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The Kallikrein-Related Peptidases hK2 and PSA with Emphasis on Genetic Variation, Secretion, and Sperm Motility

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Abstract

Prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2) are secreted by the prostate into seminal plasma and through leakage into blood. This leakage increases rapidly in prostate disease, and PSA is used worldwide as a marker of prostate cancer. PSA is activated in vitro by hK2, and cleaves the semenogelins in semen, which releases motile sperm, and low levels of PSA have been associated with low sperm motility. The present objective was to evaluate factors that might influence release of hK2 and PSA, and to study those two proteins with regard to their levels in seminal plasma and blood, and their association with sperm motility in young healthy men without prostate disease.

PSA and hK2 were measured by immunofluorometric assay. Genomic DNA was prepared from peripheral leukocytes, and genotyping was performed with the Sequenom Mass Array System.

Levels of hK2 and PSA were found to be associated with variants in the genes encoding hK2 (KLK2) and PSA (KLK3) in young men (mean age 18 years). The single-nucleotide polymorphisms (SNPs) rs198972, rs198977, rs198978, and "SNP2" in KLK2 were associated with lower hK2 levels in seminal plasma and serum in carriers of the TT genotype. In KLK3, the AA genotype in rs266882 in combination with CAG > 22 were correlated with higher total PSA (tPSA) in serum; rs2271094 and "SNP1" were associated with lower tPSA concentrations in seminal plasma from subjects homozygous for the common allele (A and G, respectively). Carriers of the common T allele in rs1058205 in KLK3, had higher amounts of tPSA in seminal plasma, higher concentrations of tPSA in serum, and a lower ratio of free to tPSA (%fPSA). Free PSA (fPSA) in blood showed ~17% co-variation with PSA in semen, whereas no relationship was found between levels of complexed PSA (cPSA) in blood and PSA in semen. In blood, levels of cPSA, but not fPSA, increased with age, which may reflect an increasing incidence of prostate disease. In men 19–40 years, an age when reproduction most commonly takes place, correlations were found between hK2 and PSA in seminal plasma ($p < 0.001$, $r = 0.47$) and between hK2 and age ($p = 0.01$, $r = -0.20$). Men in that age group who were in the lowest quartile with respect to amount of PSA in seminal plasma, semen volume, and zinc concentration had, respectively, 5.8%, 4.1%, and 3.9% fewer progressively motile sperm. These findings suggest that a decrease in secretory function of the prostate can impair sperm motility. The present results obtained by analysing the allelic variants of KLK2 and KLK3 might be useful for refining models of PSA cut-off values in PCa testing.

Key words: androgen receptor, CAG, human glandular kallikrein 2, hk2, KLK2, KLK3, prostate-specific antigen, PSA, seminal plasma, SNP, sperm motility

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I. **Sävblom C.,** Malm J., Giwercman A., Nilsson JA., Berglund G., Lilja H. Blood levels of free-PSA but not complex-PSA significantly correlates to prostate release of PSA in semen in young men, while blood levels of complex-PSA, but not free PSA increase with age. *The Prostate; 65 (1): 66–72 (2005).*

II. **Sävblom C.**, Giwercman A., Lilja H., Malm J., Halldén C., Lundin K., Giwercman Y. Association between polymorphisms in the prostate-specific antigen (PSA) promoter and release of PSA. *International Journal of Andrology; e-published ahead of print, March 10 (2008).*

III. **Sävblom C.***,* Giwercman A., Rylander L., Haugen TB., Lilja H., Malm J. Low secretory function of the prostate is associated with decreased sperm motility. *Submitted.*

IV. **Sävblom C.***,* Halldén C., Cronin AM., Säll T., Savage C., Klein R., Giwercman A., Lilja H. Allelic variation in *KLK2* and *KLK3* is associated with levels of hK2 and PSA in seminal plasma and blood in young healthy men. *Manuscript.*

Abbreviations and Acronyms

aa	amino acid
A2M	α_2-macroglobulin
ACT	α_1-antichymotrypsin
API	α_1-protease inhibitor
AR	androgen receptor
ARE	androgen response element
bp	base pair
BPH	benign prostatic hyperplasia
CASA	computer-assisted sperm analysis
CI	confidence interval
conc.	concentration
cPSA	complexed PSA
CV	coefficient of variation
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
fPSA	free PSA
%fPSA	ratio of free to total PSA
hK1	tissue kallikrein
hK2	human glandular kallikrein 2/ KLK2
hK3	PSA/KLK3
HWE	Hardy-Weinberg equilibrium
iPSA	intact (uncleaved) PSA
kb	kilo base pair
kDa	kilodalton
KLK	kallikrein gene
KLK	kallikrein protein
LD	linkage disequilibrium
Mab	monoclonal antibody
MAF	minor allele frequency
MPM	Malmö Preventive Medicine project
NAG	neutral α-glucosidase
nPSA	nicked (internally cleaved) PSA
PAI-1	plasminogen activator inhibitor 1
PAP	prostatic acid phosphatase
PCa	prostate cancer
PCI	protein C inhibitor
PCR	polymerase chain reaction
PSA	prostate-specific antigen/KLK3
SEMG	semenogelin
SHBG	sex hormone-binding globulin
SNP	single nucleotide polymorphism
tPSA	total PSA (cPSA + fPSA)
uPA	urokinase plasminogen activator
WHO	World Health Organization

Preface

The aim of the research underlying this thesis was to gain further knowledge on the physiological levels of the fertility-related proteins prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2) in seminal plasma and in serum in young men without prostate disease.

Although further work is needed to clarify the physiological functions of PSA, it is assumed that the primary action of this protein is to cleave the gel-forming proteins in seminal fluid, subsequent to its activation, which can be achieved by hK2. Such cleavage corresponds to the process of liquefaction that occurs 10 to 20 minutes after ejaculation and results in the release of spermatozoa. At least 30% of disturbed male fertility is of unknown cause and is referred to as *idiopathic infertility*. It is now believed that the condition has a genetic and biochemical basis. Since low levels of PSA have been shown to be correlated with lower percentage of motile sperm in both fertile and infertile men, better knowledge of the factors that regulate the release, and ultimately the levels, of hK2 and PSA can help explain the mechanisms behind male infertility and might also contribute to improving treatment of this condition.

PSA and hK2 are produced by the prostate, one of the male accessory sex glands, and they are released into semen in abundant concentrations and a minute proportion is also leaked into the blood. However, this leakage rapidly increases up to 10^5-fold when disease affects the prostate, which is why PSA (and to some extent also hK2) is used worldwide as a marker of prostate disorders. Over the past 20 years, researchers have made substantial efforts to enhance the testing for prostate cancer by use of these proteins. Therefore, further elucidation of the physiological secretion of PSA and hK2 will also aid comprehension of the changes in the release of these proteins that occur when disease affects the prostate.

Background

Fertility refers to the capability to conceive or to induce a pregnancy, whereas infertility refers to when a pregnancy does not occur within one year of regular unprotected intercourse. The latter condition is further divided into primary infertility, which defines the condition when no pregnancy at all has been achieved, and secondary infertility, which means that no further pregnancies have occurred.

Infertility (primary and secondary) affects one in seven couples, and it is not completely clear whether the incidence of this condition is increasing. The proportion of couples seeking medical counselling for infertility is estimated to 4–17%. Traditionally, the female partner was held responsible for the failure to conceive. In reality, male reproductive capacity was found to be deficient in 50% of infertile couples evaluated in a study conducted by the World Health Organization [1]. It is assumed that 7% of all men are confronted with the problem of disturbed fertility at some point in their lives. In Sweden, approximately 100,000 men of reproductive age have a fertility problem.

Today, at least 30% of cases of impaired male fertility still remain aetiologically unclear and are referred to as idiopathic infertility (from the Greek words *idios* meaning one's own and *pathos* for suffering), essentially indicating "a disease of its own kind". In these cases an unknown genetic or biochemical basis of infertility may exist. Laboratory investigation of the condition leaves the affected men with only a descriptive diagnosis of their semen sample (see Table I), and hence, the semen analysis is the basis of the assessment of the infertile couple.

Table I. Nomenclature for semen [2]

Normozoospermia	Normal ejaculate as defined by the reference values
Oligozoospermia	Sperm concentration less than reference values
Asthenozoospermia	Less than reference value for sperm motility
Teratozoospermia	Less than reference value for sperm morphology
Oligoasthenoteratozoospermia	Disturbance of all three parameters
	(combinations of only two prefixes can also be used)
Azoospermia	No spermatozoa in the ejaculate
Aspermia	No ejaculate

Semen analysis

At least one semen sample analysis is mandatory in the investigation of an infertile couple. This analysis includes assessment of the characteristics of the spermatozoa and seminal plasma. Seminal plasma is semen deprived of its spermatozoa, and thus it consists of a mixture of various fluids from the male reproductive tract. The production of spermatozoa, called spermatogenesis, is a two-month-long process involving differentiation of immature germ cells in the testis (Figure 1). The spermatozoa are ultimately stored for approximately 14 days in the epididymis prior to ejaculation [3].

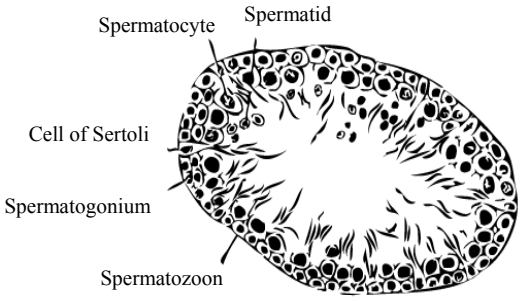

Figure 1. Spermatogenesis in the testis (from Gray's Anatomy)

Sperm morphology, sperm concentration, total sperm count, sperm motility, and semen volume are the parameters used to define semen quality according to WHO recommendations [4]. The reference values for these semen parameters are shown in Table II. If the first semen sample is normal, there is usually no need for a repeat analysis. The use of sperm count, motility and morphology as markers of male fertility has its limitations due to overlap in the levels found in fertile and infertile men [5, 6]. Semen quality is also subject to intra-individually variation [7], as well as disparities caused by intra- and interlaboratory differences in assessments [8, 9].

Table II. Reference values of semen variables [4]

Volume	≥ 2.0 ml
pH	≥ 7.2
Sperm concentration	≥ 20 x 10^6 spermatozoa per ejaculate
Total sperm number	≥ 40 x 10^6 spermatozoa per ejaculate
Motility	≥ 50% motile (grade a+b) or ≥ 25% with progressive motility (grade a) within 60 min. of ejaculation
Morphology of mature spermatozoon	Oval-shaped head, length 4.0–5.0 μm, width 2.5–3.5 μm, length/width of the head 1.50–1.75, one sperm tail (length 45 μm) attached symmetrically to the broad sperm head base. Immediately behind the sperm head is the mid-piece of the tail located, with a width of 1 μm and length of 7.0–8.0 μm.

Sperm concentration is the most widely used laboratory marker of spermatogenesis. In addition to the daily sperm production, this parameter depends on other factors such as time of abstinence [10-12] and the ejaculate volume. Sperm motility is another important parameter used to assess fertility. Inasmuch as such evaluation is influenced by subjective factors, the best developed objective approach involves tracing video images of individual sperm by computer-assisted sperm analysis (CASA), a technique that has proven successful in predicting fertility [12, 13]. A correlation has also been found between CASA-evaluated sperm motility and pregnancy rates [14] after *in vitro* fertilization (IVF) [15]. Sperm motility also depends on a multitude of factors originating from the accessory sex glands, such as the fertility-related proteins prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2) secreted by the prostate gland.

Importance of the accessory sex glands for fertilization

The prostate, the seminal vesicles, and the periurethral glands of Littré and bulbourethral glands of Cowper are the accessory sex glands (Figure 2, p. 13). These glands discharge their secretions into the urethra, where, at the time of ejaculation, they are mixed with sperm transported through the epididymis and the vas (ductus) deferens. The secretions in the seminal plasma originate largely form the seminal vesicles and constitute over 60% of the bulk volume of semen, and the remaining portions of that fluid consists of prostatic secretions (30%), secretions from the bulbourethral glands (< 10%), and spermatozoa (1%). Little is known about the significance of the accessory sex glands in fertilization. This is primarily due to the extensive variability among mammals with regard to the composition and structure of secreted proteins, and the structure of the accessory sex glands. Consequently, the use of animal models to further elucidare the importance of these glands in the fertilization process in human is limited.

What is known is that the order in which the accessory sex glands contribute to the ejaculate is fixed in fertile men. The bulbourethral glands secrete an alkaline solution that contains glycoproteins to neutralize and lubricate the urinary tract before ejaculation. The prostate, epididymis, and ductuli deferentia contract together and discharge spermatozoa from the distal cauda epidymidis and prostatic secretions. The final step occurs when the seminal vesicles contract and expel their secretions. Thus, under physiological conditions, there are few spermatozoa in the last fractions of the ejaculate.

Various chemical substances can also be measured in the ejaculate, and the levels at which they are secreted by different accessory sex glands or compartments of the male reproductive system can serve as markers of their function. However, in the case of bilateral organs, only bilateral dysfunction can cause significant changes in levels of biochemical markers.

