tillegg har det vært funnet forhøyede konsentrasjoner av suPAR i forhold til PSA hos pasienter der kreften hadde et mer aggressivt forløp. På bakgrunn av dette har det blitt foreslått at suPAR kan brukes til å overvåke prosessene som foregår ved lokal kreftinfiltrasjon, invasjon og metastasering for å kunne forutsi hvor aggressivt sykdomsforløpet vil bli.

Målet med prosjektet er å undersøke om serum-nivåene av suPAR er forhøyet hos prostatakreftpasienter i forhold til pasienter med benign prostatahyperplasi (BPH).

Informasjon og samtykke (May-Britt Sætre)

- Samtykke og informasjonsskriv leveres til Orkdal sykehus.
- Samtykke må innhentes fra hver pasient som er villig til å delta.

Prøvetaking (Laboratoriet, Eva Gundersen)

- 50 nummererte serum glass (Vacuette Z Serum Sep Clot Activator, 8 mL) leveres til Orkdal sykehus.
- Prøvetaking gjennomføres i henhold vedlagt prosedyre
- Etter innsamling skal alle personalopplysninger anonymiseres. Prøveglassene merkes KUN med fødselsår (f.eks 1950).

Prøvebehandling (Laboratoriet, Eva Gundersen)

- Sentrifugering: 2500 g i 15 minutter i romtemperatur
- Serum trenger IKKE å avpipetteres, men sendes i primærglass til Trondheim.
- Prøvene trenger IKKE fryses ned.

Forsendelse av prøver til St. Olavs Hospital (Laboratoriet, Eva Gundersen)

- Prøvene sendes med den daglige transporten mellom Orkdal sykehus og Trondheim (prøvemottak, 2. etasje, laboratoriesenteret).
- VIKTIG: Pakken merkes med ODA MOE STOKKE.

Kontaktperson:

Oda Moe Stokke

Telefon: 986 879 31

Mail: odams@stud.ntnu.no

7.3   Agreement with St. Olavs Hospital

INNSAMLING AV SERUM MATERIALE FRA PROSTATAKREFTPASIENTER VED KIRURGISK AVDELING, ST. OLAVS HOSPITAL

Masteroppgave
Oda Moe Stokke
Veileder: Haakon Skogseth

Institutt for laboratoriemedisin,
kvinne- og barne sykdommer

Beskrivelse av prosjektet

Prosjektet omhandler proteinet urokinase plasminogen activator reseptor (uPAR) og den løselige formen for reseptoren, suPAR. SuPAR er påvist ved flere kreftformer og pågående forskning viser at suPAR kan ha diagnostisk og prognostisk verdi ved prostatakreft.

Den høye forekomsten av prostatakreft og begrensingene med PSA som kreftmarkør tilsier at det er et stort behov for alternativer og/eller supplementer til PSA. Mens PSA viser en tendens til å øke med alderen, ser suPAR ut til å holde seg mer stabil hos friske individer. I tillegg har det vært funnet forhøyede konsentrasjoner av suPAR i forhold til PSA hos pasienter der kreften hadde et mer aggressivt forløp. På bakgrunn av dette har det blitt foreslått at suPAR kan brukes til å overvåke prosessene som foregår ved lokal kreftinfiltrasjon, invasjon og metastasering for å kunne forutsi hvor aggressivt sykdomsforløpet vil bli.

Målet med prosjektet er å undersøke om serum-nivåene av suPAR er forhøyet hos prostatakreftpasienter i forhold til pasienter med benign prostatahyperplasi (BPH).

Informasjon og samtykke

- Samtykke og informasjonsskriv leveres til poliklinikken.
- Samtykke må innhentes fra hver pasient som er villig til å delta.

Prøvetaking

- 50 nummererte serum glass (Vacuette Z Serum Sep Clot Activator, 8 mL) leveres til poliklinikken, ved Inger J. Stokkan.
- Prøvetaking gjennomføres i henhold til vedlagt prosedyre.
- Etter innsamling skal alle personalopplysninger anonymiseres. Prøveglasset merkes KUN med fødselsår (f.eks. 1950).

Henting av prøver

- Prøvene hentes av Oda Moe Stokke.
- Innen 2 timer fra prøven er tatt.

Kontaktperson:

Oda Moe Stokke

Telefon: 986 879 31

Mail: odams@stud.ntnu.no

7.4   Procedure for blood sampling

Prosedyre for blodprøvetaking

Hensikt: Å beskrive fremgangsmåten for venøs blodprøvetaking med vakuumrør.

Utstyr:

Desinfeksjonsmiddel

Kanyler

Kanyleholder

Vakuumrør

Stasebånd

Tupfere

Tape

Avfallsbeholder for stikkende/skjærende

Stativ til vakuumrør

Håndhygiene: Prøvetakeren må sørge for god håndhygiene gjennom bruk av alkoholbasert håndesinfeksjonsmiddel eller grundig håndvask før prøvetakingen.

Desinfeksjon av punksjonssted: Punksjonsstedet desinfiseres før prøvetaking. Stikkstedet skal vaskes fra midten og utover i sirkler eller spiral. Huden skal deretter lufttørke.

