UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

Cancer Sciences Research Division

Prostasomes: A Role in Prostatic Disease?

by

Alistair Bryce Stewart BM MRCS

Thesis for the degree of Doctor of Medicine

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ABSTRACT

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Prostasomes are vesicles secreted by the prostate into the prostatic ductal system where they form part of the ejaculate. Prostasomes contain numerous proteins, the functions of which are diverse and allow us to speculate on the biological effects of the prostasome.

Although known to possess specific biological properties, much of their physiological role and overall significance remains debatable. More evidence exists for their involvement in reproductive physiology than prostate disease.

This thesis reports the start of work to identify whether or not prostasomes have a role in the aetiology or progression of benign and malignant prostate disease. The initiation of reliable isolation of prostasomes for use in future prostasome related research within the department is reported. Part of this process involved a review various biochemical and morphological properties.

Anti-prostasome antibodies (APA) have been identified in serum of patients with prostate cancer and have been proposed as a new marker for metastatic disease (Nilsson BO 2001, Larsson A 2006). Due to the impact of this possibility a pilot study for APA in prostate cancer using an enzyme linked immunosorbent assay (ELISA) was performed.

Anti-prostasome antibodies in varying amounts were present in healthy individuals and in men with prostate cancer. Higher levels were inversely and significantly associated with prostate specific antigen (PSA). Trends, but no significant relationship between APA levels and other parameters such as age, time since diagnosis, metastatic status, Gleason histological score and hormonal treatment were noted.

The presence of APA does not appear, from this small series, to be a strong prognostic indictor for prostate cancer. However, the immune reactions to prostasomes are in any case of considerable interest both in the biology of prostate cancer and in the fertility context. Work initiated from this thesis continues in the department.

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List of Abbreviations

ACT	Alpha-1-antichymotrypsin
APA	Anti-prostasome antibodies
APTT	Activated partial thromboplastin time
ASA	Antisperm antibodies
ATP	Adenosine 5- triphosphate
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
BT	Brachytherapy
CT	Computerised tomography
CZ	Central zone (prostate)
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EBRT	External beam radiotherapy
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
HMW-SVP	High molecular weight seminal vesical proteins
HPLC	High-pressure liquid chromatography
HRPC	Hormone refractory prostate cancer
HUVEC	Human umbilical vein endothelial cells
Ig A	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGF	Insulin-like growth factor

IQR	Inter quartile range
LH	Luteinising hormone
LHRH	Luteinising hormone releasing hormone
LOH	Loss of heterozygosity
LSGS	Low serum growth supplement
M	Molar
MRI	Magnetic resonance imaging
NaOH	Sodium Hydroxide
NE	Neuroendocrine
NED	Neuroendocrine differentiation
NHP	Normal human plasma
OPD	Orthophenylene diamine dihydrochloride
PBS	Phosphate buffered saline
PIN	Prostatic intraepithelial neoplasia
PIPES	Piperazine-NN-bis-2 ethanesulphonic acid
PMN	Polymorphonuclear leucocyte
PSA	Prostate specific antigen
PZ	Peripheral zone (prostate)
RP	Radical prostatectomy
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TF	Tissue factor
TMED	N, N, N', N'-tetramethylethylenediamine
TNM	Tumour/Nodes/Metastasis *(Staging system)*
Tris	Tris(hydroxymethy)aminomethane (buffer)
TRUSS	Trans-rectal ultrasound
TSG	Tumour suppressor gene
TUIP	Transurethral incision of the prostate

TURP	Transurethral resection of the prostate.
TZ	Transition zone (prostate)
USS	Ultrasound
UV	Ultra violet
VIP	Vasoactive intestinal peptide

Chapter 1

Introduction

1.1 Embryology of the prostate

In the embryo, the Wolffian ducts, under the influence of testosterone, develop into the accessory male sex organs, namely the seminal vesicles, ejaculatory ducts and the bulbourethral glands. The prostate gland develops from the urogenital sinus in the 3rd foetal month. Its growth is controlled by dihydrotestosterone (DHT) derived from foetal testosterone under the action of 5α-reductase. Five paired buds arise from the urogenital sinus on either side of the verumontanum and go on to form the prostate gland. The top pair of buds are mesodermal in origin and form the inner part of the prostate and the 4 lower pairs are endodermal in origin and form the outer part of the prostate gland. This may be of some importance as benign prostatic hyperplasia (BPH) affects the inner, mesodermal part of the prostate, whereas the vast majority of cancers arise from the outer, endodermal part. The inner and outer parts form concentric layers around the urethra as the prostate develops. Acini and collecting ducts form within the prostate and open into the urethra. Testosterone and its active metabolite DHT are essential for prostate organogenesis. The induction of DHT is thought to be linked to multiple growth factor systems including epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF).

1.2 The prostate gland: Gross anatomy and histology

Histologically the prostate gland consists of acini of various shapes and sizes embedded in a fibromuscular stroma. (A mixture of smooth muscle and connective tissue). Macroscopically the prostate gland resembles a compressed inverted cone, lying beneath the bladder and surrounding the proximal urethra. (Figures 1.1 & 1.2). It is a solid organ weighing up to 20 grams in the normal young adult male and measures approximately 3cm from apex to base, and 3.5cm across the base in the adult male. It is composed of complex glandular elements buried in a dense fibromuscular stroma which is directly continuous with the smooth muscle of the neck of the bladder. The prostate lies behind the pubis, separated from it by the pubo-prostatic ligaments, fibro-fatty tissue and blood vessels. Flattened in its antero-posterior dimension, it extends from the bladder neck to the urogenital diaphragm, lying apex downwards and base upward abutting against the

bladder and seminal vesicles and surrounding the prostatic part of the urethra, which pierces it asymmetrically.

The prostatic urethra emerges from the apex (the lowest part of the gland) to become the membranous urethra which is surrounded by the sphincter urethrae, better known clinically as the external urethral sphincter. The ejaculatory ducts pierce the posterior surface of the prostate just below the bladder and pass through the gland to open into the mid part of the prostatic urethra. The urethral crest projects from the posterior wall of the prostatic urethra into its lumen. At the midpoint of the urethral crest the verumontanum forms a midline rounded eminence with the prostatic utricle (a small recess) in the middle. The prostatic utricle represents the fused ends of the paramesonephric ducts. Either side of the utricle open the ejaculatory ducts. The proximal prostatic urethra is surrounded by smooth muscle which contracts during ejaculation to prevent retrograde passage of semen into the bladder.

The ductus deferens is a continuation of the epidymis testis. The part lying posterior to the prostate is dilated and known as the ampulla. The ampulla stores spermatozoa but itself makes only a very minor contribution to seminal fluid. The ampullae lie parallel and medial to the seminal vesicles with which they fuse caudally to form the ejaculatory duct which passes through the prostate to open on the verumontanum.

The prostate gland comprises three distinct morphological regions or zones with functional and pathological significance: the central (CZ), transition (TZ), peripheral zones (PZ) and anterior fibromuscular stroma (McNeal 1988 &1990) (Figure 1.3). The peripheral zone occupies about 75 % of the total prostate volume and is the commonest site to develop adenocarcinoma of the prostate and prostatic intraepithelial neoplasia (PIN), both of which arise from the peripheral ducts and acini (McNeal 1969). The peripheral zone is the area most readily sampled by trans-rectal prostate biopsy. The central zone makes up 25% of the prostate volume, and closely resembles the seminal vesicles in histology and architecture. Carcinoma arising from the CZ is rare. The transitional zone usually accounts for less than 5% of the prostate gland but is the main

site of origin for benign prostatic hyperplasia (BPH) and around 20% of prostate cancers. The anterior fibromuscular stroma forms a thick sheet over the anterior part of the prostate and is completely devoid of any glandular elements. In clinical practice it is very difficult to delineate the three zones histologically in the normal prostate.

Figure 1.1 Prostate anatomy (Coronal section)

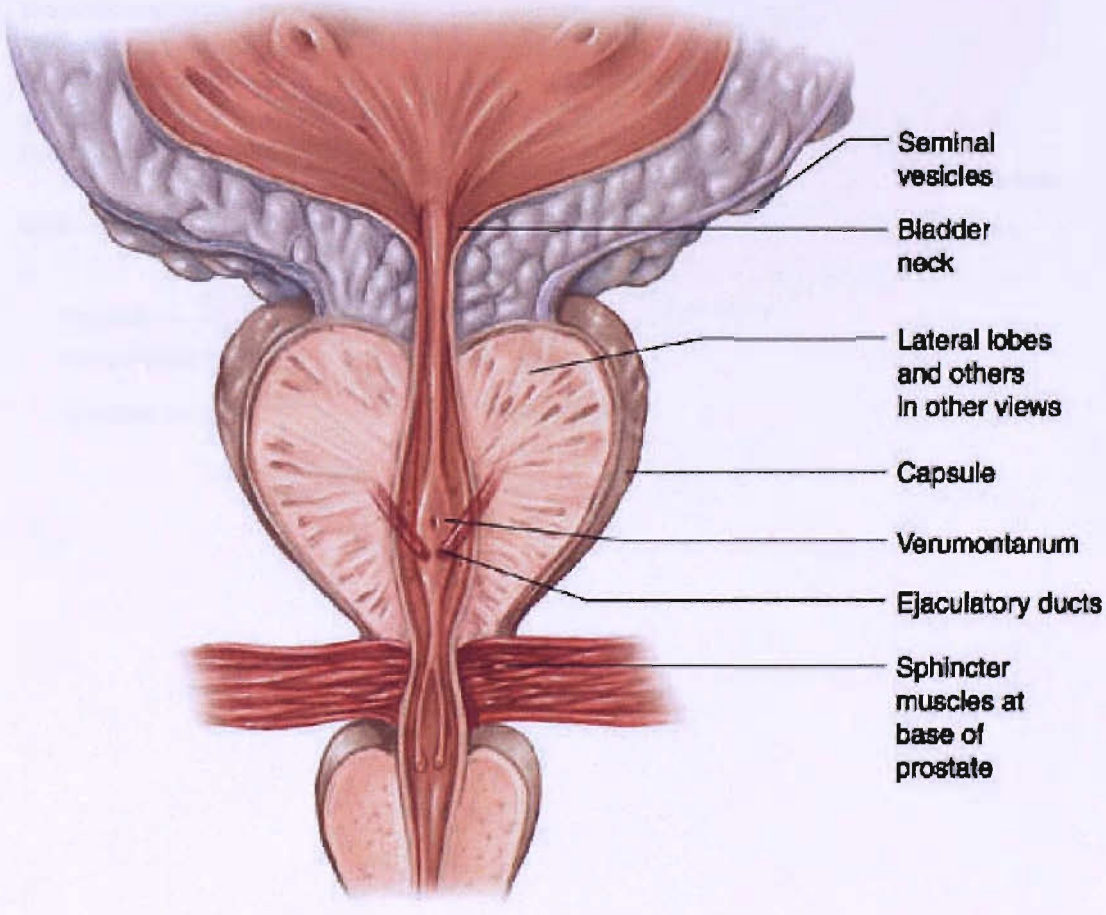


Figure 1.2 Prostate anatomy (Sagittal section)