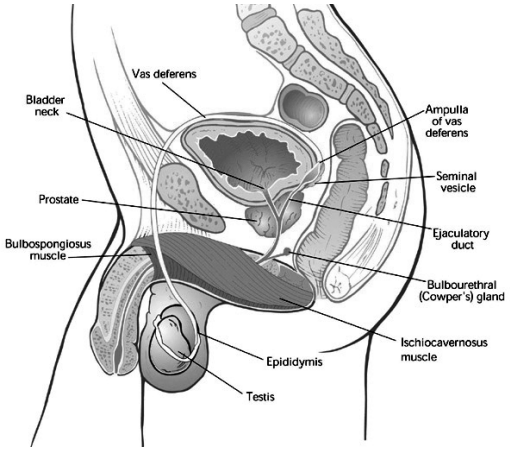

Figure 2. The male reproductive tract

Secretions from the epididymis

Epididymal secretions are rich in energy substrates such as carnitine, glycerophosphocholine, and neutral α-glucosidase (NAG). NAG offers higher specificity and sensitivity in evaluations of epididymal function compared to the other markers [16], and it is essential for maturation of sperm during their transit through the epididymis and for providing the sperm with energy [17, 18]. NAG also protects the sperm against reactive oxygen species [19]. In addition, the epididymis secretes small amounts of the gel-forming protein semenogelin 2 (SEMG2) [20], and the expression of SEMG2 is confined to the epithelium of the cauda epididymis, where the spermatozoa are collected before ejaculation [21].

Secretions from the seminal vesicles

The secretions from the seminal vesicles include fructose, semenogelins (SEMGs), and prostaglandins [22, 23]. Fibronectin, lactoferrin, and protein C inhibitor (PCI) are also secreted by the seminal vesicles but at lower concentrations [24]. Lactoferrin in complex with iron is known to display microbicidal activity, and it is assumed that fructose provides nutrition necessary for the motility of spermatozoa. Semenogelin 1 (SEMG1) and SEMG2 are the quantitatively dominating proteins in secretions of the seminal vesicles [20, 25-27], and, together with fibronectin and zinc, these proteins form the gelatinous coagulum of ejaculated semen [28]. The concentration of SEMG1 is about five times higher than the level of SEMG2 [26]. It has been suggested that SEMGs and their degradation products regulate sperm capacitation, which comprises a series of transformations that spermatozoa must undergo to become fertile [29]. Experiments *in vitro* have also shown that SEMG1 and SEMG2 are potent activators of sperm hyaluronidase, an enzyme that is released during the acrosome reaction [30], which probably facilitates sperm penetration into the ovum. Prostaglandins are thought to stimulate the movement of spermatozoa [31].

Secretions from the prostate

The prostatic secretions are composed of serine proteases, mainly PSA, but also hK2, zinc, prostatic acid phosphatase (PAP), citric acid, and β-microseminoprotein [32-35]. The release of PSA, hK2, and zinc is androgen dependent. The functions of citric acid, β-microseminoprotein, and PAP in semen have not yet been fully clarified. However, it has been reported that PAP can dephosphorylate SEMG1 and SEMG2 *in vitro* [36] and that β-microseminoprotein is a sperm-coating antigen [37].

Along with SEMGs, zinc constitutes a major component of seminal plasma, primarily in complex with citrate [38]. Zinc ions have a high binding affinity for SEMGs [39], and hence one of the major roles of zinc is to participate in the formation of the semen coagulum [40]. Zinc has also been shown to confer recovery of motility in spermatozoa that have previously been inhibited by SEMG1 [41]. Furthermore, zinc is thought to be important for the stabilization of sperm chromatin [42] and for the antibacterial activity of the seminal plasma [43]. Within the prostate, zinc binds to PSA and thereby renders it inactive. At ejaculation, zinc is redistributed from citrate to high-molecular-weight ligands released from the seminal vesicles [44, 45], which reactivates PSA [46]. Mature catalytically active PSA, possibly activated by hK2, cleaves the gel-forming proteins in seminal plasma [28], which results in the release of spermatozoa. Furthermore, it is likely that hK2 is reversibly inhibited by zinc in prostatic fluid [47]. Therefore, it is important to consider these proteins in greater detail in order to understand the process of fertilization. To address that issue, the following sections describe the characteristics, functions, regulation, and relationships of the mentioned proteins, as well as their role in fertility and various factors that influence their levels in semen and blood. To begin with is a short description of the prostate gland, where the majority of these proteins are produced.

The prostate

The prostate gland has its origin in the urogenital sinus, and initiation of prostate organogenesis requires androgens produced by the foetal testes. The human adult prostate is walnut-shaped, has a volume of 4 x 3 x 2 cm, and weighs about 20 g [48, 49]. It is located in front of the rectum, immediately below the bladder, and is wrapped around the urethra (Figure 2, p. 13). The prostate contains 30–50 tubuloalveolar glands, which empty into 15–25 independent excretory ducts that open into the urethra. The glands are embedded in a fibromuscular stroma, which consists primarily of smooth muscle separated by strands of connective tissue rich in collagenous and elastic fibres.

In clinical practice, the prostate is commonly described in terms of its three zones, although these zones are not anatomically well-defined (Figure 3, p. 15). The *central* zone surrounds the ejaculatory ducts and comprises 25% of the total volume. The *transition* zone encircles the proximal urethra, constitutes 5% of the

total volume, and is the primary location of benign enlargement of the prostate (benign prostatic hyperplasia, BPH). Finally, the subcapsular, posterior *peripheral* zone surrounds the distal urethra, represents 70% of the gland, and is the main site of prostatic tumours.

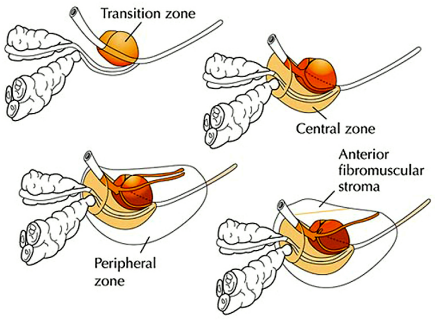

Figure 3. The zones of the prostate gland

The secretory epithelium is largely pseudostratified, and it consists of columnar exocrine cells and cuboidal basal cells situated on a basement membrane (Figure 4). The luminal cells express the androgen receptor (AR) and secrete various AR-regulated proteins, such as PSA and hK2. The volume of the basal prostatic secretions is estimated to be 0.5–2 ml/day, whereas active secretion during ejaculation corresponds to 0.5–1 ml. The presence of tight junctions between adjacent basal cells suggests that these cells form a blood-luminal barrier. It is believed that the basal layer houses prostate epithelial stem cells. The epithelium also contains scattered neuroendocrine cells, which partly control release and expulsion of prostatic secretions during ejaculation. The stroma of the prostate is considered to directly influence the behaviour of epithelial cells, partly by supplying androgen-regulated growth factors to the epithelium [50].

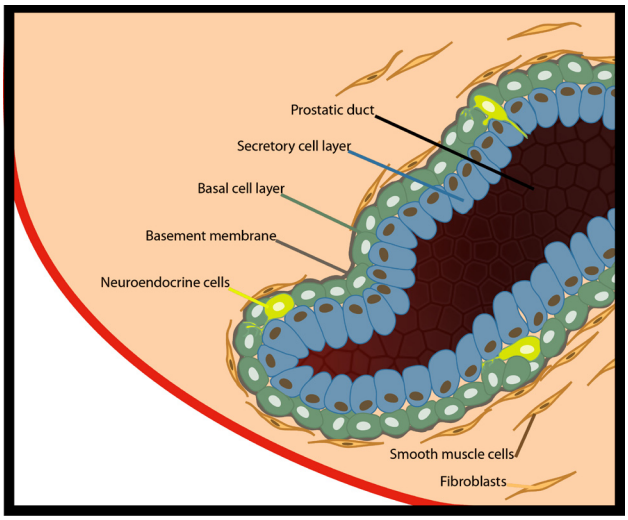

Figure 4. Schematic drawing of the prostatic epithelium

Kindly provided by Jens Ceder, PhD, Division of Urological Research, Lund University

PSA and hK2 are members of the human kallikrein family

PSA and hK2 belong to the family of proteins called the human kallikreins, which is a subgroup of the serine protease family. The name kallikrein is derived from the Greek word for pancreas, the tissue in which the first member of the family was discovered. The substance was reported to be hypotensive and was named tissue kallikrein. However, it was later designated human kallikrein 1 (hK1), and after that it was renamed glandular kallikrein to distinguish it from the subsequently discovered plasma kallikrein. It was long assumed that humans had only the three kallikreins designated hK1, hK2, and hK3 (later called kallikreins 1, 2 and 3: KLK1, KLK2, and KLK3), which are referred to as the classical kallikreins. The protein hK2 (or KLK2) is known as human glandular kallikrein 2, and hK3 (or KLK3) is recognized worldwide as PSA. PSA and hK2 are closely related, exhibiting 79% amino acid (aa) sequence identity, whereas KLK1 shows only 62% sequence similarity to PSA and 66% to hK2 [51].

The genes that encode the proteins KLK1, hK2, and PSA, are designated *KLK1*, *KLK2,* and *KLK3*, respectively, and are clustered in a 69–70-kb gene segment located on the long arm of chromosome 19 [52]. Each gene contains five exons and four introns. *KLK2* and *KLK3* are aligned 13 kb apart on 19q13.41, they have 85% identical coding regions, and 91% of their promoter regions are the same [53-56]. *KLK2* is 5,790 base pairs (bp) in length, whereas *KLK3* is 5,846 bp [57].

In 2000, the linear genomic sequences around chromosome 19q13.3–4 became available through the human genome project (HUGO), which revealed identification and the exact location of 15 members of an expanded human kallikrein gene family (Figure 5). These genes were added to the classical human kallikreins family and designated *KLK4* to *KLK15* [58-60]. All of the kallikrein genes, except *KLK3* and *KLK2,* are transcribed from telomere to centromere, and they are all relatively small, with a genomic length ranging from 4 to 10 kb. The genes *KLK4* to *KLK15* encode serine proteases, but they are less conserved than the classical human kallikreins genes, and thus research is needed to elucidate their biological functions.

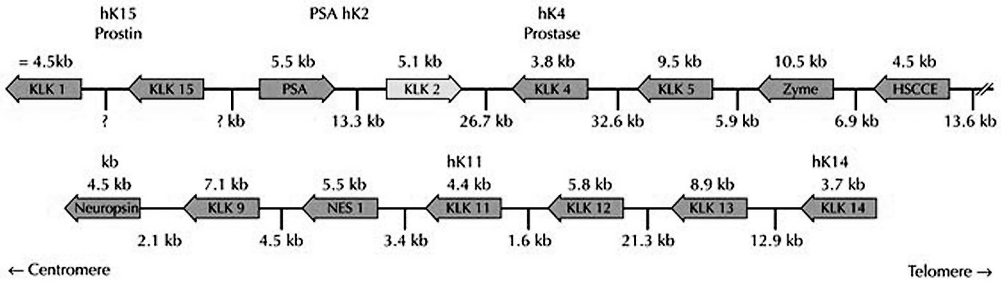

Figure 5. The human kallikrein gene locus

Several products of the kallikrein genes have also been identified. Among them is KLK5, or human stratum corneum trypsin-like serine protease, which has been suggested to be involved in desquamation of the epidermis [61]. Research has indicated that KLK6, or neurosin, may play a role in amyloid precursor processing and Alzheimer's disease [62, 63]. Moreover, it is known that KLK7, or human stratum chymotryptic enzyme, is associated with skin diseases such as psoriasis [64], and KLK8, or neuropsin, is linked to disorders of the brain, such as epilepsy [65, 66].

Studies have also evaluated the other members of the kallikrein gene family, with regard to their utility as markers of malignant disease [67]. For example, it has been reported that *KLK5, KLK10, KLK13,* and *KLK14* are down-regulated in testicular germ cell tumours [68]. Furthermore, *KLK15* may prove to be marker of advanced prostate cancer (PCa), since the gene is underexpressed in stage T3 [69], whereas *KLK4* and *KLK14* are overexpressed in PCa [70, 71]. It has also been reported that *KLK4–KLK8, KLK10, KLK11, KLK14,* and *KLK15* are highly expressed in ovarian cancer [72-75], that expression of *KLK8* is elevated in association with cervical and endometrial cancer [76] [76, 77], and that expression of *KLK14* is related to increased tumour stage and reduced prognosis for survival in breast cancer [78]. However, there is no proof that any of these genes plays a causal role in the development of cancer.

In 2006, a revised nomenclature was proposed for serine proteases with homology to tissue kallikreins giving all members new names, with the exception of KLK1 which is still called kallikrein 1 [79]. The new names for KLK2–KLK15 are kallikrein-related peptidase, followed by the number of the gene symbol, e.g. *KLK2* is kallikrein-related peptidase 2. To distinguish between proteins and the genes, the gene designations should be written in italics, as recommended by the HUGO Gene Nomenclature Committee. Despite this attempt to arrive at more rational nomenclature, the designations PSA and hK2 are still used worldwide to relate to these proteins by clinicians and researchers, as well as by the increasingly well-informed members of the general public.