Prøvetaking:

1. Utstyret som skal benyttes klargjøres
2. Sørg for at pasientens arm ligger stødig, avslappet og vendt nedover.
3. Stasebåndet plasseres 10-15 cm over stikkstedet, gjerne utenpå pasientens klær for å unngå å klemme huden. Stasen strammes og punksjonsstedet lokaliseres.
4. Stasebåndet løsnes når punksjonsstedet er valgt.
5. Desinfeksjon av punksjonsstedet utføres.
6. Sjekk at kanylens papiretikett er ubrutt.
7. Vri av den korte delen av hylsen.
8. Skru kanylen inn i kanyleholderen.
9. Vri av den lange hylsen og plasser den lett tilgjengelig.
10. Stram stasebåndet igjen.
11. Kanylen føres inn i venen med kanyleøyet opp og ca 30° vinkel i forhold til hudens overflate.
12. Skyv vakuumrøret inn i kanyleholderen.

99

13. Løsne på stasebåndet.

14. Se til at røret fylles helt.

15. Trekk røret ut fra kanyleholderen og vend forsiktig 5-10 ganger.

16. Hold en tupfer (eventuelt med tape) forsiktig over stikkstedet uten å legge noe press ned mot kanylen mens den trekkes ut av venen. Så snart kanylen er trukket ut, trykkes tupferen godt mot stikkstedet.

17. Fisk opp kanylehylsen med bruk av "enhåndsteknikk" og fest den på kanylen før kanylen skrus ut av kanyleholderen. Brukt kanyle kastes i stikkende/skjærende avfall.

18. Dersom det ikke ble brukt tupfer med tape i trinn 16, settes en tape stramt over tupferen.

19. Påse at riktig pasientidentifikasjon er utført før pasienten forlater prøvetakingsrommet.

7.5 Approval from the Regional Ethical Committee (REC)

2011/424 Etablering av referanseområde for et protein i blod og urin

Prosjektleder: Haakon Skogseth
Forskningsansvarlig: NTNU, LBK

Prosjektomtale (original):
Det er holdepunker for at uttrykket av proteolytiske enzymer i serum og urin kan være en markør for malignitet, og å beherske en metode for å måle slike enzymer kan være verdifull i fremtidig kreftforskning. Dette prosjektet har til hensikt å etablere et referanseområde for den løselige formen for urokinase plasminogenaktivatorreseptor. Vi ønsker benytte anonymisert blod og urin fra bioingeniørstudenter ved HiST som utgangspunkt. Prosjektet tenkes utført som bachloroppgave for to bioingeniørstudenter denne våren. Analysemetode er ELISA.

Vi viser til tilbakemelding mottatt 27. februar 2012. Henvendelsen er vurdert av komiteens leder. Endringene innebærer følgende:

-- utsatt sluttdato
-- økning i antall forskningsdeltakere
-- endring i rekrutteringsprosedyre

REK har vurdert endringsmeldingen, og har ingen forskningsetiske innvendinger mot endringen av prosjektet.

Vedtak
REK godkjenner prosjektet slik det nå foreligger, jfr. helseforskningsloven § 11, annet ledd. Tillatelsen er gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i opprinnelig søknad og protokollen, vedtaksbrev av 4. april 2011 og 7. april 2011, og de bestemmelser som følger av helseforskningsloven med forskrifter.

Klagerett
REKs vedtak kan påklages til Den nasjonale forskningsetiske komité for medisin og helsefag, jfr. helseforskningsloven § 10, 3 ledd og forvaltningsloven § 28. En eventuell klage sendes til REK midt. Klagefristen er tre uker fra mottak av dette brevet, jfr. forvaltningsloven § 29.

Med vennlig hilsen,

Sven Erik Gisvold
Professor, dr.med.
Leder, REK midt

7.6   Safety- and declaration of confidentiality form to the biobank

SIKKERHETS- OG TAUSHETSERKLÆRING

Jeg forstår:
- at jeg gjennom min kontakt med Regional forskningsbiobank Midt-Norge kan få tilgang til informasjon som ikke må bli kjent for uvedkommende
- at behandling av biologisk materiale og personopplysninger krever ansvarsfølelse og lojalitet, samt respekt for vern av informasjon og overholdelse av etiske normer

Jeg bekrefter:
- at jeg er kjent med innholdet i biobankens sikkerhetshåndbok
- at jeg har gjennomgått opplæring i informasjonssikkerhet ved biobanken

Jeg er kjent med innholdet i:
- § 15 i "Lov om helseregistre og behandling av helseopplysninger"
- § 13 i "Lov om behandling av personopplysninger"
- kapittel 2 i "Forskrift til personopplysningsloven"
- § 21 i "Lov om helsepersonell m.v."