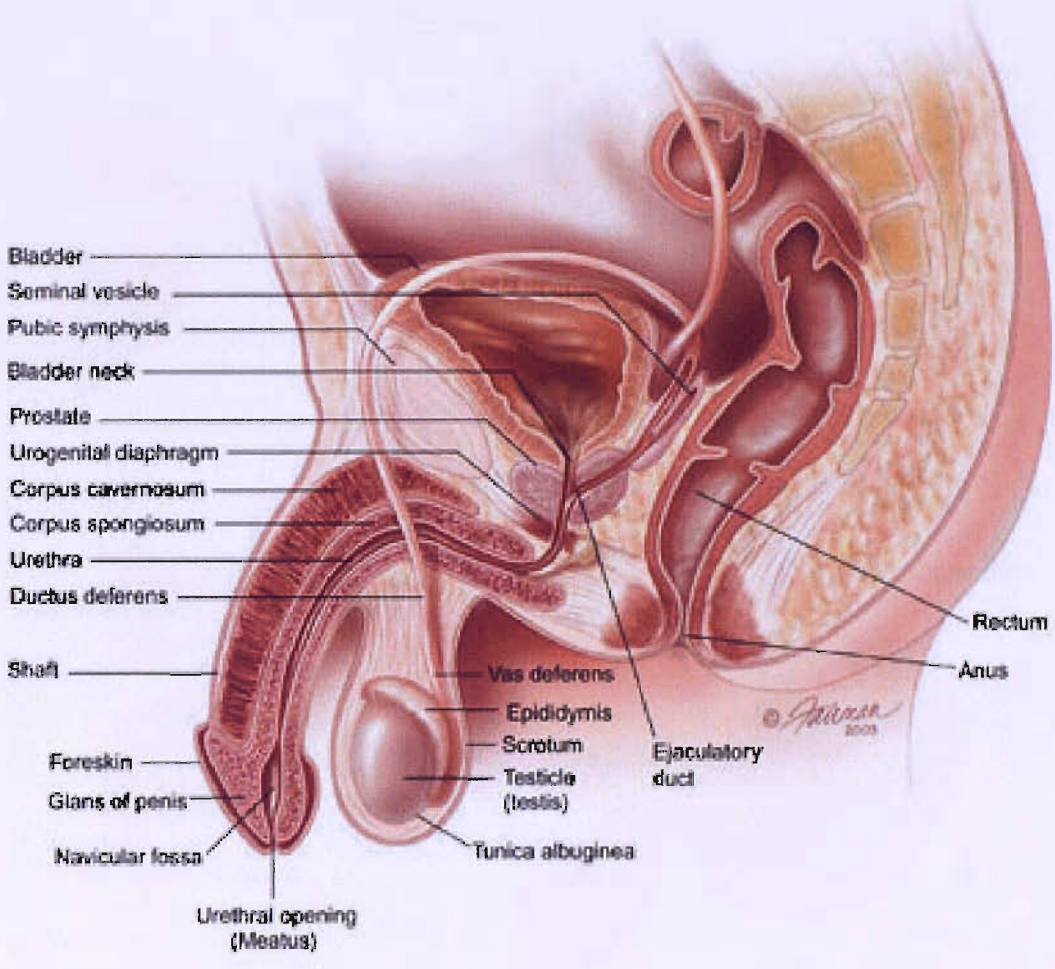
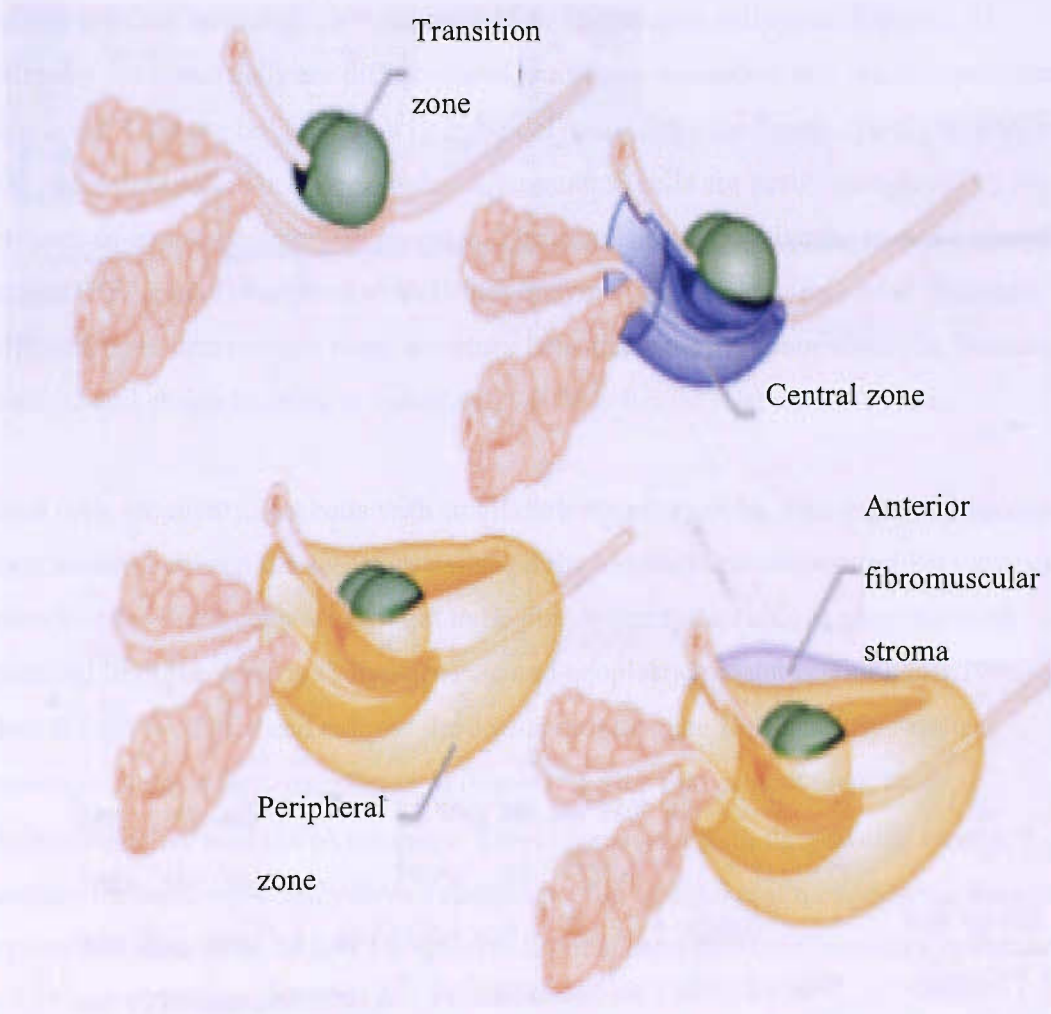


Figure 1.3 Zonal anatomy of the prostate



1.3 Prostate cell types

1.3.1 Epithelial cells

Human prostate epithelium is composed of three common cell types (Figure 1.4). Secretory (luminal) cells are differentiated, androgen-dependent and produce prostatic secretions. They are tall columnar or cuboidal cells with pale foamy cytoplasm and basal pale-staining nuclei. These terminally differentiated cells are easily recognised by the presence of abundant secretory granules. These cells stain positive for prostate specific antigen (PSA), acid phosphatase and other enzymes such as leucine amino peptidase. With androgen deprivation, these secretory cells decrease in number by 90%, become cuboidal and shrink by 80% in volume and 60% in height (Denmeade 1996).

Basal cells are scanty, flat cells with small dark-staining nuclei, forming a continuous cell layer located between the secretory cells and the basement membrane, which they lie in contact with. Basal cells are thought to be pleuripotent, thus able to give rise to all epithelial lineages in normal, hyperplastic and neoplastic prostates (Bonkhoff 1994). They are not secretory and express the anti-apoptotic gene Bcl-2 and free-radical scavenger glutathione s-transferase pi (Gst-π), factors that protect them from deoxyribonucleic acid (DNA) damage. This is consistent with the possible stem cell function for basal cells. They have a distinct profile of cytokeratin expression: basal cells express cytokeratins 5, 14 and 18, whereas luminal secretory cells express cytokeratins 8 and 18 and 19 (Sherwood 1991).

Neuroendocrine (NE) cells of the prostate are intraepithelial regulatory cells. They are found in all zones of the prostate gland, but most abundantly in the periurethral and ductal regions (Cohen 1993), where they are located amongst the luminal and basal cells (Abrahamsson 1993). They are androgen-independent cells that express chromogranin A, serotonin and various neuropeptides and are thought to provide paracrine signals that support luminal cell proliferation (Abrahamsson 1999). In the normal prostate, NE cells make up by far the minority of epithelial cells. This picture changes in prostate cancer, with an accumulation of NE-like cells amongst foci of cancer. This is referred to as NE