Prostate-Specific Antigen

History of PSA

In the late 1960s and early 1970s, the development of improved biochemical techniques enabled rapid progress in the field of immunology. Much work was focused on characterizing various antigenic proteins in semen and evaluating their relationship to fertility, because animal studies had suggested that antibodies could immobilize sperm and prevent fertilization. In parallel to this, a search was being conducted to find a "semen specific antigen" that could be used as a forensic marker in cases of rape [80].

Hara and co-workers were active in the attempts to identify a forensic "rape-marker" when they reported the finding of γ-seminoprotein in 1966 and later

characterized it in 1971 [81, 82]. However, the reports published by that group were in Japanese and unknown to the Western scientific community, where Ablin and colleagues reported the discovery of two antigens specific to the human prostate in 1970. One of those proteins was the previously identified PAP, and the other required characterization and was given the generic term prostate-specific antigen [83-85]. In 1973, Li and Beling isolated and characterized the E1 and E2 antigens in seminal plasma when performing work aimed at finding an immunological method to control fertility [86]. Five years later, as part of the continuing search for a suitable marker of rape, Sensabaugh tried to overcome the problem posed by vasectomized and azoospermic men and succeeded in isolating two proteins from seminal plasma, which he designated p41 and p30 according to their respective molecular weights in kDa [87].

In 1979, Wang and Chu and colleagues purified and characterized an antigen present in normal and malignant prostate tissue which they first called prostate antigen (PA), and, since their research also indicated that it was specific to the prostate, they used the term prostate-specific antigen, PSA [88]. Chu and co-workers further refined the immunoassay used to detect PSA and application of that technique to reveal prostate disease [89-91]. In 1980, a co-worker of Chu's named Papsidero, used the antibody against PSA in prostatic tissue and identified PSA in serum from men with metastatic prostate cancer. Papsidero also found that the PSA in prostate and that in serum were similar, which was the beginning of the use of PSA as a diagnostic tool [92].

Regarding the antigens discovered in seminal plasma, in 1985 Lilja reported that a kallikrein-like serine protease known as "PSA" could cleave the predominant protein in seminal plasma [28]. In 1986, Watt determined the primary structure of PSA [93]. A year later Lundwall and Lilja presented a molecular cloning of PSA cDNA [94] and Schaller reported the aa sequence of γ-seminoprotein [95]. In 1990, Sensabaugh and Blake used an antibody provided by Li, and were able to confirm that p30 was the same as E1 protein and that it was derived from the prostate [96]. It was subsequently found that PSA was identical to p30 [97], and in 1994 Sensabaugh could conclude that p30, E1, and γ-seminoprotein were in fact all the same protein—PSA [98].

Biological function of PSA

Further research is still needed to fully clarify the physiological functions of PSA, although it is assumed that the primary action of this protease is to cleave the gel-forming proteins SEMG1, SEMG2, and fibronectin in seminal fluid. It has also been observed *in vitro* that PSA can cleave insulin-like growth factor-binding protein (IGFBP) 3 [99, 100] and 4 [101]. Active IGFBP can bind insulin growth factor 1 (IGF-1), a protein that stimulates cell growth [102]. Therefore, hypothetically, PSA may promote cell growth, such as proliferation of prostatic cells by inactivating IGFBP 3 and 4 and thereby increasing the bioavailability of IGF-1. In addition, it has been shown that PSA stimulates the mitogenic activity of osteoblasts *in vitro*, most likely through activation of transforming growth factor-β

and proteolytic modification of cell adhesion receptors [103]. PSA has also been reported to display anti-angiogenetic activity [104, 105].

Biochemical characteristics of PSA

PSA is a serine protease with chymotrypsin-like substrate specificity and it cleaves peptide bonds on the C-terminal side of certain tyrosine and leucine residues [27, 28, 106, 107]. PSA-mediated cleavage can also occur on the C-terminal side of arginine, methionine, and phenylalanine residues [108-110]. The mature form of the protein, excluding the carbohydrate side chain, has a theoretical molecular mass of 26.1 kDa [94], and mass spectrometry has indicated a molecular mass of 28.4 kDa, when the carbohydrate side chain is included [111].

PSA is synthesized as a 261-aa pre-pro-precursor, but the mature and catalytically active form that is secreted into the seminal fluid is composed of 237 aa. A 17-aa signal peptide is removed upon transfer of the pre-pro-precursor to the endoplasmic reticulum, and ultimately a 7-aa propeptide is removed upon activation of the protein [94]. It has been demonstrated that under- or overprocessing of the propeptide results in production of enzymatically inactive PSA [112]. The protease or proteases responsible for correct processing of pro-PSA to active PSA *in vivo* are still unknown, although the presence of arginine at the cleavage site suggests that pro-PSA is activated by an enzyme with trypsin-like substrate specificity.

Studies *in vitro* have shown that the final step of the activation, which involves conversion of the PSA zymogen into an enzymatically active protein, can be achieved by adding hK2 to pro-PSA. This finding indicates that hK2 may be the physiological PSA-processing protease [113-115]. However, Denmeade and co-workers, observed that hK2 could not cleave a synthetic PSA pro-peptide, and they also found evidence of that non-kallikrein trypsin-like proteases (e.g. prostasin, hepsin), expressed in prostate tissue may be involved in the activation of PSA [28, 116]. Further research have shown that KLK4 (prostase), KLK5, and KLK15 (prostin) can activate pro-PSA *in vitro* [117-119]. Accordingly, it seems that PSA activation may require a cascade of proteases for activation.

Regulation and elimination of PSA

The activity of PSA is irreversibly inhibited by complexation to a SERPIN (serine protease inhibitor) such as α_1-antichymotrypsin (ACT), α_1-protease inhibitor (API) (or α_1-antitrypsin, AAT), and PCI. Other inhibitors are α_2-macroglobulin (A2M) and pregnancy zone protein (PZP), which are large molecules that encapsulate PSA [120]. PSA activity can be reversibly regulated by divalent cations, especially zinc, which is highly abundant in seminal plasma, but also by copper, mercury, cobalt, and cadmium [46]. In addition, the predominant seminal plasma proteins SEMG1 and SEMG2 bind zinc and can indirectly regulate the activity of PSA [39].

In seminal plasma, the major part (60–70%) of PSA is in a free active form (fPSA), and less than 5% is in complex with anti-proteases like PCI (cPSA) secreted from the seminal vesicles [108]; together, those two forms are called total PSA (tPSA) (Figure 6, p. 21). The opposite relationship exists in blood, mainly due to a $\geq 10^5$-

fold excess of protease inhibitors. The major portion (65–95%) of PSA in blood is complexed with ACT [107, 121, 122], and complexes with A2M or API are estimated to comprise only 1–2% of the PSA in that fluid [123-126]. A peculiar feature of the A2M complexation is that the inhibitor A2M envelopes PSA and thereby masks the epitopes that are recognized by commercial PSA assays, which renders PSA-A2M invisible in blood [121, 122, 127]. Only 5–35% of the PSA in blood remains in a free inert form.

The site of PSA-ACT complex formation is not fully known, although a study has localized ACT in the prostatic epithelium [128, 129], which might suggest that the formation of such complexes occurs in extracellular compartments juxtaposed to the glandular epithelium. It is plausible that the formation of these complexes occurs in the perivascular compartment, although there are no experimental data reported that have provided any detailed insight into these matters.

The free inert PSA in blood is a mixture of mature fPSA and pro-PSA [130], including some pro-PSA forms in which the propeptide is N-terminally truncated at various positions [131]. Free PSA in blood can also be divided into intact (uncleaved) form of PSA (iPSA) [132] (both mature and pro-PSA) or nicked PSA (nPSA), which is PSA that is internally cleaved (between Lys145 and Lys146) [107, 133]. Experiments have indicated that about half of the fPSA in blood is in an intact form.

.Two initial reports, determined the half-life of PSA in serum to be 2.2 +/– 0.8 days [134], and 3.2 +/– 0.1 days [135], respectively. A later study reported a linear decline in concentration of PSA-ACT complex with 0.8 ng/ml/day, whereas the concentration of fPSA decreased in a bi-exponential manner, with a mean initial half-life of 0.81 hours and a mean terminal half-life of 14 hours, suggesting the existence of different elimination pathways [136]. Due to its smaller molecular mass (28.4 kDa), fPSA is most likely eliminated by glomerular filtration. By comparison, there is evidence that receptors in the liver are involved in the elimination of the complexes PSA-A2M and PSA-ACT [137-140], and elimination PSA-ACT complex might also involve receptors in the kidney [141, 142].

Extraprostatic PSA

PSA is not produced solely by the prostate, and consequently this protein has been detected in various tissues and fluids other than the prostate and seminal plasma but at levels 10^4-fold lower than found in those two locations. To summarize, PSA have been detected in the following: plasma and saliva of healthy women [143]; mammary glands and breast milk [144]; trachea and thyroid gland [145]; salivary glands and pancreas [146]; jejunum and ileum [147]; periurethral glands [148]; anal glands [149]; ascitic fluid [150]; pleural effusions [151]; placenta [152, 153]; endometrium [154]; seminal vesicles, testis, epididymis, and amniotic fluid [155]; epidermis, kidney, and pituitary glands [156]; cerebrospinal fluid [157]; peripheral leukocytes [158, 159]. PSA has also been found in cancers of the breast [160, 161], ovaries [162], colon, parotid gland, kidney, adrenal gland, liver, and lung [163], as well as in neuroblastoma cell lines [164].

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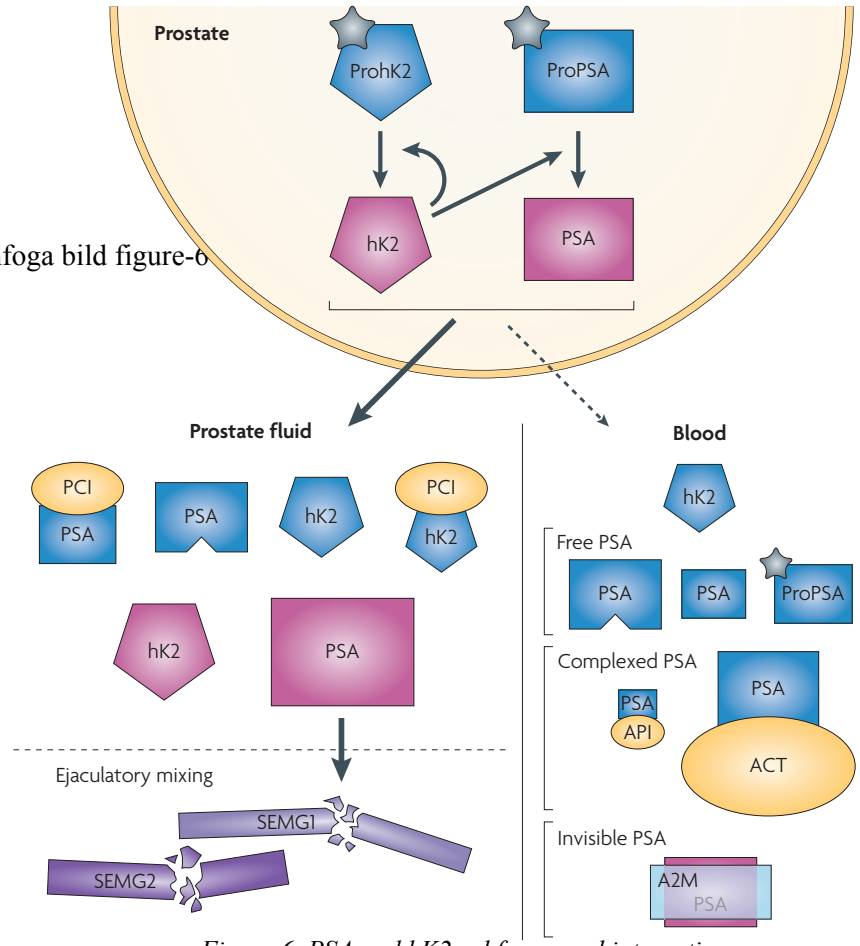

Figure 6. PSA and hK2 subforms and interactions

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Human glandular kallikrein 2

History of hK2

In 1987, Schedlich and co-workers identified the gene coding for the protein hK2, which at that time was called hGK1 [51]. Two years later it was found that hK2 is expressed mainly in the prostate in an amount that corresponds to 10–50% of the expression of PSA [166, 167]. A correlation between expression of the genes for hK2 and PSA was reported in 1990 [168], and in 1995, hK2 was isolated in seminal plasma, where it was found in complex with PCI [169]. The first assay for measurement of serum hK2 was developed in 1996 [170]. However, the extensive structural similarity of PSA and hK2 has made it a challenging task to develop specific hK2 assays with sufficiently low functional detection limits and negligible immunological cross-reactivity to PSA [171, 172].