Jeg erkjenner min plikt til å:
- bevare taushet om personopplysninger jeg får kjennskap til
- bevare taushet om andre opplysninger og forhold jeg får kjennskap til og som jeg forstår ikke bør bringes videre
- vise aktsomhet i behandlingen av alle oppgaver
- følge biobankens regler for informasjonssikkerhet

Jeg er klar over:
- at forsettlig eller uaktsomt brudd på taushetsplikten, eller medvirkning til dette, kan medføre straffeansvar
- at taushetsplikten også gjelder etter at kontakten med Regional forskningsbiobank Midt-Norge er avsluttet

Sted:....................................   Dato:....................................

....
underskrift

7.7 Declaration of confidentiality form to HEMIT

TAUSHETSERKLÆRING
for tilgang til informasjonssystem HEMIT er databehandler for.

Firmanavn	Adresse	Telefonnr.
Kontaktperson	Referanse til kontrakt	Leveranse

Tilgangen gjelder:

Utstyr	Område	Program
Tidsrom det er behov for tilgang		

Leverandørens representanter er kjent med at ansatte og enhver som er i vår tjeneste som får kjennskap til folkeregisteropplysninger, personopplysninger eller pasientopplysninger har taushetsplikt etter spesialisthelsetjenestelovens § 6-1 (se side 2), folkeregistreringslovens § 13 første ledd (gjengitt på side 2), personopplysningsloven § 9 med forskrift (se side 2) og forvaltningsloven §§ 13 til 13 e.

Taushetsplikten gjelder også andre opplysninger av konfidensiell art om sykehusets drift.
Leverandørens representanter forplikter seg til å respektere taushetsplikten.

Leverandørens representanter forplikter seg også til å sikre at utstyr som er tilkoblet St. Olavs Hospitals datanett ikke samtidig er tilkoblet andre nettverk.

Leverandørens representanter er kjent med at brudd på taushetsplikten er straffbart etter folkeregistreringslovens § 16 første ledd (gjengitt på side 2).

Ved eventuelle behov for lagring av informasjon lokalt på maskiner hos Leverandør, forplikter Leverandør seg til å fjerne all slik informasjon når arbeidet er utført.

Undertegnede erklærer seg kjent med innholdet av denne meddelelsen og forplikter seg til å overholde taushetsplikten om det undertegnede får kjennskap til under tjenesten eller arbeide ved sykehuset. St. Olavs Hospitals datanett - oversikt over leverandører/tilkoplinger

Underskrift av Leverandørens ansatte og andre i Leverandørens tjeneste som deltar i overnevnte arbeid:

Navn på berørt personell	Underskrift av berørt personell	Født	Dato

Sted: _____   Dato: _____

_____
Underskrift leder

7.8   IMUBIND® uPAR ELISA procedure

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SEKISUI

IMUBIND® uPAR ELISA

Product No. 893

For Research Use Only.

INTENDED USE

The IMUBIND® uPAR ELISA is an enzyme-linked immunoassay for the measurement of human urokinase-type plasminogen activator receptor, uPAR, in tissue extracts, human plasma and cell culture supernatants.

This assay is for research use only. It is not intended for diagnostic or therapeutic procedures.

EXPLANATION OF THE TEST

The urokinase-type plasminogen activator receptor, uPAR, is a GPI-linked single chain glycoprotein having a molecular weight between 50 kD and 60 kD. It also exists as a deglycosolated protein having a molecular weight of 35 kD. uPAR is composed of three domains: Domain I is involved with uPA binding, Domains II and III aid in orienting the uPAR molecule on the cell membrane. The presence of a cellular receptor for uPA was first demonstrated by Vassalli[1], who observed a saturable specific binding of uPA to the surface of monocytes. uPAR binds both the enzymatically inactive single chain pro-uPA and the enzymatically active two-chain HMW-uPA with high affinity (0.1-1.0 nM).[2] Binding occurs via the Growth Factor Domain in the Amino Terminal Fragment of uPA (amino acids 10-30). The enzymatically active two-chain LMW-uPA, which lacks the Amino Terminal Fragment, does not bind to uPAR. Recent studies have demonstrated inhibition of uPAR reduces the metastatic potential of human PC3 prostate carcinoma cells.[3]

PRINCIPLE OF THE METHOD

The IMUBIND uPAR ELISA employs a mouse monoclonal antibody against human uPAR as the capture antibody. Samples incubate in microwells precoated with this monoclonal antibody and a second, biotinylated antibody is used to recognize the bound uPAR molecules. Adding streptavidin conjugated horseradish peroxidase (HRP) completes the formation of the antibody-enzyme detection complex.

The addition of a perborate/3,3',5,5' - tetramethylbenzidine (TMB) substrate, and its subsequent reaction with the HRP creates a blue colored solution. Sensitivity is increased by addition of a sulfuric acid stop solution, yielding a yellow color. uPAR levels are quantified by measuring solution absorbances at 450 nm and comparing the values with those of a standard curve.