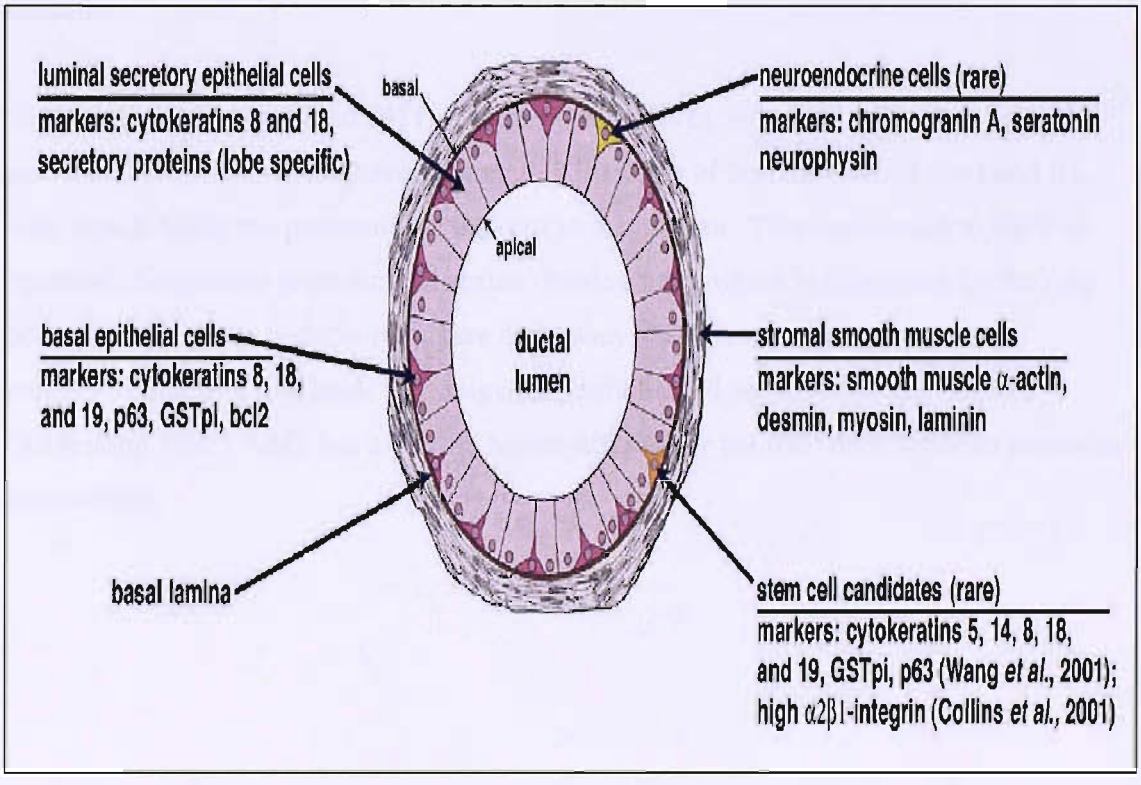
differentiation (NED) (di Sant'Agnese 1992) and is generally associated with disease progression (Abrahamsson 1998, Cussenot 1998). The exact role of neuroendocrine cells in the prostate remains unknown, however it is hypothesised that they regulate growth and differentiation as well as exocrine secretory activity through various paracrine and endocrine mechanisms (di Sant'Agnese 1998).

1.3.2 Stromal cells

The stromal compartment consists of a variety of cells, including fibroblasts, vascular and lymphatic endothelial cells, smooth muscle cells, neuroendocrine cells and axons (Mawhinney 1989). Smooth muscle cells are located around the acini and prostate capsule. They are believed to be involved in the mechanical secretion of ejaculate following neuronal stimulation. In the stroma that surrounds the distal segments of the ducts, a single layer of smooth muscle surrounds the epithelial cells whereas four to five layers of smooth muscle surround cells of the proximal ductal region (Griffiths 1994). Fibroblasts are evenly distributed throughout the stroma of the ductal system and can be distinguished by immunostaining (vimentin positive and actin negative) whereas the reverse is observed for smooth muscle cells (Lee 1996).

The tissue matrix is a biological scaffold that forms an interacting framework between many components including the extracellular matrix, cytoskeleton and nuclear matrix. It forms an interface with the stromal compartment and is thought to play an integral role in the epithelial-stromal interactions that are required for normal prostatic development. Connected via integrin receptors, epithelial cells rest on the basement lamina or membrane which surrounds the acini. The basement membrane is composed of a complex meshwork of collagen types IV and V, glycosoaminoglycans, complex polysaccharides and glycolipids. Inside the cells the cytomatrix or cytoskeleton network, formed from a meshwork of microtubules, actin microfilaments and intermediate filaments, binds directly to the nuclear matrix which is in connection with the cellular DNA. This allows the cells of the prostate to have direct structural linkages with one another via the cytoskeletal-nuclear matrices from the DNA to the plasma membrane.

Figure 1.4 Prostate cell types. Diagram showing the arrangement of prostate cells in a prostatic duct, with specific markers for each cell type (Adapted from Marker 2003).



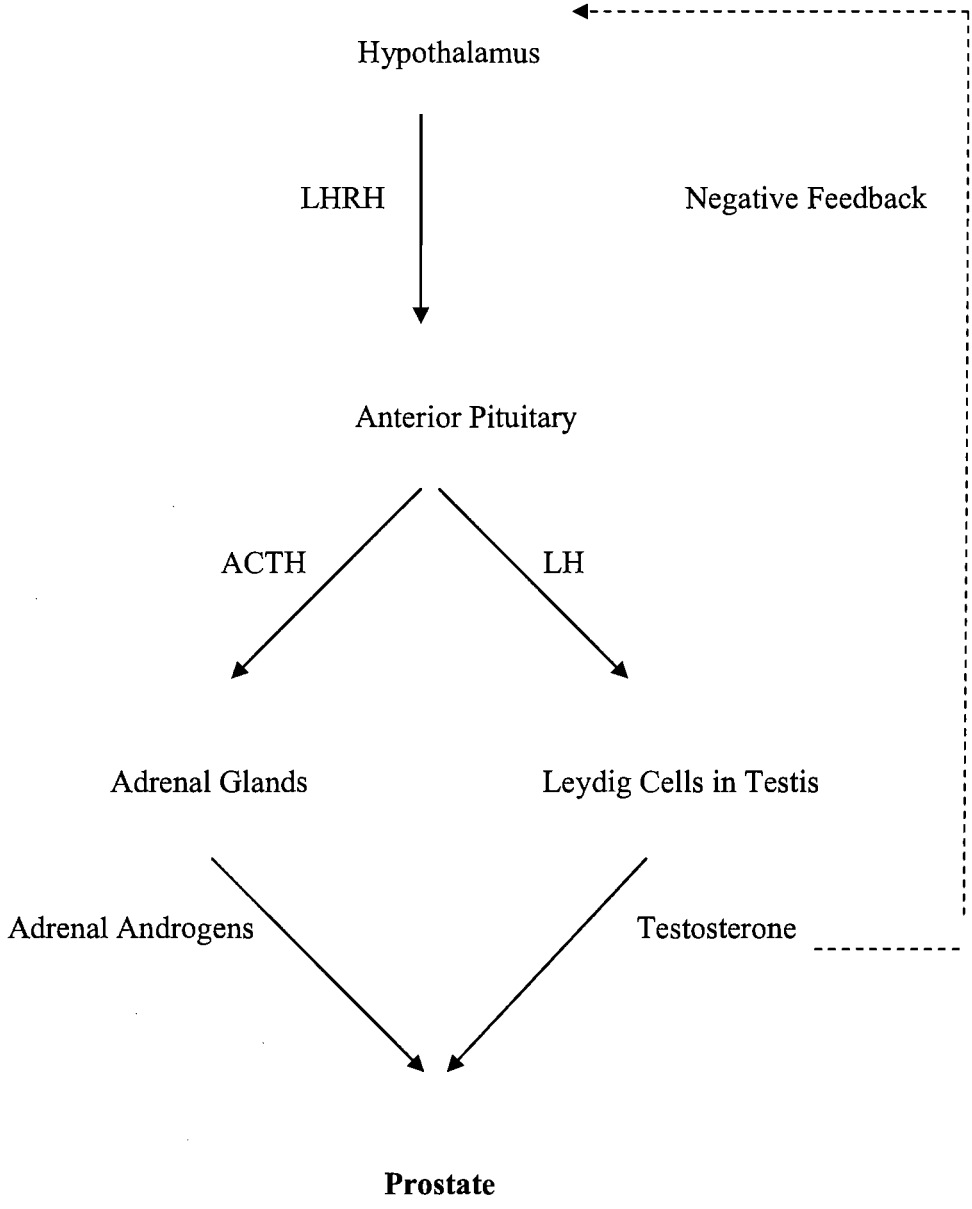
1.4 Endocrinology of the prostate

Prostate development, growth and homeostasis are regulated by dihydrotestosterone (DHT), the active metabolite of testosterone. The majority of testosterone is produced directly from Leydig cells in the testes (figure 1.5), with a small amount produced indirectly from the conversion of adrenal androgens to testosterone. Testosterone is essential for prostate growth. Removal of circulating androgens induces prostate involution.

Testosterone release is controlled by the hypothalamic pituitary axis. Luteinising hormone releasing hormone (LHRH) from the hypothalamus causes release of Luteinising hormone (LH) from the pituitary gland. This is released into the systemic circulation and causes the Leydig cells in the testes to release testosterone. The majority (98%) of circulating testosterone is bound to plasma proteins. Approximately 60% of the bound testosterone is bound to sex hormone binding globulin and the other 40% to albumin.

Testosterone is converted to DHT in the prostate cells by the nuclear membrane associated enzyme 5α-reductase. There are 2 isoforms of 5α-reductase (Type I and II), with type II being the predominant isoform in the prostate. This conversion to DHT is necessary for normal prostatic and sexual development, which is illustrated by the rare occurrence of males with 5α-reductase deficiency. This is a sex linked autosomal recessive condition that leads to ambiguous genitalia and pseudohermaphroditism (Andersson 1991). DHT has a 10 fold higher affinity for the AR and is twice as potent as testosterone.

Figure 1.5 Androgen physiology. Secretion by the testicular leydig cells is stimulated by the pulsatile release of luteinising hormone (LH) from the anterior pituitary. LH release is stimulated by hypothalamic LHRH whose secretion in turn is controlled by negative feedback responsive to serum testosterone concentration. Adrenal androgens, androstenedione and dehydroepiandrosterone are under pituitary control. Through adrenocorticotropic hormone (ACTH), they are metabolized to testosterone and dihydrotestosterone (DHT) accounting for approximately 10% of total androgen concentrations within the prostate.



1.5 Prostatic secretion

The prostate provides approximately 30% of the volume of seminal fluid. Prostatic secretion is a slightly acidic liquid that depends on the synthesising activity of prostatic epithelial cells and translation from blood serum. (Zaneveld et al 1981). Prostatic epithelial cells contain an abundance of rough endoplasmic reticulum and golgi apparatus that synthesise and store specific intracellular components that become secreted into the prostatic ductal lumen. (Ronquist et al 1985). Human prostatic secretion is rich in monovalent and divalent cations such as sodium, potassium, calcium, magnesium and zinc. It also contains citric acid and many enzymes. (Zaneveld et al 1981). Prostatic secretion contains high concentrations of the glycoprotein prostate specific antigen (PSA). This is a powerful protease which aids semen liquefaction after ejaculation by degrading seminogelin, thus freeing spermatozoa to migrate within the female genital tract. (Lilja H et al 1984). Prostatic epithelial cells also secrete prostasomes which will be described in detail later.

Prostatic fluid and prostasomes are excreted during ejaculation although prostatic secretion into the acinar lumen is a continuous process. (Isacs JT 1983). Prostatic fluid comprises approximately 30% of the ejaculate volume. The seminal vesicles produce up to 66% of the ejaculated volume. Seminal secretion is rich in basic proteins and prostaglandins. (Eliason E 1968). Spermatozoa, epididymal, ampullary and Cowpers gland secretion account for only 5% of the total ejaculate volume. (Zaneveld 1981). During ejaculation prostatic fluid with spermatozoa, ampullary, epididymal and Cowpers secretions is excreted first, followed by the seminal vesicle contibution. This does make it possible to study the different components of semen by splitting the ejaculate into initial and terminal parts. (Tauber et al 1975).