Biological function of hK2

Studies *in vitro* have shown that hK2 can convert the zymogen form of PSA into the enzymatically active protein [47, 113-115]. HK2 can also cleave the semenogelins and fibronectin [47, 173]. Furthermore, it has been reported that hK2 can activate urokinase plasminogen activator (uPA), and inactivate its primary inhibitor Plasminogen activator inhibitor-1 (PAI-1) through complex formation [174, 175]. Activated uPA cleaves the zymogen plasminogen to yield the active form plasmin, an enzyme that is responsible for breaking down the fibrin polymers in blood clots. Moreover, hK2 has also been found to cleave IGFBPs [101].

Biochemical characteristics of hK2

The protein hK2 is a serine protease with trypsin-like substrate specificity, and it cleaves its substrates on the C-terminal side of certain single and double arginines, and less often lysines and histidines [47, 51]. Mature hK2 has a theoretical molecular mass of 26.2 kDa excluding the carbohydrate chain and a molecular mass of 28.5 kDa determined by mass spectrometry [176]. The protein is synthesized as a 261-aa prepro form that includes a 17-aa signal peptide. Upon transfer to the endoplasmic reticulum, the signal peptide is removed, and ultimately a 7-aa propeptide is removed upon activation which yields the mature protein composed of 237 aa. It is believed that the activation of hK2 is an autocatalytic process that occurs after secretion [114, 177, 178].

Regulation and elimination of hK2

Most of the hK2 in seminal plasma is in complex with PCI and therefore inactive [169]. Studies *in vitro* have demonstrated that hK2 can form complexes with several extracellular protease inhibitors, such as α_2-antiplasmin, A2M, ACT, antithrombin III, C1-inactivator, and PAI-1 [174-176, 179, 180]. The enzymatic activity of hK2 can also be reversibly regulated by the micromolar levels of zinc that are present in both the prostate and seminal plasma [47]. Moreover, in a study conducted by Mikolajczyk et al. it was found that 10% of the hK2 in prostatic tissue was in complex with the intracellular serine protease inhibitor-6 (PI-6) [181]. In serum, most of the hK2 (80–95%) is in a free form, and only a minor fraction is in complex with ACT [170, 179, 182, 183], which suggests clearance of the protein through glomerular filtration in the kidneys. In addition, an inactive zymogen form of hK2 (pro-hK2) has been found in blood [179, 184].

Extraprostatic hK2

Like PSA, hK2 is not produced solely by the prostate. Studies have demonstrated hK2 in the following tissues and fluids: endometrium [154]; pituitary gland [156]; thyroid gland [145]; saliva, amniotic fluid, breast milk, nipple aspirate, and breast cyst fluid [185, 186]; trachea, skin, salivary gland, jejunum, ileum, urethra, testis, seminal vesicles, and epididymis [147, 159].

PSA and hK2 in relation to fertility

Observations made both *in vitro* and *in vivo* indicate that the release of PSA and hK2 from the prostate into semen is necessary for normal sperm motility. As mentioned above, semen is composed of secretions from the prostate gland and seminal vesicles, along with spermatozoa that have been stored in the epididymis. The prostate secretions constitute one third of the total semen volume, and along with most of the spermatozoa, are included in the first part of the ejaculate [187]. Studies of spermatozoa ejaculated erroneously together with secretions from the seminal vesicles have indicated decreased sperm motility and survival, and also a reduced zinc content and stability of sperm chromatin [188-190], which indicates that factors in the prostatic secretions are essential for normal fertility.

After ejaculation, semen is immediately transformed into a sperm-entrapping gel through the actions of the proteins SEMG1, SEMG2 and fibronectin produced by the seminal vesicles. Zinc also participates in the process of gel formation [40]. SEMG1 and SEMG2 are in disulphide complexes in the gel and cleavage of the bonds in those associations does not lead to liquefaction of the coagulum [25, 191]. However, addition of denaturing concentrations of urea causes macroscopic dissolution, which indicates that non-covalent bonds are essential for gel formation.

Concomitant with gel formation, the process of dissolution of the gel (liquefaction) begins. Semen is normally liquefied within approximately 10 to 20 minutes after ejaculation, both *in vitro* and *in vivo* in the vagina [192, 193]. The sperm motility pattern changes as liquefaction proceeds, possibly due to attachment of SEMG fragments to the spermatozoa. Assays *in vitro* have shown that PSA is responsible for proteolysis of the gel-forming proteins SEMG1 and SEMG2 into multiple soluble fragments, which in turn induces sperm motility [24, 28, 194-197].

In vitro, PSA is converted to its enzymatically active form by hK2 [113-115]. The presence of hK2 in seminal plasma suggests that it is also a physiological activator of PSA [169]. Furthermore, research has shown that hK2 can cleave fibronectin and the semenogelins [47, 114, 173].

Regulation of both PSA and hK2 in semen is effected mainly by divalent cations, especially zinc [46, 47]. Moreover, it has been shown that zinc-inhibited PSA is activated by exposure to semenogelins, which suggests that the semenogelins are also involved in controlling the activity of PSA in seminal plasma [39]. In addition to the important regulators of PSA and hK2 activity in semen factors that affect the secretory function of prostate are known to have an impact on normal sperm motility, which agrees with results of previous studies. For example, Elzanaty et al. investigated young men in the general Swedish population and observed a significant positive correlation between PSA concentration in seminal plasma and the percentage of motile sperm determined by CASA [198]. Low levels of PSA in semen have also been associated with a reduced percentage of motile sperm in both infertile men [199] and men with spinal cord injuries [200].

Factors affecting levels of PSA and hK2 in semen and blood

PSA and hK2 levels in serum are known to increase rapidly in men with prostate disease, and under normal physiological conditions concentrations of PSA vary according to age and between men of different ethnic origin. Furthermore, some studies have indicated that ejaculation has an impact on PSA levels in serum, although that conclusion has been contradicted by other investigations. Associations between levels of PSA and hK2 and genomic variations called single nucleotide polymorphisms (SNPs) in the genes encoding respective proteins have also been reported. Notwithstanding, it is indisputable that levels of PSA and hK2 in seminal plasma and serum are determined by androgen action mediated through the androgen receptor.

Androgens

Androgens are largely responsible for regulating the expression of PSA and hK2 in the glandular epithelial cells of the prostate [201, 202]. It has been shown that androgen suppression (e.g. by use of 5α-reductase inhibitors such as finasteride and dutasteride) lowers the levels of PSA in blood by approximately 50% during the first year of use and further with longer-term use [203-205]. Research has also demonstrated that androgen-mediated release of PSA and hK2 from the prostate results in PSA levels ranging from 0.2 to 5 mg/ml in seminal plasma [91, 199, 206-210], and the concentration of hK2 corresponds to about 1% (2-12 μg/ml) of the level of PSA in that fluid [159]. Under normal conditions median PSA level in blood is 0.57 ng/ml [211], and median hK2 level is 0.026 ng/ml [212], and the concentration of hK2 has been reported to correspond to about 3.6% of the level of PSA [213]. Thus, PSA levels are ~10^6-fold higher in seminal plasma than in blood, whereas levels of hK2 are ~10^4-fold higher in seminal plasma than in blood [165].

Testosterone is the major circulating androgen, and it is synthesized through conversion of cholesterol in five enzymatic steps. This process occurs continuously in the Leydig cells of the testis, which secrete 6–7 mg/day, to yield 95% of all testosterone in the male body; the remaining 5% is derived from the adrenal glands [214]. Testosterone is secreted primarily to the blood, where it is transported bound either to albumin (~55%) or to sex hormone-binding globulin (SHBG) (~45%), and only about ~3% circulates in a free form. The half-life of testosterone in plasma is approximately 12 minutes. In its target cells, testosterone is converted to the highly biologically active hormone dihydrotestosterone (DHT) through 5α-reduction mediated by the enzyme SRD5A and also to estradiol via aromatization [215]. DHT is the main biologically active androgen in the prostate [216], and the first step in androgen action involves binding of this hormone to the androgen receptor (AR) (Figure 7, p. 25).

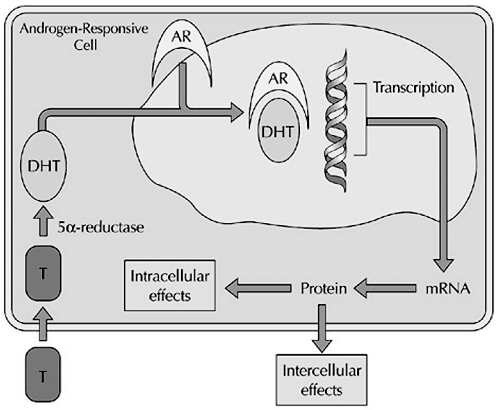

Figure 7. Androgen action in androgen-responsive cells in the prostate

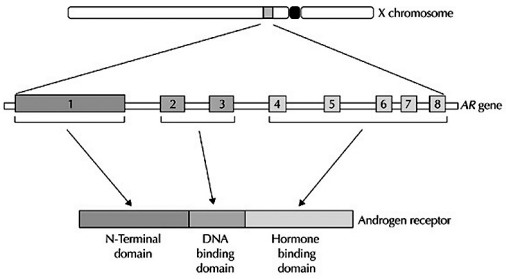

Figure 8. Androgen receptor gene and protein

The androgen receptor

Testosterone and DHT exert their effects through the AR, which belongs to the family of steroid hormone receptors. The function of AR is essential for normal male sex differentiation, development of secondary sex characteristics at puberty, and spermatogenesis.

AR is activated by binding of DHT, and thereafter the AR-DHT complex enters the cell nucleus, where AR recognizes and binds to specific nucleotide sequences called androgen response elements (AREs) in the promoter regions of androgen-regulated genes (Figure 7). Then transcription begins, which results in secretion of the respective gene products, such as PSA and hK2. Studies have shown that the PSA gene *KLK3* comprises several AREs, two of which have been identified in the proximal region of the *KLK3* promoter: ARE I centred at –170 bp and ARE II at –394 bp, with respect to the transcription start site. An ARE III has been identified far upstream, at –4200 bp [217, 218], and the presence of additional AREs has also been detected in the 5´ upstream enhancer region of the *KLK3* promoter [219]. Both ARE I and III, but only one of the non-consensus AREs found in *KLK3* are conserved in *KLK2* [220-222]. ARE III has been shown to be the principal control region for hK2 expression, but it exhibits lower androgen inducibility compared to the *KLK3* promoter.

The AR gene is located on chromosome Xq11–12. It consists of eight exons and is divided into three functional domains: the N-terminal transactivating domain (exon 1), the DNA-binding domain (exon 2–3), and the C-terminal hormone binding domain (exon 4–8) [223, 224] (Figure 8).

There are multiple allelic variants of the AR gene in the general population. These involve polymorphisms that affect the N-terminal transactivating domain of AR such that it contains two polymorphic polynucleotide repeats, a $(CAG)_nCAA$ repeat

sequence encoding a polyglutamine stretch [225] and a $(GGT)_3GGG(GGT)_2$-$(GGC)_n$ repeat sequence encoding a polyglycine stretch, generally designated the GGN repeat [226].

These variations are known to affect AR function both *in vivo* and *in vitro* [227, 228]. For example, it has been reported that increased CAG repeat length is associated with diminished co-activator interaction and transcriptional activity. Moreover, abnormal expansion of the CAG repeat to > 40 repeats has been found to be connected with spinal and bulbar muscular atrophy, as well as with mild to moderate androgen insensitivity, also known as Kennedy's disease [229, 230]. The androgen insensitivity in Kennedy's disease is characterized by low virilization, reduced sperm production, testicular atrophy, and infertility.

There are also ethnic differences in the distribution of CAG and GGN repeats. The median number of CAG repeats is 21 +/– 2 (range 10-35) in Caucasian populations. African populations have shorter repeats and also carry the broadest range of repeats, whereas longer repeats are seen in Asian populations [231-234]. Furthermore, it has been found that 85% of ethnic Swedish population has 23 or 24 GGN repeat [235]. GGN repeats are shortest in African populations (mean and median length), but longer in Asian and Caucasian populations [232].

With regard to effects on fertility, long CAG repeat length has been associated with decreased sperm concentration [228, 236, 237], whereas no connection has been discovered between the GGN repeat length and infertility [228]. However, in a study of young Swedish men, GGN < 23 was associated with lower semen volume in comparison with GGN ≥ 23 [238].

Research has also revealed significant negative associations between length of CAG repeats and PSA levels in serum [239, 240], and in seminal plasma [241]. By comparison, a study of men with PCa did not reveal any difference in mean serum concentrations of PSA in relation to CAG or GGN repeat length [242], and similarily there are no reports so far of associations between such repeat length and hK2 levels in seminal plasma and serum.

On the other hand, it has been observed that PCa is related to shorter CAG repeat length (< 20) alone or in combination with a GG genotype in a SNP in ARE I in *KLK3* (rs266882) [243], and also to shorter CAG repeats (< 22) in combination with a CC genotype in a SNP (rs198977) in *KLK2* [242]. However, some reports have indicated no significant relationship between PCa and CAG repeat length considered alone [244-246] or in combination with GGN repeats [247, 248], although there are some findings that suggest a connection between PCa and short GGN repeat length [249] and linkage disequilibrium (LD) between AR polymorphisms [234]. In any case, one thing that is clear is that over the last decade an increasing amount of research interest has been focused on AR polymorphisms and SNPs in relation to PCa.