REAGENTS (Sufficient to assay 40 samples in duplicate)

96 uPAR antibody coated microwells with holder and cover sheet
6 vials of uPAR Standards, 0-3 ng/mL
2 vials of Detection Antibody, biotinylated anti-human uPAR (lyophilized)
1 vial of Enzyme Conjugate, Streptavidin-horseradish peroxidase (60 µL)
1 vial of Enzyme Conjugate Diluent (lyophilized)
1 vial of Substrate, TMB (12 mL)
1 vial of Detergent, 25% Triton X-100 (10 mL)
2 packets of PBS Buffer, pH 7.4

REAGENT PREPARATION AND STORAGE

Unopened and lyophilized reagents are stable until the expiration date printed on the box when stored as instructed.

1. **Antibody Coated Microwells:** Once removed from the foil pouch, the microwell strips must be used within 30 minutes. Unused strips may be stored at 2°-8°C for 4 weeks when sealed in the original pouch with the desiccant present and protected from moisture.

2. **uPAR Standards:** Reconstitute the 0.25, 0.75, 1.5, 2.0, and 3.0 ng/mL standard vials with 1.0 mL of filtered deionized . Reconstitute the 0 ng/mL standard vial with 2.0 mL of filtered deionized . Mix gently for 3 minutes. Do Not Vortex. uPAR Standards may be aliquoted and stored for up to 4 weeks at -20°C.

3. **Detection Antibody:** Reconstitute with 5.5 mL of filtered deionized water. Mix gently for 3 minutes. Once reconstituted, Detection Antibody may be stored for up to 4 weeks at -20°C.

4. **Enzyme Conjugate, SA-HRP:** Supplied as a concentrate, Enzyme Conjugate may be used until the expiration date stated on the vial when stored at 2°-8°C. Dilute the Enzyme Conjugate 1:1000 with Enzyme Conjugate Diluent just prior to use. For using all 96 microwells at one time, dilute 12 µL of Enzyme Conjugate to 12 mL in Enzyme Conjugate Diluent. If all 96 microwells are not to be used, dilute 2 µL of Enzyme Conjugate to 2 mL in Enzyme Conjugate Diluent each 16 well strip that will be used. Working strength Enzyme Conjugate is stable for 2 hours at 2°-8°C. Discard any unused working strength Enzyme Conjugate.

5. **Enzyme Conjugate Diluent:** Reconstitute with 20 mL of filtered deionized water and mix well. Once reconstituted, Enzyme Conjugate Diluent may be stored for up to 4 weeks at 2°-8°C.

6. **Substrate, TMB:** Supplied ready to use, the Substrate may be used until the expiration date stated on the vial when stored in the dark at 2°-8°C.

7. **Detergent:** Supplied as a 25% solution, the Detergent may be used until the expiration date stated on the vial when stored at 2°-8°C. A 10% Triton X-100 solution is prepared by adding 4 mL of Detergent (25% Triton X-100) to 6 mL of filtered deionized water.

8. **Wash Buffer:** Wash Buffer is prepared by dissolving the contents of a packet of PBS Buffer with 1 L of filtered deionized water. After dissolving, add 4 mL of Detergent (25% Triton X-100) to the PBS Buffer. Mix well. Wash Buffer may be used for up to 4 weeks when stored at 2°-8°C.

9. **Sample Buffer:** Prepare an appropriate amount of Sample Buffer by adding BSA to Wash Buffer to a final concentration of 1 % w/v (1 gm BSA/100 mL Wash Buffer).

10. **10% Triton X-100:** Prepare a 10% Triton X-100 solution by adding 4 mL of 25% Triton X-100 to 6 mL of filtered deionized .

SPECIMEN COLLECTION AND PREPARATION

A. **Detergent Extraction of Homogenized Tissue Samples**

1. Suspend powder from pulverized frozen tissue samples (100-300 mg wet weight) in 1.8 mL TBS, pH 8.5.
2. Add 0.2 mL 10% Triton X-100 in TBS, pH 8.5, to the tissue suspension to yield a 1% Triton X-100 final preparation.
3. Stir for 12 hours at 2°-8°C.
4. Centrifuge the suspension at 100,000 x g for 60 minutes at 2°-8°C to separate cell debris.
5. Decant the supernatant/tissue extract and measure the total protein content of the extract using a BCA protein assay. If necessary, adjust the total protein content to 2-3 mg/mL with TBS, pH 8.5. Aliquot the extract into 100 µL portions.
6a. For storage, freeze at -80°C or in liquid nitrogen.
6b. For immediate use in the ELISA, dilute the tissue extract 1:20 in Sample Buffer.

B. **Tissue Culture Supernatant Samples**

Dilute samples 1:5 (recommended initial dilution) in Sample Buffer. Note: some cell systems may require a higher dilution factor (up to 1:500).

C. Human Plasma

Only citrate collected platelet poor plasma may be used for this assay. Do Not Use EDTA collected plasma. See "Collection, Transport and Processing of Blood Specimens for Testing Plasma-based Coagulation Assays and Molecular Hemostasis Assays; Approved Guidelines-Fifth Edition", NCCLS Document H21-A5, Vol. 28, No. 5, January 2008. Plasma collection should be performed as follows:

1. Collect 9 parts of blood into 1 part of 3.2% (0.109 M) trisodium citrate anticoagulant solution.

2. Centrifuge the blood sample at 10,000 x g for 15 minutes.

3. Plasma should be stored at room temperature and assayed within 2 hours. Alternatively, plasma may be stored at –80°C for up to 6 months

4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasmas should be stored at room temperature and assayed within 2 hours.