Stimulated prostatic secretion obtained from ejaculation has a slightly different composition to resting prostatic fluid. (Obtained from prostatic massage). (Gutman 1941). It contains for example an increased concentration of acid phosphatase.

1.6 Benign prostatic hyperplasia

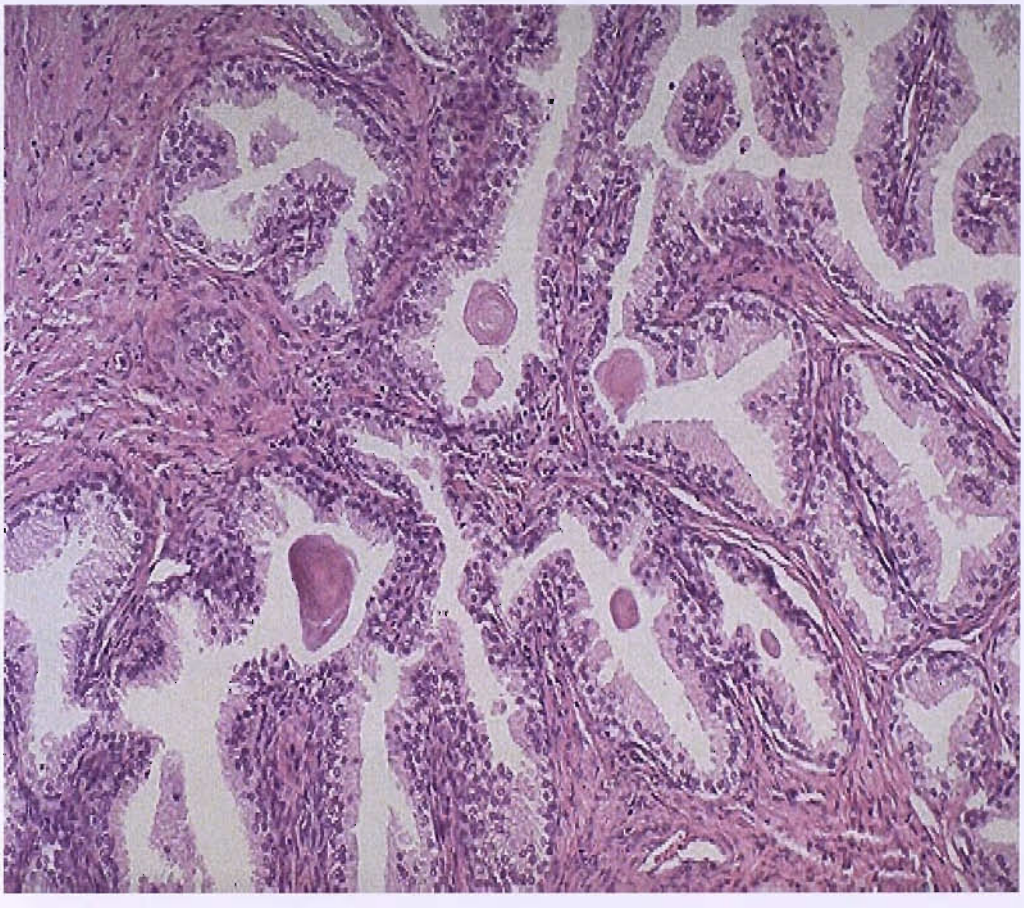
BPH is one of the commonest disease processes affecting the ageing male. BPH has no distinct definition, but encompasses a group of disorders whose symptoms include prostatic enlargement, histological hyperplasia, symptoms of the lower urinary tract or urinary obstruction.

BPH occurs exclusively in the transition zone and periurethral gland of the prostate. Both microscopic and clinical BPH have been shown to arise from histologically similar tissues. The first signs of BPH are the presence of microscopic nodules of fibromuscular hyperplasia consisting of variable proportions of glandular epithelium, smooth muscle and fibrous tissue (Figure 1.6) (Berry 1984). The majority of BPH stroma is connective tissue, approximately 50%, the remainder is smooth muscle (25%) and epithelium (25%) (Shapiro 1992).

Microscopic BPH can be found in males at a young age (25-30 years old) while clinical BPH is identifiable at later ages with an incidence of approximately 25% at 85 years of age (Oesterling 1996). The incidence of prostate cancer and BPH increases with age and both diseases are androgen-associated conditions. The only proven risk factors for developing BPH are ageing and the presence of functional testes (Glynn 1985). There are no reported differences in the incidence of BPH between different countries.
The aetiology of BPH remains largely unknown, however it is considered to arise due to stromal-epithelial interactions. Indeed, in BPH the stromal:epithelial ratio increases (Bartsch 1979). Normally it is within the region of 2:1, but can increase to 3 or 4:1 in BPH. Whether androgens have a causative role in the development of BPH is debatable. There is no doubt that androgens have a permissive role in BPH. For example, men castrated prior to puberty do not develop BPH.

The 'gold standard' surgical treatment for BPH is TURP (Trans-Urethral Resection of the Prostate), although pharmaceutical blockade using alpha-blockers and or 5 alpha-reductase inhibitors is now an acceptable alternative in uncomplicated disease. (McConnell 2003).

Figure 1.6 Microscopic appearance of BPH



Microscopic appearance of nodular prostatic hyperplasia at medium power (×200).

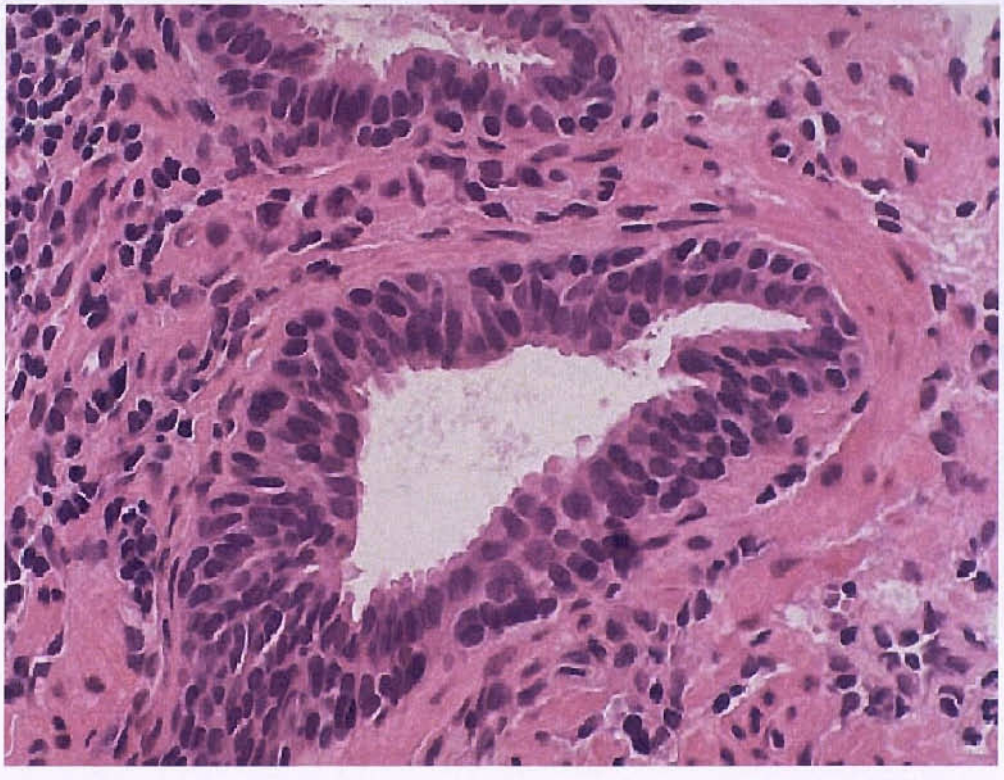
The columnar arrangement of cells near the gland lumina is preserved.

(Image from The internet Pathology Laboratory for Medical Education: http://www-medlib.med.utah.edu/WebPath/webpath.html).

1.7 Pre-malignant lesions of the prostate

Prostatic intraepithelial neoplasia (PIN) was first described as a pre-malignant condition of the prostate in 1986 by McNeal. PIN refers to the dysplastic changes in the prostatic ducts or acini that mimic cancer, with nuclear and nucleolar enlargement (Figure 1.7). PIN is distinguished from prostate cancer by the fact that it maintains an intact basal cell layer. PIN was originally described as grades 1-3, but is now termed low-grade PIN (grade 1) and high-grade PIN (grade 2-3). Low grade PIN is not reported by pathologists as it does not mean there is any increased risk of developing prostate cancer (Brawer 1991). High grade PIN is held to be pre-malignant. The exact rate of progression of high grade PIN to invasive carcinoma is not known. However, high grade PIN at trans-rectal ultrasound (TRUSS) biopsy is associated with a 23% to 35% risk of prostate cancer at subsequent biopsy (Davidson 1995, O'Dowd 1999, Kronz 2001). The majority of high grade PIN is also found in the peripheral zone, the main site of prostate cancer. As PIN has no affect on serum PSA (Ronnette 1993), clinical monitoring of the disease is difficult and men with high grade PIN are therefore recommended to have a repeat biopsy.

Figure 1.7 Prostatic intraepithelial neoplasia (PIN).



1.8 Carcinoma of the prostate

Prostate cancer is the most commonly diagnosed male malignancy in the European Union and the USA. In Europe more than 100,000 men are found to have prostate cancer and 35,000 men die from the disease each year. (Greenlee 2001). Prostate cancer has become one of the leading causes of cancer related death in men. It is estimated that the lifetime risk of dying from prostate cancer is approximately 3%. Post-mortem studies suggest that a pathological diagnosis of the disease can be made in up to 30% of men over 50 years old and 70% of men over 80 (Dijkman 1996).

Prostate cancer can be classified as:

- Symptomatic or asymptomatic
- Localised, locally invasive or metastatic
- Histologically, poorly, moderately or well differentiated
- Hormone sensitive or refractory

Prostate cancer stage and grade combined with a patients age, co existing morbidity and treatment preference all need to be taken into consideration when evaluating prognosis and planning therapy. Current treatment options for prostate cancer include;

- Watchful waiting/ Active surveillance
- Surgery
    - Radical prostatectomy
    - Transurethral resection of the prostate for relief of lower urinary tract symptoms
- Radiotherapy
    - External beam
    - Brachytherapy

- Cryotherapy
- High intensity focused ultrasound
- Drug therapy
- Palliative treatment

Early localised prostate cancer is potentially curable by either radical prostatectomy or radiotherapy (external beam or internal seed brachytherapy). However, not all early prostate cancers progress or prove fatal, therefore a policy of active surveillance can be justified in selected cases.