Single nucleotide polymorphisms

A SNP (pronounced *snip*) is the most common form of polymorphism in the human genome, representing about 90% of all known genetic variation. It is assumed that most SNPs have no effect on cell function, but the current interest lies in the possibility that these polymorphisms can predispose individuals to disease or influence responses to drugs. SNPs are also evolutionarily stable and do not change extensively from one generation to another, and therefore they are easier to follow in population studies.

It has been estimated that around one in every 500 to 2000 bases in the human genome exhibit variation [250]. SNPs arise due to errors in DNA replication and repair, and consist of a difference in a single nucleotide (A, T, C, or G) and consequently they are shared among individuals by descent [251]. For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs can also be assigned a minor allele frequency (MAF), which refers to the lowest allele frequency at a locus that is observed in a population. For SNPs, this is simply the lesser of the two allele frequencies. Studies have estimated that a common SNP (i.e. with a MAF > 10%), occurs once approximately every 600 bp [252].

SNPs can occur within coding sequences or non-coding regions of genes, or in intergenic regions. SNPs located within a coding sequence will not necessarily change the aa sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed *synonymous* (or called silent mutation), and if a different polypeptide sequence are produced it is designated a *non-synonymous* SNP. Non-synonymous SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA. Inasmuch as only approximately 3–5% of the human DNA sequence codes for proteins most SNPs are found in non-coding regions.

In recent years, it has been reported that SNPs located in the genes *KLK3* [239, 253, 254] and *KLK2* [255, 256], are associated with variations in serum levels of PSA and hK2, respectively, and with risk of PCa. A higher mean serum PSA concentration has been observed in one study in men with an AA genotype at -158 bp (rs266882) relative to the transcription start site of *KLK3* in combination with short AR CAG repeat length [239]. In addition, research has shown that the A allele increases the binding of ARE I to AR and the transcriptional reponse to androgens, and also that the AA genotype in related to an elevated risk of PCa [257].

However, the finding regarding rs266882 has been questioned. For instance, two studies revealed no association between this SNP and serum levels of PSA [248, 253], and an investigation *in vitro* showed no difference between the PSA promoter variants with respect to their abilities to activate a reporter gene [258]. Furthermore, Cramer et al. [254] observed connections between three other SNPs in *KLK3* and levels of PSA in serum. More precisely, those authors reported that the C allele at –5412 bp (rs2739448), and the G allele at –5429 bp (rs2569733) were associated with an increase in serum PSA levels and were in LD, and that the

G allele at position –4643 bp (rs925013) was correlated with increased serum PSA. However, the results regarding the G allele and rs925013 were later challenged by other investigators [259].

Regarding hK2, a SNP in exon 5 of *KLK2* (rs198977) results in a nucleotide change from cytosine to thymine, and *in vitro* the two alleles code for an active and an inactive form of hK2 [260]. The common allele codes for Arg^{226}-hK2, which has trypsin-like activity, whereas the variant allele codes for Trp^{226}-hK2, which has no detectable activity. In a study of men referred to a PCa center due to elevated serum PSA levels (≥ 4.0 ng/ml) or abnormal digital rectal examination (DRE), it was found that those carrying the TT genotype had significantly lower median hK2 concentrations in serum [255]. Later, the same research group observed that a SNP located in intron 1 in *KLK2* (rs2664155) was associated with significantly lower serum hK2 in men with the GG genotype as compared to carrying the A allele. Both of these SNPs have also been shown to be positively correlated with PCa [256].

Age

Oesterling et al. evaluated the association between age, prostate size, and serum levels of PSA in 478 healthy men aged 40–70 years old and found that PSA concentration in serum increased with age [261], an effect that is considered to be due to the concomitant increase in prostate size. They also established age-specific reference ranges for serum PSA in men: 40–49 yrs, 0.0–2.5 ng/ml; 50–59 yrs, 0.0–3.5 ng/ml; 60–69 yrs, 0.0–4.5 ng/ml; 70–79 yrs, 0.0–6.5 ng/ml. Preston and co-workers, later studied black and white men in the age groups 20–29, 30–39, and 40–45 years and reported a median serum PSA levels of 0.38, 0.45, and 0.52 ng/ml for black men and 0.38, 0.45, and 0.40 ng/ml for white men [262].

In contrast, there are reports that the levels of PSA in seminal plasma decrease with age. Matsuda et al. found a significant lower concentration of PSA in seminal plasma in men in their forties compared to those in their twenties, and likewise between men in their forties and thirties [263]. Furthermore, a study of men assessed for infertility revealed that those older than 50 had significantly lower amounts of PSA in seminal plasma and a lower percentage of progressively motile sperm compared to men aged 21–30 years [264]. It has also been demonstrated that levels of hK2 in serum increase with age [183, 212].

Ethnicity

Ethnicity has been shown to influence serum levels of PSA, and the investigations that have been conducted have focused primarily on men 40 years of age and older due to the interest in improving PCa testing. Thus far, no studies have reported that levels of hK2 in serum or PSA or hK2 in seminal plasma vary according to race.

Considering PSA in serum, an early study showed lower concentrations in healthy Japanese men aged 40–79 years than in comparable group of white men, and this was concluded to be due to smaller prostate glands in the Japanese [265]. A large community-based investigation of 77,700 men aged 40–70 years found significant

pairwise differences in mean serum PSA concentration between white and black men, between white and Hispanic men, between black and Asiatic men, and between Asiatic and Hispanic men [266]. In addition, African American men have been found to have higher mean serum PSA levels than white men [267, 268] or men of Hispanic origin [269]. As mentioned above, Preston et al. measured PSA levels in serum from white and black men aged 20–40 years, and their results indicated higher mean baseline levels in black subjects than in those who were white [262].

Ejaculation

The effect of ejaculation on serum PSA levels has also been investigated in men in different age groups, but results are conflicting (Table III). Ejaculation has been claimed to both decrease [270] and increase [271, 272] serum PSA, with rises seen up to 48 h after ejaculation [273]. There are also numerous reports indicating that ejaculation has no significant effect on serum PSA levels [274-278]. No studies have addressed the impact of ejaculation on serum levels of hK2.

Table III. Studies on the effect of ejaculation on serum PSA levels

Main Author (Year)	N of Subjects	Age of Subjects	Effect on PSA levels
Glenski (1992)	100	20-29, mean 26.3	no effect
Heidenreich (1997)	100	25-35	no effect
Herschman (1997)	20	mean 59	increase
McAleer (1993)	116	mean 57	no effect
Netto (1996)	40	50-60, mean 55	no effect
Simak (1993)	18	30-39	decreased
Stenner (1998)	89	mean 60.4	no effect
Tchetgen (1996)	64	49-79	increase
Zisman (1997)	18	mean 32.5 +/- 5.5	increase

PSA and hK2 as markers of prostate disease

Even though the physiological roles of PSA and hK2 are regarded as being related to fertility, these proteins are better known as markers of prostate disease, because there is a highly increase in the leakage of PSA and hK2 to the blood in men with prostate disease. The discovery of PSA has revolutionized the diagnosis and management of benign and malignant disease of the prostate and PSA is the most widely used of all known markers for diagnosis and follow-up of any type of cancer [279]. In the last decade, the usefulness of hK2 in diagnosis and staging of PCa has been evaluated as well.

Epidemiology and risk factors of prostate disease

Non-malignant prostate diseases, such as BPH and prostatitis are relatively unusual in young men but common in older men. Population-based studies have shown a

prevalence of 2–3% for symptomatic disease in men aged 44–50 years compared to 20–25% in those 65–75 years old [280]. PCa is a disease of the elderly (mean age at diagnosis 72–74 years), and it is also the third most common cancer in men and the fifth most common malignancy overall [281]. It has been estimated that 700,000 new cases occurred worldwide in 2002, and 10,000 cases were diagnosed in Sweden in 2004 (Swedish National Board of Health and Welfare). The incidence of PCa varies considerably between ethnic populations and countries, and is highest in North America and lowest in China. Accepted risk factors for PCa include familial predisposition, lifestyle or dietary intake. The progression of prostate tumours is also influenced by androgens [282].

PSA in prostate cancer

In patients with PCa, the level of PSA is slightly decreased in the prostatic tissue [283], although in advanced stages of the disease extracellular release of the protein can increase the serum concentrations as much as 10,000-fold compared to levels found in healthy men. In 1987, Stamey and co-authors observed that serum PSA in PCa patients was correlated with the stage of disease, was proportional to the estimated volume of the tumour, and became undetectable after radical prostatectomy. Therefore those investigators proposed that PSA could serve as a marker to monitor either the response to radiation therapy, or the occurrence of residual or recurrent disease [134]. The first evaluation of measuring PSA as a screening test for PCa was conducted in 1991 by Catalona and colleagues [284]. Based on their results, those investigators suggested that a serum PSA level of 4 ng/ml was an optimal cut-off value to achieve both the best rate of detection of curable PCa and a reduction in the number of unnecessary prostate biopsies. Later on it was found that the concentration of PSA in serum also depends on age and ethnicity, and researchers established age- and race-specific reference ranges to make PSA as a tumour marker more sensitive for men younger than 60 years and more specific for men over 60 years [261, 266]. In addition to this, early models introduced to enhance the PSA testing involved the kinetics of PSA (PSA velocity and PSA doubling time) [285, 286], and studies also considered the concentration of PSA in blood in correlation to prostate volume (i.e. PSA density (PSAD), and PSA density of transition zone (PSAT)) [287-289]. Furthermore, PSA velocity was recently evaluated concerning the usefulness for prediction and diagnosis of PCa [290, 291].

Although it is known that PSA is associated with both benign and malignant processes in the prostate, there is evidence that levels of the ratio of free to total PSA (%fPSA) and hK2 are more closely related to malignant disease. Based on the finding that the proportion of PSA-ACT complex is higher in men with PCa compared to those with BPH [121, 122], Christensson et al. were able to demonstrate that the separation of patients with BPH and PCa could be improved by analysing %fPSA [292]. Further research showed that PCa cells, in contrast to BPH cells, synthesize increased amounts of ACT, which can lead to early PSA-ACT complexation [293]. Determination of %fPSA has also been found to increase diagnostic accuracy, in particular at serum PSA levels ranging from 4 to 10 ng/ml

[294-300]. Moreover, it was recently reported that a PSA value of ≥ 0.9 ng/ml for patients ≤ 50 years of age, can predict a diagnosis of advanced PCa up to 25 years later [301], which might offer a method of risk stratification in PCa screening.

Possible associations of other molecular forms of PSA with PCa have also been evaluated with the aim of improving the accuracy of this protein as tumour marker. Studies have demonstrated that BPH patients have a higher proportion of nPSA [132, 302, 303], and that PCa patients have a larger proportion of iPSA, and thus analysis of nPSA and iPSA might enhance discrimination of men with moderately elevated PSA levels [304]. The use of cPSA has not proven to be of any convincing supplementary value compared to %fPSA [297, 305-307].

hK2 in prostate cancer

It has been found that hK2 is expressed more extensively in malignant prostatic tissue than in benign tissue [308], and serum levels of this protein are higher in men with PCa than in those with BPH [309-312]. It has been reported that hK2 can be used to distinguish between organ-confined PCa and extracapsular disease stages in men with total serum PSA values < 10 ng/ml [313]. It has also been proposed that analysing the concentration of hK2 might improve identification of poorly differentiated tumours [314], and help predict unfavourable prognosis in PCa [315].

The Present Studies

Objectives

The overall aim of the research underlying this thesis was to investigate the physiological levels of the fertility-related proteins PSA and hK2 in seminal plasma and in serum in young men without prostate disease.

The specific aims of the present studies were as follows:

- To investigate the physiological relationship between PSA in seminal plasma and blood in young healthy men (Paper I).
- To compare age-related changes in PSA forms in blood from young men (mean age of 18.1 years) and from older men (mean age of 46.5 years) without a diagnosis of PCa during long-term follow-up (Paper I).
- To analyse SNPs at –158 bp and –4643 bp, relative to the transcription start site within the promoter of the PSA gene *(KLK3)* on chromosome 19q13.4, separately or in combination with the AR CAG microsatellite to determine their association to secretion of PSA into seminal plasma and blood in young healthy men (Paper II).
- To elucidate the association between genetic variation (SNPs) in genes in the kallikrein locus on chromosome 19q13.4 and the levels of PSA and hK2 released into ejaculate and blood in young healthy men (Paper IV).
- To determine whether age and PSA in seminal plasma are correlated with levels of hK2 in seminal plasma in men at an age when reproduction commonly occurs (Paper III).
- To evaluate the impact on age, hK2, PSA, zinc and semen volume on sperm motility in men at an age when reproduction commonly occurs (Paper III).

Study populations

The work presented in this thesis was based on samples collected from three cohorts: Swedish male army conscripts, the Malmö Preventive Medicine Project, and the Norwegian Light Project.