5. Dilute plasma 1:10 with Sample Buffer.

PROCEDURE

Materials Provided – See Reagents

Materials Required But Not Provided

0.22 µm filtered deionized water
50-200µL eight channel multi-pipette
10-200µL single pipette
microwell plate reader at 450 nm
microwell plate washer (optional)
0.5M
Bovine Serum Albumin (BSA, e.g. Sigma A-7030)
Tris Buffered Saline (TBS), pH 8.5

Assay Procedure

Day One

1. Remove the required number of antibody coated microwells from the foil pouch and place them in the plate holder. The microwells are supplied in 16 well strips (2 columns x 8 rows). Tightly reseal the foil pouch with the desiccant inside and store at 2°-8°C.

2. Add 100 µL of uPAR Standard or diluted sample to a microwell, cover and incubate overnight at 2°-8°C. It is recommended to perform measurements in duplicate.

DAY TWO

3. Wash the microwells 4 times with Wash Buffer.

4. Add 100 µL of Detection Antibody to each microwell, cover and incubate for 1 hour at room temperature (18°-25°C).

5. Wash the microwells 4 times with Wash Buffer.

6. For running all 96 microwells at one time, add 12 µL of Enzyme Conjugate to 12 mL of Enzyme Conjugate Diluent (add 2 µL. conjugate to 2 mL of diluent for each 16 well strip when running less than 96 microwells). Add 100 µL of diluted enzyme conjugate to each well, cover with lid and incubate for 1 hour at room temperature (18°-25°C).

7. Wash the microwells 4 times with Wash Buffer.

8. Add 100 µL of Substrate solution to each well, cover and incubate for 20 minutes at room temperature (18°-25°C). A blue color will develop.

9. Stop the enzymatic reaction by adding 50 µL of 0.5N . Tap the sides of the microwell frame to help ensure even distribution of the . The solution color will turn yellow. Read the absorbances on a microwell plate reader set at a wavelength of 450 nm within 30 minutes. Deduct the background average of the blanks from the standards and sample readings.

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RESULTS

Construct a standard curve by plotting the mean absorbance value for each uPAR standard versus its corresponding concentration. A standard curve should be generated each time the assay is performed. The following standard curve is for demonstration purposes only.

Representative Standard Curve

IMUBIND® uPAR ELISA

$$y = -0.0726x^2 + 0.8491x + 0.1943$$
$$R^2 = 1$$

Absorbance, 450 nm vs. uPAR concentration, ng/mL
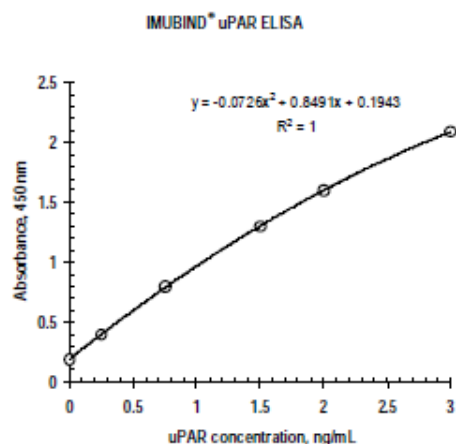
Calculations

Using the mean absorbance value for each diluted sample, determine its corresponding uPAR concentration in ng/mL obtained from the standard curve.

A. Tissue Samples

1. Multiply the sample value by 20 to obtain the uPAR concentration of the tissue extract as the extract was diluted 20-fold in the assay.

2. Divide the tissue extract uPAR concentration by the protein concentration (mg/mL) of the tissue extract to convert ng uPAR/mL of sample to ng total uPAR/mg protein.

B. Plasma and Tissue Culture Supernatant Samples

Multiply the sample value by the dilution factor to determine the uPAR concentration of the plasma/tissue culture supernatant.

ANALYTICAL PERFORMANCE

Native and recombinant uPAR as well as uPAR/uPA and uPAR/uPA/PAI-1 complexes are all recognized by this assay. Antigen recognition was demonstrated by Western blot analysis of full length wild type uPAR secretions from a transfected osteosarcoma cell (2A2) and an RKO cell line, as well as recombinant uPAR from Baculovirus and CHO cell lines.