1.8.1 Epidemiology

Prostate cancer is the second commonest cancer in men behind non-melanoma skin cancers. It is the second commonest cause of cancer related death behind lung cancer and its incidence is increasing (Woolf 1995, Boyle 1996). In the UK, 23,109 new cases of prostate cancer were reported in 2000, this figure has risen from 13,481 in 1990. This was the most commonly diagnosed cancer in men (excluding non-melanoma skin cancers) and accounted for approximately 25% of all new cancers diagnosed in men for the year 2000. In England and Wales there were 8531 registered deaths from this disease in 1997, over 95 percent in men aged over 55 years (Office of National Statistics). In the USA, approximately 95,000 newly registered cases and 35,000 men die from the disease every year. Since 1985, the incidence of prostate cancer has risen by approximately 6% per annum, but recently this has levelled off and the incidence slightly reduced between 1992-1995.

1.8.2 Aetiology and risk factors

1.8.2.1 Age

Prostate cancer is predominantly a disease of the elderly. It is rare to find prostate cancer in men under the age of 50 and 75% of prostate cancer occurs in men over the age of 65.

1.8.2.2 Race

In the USA, the incidence varies between different ethnic groups. The incidence is highest in African-American men (Table 1.1).

Worldwide incidence and mortality from prostate cancer varies widely. It tends to be higher in western countries than developing countries. In Europe, there is a marked difference between north and south, with Norway having double the mortality of Spain (Landis 1999). Asian countries, in particular Japan and China, have the lowest incidence and mortality from prostate cancer (Landis 1999).

Table 1.1 Incidence of prostate cancer, in different ethnic groups in the USA in 1992.

Ethnic group	Prostate cancer incidence per 100,000
African-American	250
White-American	179
Hispanic-American	104
Asian-American	82

1.8.2.3 Hereditary

Several studies have shown that men with first-degree relatives with prostate cancer have a significant increase in risk of developing prostate cancer. The risk increases with the number of affected first-degree relatives. With 1, 2 and 3 first degree relatives with prostate cancer, the increased risk of developing the disease is 2, 5 and 11 fold (Carter 1993). This is also linked with age, in that the younger the relative with prostate cancer the higher the risk of developing the disease. It has been proposed that familial prostate cancer inheritance occurs in approximately 9% of prostate cancers but accounts for 43% of prostate cancers in men under the age of 55 (Carter 1992).

1.8.2.4 Environmental and occupational factors

There is some evidence that the risk of developing prostate cancer may be increased by factors such as increased cadmium exposure and reduced ultraviolet light (sunlight) exposure. The latter probably being related to a possible protective effect of vitamin D.

There was evidence that mortality from prostate cancer appeared to be increased among men living in urban areas where there is higher exposure to environmental pollutants such as automobile exhaust fumes, chemical fertilizers, cadmium and other industrial chemical carcinogens. It appears, from the available evidence, that the association between cadmium exposure and the risk of prostate cancer is weak (Elghany 1990). Exposure to radioactivity may be significant in some cases. The risk of prostate cancer was reported to be significantly increased in men who were exposed to tritium, Cr51, Fe59, Co60 or Zn65 over a 40-year period (Rooney C 1993). In this analysis the relative risk of developing prostate cancer appeared to increase with increasing level and duration of potential exposure to each of these radionuclides.

1.8.2.5 Testosterone

It is clear that androgen is required for normal development and growth of the prostate gland. Androgen also plays a significant role in prostate carcinogenesis. Androgen ablation therapy is the treatment of choice for men with advanced/metastatic prostate cancer. Men castrated at an early age or with congenital 5α-reductase deficiency have a very low risk of prostate cancer. (Huggins 1941, Imperato-McGinley 1992).
The exact relationship between androgen and prostate cancer is not fully understood. It has been hypothesised that the increased ratio of oestrogen to androgen that occurs with the age related decrease in testosterone may contribute to prostate cancer development (Prehn 1999).

1.8.2.6 Diet

Diet plays an important part in the development of prostate cancer. Convincing evidence for this is illustrated in migration studies. First-degree relatives from areas of low prostate cancer incidence (Japan and China) have increased prostate cancer rates when they

migrate to a Westernised society (Muir 1991, Shimizu 1991). However, this is an increase in clinical prostate cancer rates. Latent prostate cancer rates are similar between east and west and diet would therefore influence progression rather than development of prostate cancer. A summary of the dietary factors involved in prostate cancer is given in Table 1.2.

There appears to be a strong positive correlation between dietary animal fat intake and the risk of prostate cancer (Rose 1986). Giovanucci (1995) also showed an association between advanced stage disease and high dietary intake of fat from red meat. They postulated that dietary fat intake effects the production of hormones with increased risk of prostate cancer. Fat intake also effects the absorption of vitamins A, D and E, all of which are reported to lower the risk of prostate cancer (Table 1.2).

Table 1.2 Diet and the risk of prostate cancer.

*The discrepancy between reported effects of dietary vitamin A on the risk of prostate cancer is thought to be due to the source of vitamin A. In Asia, vegetables are the main source, whereas in the USA it is animal fat. The increased risk reported may be due to the higher fat intake.

Dietary component	**Risk of prostate cancer**	**Reference**
Red meat/ unsaturated fat	↑	Giovannucci 1995
Phytooestrogens (e.g isoflavanoids)	↓	Aldercreutz 1993
*Vitamin A**	↓/↑	Hsing 1990 Hayes 1999
Vitamin D	↓	Lieberman 2001
Vitamin E	↓	Lieberman 2001
Selenium	↓	Clark 1996
Tomatoes (lycopenes)	↓	Giovannucci 1995

1.8.3 Molecular genetics

Prostate cancer exhibits aneuploidy (presence or absence of DNA outwith the normal diploid number) in 40-50% of cases. This has been associated with advanced clinical stage, high disease grade, and poor patient prognosis (Visakorpi 1993). The commonest aneuploidy changes in prostate cancer include loss of chromosomes 1, 2, 4, 5 and Y and gains of chromosomes 7, 14, 20, and 22. Structural re-arrangements involving chromosome arms 2p, 7q and 10q and deletions at 7q, 8p and 10q have been reported. (Lundgren 1992, Sandberg 1992). Loss of heterozygosity (LOH) is considered to indicate areas of chromosomes that may harbour tumour suppressor genes (TSGs). In prostate cancer many areas of LOH have been identified. The commonest chromosomal regions involved are 5q, 6q, 7, 8p, 10, 13q, 16q, 17q and 18q (Carter 1990, MacGrogan 1994, Kunimi 1991, Cooney 1996 and Cunningham 1996). LOH of 8p is the usual defect in prostate cancer and occurs in up to 70% of prostate carcinomas and PIN (Bova 1993, Trapman 1994, Emmert-Buck 1995).

The tumour suppressor gene *PTEN/MMAC-1/TEP-1* maps to chromosome 10q23 and encodes a dual specificity phosphatase. PTEN protein negatively regulates cell migration and cell survival and induces a G_1 cell cycle block *via* the negative regulation of the phosphatidylinositol 3'-kinase/protein kinase B/Akt signalling pathway. PTEN is frequently mutated or deleted in primary prostate cancers and prostate cancer cell lines (Cairns 1997). In a survey of over one hundred cases, a loss of PTEN expression in paraffin-embedded primary prostate cancers correlated with advanced stage cancer and high Gleason score (McMenamin 1999).

1.8.4 Pathology

Prostate cancer is usually found in the peripheral zone of the gland (McNeal 1969, Byar 1972). The remainder are predominantly in the transitional zone. Prostate cancer is multi-focal in 85% of cases (Ayala 1989). Prostate cancer can spread locally or distally. Local extension is usually posteriorly or postero-laterally, in keeping with the location of the peripheral zone. Local extension is termed extra-prostatic extension, rather than capsular penetration, as the prostate lacks a distinct histological capsule (Gleason 1974).

Metastatic spread usually occurs to the pelvic lymph nodes or bones. However, spread to the lungs, bladder, liver and adrenal glands can occur. As metastatic spread is usually lymphatic in origin, metastatic tumour deposits tend to be diffuse small lesions rather than large discreet masses.

1.8.4.1 Grade

Various grading systems are used for prostate cancer, but the system described by Gleason in 1974 is the most universally accepted (Gleason 1974) (Figure 1.8). This is based on the architectural pattern of the tumour and is assigned a grade of 1 to 5. Grade 1 represents a well differentiated tumour, whereas grade 5 represents a poorly differentiated tumour. Combining the most prevalent pattern of Gleason grade with the second most prevalent pattern (Gleason sum score) produces good correlation with patient survival from prostate cancer (Gleason 1974, Chodak 1994). Therefore the Gleason sum score ranges from 2 (1+1, well differentiated) to 10 (5+5, undifferentiated).

Care has to be taken interpreting Gleason sum in needle biopsies of the prostate. As prostate cancer is heterogenous, a needle biopsy may not represent the true overall pattern of disease within the prostate. Indeed, there is a tendency for under-grading when needle biopsies are compared to radical prostate specimen (Bostwick 1994). Up to 33% of needle biopsies under-grade prostate cancer, whereas only 8% were over-graded (Catalona 1982).

1.8.5 Detection of prostate cancer

Early prostate cancer has virtually no clinical symptoms to aid detection. Local symptoms that can arise are due to obstruction of urine flow by the prostate cancer mass. Other localised symptoms can include haematospermia and ureteric obstruction. These tend to indicate locally advanced disease. Systemic symptoms of malaise, weight loss, bone pain, anaemia and lower limb oedema represent metastatic disease. A high proportion of men presenting with prostate cancer used to have metastatic disease due to the lack of clinical signs and general awareness of the disease. Since the discovery of PSA, its use has become widespread since the late 1980's. This has lead to a dramatic decrease in the number of men presenting with metastatic disease (Hankey 1999).

Clinical evaluation of prostate cancer is carried out by digital rectal examination (DRE), serum prostate specific antigen (PSA) and trans-rectal ultrasound guided biopsy of the prostate gland (TRUSS biopsy).