Swedish Male Army Conscripts (Papers I, II, IV)

From May to December 2000, 305 men undergoing compulsory medical examination for military service were enrolled in a study of reproductive function in young Swedish males [316]. Approximately 95% of all 18-year-old men in Sweden undergo this health examination, and only those with serious chronic diseases are excluded. The conscripts in the current studies were born in 1979–1982, and had a mean age of 18.1 +/–0.4 years. The rate of participation was 13.5% (305/2,255). Semen samples were obtained by masturbation, and a blood sample was subsequently drawn. The semen was analysed for sperm characteristics, and then both semen and serum samples were frozen at –70ºC pending further analysis. The study was approved by the Ethics Committee of Lund University, Sweden. All of the men who participated signed written informed consent.

The Malmö Preventive Medicine Project (Paper I)

In 1974, the Section of Preventive Medicine, Lund University, was formed at Malmö University Hospital in Malmö, a city in southern Sweden with a population of approximately 250,000. During the period 1974–1986, the section invited all men living in Malmö and born between 1921 and 1944 to undergo baseline venipuncture and examination of anthropometric and lifestyle variables (e.g. body mass index, blood glucose, and insulin) within an undertaking that was called the Malmö Preventive Medicine Project (MPM) [317]. Altogether 22,444 men (74%) accepted the MPM invitation, and their mean age at baseline was 44 years (ranging from 33–61 years). One anti-coagulated EDTA blood sample was collected from each participant, and then centrifuged and stored as plasma at –20^{o} C until analysed.

In the study reported in Paper I, 1,389 men from the MPM who were not diagnosed with PCa according to the Swedish Cancer Registry (updated by 31 December 1999) were used as controls for the Swedish male army conscripts. The number of controls (1,389) corresponded to plasma samples collected from a minimum of three healthy men in the MPM that were selected as controls for 442 men who were diagnosed with PCa and were younger than 53 years at the time of the baseline MPM examination.

The Norwegian Light Project (Paper III)

A total of 207 Norwegian men aged 19–40 years and living in Oslo or Tromsø were recruited through radio and newspaper advertisements to participate in a study of the effect of seasonal changes in length of daylight on human semen

quality in men living south and north of the Arctic Circle [318]. Of the 207 recruited men, 93 living in Tromsø and 114 in Oslo gave an initial semen sample, and a second sample was provided by 92 and 112, respectively. All participants completed a questionnaire concerning reproductive history (e.g. infertility, proven fertility, and genital disorders), smoking habits, and chronic or endocrine diseases that might influence their reproductive function.

Semen sample collection periods were based on the time of sunrise and sunset in the respective area, and thus they were conducted 2–3 months after the first day of the polar night. Blood samples were obtained in the morning, 50–60 days before obtaining the ejaculate. After a delay corresponding to the duration of spermatogenesis and epididymal spermatozoa maturation, the first semen collection period in Tromsø was July 30 to August 10, 2001, and the second was January 21 to February 1, 2002. The corresponding dates in Oslo, were September 3–14, 2001, and March 4–15, 2002. Semen samples were obtained by masturbation and analysed for sperm characteristics. Thereafter, both the blood and semen samples were frozen at –70ºC pending analysis. The subjects were included after having given written informed consent. The study was approved by the Ethics Committee of Lund University, Sweden, and the Regional Committee for Medical Research Ethics of Southern Norway.

Samples collected from July to September 2001 were used in the investigation described in Paper III. Due to lack of seminal plasma, sperm motility and PSA could be assessed only in 205 and 185 of the subjects, respectively. Information about age was provided by 203 subjects, and data on all three of the parameters age, PSA, and sperm motility were accessible for 182. One person was excluded due to high concentration and amount of PSA (9.1 mg/ml and 27 mg, respectively) in seminal plasma. The final study group comprised 181 men with a mean age of 28.3 years (range 19–40 years).

Methods

Biochemical analyses

Time-resolved immunofluorometric assay (TRIFA) was performed to analyse the different forms of hK2 and PSA. Seminal plasma samples were analysed for hK2 by use of an improved in-house immunofluorometric research assay [182] in which both the sample volume and labelling degree of the tracer antibody had been increased. Capture of hK2 was achieved with the biotinylated monoclonal antibody (Mab) 6H10, and blocking of tPSA was enhanced with the three PSA-specific anti-PSA Mabs 2E9, 5F7, and 5H6, which do not cross-react with hK2. Finally, the antibodies 5H6 and 7G1-Eu hK2 were used to detect hK2. The coefficient of variation (CV) for measurement of hK2 in semen was 12% at a mean concentration of 8.5 microg/ml. The detection limit for hK2 in serum was 0.004 ng/ml. CV values for high (mean conc. 0.54 ng/ml) and low (mean conc. 0.02 ng/ml) hK2 controls were 10.3% and 6.4%, respectively.

Measurements of f- and tPSA in serum and seminal plasma were performed using a commercially available dual-label immunofluorometric assay Prostatus™ PSA Free/Total kit (Delfia® Reagents, Wallac Oy, Turku, Finland). The combination of Mab H117 and H50 provides equimolar detection of fPSA, and cPSA, but it also results in cross-reaction with hK2. Measurement of fPSA can be achieved by using a combination of Mab H117 and 5A10, and those antibodies do not show any significant cross-reactivity with cPSA or hK2. The analysis of tPSA in seminal plasma measures the sum of fPSA (> 95%), PSA in complex with PCI (1–3%), and hK2 (< 1%). The assay for fPSA in seminal plasma determines the sum of active single-chain fPSA and inactive internally cleaved two-chain fPSA. The CV for PSA measurements in semen was 12% at a mean concentration of 0.66 mg/ml. The detection limit was 0.05 ng/ml for tPSA in serum (CV 5% at a mean conc. 2.3 ng/ml), and 0.04 ng/ml for fPSA (CV 5.9% at mean conc. 0.25 ng/ml).

Genetic analyses

In the analyses described in Papers II and IV, genomic DNA was prepared from peripheral leukocytes using a QIAamp DNA Maxi Kit (Qiagen Gmbh, Hilden, Germany). The DNA concentrations were determined by Pico Green™ DNA assay (Cambio Ltd., Cambridge, UK), and all the samples were normalized to the same DNA concentration. The genotypes were determined by the Sequenom MassARRAY MALDI-TOF and the assay design was made using MassARRAY Assay Design 2.0 software (Sequenom Inc., San Diego, CA, USA). Primers were obtained from Metabion GmbH (Planegg-Martinsried, Germany), and all reactions were run under the same conditions, with the exception of the primer annealing temperature of the primary PCR. PCR reactions were performed in a total volume of 6 µl containing 2.5 ng of template DNA, 1.25X Taq PCR buffer (HotStar, Qiagen Gmbh, Hilden, Germany), 0.15 units of Taq polymerase (HotStar, Qiagen Gmbh, Hilden, Germany), 3.5 mM $MgCl_2$, 0.5 mM dNTPs, and each primer at a concentration of 100 nM. Amplifications were performed using an Applied Biosystems GeneAmp® PCR System 9700 (Foster City, CA, USA) with dual 384 heads as follows: 95 °C for 15 min: 45 cycles at 95 °C for 20 s; 64 °C for 30 s; 72 °C for 60 s; and finally 72 °C for 3 min. Dephosphorylation of unincorporated dNTPs was achieved using shrimp alkaline phosphatase. Concentrations of individual hME primer pairs were adjusted to even out peak heights in the mass spectrum. The extension reactions were then performed by mixing the adjusted MassEXTEND primer mix (each primer at a conc. of approximately 1 uM) with hME EXTEND mix containing buffer, d/ddNTP mix with 50 µM of each nucleotide and 1.25 units of Thermo Sequenase. PCR amplification of hME reactions was done as follows: 94 °C for 2 min and 99 cycles at 94 °C for 5 s, 52 °C for 5 s and 72 °C for 5 s. The samples were then manually desalted with 6 mg of Clean Resin on a dimple plate and were subsequently transferred to a 384-well SpectroCHIP by use of a nanodispenser. In the study reported in Paper II, the AR gene CAG repeats were analysed as described by Lundin and co-authors [235].

Semen samples

All of the participating Swedish male army conscripts and the men in the Norwegian Light Project were asked to abstain from sexual activity for at least 48 hours and to note the actual time of abstinence. Semen volume was measured by weighing, assuming the density of semen to be 1 g/ml. After collection, a 450-µl aliquot of each semen sample was mixed with 50 µl of benzamidine (0.1 M) to inhibit liquefaction. Seminal plasma was obtained by centrifuging the benzamidine-treated sample at 10,000 x g for 10 min.

Sperm motility analysis

In the study presented in Paper III, the semen samples were obtained in the laboratory and analysed according to WHO recommendations [4]. Using a positive displacement pipette, 10 µl of well-mixed semen fluid was placed on a clean microscope slide and then covered with a 22 x 22 mm coverslip. At least 200 spermatozoa were classified using a phase contrast microscope at a magnification of 400x and a temperature of 37 °C. Each spermatozoon was graded according to the character of its motility: (A) rapid progressive (≥ 25 µm/s), (B) slow progressive, (C) nonprogressive (< 5 µm/s), or (D) immotile. The assessment of 200 spermatozoa was repeated on a separate 10-µl aliquot of the same semen sample, and the average percentage and the difference between percentages in the most prevalent category in the independent counts were calculated. The motility analysis was repeated if this difference exceeded 10% of the mean value. Two laboratory technicians analysed the ejaculates in both Tromsø and Oslo. The inter-observer CV was 5% for the motility assessment.

Statistical analyses

Paper I

Statistical analysis was performed using Statview® 5.0.1 (SAS Institute, Inc. NC, USA). Logarithmic transformation was done to obtain a normal distribution of the residuals, and the influence on PSA levels in serum and in seminal plasma was evaluated by simple regression analysis. Student's t-test was used to compare the MPM-derived control group and the conscript cohort with regard to PSA levels.

Papers II–IV

For the SNPs in *KLK2-KLK3*, the observed genotype distribution was tested for consistency with Hardy-Weinberg equilibrium (HWE) expectations by applying Pearson's chi-square test (Paper II) or Fischer's exact test using Haploview 4.0 (Paper III). Lewontin's D' statistics were calculated to estimate the strength of LD between all possible pairwise combinations of SNPs by use of Haploview 4.0.

The SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA) was used to construct the linear regression models described in Paper II. Logarithmic transformation was done to obtain normal distribution of the residuals. The potential confounders were included in the final models if they changed the effect estimates by ≥ 15% between the PSA genotypes and the endpoints. To test for a possible impact of AR polymorphism on PSA levels, the interaction terms (PSA

promoter genotype * AR genotype) were introduced into the regression model. $P < 0.05$ was considered statistically significant. To check the robustness of the results, a Pascal program was constructed to conduct a permutation test.

The group characteristics reported in Paper IV was expressed using SPSS 13.0 software package. In all, the associations between nine SNP genotypes and serum and semen levels of PSA and hK2 were analysed. All p values were obtained using the Kruskal-Wallis test and Stata 9.0 (Stata Corp., College Station, TX).

Paper III

The SPSS 13.0 software package was used for statistical analysis. Linear regression models were applied to evaluate the hypothesized associations between sperm motility and the potential determinants age, semen volume, zinc concentration, and amounts and concentrations of hK2 and PSA. The potential determinants were analysed as continuous variables, and semen volume, hK2, PSA, and zinc were further categorized into quartiles according to increasing amounts and concentrations.

It was assumed that hK2 and zinc, respectively activate and inhibit PSA, and hence they were not included in the models with PSA. Age was dichotomized (< 30 and ≥ 30 years). The determinant showing the strongest association with sperm motility was kept in the model, and the other determinants were included one by one. The procedure was continued until all additional determinants showed no associations (determined as $p > 0.05$). An interaction term was included in the model to ascertain whether the determinant age modified the effect of PSA or hK2. Potential confounders (time of abstinence, free testosterone, fructose, and testosterone) were included as continuous variables, and a potential confounder was kept in the model if it changed the effect estimates by $\geq 15\%$. Model assumptions were evaluated by means of residuals analyses. The explained variance was presented, and Pearson's and Spearman's correlation coefficients were used to investigate whether age and PSA were correlated with hK2. To ensure the reasonableness of linear assumptions (and thus also of the use of Pearson's r), scatter plots of all the bivariate comparisons were assessed.

Summary of the studies

Paper I

The objective of this study was to investigate the physiological relationship between PSA in seminal plasma and blood in young healthy men and to compare age-related changes in different forms of PSA in blood. To achieve this goal, tPSA, fPSA, and cPSA in blood and tPSA in semen were measured in samples obtained from 289 young male army conscripts (mean age of 18.1 years). The indicated analyses in blood were also done on a population-based study cohort comprising 1,389 men from the MPM who had a mean age of 46.5 years and no PCa diagnosis during long-term follow up.