REFERENCES

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7.9 Raw data: Normal probability plot and Normal Box Cox probability plot

Lambda	0,65

Prostate cancer group

Probability	Z-score	Sorted suPAR	Box Cox
0,03	-1,87	0,00	-1,54
0,08	-1,40	0,00	-1,54
0,13	-1,13	0,00	-1,54
0,18	-0,92	0,00	-1,54
0,23	-0,74	0,04	-1,35
0,28	-0,59	0,08	-1,24
0,33	-0,45	0,10	-1,19
0,38	-0,31	0,10	-1,19
0,43	-0,19	0,11	-1,17
0,48	-0,06	0,12	-1,15
0,52	0,06	0,22	-0,96
0,57	0,19	0,23	-0,95
0,62	0,31	0,23	-0,95
0,67	0,45	0,24	-0,93
0,72	0,59	0,26	-0,90
0,77	0,74	0,26	-0,90
0,82	0,92	0,41	-0,68
0,87	1,13	0,49	-0,57
0,92	1,40	0,52	-0,53
0,97	1,87	0,56	-0,48

BPH group

Probability	Z-score	Sorted suPAR	Box Cox
0,03	-1,89	0,00	-1,54
0,08	-1,43	0,03	-1,38
0,12	-1,16	0,04	-1,35
0,17	-0,95	0,09	-1,22
0,22	-0,78	0,09	-1,22
0,26	-0,63	0,17	-1,05
0,31	-0,49	0,19	-1,02
0,36	-0,36	0,19	-1,02
0,41	-0,24	0,20	-1,00
0,45	-0,12	0,20	-1,00
0,50	0,00	0,22	-0,96
0,55	0,12	0,23	-0,95
0,59	0,24	0,27	-0,88
0,64	0,36	0,35	-0,76
0,69	0,49	0,36	-0,75
0,74	0,63	0,36	-0,75
0,78	0,78	0,37	-0,73
0,83	0,95	0,37	-0,73
0,88	1,16	0,40	-0,69
0,92	1,43	0,46	-0,61
0,97	1,89	0,47	-0,60

7.10 Raw data: Test run

Data

Project	Optimization of ELISA
Date	08.05.2013
Kit	IMUBIND uPAR ELISA
Lot	111301-1
Expiration	30.09.2013

Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 5	Sample 1, 1:4	Sample 3, Undiluted								
B	Std 1	Std 5	Sample 1, 1:4	Sample 3, Undiluted								
C	Std 2	BLANK	Sample 2, Undilute	Sample 3, 1:2								
D	Std 2	BLANK	Sample 2, Undilute	Sample 3, 1:2								
E	Std 3	Sample 1, Undilute	Sample 2, 1:2	Sample 3, 1:4								
F	Std 3	Sample 1, Undilute	Sample 2, 1:2	Sample 3, 1:4								
G	Std 4	Sample 1, 1:2	Sample 2, 1:4									
H	Std 4	Sample 1, 1:2	Sample 2, 1:4									

Raw rata Abs 450

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,544	0,159	0,211	0,280								
B	0,568	0,155	0,258	0,477								
C	0,441	0,100	0,300	0,253								
D	0,447	0,102	0,368	0,256								
E	0,373	0,404	0,261	0,195								
F	0,372	0,373	0,314	0,192								
G	0,247	0,339	0,205									
H	0,255	0,316	0,216									

BLANK	0,101

Abs - Blank

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,443	0,058	0,110	0,179								
B	0,467	0,054	0,157	0,376								
C	0,340	-0,001	0,199	0,152								
D	0,346	0,001	0,267	0,155								
E	0,272	0,303	0,160	0,094								
F	0,271	0,272	0,213	0,091								
G	0,146	0,238	0,104									
H	0,154	0,215	0,115									

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Results

No	DF	Mean (ng/mL)	Mean x DF	CV
1	1	1,74	1,74	8,3 %
1	2	1,34	2,68	4,0 %
1	4	0,73	2,92	7,5 %
2	1	1,38	1,38	22,8 %
2	2	1,08	2,16	11,4 %
2	4	0,57	2,29	2,2 %
3	1	1,68	1,68	54,5 %
3	2	0,86	1,72	0,8 %
3	4	0,46	1,85	0,8 %

Trendline formula through intercept and the standard curve point is used to calculate the unknown samples

Line fomula: y=ax + b > suPAR concentration (ng/mL) = ((ABS - blank)-b)/standard curve slope

Slope of line	0,1524
Intercept	0,0221
Correlation coefficient, R2	0,987

Standard kurve		Mean ABS	CV
Std 1	**3,00**	0,455	3,7 %
Std 2	**2,00**	0,343	1,2 %
Std 3	**1,50**	0,272	0,3 %
Std 4	**0,75**	0,150	3,8 %
Std 5	**0,25**	0,056	5,1 %
Std 6	**0,00**	0,000	0,0 %

Acceptance criteria: CV < 10%
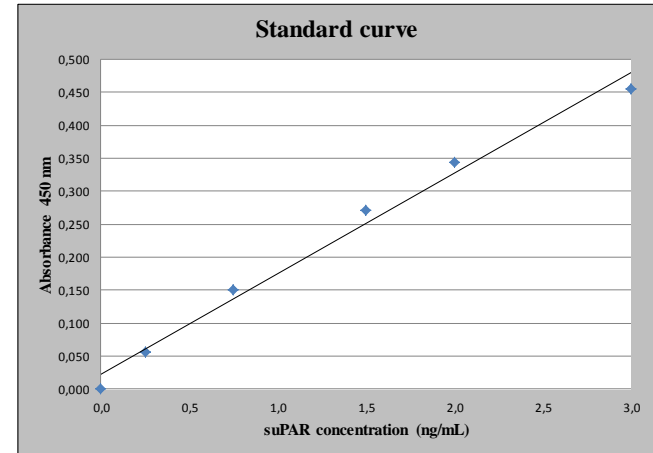
Standard curve

suPAR concentration from the Standard curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,76	0,24	0,58	1,03								
B	2,92	0,21	0,89	2,32								
C	2,09	-0,15	1,16	0,85								
D	2,13	-0,14	1,61	0,87								
E	1,64	1,84	0,90	0,47								
F	1,63	1,64	1,25	0,45								
G	0,81	1,42	0,54									
H	0,87	1,27	0,61									