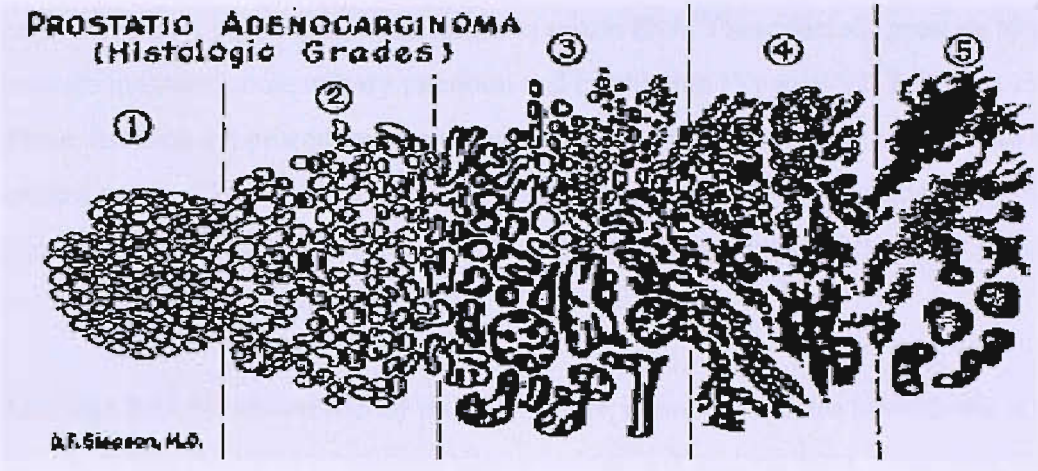
1.8.5.1 Digital rectal examination

The prostate gland is easily palpable through the anterior wall of the rectum. Obviously, this is only examining the posterior aspects of the gland, but the peripheral zone lies here and is the area most likely to be involved with prostate cancer. DRE is an important tool in prostate cancer. A palpable abnormality on DRE is cancer in 50% of cases (Jewett 1975). It is shown not to be as accurate as PSA in the detection of cancer (Catalona 1994). However, a study has shown 17% of cancers were missed using PSA screening alone (Schroder 1998). It is recommended that all men with a DRE suspicious of malignancy should have a TRUSS, as 25% of prostate cancers have a normal PSA.

1.8.5.2 Prostate specific antigen

PSA is a 34 kDa glycoprotein secreted by prostatic ductal cells, first described by Wang et al in 1981. It is a serine protease and is thought to liquefy seminal fluid. It is secreted at high concentrations in the mg/ml range in the seminal fluid, but only small amounts in the ng/ml range are detected in the serum. In the serum, most is bound to serum anti-proteases (65-90%), although a small amount (10-35%) remains free in the serum (Lilja 1991). Bound PSA is cleared by the liver and has a half life of 2-3 days, whereas free PSA is cleared by the kidney, and has a half life of 2-3 hours (Stamey 1987, Bjork 1998).

Figure 1.8 Gleason grading system of prostate cancer



Original drawing of Dr. Gleason's, showing the five distinct patterns of the Gleason grading system. The 2 most prevalent Gleason grades are added together to give the Gleason score. Where 2 is the most differentiated and 10 is the least differentiated.

Gleason grade 1 and 2: These two grades closely resemble normal prostate. They are the least important grades because they seldom occur in the general population and because they confer a prognostic benefit which is only slightly better than grade 3. In grade 2 glands are more loosely aggregated, and some glands invade into the surrounding stroma.

Gleason grade 3: This is the most common grade and is also considered well differentiated (like grades 1 and 2). All three grades have normal glandular appearances. The lumen contains prostatic secretion like normal prostate. Each gland unit is surrounded by prostate muscle which keeps the gland units apart. Invasion into the stroma is very prominent and is the main defining feature. The cells are dark rather than pale and the glands often have more variable shapes.

Gleason grade 4: Here there is a loss of architecture with disruption and loss of the normal gland unit. This is the main class of poorly differentiated prostate cancer, and its distinction from grade 3 is the most commonly important grading decision.

Gleason grade 5: This usually predicts another significant step towards poor prognosis. It is less common than grade 4, and it is seldom seen in men whose prostate cancer is diagnosed early in its development. This grade too shows a variety of patterns, all of which demonstrate no evidence of formed glands. This grade is often called undifferentiated, because its features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.

Unfortunately, PSA is not specific for prostate cancer. Prostatitis and BPH can cause elevated serum PSA (Dalton 1989, Carter 1992). Several urological conditions and procedures can cause transient increases in serum PSA. These include prostate biopsy, prostate massage, acute urinary retention and ejaculation (Yuan 1992, Tchetgen 1996). These diseases and procedures are thought to disrupt normal prostate architecture and cause a surge of PSA in the serum. PSA is also not specific to the prostate gland, with expression demonstrated in breast tissue, peri-urethral and anal glands, sweat glands and even renal tumours (Levesque 1995).

Although PSA is not specific for prostate cancer, numerous studies have shown it to be the single best predictor of prostate adenocarcinoma (Catalona 1994, Ellis 1994, Stone 1994). It is also independent of user expertise, unlike TRUSS and DRE. The risk of prostate cancer increases as the serum PSA increases. Normal PSA level is typically set at 4 ng/ml, which gives a 1 in 50 chance of having prostate cancer on biopsy (Schroder 1998). Increasing the range of PSA to 4 - 10 ng/ml results in a 1 in 4 chance of having prostate cancer, which increases to 1 in 2 for PSA levels greater than 10 ng/ml (Schroder 1998).

Several methods have been investigated to try to improve the sensitivity and specificity of PSA testing for prostate cancer.

Specific age ranges for PSA

The recommended reference range for serum PSA (95th percentile) for men aged 40 to 49 years is 0.0 to 2.5 ng/ml; for 50 to 59 years, 0.0 to 3.5 ng/ml; 60 to 69 years, 0.0 to 4.5 ng/ml; and 70 to 79 years, 0.0 to 7.5 ng/ml (Oesterling 1993).

PSA density

The commonest reason for a marginally elevated PSA level in the range of 4 – 10 ng/ml is BPH. PSA density is calculated by dividing the serum PSA (ng/ml) by the prostate volume on TRUSS (ml). It is suggested that PSA densities of 0.15 or greater are an indication for a TRUSS guided biopsy in patients with a normal DRE (Benson 1992).

However, several studies have since disputed this cut off as a significant number of prostate cancers will be missed (Brawer 1993). PSA density is however a useful tool for monitoring patients with persistently raised PSA despite normal biopsy, to determine who may need a further TRUSS guided biopsy.

PSA velocity

Carter et al looked at PSA levels in archival frozen serum samples obtained from men who were subsequently followed up to determine who did or didn't develop prostate cancer. Individuals whose serum PSA increased by more than 0.75 ng/ml per year had a 72% risk of developing prostate cancer compared to only a 5% risk if PSA velocity was 0.75 ng/ml or less per year. This data is particularly relevant for patients with PSA between 4-10 ng/ml. Several other studies have shown similar results (Carter 1992, Carter 1995, Lujan 1999).

Free/total PSA

The majority of PSA in the serum is bound to alpha-1-antichymotrypsin (ACT), with a lesser amount free in the serum. Men with prostate cancer have relatively more PSA bound to ACT and therefore the ratio of free PSA to total PSA is higher in men without prostate cancer (Christensson 1993). Several studies have shown increased sensitivity using this method. However the exact cut off point has varied between the studies. Catalona et al used a 25% (% free PSA) or less as an indication for biopsy in men with a PSA of 4 – 10 ng/ml, detecting 95% of cancers and avoiding 25% of unnecessary biopsies (Catalona 1998).

1.8.5.3 Trans-rectal ultrasound scan and prostate biopsy

The prostate gland is scanned by an ultrasound (USS) probe placed in the rectum, either in transverse or longitudinal plane. Prostate cancer lesions have traditionally been described as hypoechoic. Hypoechoic lesions are twice as likely to contain prostate cancer as isoechoic lesions (Hammerer 1994). However, most hypoechoic lesions seen on TRUSS are not malignant and approximately 50% of prostate cancer lesions are not visualised by TRUSS (Carter 1989). TRUSS is therefore not a diagnostic tool, but is a

very useful tool to image the prostate for accurate sampling of biopsies as well as targeting suspicious areas for biopsy.

1.8.6 Staging of prostate cancer

Following diagnosis of prostate adenocarcinoma, accurate staging of the disease is important to determine the most appropriate treatment option for individual patients. The staging modalities currently used in prostate cancer are DRE, radiological imaging and pelvic lymphadenectomy. The TNM system (Tumour/Nodes/Metastasis) is the most widely used for both clinical and pathological staging of prostate cancer (Table 1.3). Pathologic staging is more accurate than clinical staging in predicting outcome, as multiple factors are included. The more important pathological criteria for prognosis are surgical margins, Gleason grade, extra capsular spread, and seminal vesicle or pelvic lymph node involvement. Tumour grade is an independent predictor of prognosis. Significantly worse survival is seen in identically staged disease when comparing Gleason 7 and above to Gleason 6 and below (Epstein 1993a, Epstein 1993b). Seminal vesicle and pelvic lymph node metastases are both associated with a very low probability of a complete surgical cure (Pound 1997).

DRE may under-stage prostate cancer. A study looked at a large series of prostate cancers assessed as clinically organ confined. Fifty two percent had organ confined disease, 31% had extra-prostatic extension and 17% had seminal vesicle invasion of pathological examination. The same study also showed 19% of prostate initially staged as T3a on DRE were actually organ confined (Partin 1993).

PSA on its own is a poor predictor of prostate cancer stage as there is considerable overlap between PSA levels and disease stage. However PSA levels do give a rough guide to prostate cancer stage. Prostate cancer with a serum PSA less than 4 ng/ml has an 80% chance of being organ confined. PSA 4 - 10 ng/ml has a 66% of being organ confined, whereas PSA greater than 10 ng/ml has a less than 50% probability of being organ confined. A PSA over 20 ng/ml carries a 20% risk of pelvic lymph node

metastases, and a PSA of over 50 ng/ml carries a 75% risk of pelvic lymph node metastases (Partin 1993, Rietbergen 1999).

Prediction of organ confinement of prostate cancer can be further enhanced by combining PSA, grade and local stage. This data exists in the form of Partin's tables (Partin et al 1997).

Various imaging techniques have been used to stage prostate cancer including TRUSS, isotope bone scan, computerised tomography (CT) and magnetic resonance imaging (MRI). TRUSS is an insensitive method of detecting organ confinement. Isotope bone scan is used to screen the skeleton for evidence of bony metastases with an 8% false negative rate reported (Terris 1991, Ball 1979). Areas of suspicion on bone scan are not specific of prostate cancer, and if the positivity of result is questioned, then plain radiography or MRI of the area should be performed. The routine use of isotope bone scan in patients with PSA less than 20 ng/ml has been questioned due to the very low pickup rate (Chybowski et al 1991). CT and MR scans are used to examine for evidence of extra capsular spread and evidence of pelvic lymph node involvement. However both modalities are insensitive in picking up microscopic lymph node metastases.