Simple regression analysis showed that levels of tPSA and fPSA in serum were significantly correlated with the concentration of tPSA in seminal plasma (respectively: $r = 0.22$, $p = 0.0001$: $r = 0.41$, $p < 0.0001$). Levels of tPSA and fPSA in serum were also correlated with the amount of tPSA in seminal plasma. However, no significant correlation was found between concentration of cPSA in serum and concentration or amount of tPSA in seminal plasma.

Considering age-dependent changes in blood PSA, the geometric mean concentration of tPSA was significantly lower in conscripts than in the control group of middle-aged men ($p = 0.0008$), and the same was true for the geometric mean concentration of cPSA ($p < 0.0001$). There was no significiant difference between the two groups with regard to age-dependent change in the geometric mean concentration of fPSA in serum.

The conclusions drawn from this study were that levels of fPSA in blood may reflect the normal physiological function of the prostate gland, and it might be possible to analyse cPSA in blood as a marker of prostate disease. The rise in blood of cPSA with age might reflect an increasing incidence of prostate disease and also be due to a prolonged elimination rate, since the size of cPSA precludes its elimination by glomerular filtration.

Paper II

The aim of the second study was to analyse two SNPs located at –158 bp (rs266882) and –4643 bp (rs925013), relative to the transcription start site within the promoter of the PSA gene *(KLK3)*, separately or in combination with an AR CAG microsatellite to determine their association with PSA secretion into seminal plasma and blood in young healthy men. The subjects consisted of the cohort from the Swedish male army conscripts.

Genotyping included 291 conscripts, since earlier studies had reported higher promoter activity in homozygotes with the A variant than in subjects with G at position –158, A/G and G/G genotypes were combined into one group, which was compared with the A/A genotype. Based on the median value of 22 for CAG repeat length in the population under consideration, the subjects were dichotomized into ≤ 22 or > 22. In case a significant interaction was found, the differences in the level of outcome variable between the A/A and A/G +G/G promoter groups were tested for each CAG category.

Linear regression analysis did not reveal any significant association between the two SNPs and levels of tPSA in seminal plasma or blood. However, homozygotes for the A allele in rs266882 in combination with a CAG > 22 were found to have significantly higher serum levels of tPSA compared to subjects carrying the G allele ($p = 0.01$). This result was further verified by a permutation test that gave an empirical p value of 0.0083.

This study indicated that SNPs at position –158 bp and –4643 bp, relative to the transcription start site within the promoter of *KLK3,* did not have any substantial influence on levels of tPSA in seminal plasma or blood. However, the levels of

tPSA in serum were influenced by the interaction between PSA promoter variants and AR CAG polymorphisms, i.e. higher tPSA was seen with AA genotype in rs266882 and CAG > 22.

Paper III

The objective of the third study was to ascertain whether age and seminal PSA are correlated with hK2 in seminal plasma, and to evaluate the impact of age, hK2, PSA, zinc, and semen volume on sperm motility in men 19–40 years old, the age when reproduction commonly occurs. The Norwegian Light Project cohort served as subjects in this study.

One hundred eighty-one men were categorized according to age (< 30 and ≥ 30 years), semen volume, and levels of hK2, PSA, and zinc in seminal plasma. Sperm motility was assessed according to WHO recommendations. The impact of the determinants on sperm motility was evaluated by linear regression analysis, and correlations were found between hK2 and PSA in seminal plasma ($p < 0.001$, $r = 0.47$) and between hK2 and age ($p = 0.01$, $r = –0.20$). Compared to other subjects, those in the lowest quartile with respect to amount of PSA, semen volume, and zinc level had, respectively, 5.8%, 4.1%, and 3.9% fewer progressively motile sperm. Age and hK2 were not associated with sperm motility.

The results of this study led to the conclusion that there was a significant association between PSA and hK2 in seminal plasma in the men that were investigated. Furthermore, a low amount of PSA in seminal plasma and reduced semen volume, and concentration of zinc had an effect on sperm motility. These findings suggest that a decrease in the secretory function of the prostate can impair sperm motility.

Paper IV

The forth study was a continuation of the investigation started in Paper II, and the objective was to determine whether the newly identified SNPs in *KLK2* and *KLK3* were associated with the physiological prostatic secretion of hK2 and PSA. Accordingly, the Swedish male army conscripts were once again used as subjects. Genotyping was done for 303 of those young men, and the Kruskal-Wallis Test was used to assess associations between allelic frequencies for SNPs and levels of hK2 and PSA in seminal plasma and serum. Apart from one haplotype block from rs198977 to the previously undescribed “SNP2” (1541int), there was moderate LD in the region.

The results for *KLK2* showed that four SNPs in LD (rs198972, rs198977, rs198978, and SNP2, were associated with significantly lower levels of hK2 in seminal plasma and serum in carriers of the TT genotype (all p values ≤ 0.0007). By comparison, subjects carrying the CC genotype in rs3760728 had a lower concentration of tPSA in seminal plasma ($p = 0.01$). The analysis of *KLK3,* demonstrated that rs2271094 was associated with lower tPSA in seminal plasma in subjects with the AA genotype ($p = 0.01$), and rs1058205 was associated with higher tPSA amount in seminal plasma ($p = 0.02$) and in serum ($p = 0.001$), and a

lower %fPSA ($p \leq 0.02$) in carriers of the TT genotype. Furthermore, a newly identified SNP in *KLK3,* designated "SNP1" showed a significant association with lower amount and concentration of hK2 in seminal plasma in subjects with the GG genotype.

These results indicate that genetic variants in *KLK2* and *KLK3* are indeed associated with the physiological secretion of hK2 and PSA by the prostate. This is of interest, since it has been shown that low levels of PSA are correlated with lower percentage of motile sperm in both fertile and infertile men. The findings might also be useful for refining models of PSA cut-off values for PCa testing.

Discussion

The present studies generated new knowledge on factors that affect the physiological release of the fertility-related proteins PSA and hK2 in seminal plasma and the levels of those proteins in serum of young men without prostate disease. The results are the first to show that SNPs in *KLK3* and *KLK2* are associated with seminal plasma levels of PSA and hK2, respectively, in such male subjects. The current research also provided novel information about how different molecular forms of PSA in blood reflect the physiological release of PSA into seminal plasma in healthy young men. A correlation was noted between serum fPSA and levels of tPSA in seminal plasma, whereas blood levels of cPSA, but not fPSA, were found to increase with age. The present studies also showed that men with low amounts of PSA in seminal plasma had fewer progressively motile sperm compared to those with higher amounts of PSA. Similar observations were made regarding ejaculate volume and concentration of seminal zinc.

Considering allelic variation, a total of six SNPs in *KLK2* (rs3760728, rs11670728, rs198972, rs198977, rs198978, and SNP2) and five SNPs in *KLK3* (rs925013, rs266882, rs2271094, SNP1, and rs1058205), were evaluated with regard to possible associations with levels of hK2 and PSA. Two SNPs, one in *KLK3* (SNP1) and one in *KLK2* (SNP2), had not previously been genotyped. Two SNPs in *KLK3* (rs925013 and rs266882) were also assessed concerning association with serum and seminal plasma levels of PSA in combination with the AR CAG polymorphism.

The findings for *KLK3* showed that two SNPs rs2271094 and SNP1 were correlated with significantly lower concentrations of PSA in seminal plasma in subjects homozygous for the common allele (AA and GG genotype, respectively). For rs2271094 this was also valid regarding the amount of PSA. Considering, rs1058205, there was a trend towards a higher concentration of tPSA in seminal plasma in subjects homozygous for the common T allele. In addition, the same genotype was associated with significantly higher amounts of tPSA in both seminal plasma and serum and with lower %fPSA. The decrease in %fPSA is interpreted as being a consequence of the increase in serum levels of tPSA due to the differences in elimination of fPSA and cPSA.

Two of the SNPs first described in *KLK3* were an A/G polymorphism (rs266882) located at position –158 bp and a G/A polymorphism (rs925013) at position –4643 bp relative to the transcription start site within the promoter. Some earlier studies had reported that the A allele in rs266882 was associated with increased serum PSA and with risk of PCa [239, 319], although there were also reports that contradicted those results with regard to both PSA [253, 258] and the risk of PCa [320]. In the present investigations, no connection was found between this SNP and seminal plasma or serum levels of PSA. However, rs266882 AA genotype in combination with CAG > 22 showed significantly higher serum levels of tPSA than seen in subjects carrying the G allele. Regarding rs925013, it has been reported that the G allele is associated with higher serum levels of PSA [254]. However, the current data confirmed the results of a study that found no connection between that SNP and serum levels of PSA [259], and they also revealed no association between seminal plasma levels of tPSA and rs925013 or the combination with AR CAG polymorphism.

In *KLK2,* the SNPs rs198972, rs198977, rs198978 and the newly genotyped SNP2 were correlated with lower hK2 levels in both seminal plasma and serum in subjects homozygous for the mutant T allele. A haplotype block was identified between rs198977 and SNP2, and the results for rs198972, rs198978, and SNP2 can most likely be explained by LD with rs198977. Also, the recently identified SNP1 in *KLK3* showed a significant association with lower amount and concentration of hK2 in seminal plasma in subjects with the GG genotype. SNP1 was in moderate LD with the SNPs in the haploblock as well as with rs198972.

Regarding rs198977, the mutant T for wild-type C allele substitution results in a nucleotide change from cytosine to thymine on exon 5 and is known to be a *non-synonymous* SNP. As previously mentioned, the common allele codes for Arg^{226}-hK2, which has trypsin-like activity, whereas the variant allele codes for Trp^{226}-hK2, which has no detectable enzymatic activity *in vitro* [260]. In the present subjects, the TT genotype was associated with lower hK2 levels. That finding agrees with results reported by Nam et al. [255, 256], which also demonstrated that the T allele was related to a higher risk of PCa in a population composed mostly of Caucasian men. In contrast, Chiang et al. [321], found that a population of carriers of the C allele were at significantly higher risk of PCa. The observation that the inactive T allele in *KLK2* is associated with lower levels of hK2 but a higher risk of PCa might be explained by the fact that men with the T allele inherit a predisposition to produce less hK2 in normal and malignant cells, and hence they have a low baseline level of hK2 in serum. Another possibility is that the mutant form of hK2 is not detected by the Mab used to capture hK2 in the immunofluorometric assays that are currently available. In the *KLK2* gene, four SNPs (rs3760728, rs198972, rs198977, and rs198978) were also found to be correlated with PSA levels, although further studies are needed to correctly interpret and substantiate or dismiss those results.

The present studies also gave new information on how different molecular forms of PSA in blood reflect the physiological release of PSA into seminal plasma. Serum

fPSA was found to be correlated with levels of tPSA in seminal plasma in healthy young men. The major portion of fPSA in seminal plasma is in a free form of which 30–40% is estimated to be inactive [107]. In blood, approximately 5–35% of PSA is in free form. Free inactive PSA in prostatic secretions does not form complexes and remains as fPSA after leaking into the blood circulation. In young healthy men, the frequency of this retrograde release into the bloodstream is less than one PSA molecule per million secreted PSA molecules. Furthermore, no increase in fPSA in blood was detected when comparing healthy adolescent men with healthy middle-aged men. The present results corroborate earlier reports indicating an overall increase in median serum tPSA values with age [261, 262, 322, 323]. A rise in tPSA levels in blood with age will lead to increased levels of fPSA, although that effect might be counteracted by the fact that fPSA is rapidly eliminated from the circulation.

The concentration of cPSA in serum was not correlated with levels of tPSA in seminal fluid in healthy young men. This was probably due to a prolonged elimination time for cPSA, which might also explain the observed increases in levels of cPSA in blood from healthy middle-aged men as compared to healthy adolescent males. Elevated cPSA in serum may also be the result of BPH or an early sign of PCa. Most of the PSA in serum is normally in complexed form, primarily with ACT. The site of PSA-ACT complexation is not fully known, but a study has localized ACT in the prostatic epithelium [128], which suggests that the PSA-ACT complexation occurs in extracellular compartments juxtaposed to the glandular epithelium. It is also possible that this complex is formed in the perivascular compartment, although no experimental data published thus far have provided any detailed information on this matter. However, high serum levels of cPSA as a consequence of prostatic disease may result in delayed elimination, which would subsequently further increase the serum levels of the protein. This agrees well with observations of PSA clearance from the circulation showing that the size of the PSA-ACT complex precludes elimination by glomerular filtration [136].

The present studies showed that men with a low amount of PSA in seminal plasma (< 2.71 mg), corresponding to the lowest quartile (quartile 1) of PSA amount, had approximately 6% fewer progressively motile sperm compared to men with a higher amount of PSA. Analogously, men in the lowest quartile for ejaculate volume and concentration of seminal zinc, had approximately 4% fewer progressively motile sperm compared to those with higher values for those two parameters. Inasmuch as PSA and zinc in seminal plasma are markers of the secretory capacity of the prostate, the current results support the importance of prostate secretions for fertility.

Notably, the present research did not demonstrate any association between concentration of PSA in seminal plasma and sperm motility. By comparison Elzanaty et al. [198] found a 2.4% covariance between the concentration of PSA in seminal plasma and the percentage of motile sperm determined by CASA. This discrepancy might be explained by the fact that in the current studies the

methodology involved in manual assessment of sperm motility, which might entail more pronounced inter-observer variation than CASA does, and the men that were investigated were on average ten years older [324, 325].