7.11 Raw data: All samples

Data

Project	Master thesis OMS, Run 1
Date	10.05.2013
Kit	IMUBIND uPAR ELISA
Lot	111301-1
Expiration	30.09.2013

Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 5	Sample 3 PCa	Sample 7 BPH	Sample 11 PCa	Sample 15 BPH	Sample 19 PCa	Sample 23 BPH	Sample 27 PCa	Sample 31 BPH	Sample 35 PCa	Sample 39 PCa
B	Std 1	Std 5	Sample 3 PCa	Sample 7 BPH	Sample 11 PCa	Sample 15 BPH	Sample 19 PCa	Sample 23 BPH	Sample 27 PCa	Sample 31 BPH	Sample 35 PCa	Sample 39 PCa
C	Std 2	BLANK	Sample 4 PCa	Sample 8 PCa	Sample 12 PCa	Sample 16 BPH	Sample 20 PCa	Sample 24 BPH	Sample 28 BPH	Sample 32 BPH	Sample 36 PCa	Sample 40 BPH
D	Std 2	BLANK	Sample 4 PCa	Sample 8 PCa	Sample 12 PCa	Sample 16 BPH	Sample 20 PCa	Sample 24 BPH	Sample 28 BPH	Sample 32 BPH	Sample 36 PCa	Sample 40 BPH
E	Std 3	Sample 1 BPH	Sample 5 BPH	Sample 9 PCa	Sample 13 PCa	Sample 17 PCa	Sample 21 PCa	Sample 25 BPH	Sample 29 BPH	Sample 33 BPH	Sample 37 PCa	Sample 41 BPH
F	Std 3	Sample 1 BPH	Sample BPH	Sample 9 PCa	Sample 13 PCa	Sample 17 PCa	Sample 21 PCa	Sample 25 BPH	Sample 29 BPH	Sample 33 BPH	Sample 37 PCa	Sample 41 BPH
G	Std 4	Sample 2 BPH	Sample 6 BPH	Sample 10 PCa	Sample 14 BPH	Sample 18 PCa	Sample 22 BPH	Sample 26 PCa	Sample 30 BPH	Sample 34 BPH	Sample 38 PCa	
H	Std 4	Sample 2 BPH	Sample 6 BPH	Sample 10 PCa	Sample 14 BPH	Sample 18 PCa	Sample 22 BPH	Sample 26 PCa	Sample 30 BPH	Sample 34 BPH	Sample 38 PCa	

Raw rata Abs 450

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,482	0,145	0,154	0,132	0,133	0,161	0,117	0,166	0,137	0,138	0,131	0,191
B	0,514	0,150	0,165	0,125	0,149	0,185	0,125	0,194	0,142	0,135	0,126	0,212
C	0,431	0,094	0,195	0,155	0,134	0,182	0,153	0,170	0,154	0,150	0,108	0,173
D	0,432	0,097	0,198	0,165	0,142	0,194	0,157	0,181	0,155	0,161	0,108	0,175
E	0,380	0,150	0,130	0,158	0,155	0,142	0,195	0,194	0,152	0,149	0,135	0,153
F	0,384	0,150	0,125	0,155	0,155	0,133	0,189	0,185	0,148	0,145	0,134	0,151
G	0,228	0,163	0,177	0,122	0,129	0,157	0,179	0,196	0,157	0,141	0,121	
H	0,239	0,159	0,174	0,115	0,116	0,152	0,170	0,166	0,147	0,132	0,125	

BLANK	0,096

Abs - Blank

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,387	0,050	0,059	0,037	0,038	0,066	0,022	0,071	0,042	0,043	0,036	0,096
B	0,419	0,055	0,070	0,030	0,054	0,090	0,030	0,099	0,047	0,040	0,031	0,117
C	0,336	-0,002	0,100	0,060	0,039	0,087	0,058	0,075	0,059	0,055	0,013	0,078
D	0,337	0,002	0,103	0,070	0,047	0,099	0,062	0,086	0,060	0,066	0,013	0,080
E	0,285	0,055	0,035	0,063	0,060	0,047	0,100	0,099	0,057	0,054	0,040	0,058
F	0,289	0,055	0,030	0,060	0,060	0,038	0,094	0,090	0,053	0,050	0,039	0,056
G	0,133	0,068	0,082	0,027	0,034	0,062	0,084	0,101	0,062	0,046	0,026	
H	0,144	0,064	0,079	0,020	0,021	0,057	0,075	0,071	0,052	0,037	0,030	