1.8.7 Management of organ-confined prostate cancer

Currently there are 3 distinct treatment modalities for early prostate cancer that is clinically organ confined. It is generally accepted that a life expectancy of 10 years or more is required for a patient to significantly benefit from radical treatment of prostate cancer.

1.8.7.1 Radical prostatectomy (RP).

In many European countries and in the USA, radical surgery is the preferred treatment for localised prostate cancer, with 95% of American Urologists recommending it as a first line treatment for localised prostate cancer in suitable patients (Gee 1995). In the UK only 29-44% of Urologists would recommend this treatment, depending on tumour grade (Savage 1997).

Table 1.3 TNM Classification for Prostate Cancer UICC sixth edition 2002

T- Primary Tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically unapparent tumour not palpable or visible by imaging **T1a** Tumour incidental histological finding in 5% or less of tissue resected from TURP **T1b** Tumour incidental histological finding in more than 5% of tissue resected from TURP **T1c** Tumour identified by needle biopsy (eg because of elevated PSA)
T2	Tumour confined within the prostate **T2a** Tumour involves one half of one lobe or less **T2b** Tumour involves more than half of one lobe but not both lobes **T2c** Tumour involves both lobes
T3	Tumour extends through the prostatic capsule capsule **T3a** Extracapsular extension (unilateral or bilateral) **T3b** Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than the seminal vesicles: bladder neck, external sphincter, rectum, levator muscles, or pelvic wall
N- Regional Lymph Nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M- Distant metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis **M1a** Non regional lymph nodes **M1b** Bone(s) **M1c** Other site(s)

Surgery involves the removal of the entire prostate gland with attached seminal vesicles, followed by reconstructing the lower urinary tract by anastomosing the bladder onto the distal urethra. Immediate post-operative complications are rare with a mortality rate of 0.3%. The operation can be performed through an abdominal or a perineal incision. Late complications include bladder neck stricture in 0.5-9%. Urinary incontinence is a significant late complication and occurs in 5-20% of patients, with the accepted rate being around 10% (Murphy 1994). The majority of men with incontinence following radical prostatectomy respond to conservative treatment, with a chance of improvement up to 1 year after surgery (Leandri 1992). Erectile dysfunction occurs in 30-100% of men post-operatively and is dependant on patient age and use of neuro-vascular bundle sparing techniques during surgery (Quinlan 1991, Murphy 1994). Radical prostatectomy gives 5 year PSA free survival rates of >70% (Epstein 1993).

1.8.7.2 External beam radiotherapy (EBRT)

EBRT is probably still the modality that is most commonly used in the UK for radical treatment of prostate cancer. It usually involves a 6-week course of radiotherapy treatments to the prostate gland and has similar progression-free and overall survival rates to those seen after RP. However it also has its drawbacks, including urinary incontinence (2-3%), rectal inflammation and bleeding (2-10%), and impotence (20-30%).

1.8.7.3 Brachytherapy (BT)

BT involves a one off treatment to implant either iodine-125 or palladium-103 radioactive seeds into the prostate directly through the perineum. Although it has a low morbidity, there is no conclusive evidence that brachytherapy results are better than the results of the other two radical treatments. Additionally, the selection criteria for brachytherapy tend to be more exclusive.

1.8.7.4 Active surveillance

Another option for management of early prostate cancer is 'watchful waiting', now more commonly referred to as 'active surveillance'. This essentially involves clinical

assessment and PSA monitoring of patients. Primary intervention is avoided unless there is evidence of disease progression either clinically or biochemically.

1.8.8 Management of locally advanced/metastatic prostate cancer

Hormonal manipulation (androgen ablation) has remained the mainstay of treatment in advanced or metastatic prostate cancer (Huggins & Hodges 1941). Approximately 80% of patients will respond to androgen blockade. Common drugs used include LHRH analogues such as Goserelin and Leuprolide. Androgen receptor antagonists such as Bicatulamide (Casodex) and Flutamide, both non-steroidal anti-androgens, are competitive inhibitors of the androgen receptor. Both are commonly used in the clinical treatment of prostate cancer. However, hormone refractory disease develops within a median of two years following the initiation of androgen blokade and the prognosis is then generally poor.

1.9 Prostasomes

In the late 1970's, at the University of Uppsala, Sweden, Ronquist and colleagues described the existence of microscopic vesicles present in seminal plasma, which they subsequently termed prostasomes due to their prostatic origin.(Ronquist 1977, Ronquist 1978, Stegmayr 1982 & Ronquist 1985). Much of the subsequent research has been conducted at the department of Medical Sciences, Uppsala, Sweden and also in the Istituto di Biochimica Medica, Perugia, Italy.

Prostasomes are membrane-bound secretory vesicles, in the nanometer diameter range. (Ronquist 1985). They are secreted by the prostatic ductal epithelium, into the lumen where they form part of the ejaculate. Although known to possess specific biological properties, their physiological role and overall significance remain far more debatable. There has been comparatively little written about these structures since their detection two decades ago and this may help explain the relative lack of awareness of prostasomes within the urological community.

In 1977 Ronquist et al described high ATPase activity in human prostatic fluid that was linked to membranous structures. The ATPase was found to be a membrane associated phospholipoprotein that was magnesium/calcium dependant (Ronquist 1977 & 1978). Secretion from the seminal vesicles was found not to contain ATPase activity or magnesium or calcium cations. This suggested that prostasomes were prostatic in origin.

1.9.1 Origin and physical properties

1.9.1.1 Production and secretion of prostasomes.

Prostasome production occurs in the apical part of the prostatic ductal epithelial cell where golgi apparatus is abundant. Multiple prostasomes appear together within larger storage vesicles prior to secretion into the prostatic ductal lumen. There are believed to be two main modes of prostasome secretion. (Ronquist 1985). In exocytosis larger storage vesicles fuse with the cell membrane to discharge their contents, followed by recycling of the vesicular membrane. Secretion in membrane-bound form, or diacytosis, involves the

whole storage vesicle together with its contained prostasomes being translocated through the membrane (as happens with hepatic phospholipids). They are thought to occur with approximately equal frequency. (Ronquist 1985 & Brody 1983). This is supported by the morphological finding of both free prostasomes and membrane bound groups of prostasomes within the ductal lumen.

1.9.1.2 Androgen dependency of prostasome occurrence in seminal plasma.
Prostatic epithelial cell activity among other things is stimulated by testosterone. There is evidence to suggest that prostasome production is also androgen dependant. Stegmayr et al (1980) reported a 30% decrease in prostasomal ATPase activity in 22 oligo-zoospermic men with reduced testosterone levels compared with 30 normo-zoospermic men. In addition Ronquist et al (1984) described reduced prostasomal ATPase activity in a patient with well-differentiated prostate cancer after the initiation of hormonal anti-androgen therapy.

1.9.2 Prostasomal structure

Prostasomes are found in high concentrations in seminal plasma. Under electron microscopy there appear to be two distinct morphological types: smaller, 'dark' prostasomes with tightly packed electron dense contents, and larger 'light' less dense structures. Their diameter varies from 40-500 nm (Brody 1983 & Ronquist 1985). They possess a lipid bi-layer membrane which may be arranged in a multi-lamellar fashion (Ronquist 1985) and has a characteristically high concentration of cholesterol. The ratio of cholesterol to phospholipid is approximately 2:1 (Arvidson 1989) compared to 1:1 in a typical mammalian cell membrane. Consequently, prostasomes have a membrane which is less permeable to small water-soluble molecules. Analogous structures are also shed by prostate cell lines into culture media and by prostate cancer metastasis. (Nilsson 1999).

1.9.3 Preparation of prostasomes

Prostasomes can be prepared from both *in-vitro* and *in-vivo* sources, using semen, prostatic tissue (benign, primary and secondary malignancy) or prostate cancer cell lines.

Although the finer details of extraction will depend upon the source, the protocols used to isolate prostasomes contain the following elements:

1. Removal of cells from semen by low speed centrifugation.
2. Ultracentrifugation of seminal plasma, e.g. 100,000G for 2 hours.
3. Re-suspension of prostasome pellet, typically in isotonic Tris-HCl buffer.
4. Gel chromatography, e.g. using a Superdex column, to purify the re-suspended prostasome containing fractions.

1.9.4 Prostasome biochemistry

1.9.4.1 Lipids

One of the main lipid components of prostasomes is sphingomyelin (Ronquist 1985), a group of phospholipids, which contains 1-phosphocholine combined with a ceramide (an N-acyl long-chain base) such as sphingosine. Sphingomyelin is one of the main constituents of the myelin sheath of neurones. More than 50% of the phospholipid component of the prostasomal membrane is either palmitic ($COOH\ (CH_2)_{14}\ CH_3$) or stearic acid ($COOH\ (CH_2)_{16}\ CH_3$).

1.9.4.2 Proteins

Proteomic analysis using a high-pressure liquid chromatography -mass spectrometry approach, trawled 139 associated proteins in prostasomes isolated from pooled semen (Utleg 2003). The author's hypothesise that prostatic diseases may present unique phenotypes at the level of their respective prostasomal proteomes. Prior to this analysis approximately 80 different protein entities had been detected using 2-D chromatography and gel electrophoresis. (Lindahl 1987 & Renneberg 1997). Some of these previously identified proteins (e.g. CD142 – see below) do not appear in the recent HPLC analysis. Of the 139 proteins identified using HPLC nine percent appeared to be novel. The functions of the "known" molecules are diverse and include structural membrane proteins, enzymes, chaperone proteins and signal transduction molecules. Some of the enzymes are linked to the prostasomal membrane via a glycosylphosphatidylinositol anchor. (Fabiani 1995). Some of the prostasomal proteins have particular relevance in the

genito-urinary tract and inform speculation on the biological effects of the prostasome. Table 1.4 lists some of the more interesting candidates. The exact function of many prostasomal proteins however, remains unclear.

In addition prostasomes contain the following enzmyes: protein kinases, phospholipase A2, lactic dehydrogenase, alkaline phosphatase and alkaline phosphodiesterase.

Tissue Factor (TF) has been a subject of interest within our unit for many years. TF *alias* thromboplastin or CD 142, is a single chain transmembrane glycoprotein with a molecular weight of approximately 43-45kDaltons. (Bach 1981 & 1988, Broze 1985, Ghua 1986 & Kang 1988). It is a receptor and essential cofactor for clotting factors VII and VIIa and acts as the main physiological initiator of the coagulation cascade. (Guha 1986, Kang 1988 & Bach 1988). TF is present in human semen, predominantly in the prostasomal fraction and has been located on the prostasomal surface using immuno-electron microscopy. (Fernandez 1997).