In the present study, seventeen men had seminal plasma PSA below reported physiological levels (i.e. < 0.5 mg/ml) [207] and fifteen of those subjects were in quartile 1. Further analysis showed no difference in sperm motility between those with < 0.5 mg/ml PSA and the other men in quartile 1. An additional evaluation was subsequently performed on a subgroup of the participants with an abstinence time according to WHO recommendations (i.e., ≥ 48 to < 169 hours) [4], and with a semen volume corresponding to the mean +/– SD for the group studied, but that did not reveal any association between PSA concentration and sperm motility. This finding implies that the impact of the prostate on sperm motility is mediated not only through the action of PSA, but also via a complex interaction between various seminal factors.

There is some proof that older age is accompanied by lower sperm motility [326, 327], and an age-dependent decrease in PSA levels has been observed in seminal plasma [263]. However, the present research did not detect any age-associated decline in PSA levels or any significant difference in sperm motility between men < 30 and ≥ 30 years of age. This lack of a more pronounced connection between age and prostate function was probably due to the limited age span of 19–40 years in the subjects. Nevertheless, a significant negative correlation was found between age and levels of hK2 in seminal plasma, although no relationship was noted between levels of hK2 in seminal plasma and sperm motility.

The men in the quartile with the lowest concentration of zinc in seminal plasma had about 4% fewer motile sperm. This observation does not confirm earlier results showing a negative correlation between zinc levels in seminal plasma and percentage immotile sperm determined by CASA [198].

Naturally, there are factors that require special attention when interpreting the results of the current research. Regarding the study populations, one weakness concerning the cohort of Swedish male army conscripts is selection bias, since only 13.5% of the conscripts agreed to participate. However, the possibility of such bias was negligible, because the conscripts that did accept participation were healthy and, due to their young age, had limited knowledge of their fertility status. Unfortunately, during sample handling, 84 consecutive seminal plasma specimens (sample nos. 42–125) were lost, which made it impossible to evaluate hK2 and fPSA levels for the entire group.

In the cohort derived from the Norwegian Light Project, bias of infertile men volunteering to participate might have been higher, because at the time of recruitment those subjects were at an age when reproduction most commonly takes place (i.e. 19–40 years old). Unfortunately, 10% of the participants could not be analysed for hK2 due to insufficient volumes of seminal plasma, and this subcohort was also characterized by lower amounts of PSA and lower sperm motility. Therefore, the results concerning an age-related decrease in seminal plasma hK2,

and the lack of an association between sperm motility and hK2 levels need to be confirmed.

Another plausible objection to the study of the age-dependent changes in molecular forms of PSA in blood is that the levels of PSA in serum from young men were compared with PSA in plasma samples that had been collected from middle-aged men and stored for a minimum of 15 years, and thus the PSA in those specimens might have undergone degradation. However, it has been shown that plasma-EDTA samples are less sensitive to storage at –20ºC than serum samples are [328, 329], and in a study including the same MPM-derived cohort as used in the present research, Ulmert et al. [330] found that the results of analyses of fPSA and tPSA in samples stored for up to 25 years were not significantly different from measurements performed on age-matched freshly collected control samples.

Finally, considering the current investigation of associations between allelic variation in *KLK3* and *KLK2* and levels of PSA and hK2, it should be said that all such studies have limitations related to the fact that correlations of that nature cannot claim to be the direct cause of the effects that are observed. The weak co-variance that was found between tPSA in seminal fluid and fPSA in serum also illustrates that factors other than rates of transcription, translation, and secretion of PSA by the prostate epithelium have a substantial impact on the levels of that protein in serum.

Conclusions

Several conclusions can be drawn from the present findings:

- The physiological secretion of hK2 and PSA from the prostate in young men is associated with genetic variation in *KLK2* and *KLK3*. The results of the analyses of the allelic variants might be used to refine models of PSA cut-offs values for PCa testing as indicated by the following observations:
    - It was found that rs198972, rs198977, rs198978 and SNP2 in *KLK2* were associated with lower hK2 levels in both seminal plasma and serum of carriers of the TT genotype.
    - Homozygotes for the A allele in rs266882 in *KLK3* in combination with CAG > 22 had significantly higher serum levels of tPSA than did subjects carrying the G allele.
    - It was observed that rs2271094 and the newly genotyped SNP1 in *KLK3* were associated with significantly lower concentrations of tPSA in seminal plasma in subjects homozygous for the common allele (AA and GG genotype, respectively).
    - The SNP rs1058205 in *KLK3* was associated with a significantly higher amount of tPSA in both seminal plasma and serum, and with lower %fPSA in homozygotes for the common T allele, as well as a trend towards a higher concentration of tPSA in seminal plasma.
- In healthy young men, there was a 17% co-variation between fPSA in blood and PSA in semen, whereas no association was found between levels of cPSA in blood and PSA in semen.
- Levels of cPSA, but not fPSA, in blood increased with age, which may reflect an increasing incidence of prostate disease.
- Correlations were found between hK2 and PSA in seminal plasma ($p < 0.001$, $r = 0.47$) and between hK2 and age ($p = 0.01$, $r = –0.20$).
- Considering male subjects of an age when reproduction most commonly takes place, those who were categorized in the lowest quartile with respect to amount of PSA, semen volume, and zinc concentration in seminal plasma had, respectively, 5.8%, 4.1%, and 3.9% fewer progressively motile sperm. These findings suggest that a decrease in secretory function of the prostate can impair sperm motility.

Populärvetenskaplig sammanfattning på svenska

Syftet med denna avhandling har varit att få ökad kunskap om de fertilitetsrelaterade proteinerna (äggviteämnena) prostata specifikt antigen (PSA) och humant glandulärt kallikrein 2 (hK2) i sädesvätska och i blod hos unga män utan prostatasjukdom. Detta mot bakgrund av att genetiska och biokemiska orsaker anses föreligga hos 30 % av de män som idag genomgår infertilitetsutredning och där ingen orsak kan säkerställas. Emellertid har låga nivåer av PSA i sädesvätskan korrelerats till en lägre procentandel rörliga spermier hos både fertila (fruktsamma) och infertila (icke fruktsamma) män. Genom ökad kunskap om genetiska och biokemiska processer i den normala fortplantningen är förhoppningen förstås att diagnostik och behandling av manlig infertilitet skall kunna förbättras.

PSA och hK2 bildas huvudsakligen i prostata (blåshalskörteln) som är en av de accessoriska könskörtlarna (Figur 2, sidan 13). PSA och hK2 utsöndras i prostatasekretetet som utgör ca 30 % av mängden i mannens sädesvätska. Proteinerna läcker även över till blodcirkulationen. I blodet cirkulerar PSA bundet till hämmarproteiner och endast en mindre del förekommer i fri form, i sädesvätskan är förhållandet det omvända. För hK2 är majoriteten bundet till hämmarproteiner i sädesvätskan och i blodet är hK2 i fri form. Den fria formen av PSA och hK2 i blod möjliggör snabb utsöndring via njurarna.

Läckaget av PSA och hK2 till blodet ökar snabbt då sjukdom drabbar prostatakörtlen och PSA, men även hK2, används därför som markörer för prostatasjukdom. Därmed är kunskap om dessa proteiner och deras normala (fysiologiska) sekretion och nivåer i sädesvätska och i blod hos friska unga män även av stor vikt för att förstå de förändringar som sker i samband med sjukdomsutveckling.

Trots att PSA:s fysiologiska funktion ytterligare kan klargöras så anses proteinets huvudsakliga funktion vara att klyva de gelbildande proteinerna i sädesvätskan. Vid sädesuttömningen (ejakulationen) blandas spermierna från bitestiklarna med sekret från prostata och sädesblåsorna vilket bildar en gel. Gelen består av de gelbildande proteinerna semenogelin I, semenogelin II och fibronektin från sädesblåsorna. De molekylära mekanismerna bakom gelbildandet är idag okända. Processen sker på några sekunder och både kovalenta och icke-kovalenta bindningar är viktiga.

Samtidigt som gelen bildas startar processen med sönderflytning, s.k. likvefiering, av sädesvätskan. Likvefieringen sker då PSA klyver de gelbildande proteinerna till ett flertal lösliga fragment. PSA bildas som en inaktiv föregångare (proPSA) och aktiveras till aktivt PSA, genom klyvning och frisättning av en kort aktiveringspeptid. PSA aktiveras troligen av hK2. PSA:s spjälkning av gelproteinerna leder till att gelstrukturen löses upp. Spermiernas rörelsemönster förändras vid denna likvefieringsprocess och progressivt rörliga spermier frisläpps.

HK2 kan liksom PSA spjälka både semenogeliner och fibronektin men mönstret för hK2:s spjälkning är distinkt skilt från PSA:s klyvning. Den fysiologiska betydelsen av hK2 medierad klyvning av gelproteinerna är fortfarande okänd.

Således kan faktorer som påverkar sekretionen av PSA och hK2 eller deras aktivitet indirekt påverka spermarörligheten. Zink som också bildas i prostata hämmar både PSA och hK2. Blodnivåerna av PSA och hK2 stiger som tidigare nämnts hastigt vid prostatasjukdom, men även vid stigande ålder. Etnisk bakgrund har visat sig samband med blodnivåer av PSA, och det har även rapporterats att PSA i blodet påverkas av sädesuttömning. I sädesvätskan sjunker mängden PSA med stigande ålder. I tillägg har samband rapporterats mellan nivåer av PSA och hK2 i blodet och s.k. enbaspolymorfi (eng. single nucleotide polymorphism (SNP) uttalas "snipp") i generna som styr produktionen för respektive protein (en SNP är en positionsbestämd variation i arvsmassan som berör en enda nukleotid och variationen skall finnas i > 1 % av populationen). Vedertaget är dock att nivåerna av PSA och hK2 i sädesvätska och i blod är resultatet av de manliga könshormonernas – androgenernas – (d v s testosteron och dihydrotestosterons) aktivering av androgen receptorn (AR).

AR är i sig ett protein som finns i nästan alla kroppens celler och som verkar genom att slå av eller på funktionen av vissa gener vilket innebär att produktionen av proteiner avbryts eller påbörjas. Då AR genen är lokaliserad på X-chromosomen har män bara en kopia och kan därmed drabbas om genen är defekt. I genen till AR finns också två variabla områden där samma bastripletter upprepas om och om igen, s.k. ”repeat”. Antalet av dessa ”repeat” påverkar receptorns funktion och varierar även mellan olika individer och populationer. Det första ”repeatet” kallas CAG och kodar för aminosyran glutamin. I en kaukasisk befolkning har männen ca 10–30 CAG och ett förlängt CAG-repeat (> 40) är associerat med en neuromuskulär sjukdom kallad Kennedys sjukdom. Män med Kennedys sjukdom har även försämrad spermieproduktion vilket indikerar att CAG-repeatet är involverat i reproduktionsfunktionen. Negativa samband har även rapporterats mellan nivåer av PSA i blod och sädesvätska och ”repeat-längd” av CAG.

I detta arbete kunde konstateras att flera SNP:ar i generna för PSA och hK2 hade samband med nivåer av respektive protein i både sädesvätska och i blod hos unga män utan prostata sjukdom (medelålder 18 år). Två SNP:ar undersöktes även för möjligheten av en kombinerad betydelse av CAG repeat-längd i AR och PSA genen. En SNP (rs266882) i kombination av AR polymorphism CAG repeat-längd > 22 hade samband med högre blodnivåer av PSA.

Det kunde även klargöras att nivåer av fritt PSA i blodet hade 17 % samvariation med nivåer av PSA i sädesvätskan hos unga män medan nivåerna av PSA bundet till hämmarproteiner i blodet inte hade något samband med nivåerna i sädesvätskan. Detta beror säkerligen på skillnader i bortförskaffandet av PSA från blodcirkulationen där PSA bundet till hämmarproteiner inte kan utsöndras via njurarna. Vidare kunde klargöras att koncentrationen av PSA bundet till hämmarproteiner ökar i blodet redan hos medelålders män (som ej diagnosticerats med prostata cancer) vilket troligen är uttryck för underliggande prostatasjukdom.

Slutligen kunde noteras att män 19–40 år gamla (således i en ålder då familjebildning ofta sker), och kategoriserade i den lägsta kvartilen avseende PSA mängd i sädesvätska, ejakulat volym och zink koncentration hade 5.8, 4.1, och 3.9 % färre progressivt rörliga spermier i jämförelse med övriga män. Nivåer av PSA och hK2 i sädesvätskan visade en 22 % samvariation och hK2 nivåerna var negativt korrelerade till ålder. Inget samband kunde dock ses mellan koncentration av PSA eller hK2 i sädesvätska och spermarörlighet hos män. Detta tyder på PSA ej ensamt har en avgörande betydelse för spermarörligheten utan att detta är ett resultat av en komplex interaktion mellan olika faktorer i sädesvätskan. Dock kan en nedsatt sekretorisk funktion i prostata försämra rörligheten hos spermierna.

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