Results

No	Sample ID	Diagnosis	Alder	Mean (ng/mL)	CV
1	11308	BPH	82	0,19	0,0 %
2	11992	BPH	60	0,27	7,6 %
3	52483	PCa	57	0,26	21,7 %
4	52840	PCa	64	0,52	2,9 %
5	53496	BPH	67	0,03	90,7 %
6	53853	BPH	65	0,37	4,1 %
7	54410	BPH	77	0,04	101,0 %
8	55066	PCa	67	0,26	19,4 %
9	55651	PCa	71	0,24	6,4 %
10	78372	PCa	68	-0,04	-97,1 %
11	78957	PCa	64	0,12	65,0 %
12	94230	PCa	55	0,10	39,3 %
13	94815	PCa	66	0,23	0,0 %
14	96613	BPH	59	-0,01	-835,7 %
15	97497	BPH	69	0,35	34,4 %
16	98054	BPH	75	0,46	13,2 %
17	98639	PCa	62	0,10	45,8 %
18	99295	PCa	61	0,22	11,4 %
19	99880	PCa	65	-0,02	-217,6 %
20	16969	PCa	67	0,23	9,0 %
21	17526	PCa	62	0,49	6,2 %
22	37664	BPH	63	0,36	12,5 %
23	38221	BPH	80	0,40	35,1 %
24	38920	BPH	72	0,37	15,0 %
25	39576	BPH	69	0,47	9,7 %
26	40114	PCa	61	0,41	37,0 %
27	40798	PCa	62	0,11	22,2 %
28	41469	BPH	79	0,22	2,3 %
29	42026	BPH	69	0,19	10,7 %
30	42611	BPH	70	0,20	24,9 %
31	43267	BPH	65	0,09	16,4 %
32	43852	BPH	69	0,23	24,4 %
33	44409	BPH	76	0,17	12,1 %
34	45065	BPH	65	0,09	49,3 %
35	45650	PCa	63	0,04	72,2 %
36	46321	PCa	55	-0,11	0,0 %
37	46906	PCa	65	0,08	6,5 %
38	47562	PCa	63	0,00	-471,4 %
39	48119	PCa	75	0,56	19,1 %
40	48704	BPH	82	0,36	2,8 %
41	49360	BPH	75	0,20	5,0 %

Trendline formula through intercept and the standard curve point is used to calculate the unknown samples

Line fomula: y=ax + b > suPAR concentration (ng/mL) = ((ABS - blank)-b)/standard curve slope

Slope of line through (0,0281)	0,1395
Intercept	0,0281
Correlation coefficient, R2	0,954

Standard kurve		Mean ABS	CV
Std 1	3,00	0,403	5,6 %
Std 2	2,00	0,336	0,2 %
Std 3	1,50	0,287	1,0 %
Std 4	0,75	0,138	5,6 %
Std 5	0,25	0,052	6,8 %
Std 6	0,00	0,000	0,0 %

Acceptance criteria: CV < 10%
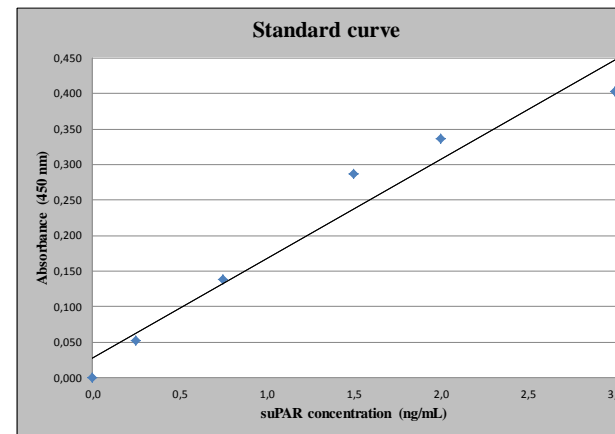
Standard curve

Calculated suPAR concentration from the Standard curve

	1	2	3	4	5	6	7	8	9	10	11	12
A	2,57	0,15	0,22	0,06	0,07	0,27	-0,05	0,30	0,10	0,10	0,05	0,48
B	2,80	0,19	0,30	0,01	0,18	0,44	0,01	0,50	0,13	0,08	0,02	0,63
C	2,20	-0,21	0,51	0,23	0,07	0,42	0,21	0,33	0,22	0,19	-0,11	0,35
D	2,21	-0,19	0,53	0,30	0,13	0,50	0,24	0,41	0,23	0,27	-0,11	0,37
E	1,84	0,19	0,05	0,25	0,23	0,13	0,51	0,50	0,20	0,18	0,08	0,21
F	1,87	0,19	0,01	0,23	0,23	0,07	0,47	0,44	0,17	0,15	0,07	0,20
G	0,75	0,28	0,38	-0,01	0,04	0,24	0,40	0,52	0,24	0,12	-0,02	
H	0,83	0,25	0,36	-0,06	-0,05	0,20	0,33	0,30	0,17	0,06	0,01	