Neuroendocrine components, such as neuropeptide Y, chromogranin A and B, and vasoactive intestinal peptide (VIP) have also been demonstrated in prostasomes by radioimmunoassay and immunoelectron microscopy. (Stridsberg 1996). Skibinski 1994 identified the secretory granule protein granulophysin, which is structurally similar to the neuroprotein synaptophysin. (Shalev 1992). The role that these neuro-components play in prostasomal activity is still not fully understood but they are thought to facilitate molecular interactions by increasing the formation and stability of functional signalling complexes. (Maecker 1997).

Table 1.4 Prostasomal proteins

Protein	Putative Function
CD 13. Aminopeptidase.	Zinc dependant proteolytic enzyme. Liquefaction of semen. (Lilja 1984)
CD 26. Dipeptidyl peptidase IV. (T-Cell activating antigen).	Peptide metabolism, T-Lymphocyte activation and the regulation of DNA synthesis, cell proliferation and the production of cytokines. HIV binding. (Valenzuela 1997, Shrimph 1999, Vanhoof 1992)
CD 10. Enkephalinase. (Neutral endopeptidase).	Membrane Anchored ectoenzyme (peptidase). Regulation of growth stimulatory capacity. Lost in hormone insensitive invasive prostate cancer cell lines. (Papandreou 1998)
CD 143. (Angiotensin converting enzyme).	Angiotensin 2 and bradykinin metabolism. (Erdos 1985)
CD 46, CD 55, CD 59.	Complement inhibitors. Sperm motility. Protection of sperm against complement attack in the female genital tract. (Rooney 1993 Kitamura 1995
CD 69. Granulophysin.	Facilitate molecular interactions. Increasing the formation and stability of functional signalling complexes. (Skibinski 1994)
CD 142. Tissue factor.	Rapid activation of clotting preventing bleeding and transfer of infection. (Fernandez 1997)
ATPase. Calcuim/magnesium dependant.	Vectorial calcium transport into prostasomes. Prostasomes are rich in Ca2+, and participate in the maintenance of Ca2+ homeostasis which is necessary for sperm motility. (Arienti 2002)
Heat shock proteins including HSP70 & HSP 90.	Chaperon proteins. Promotion of protein folding, assembly, translocation and secretion of newly formed polypeptides. HSP 90 targets signal transduction proteins such as steroid hormone receptors and signalling kinases. (Young 2001)
PSA. Prostate specific antigen.	Tumour marker for prostate cancer. Semen liquefaction. Crawford ED 1993

1.9.5 Prostasomal function

Function depends on the source of prostasomes. For instance, if one considers sperm motility, prostasomes isolated from PC3 cell lines offer similar properties to those isolated from seminal plasma (Wang 2001); whereas amino-peptidase activity differs (Ronquist 1978).

The identification of prostasome associated proteins sheds light on the possible function of prostasomes. (Table 1.4). It is postulated that they have a role in sperm motility, semen calcium homeostasis, semen liquefaction, complement inhibition, immunosupression, prevention of haematogenous infection, clotting activation, and even a role in HIV transmission. (Valenzuela 1997). They are also reputed to have antioxidant properties. (Saez 1998).

1.9.5.1 Sperm – prostasome interactions

The evidence suggests that by sperm prostasome interaction, advantageous effects of prostasomal membrane proteins are conferred to the sperm cell resulting in an improved microenvironment and an increased chance of the sperm cell achieving successful fertilisation. Sperm fusion with prostasomes has been an area of some interest. Ronquist has demonstrated that both the spermatozoa and prostasomes display a net-negative surface charge, with the prostasome being less negative (Ronquist 1990). Fusion occurs despite the expected repulsion, raising further questions regarding sperm prostasomal interaction. It has been shown that fusion between sperm and prostasomes is Ca^{2+} dependent and involves sperm intra-cytoplasmic Ca^{2+} concentration increasing. (Arienti 2002).

Although the mechanisms involved in the acrosome reaction are not completely understood, Arienti (1998 & 2002) has suggested that stabilisation of the sperm membrane appears to occur on fusing with prostasomes due to enrichment with cholesterol, sphingomyelin, and saturated glycerophospholipid. It is argued that this may "prevent the untimely occurrence of the acrosome reaction". The significance of other molecules, including arachidonic acid 15-lipoxygenase has also been brought to light. It

has been implicated in the acrosome reaction of bull semen and was found by Oliw and colleagues to be present in purified human prostasomes (Oliw 1993).

1.9.5.2 Sperm motility

Fabiani et al (1994 & 1995) and (Stegmayr 1982) have demonstrated an increase in sperm motility in semen samples treated with prostasomal preparations, with a response rate of up to 70%. Motility may even occur in sperm that are initially deemed to be immotile. The increased sperm motility attributable to prostasomes is pH-dependant. The pH dependency may be physiologically significant as sperm encounter an acidic environment on ascending the female genital tract. Sperm motility was shown by Arienti et al (2002) to be decreased in an acidic environment, but this was improved with the addition of prostasomes. Thus, one may speculate that prostasomes offer some buffering protection for the sperm against the relatively harsh vaginal/ uterine environment. However, these discoveries do not appear to have led to widespread clinical application within infertility management perhaps raising doubts as to the overall significance of the results.

1.9.5.3 Calcium homeostasis

It is believed that prostasomes promote sperm motility by modulating calcium ion concentration. Prostasomes are rich in calcium ions and the prostasome associated Calcium/Magnesium ATPase system may control their transport across the prostasome membrane. Annexins and calmodulin are other proteins involved with Ca^{2+} regulation that are present in prostasomes. (Utleg 2003).

1.9.5.4 Semen coagulation and liquefaction

Immediately after ejaculation normal human semen coagulates. The coagulum is largely composed of sialo-glycoprotein-metal complexes (semenogelins), glycerylphosphorylcholine and fibronectin, the whole being known as High Molecular Weight Seminal Vesical Proteins (HMW-SVP). (Gonzales 2000). Within approximately twenty minutes spontaneous liquefaction occurs due to the action of prostate specific antigen (a serine protease present in seminal plasma both free and prostasome associated

Utleg 2003) and possibly the involvement of prostasome aminopeptidase, a zinc dependant proteolytic enzyme. (Lilja 1984).

1.9.5.5 The haemostatic system in semen

There is evidence for most of the components of the blood coagulation and fibrinolytic system in semen. (Lwaleed 2003). There are points of contact between these and the HMW-SVP system, notably PSA, which binds to Protein-C inhibitor. Moreover, digestion of semenogelin with PSA produces fragments which cross-link into complexes with clotting factor XIIIa. (Lwaleed 2003). This circumstance is a reminder not to assign exclusive pigeon-holes to groups of molecules; semen is awash with interrelating proteases. Due to its prostasomal (and perhaps some soluble) TF content semen has potent blood clotting activity. The ability to coagulate blood rapidly from abrasions during intercourse is postulated to prevent sperm and seminal components, including infectious agents such as human immunodeficiency virus, from entering the blood stream, generating antibodies, or promoting infectious disease. (Fernandez 1997).

1.9.5.6 Effects on the lymphoreticular system and immunity

Human semen has potent immunosuppressive activity. (Stites 1975). This is believed to help protect sperm from immunological attack within the female reproductive tract. Local immunosuppression may also help protect the female genital tract from becoming sensitised to the alloantigenic proteins present in seminal plasma and on sperm.

Prostasomes inhibit lymphocyte proliferation and protect sperm cells from macrophage phagocytosis. (Liszewski 1996, Jiang 1998, Kelly 1991 & Skibinski 1992). CDs 46,55 and 59 on prostasomes have been shown to regulate complement pathways and opsonisation. (Kitamura 1995 & Rooney 1993). CD 46 causes proteolytic inactivation of factors C3b and C4b of the complement cascade. CD 55, the decay accelerating factor and CD59, an inhibitor of the membrane attack complex both protect from complement mediated lysis and phagocytosis. (Babiker 2002).

1.9.5.7 HIV infection

Prostasomes may facilitate HIV binding and survival in human semen. CD 26 (Dipeptidylpeptidase), a prostasomal membrane protein, binds HIV. (Subramanyan 1993 & Valenzuela 1997). Secondly the complement inhibitors CD's 46,55 and 59 that protect sperm may also protect the HIV virus. Indeed CD 55 and CD 59 might become transferred from the prostasome to the HIV membrane. (Saifuddin 1995).

1.9.5.8 Antioxidant properties

Reactive oxygen species (ROS) or free radicals are pivotal in inflammation and carcinogenesis. They are harmful to sperm and a major cause of idiopathic male infertility. Polymorphonuclear leucocytes (PMNs) in semen are believed to be a major local source of ROS. The addition of prostasomes to PMNs caused a reduction of ROS (Saez 1998). They attributed this decrease not to free radical scavenging, but the incorporation of 16-doxyl-stearate into the cell membranes, increasing rigidity.

1.9.6 The role of prostasomes in urological disease and current research

Prostate cancer cells generate prostasome like granules. (Nilsson 1999). Antibodies to prostasomes have been detected in the serum of patients with prostate cancer. A pilot study by Nilsson et al (2001), using ELISA technology, demonstrated markedly raised serum levels of antibodies to prostasomes in patients with prostate cancer compared to control subjects. Nilsson's work used a prostasome-like preparation prepared from prostate tissue. Further work is required to establish the role of antibodies to prostasomes as prognostic indicators and determinants in tumour progression. The antigenic target(s) need identifying. Nilsson's group chose a monoclonal antibody for their studies and are thus detecting antibodies to one epitope only on these complex structures. (Nilsson 1998). It is not apparent how many specificities emerged from their fusions. The metastatic status of the patients in this study was not recorded. Indeed further work by Larsson et al (2006) indicated that antiprostasome antibody titre had a significant but inverse relationship to prostate cancer metastasis.

Local invasion or metastasis should facilitate prostasomes escaping into the vascular and lymphatic systems. Leaked prostasomes may also impinge on the course of whatever

pathological process caused their presence there, for good or ill. The ease with which prostasomal membrane elements apparently fuse with cells and pathogens gives much scope for them to influence events.

Whether or not prostasomes have a mechanistic role in the aetiology or progression of prostate cancer is not clear. Relatively little has been published on the potential role of prostasomes in urological disease. Recent work by Carlsson et al suggests that prostasomes have a growth inhibitory effect on prostatic cancer cell lines in culture with the effect being most marked with DU145 cells. (Carlsson 2000). Heat treating the prostasomes prior to plating them with the cells destroys this inhibition. The possibility of in-vivo growth-inhibition should therefore be considered.

Tissue Factor has been implicated in breast cancer as a marker for the switch to an angiogenic phenotype. (Contrino 1996). The role, if any, of prostasome bound tissue factor in prostate cancer angiogenesis has yet to be determined. Malignancy has long been associated with hypercoagulability, presumably involving TF. Urinary exosomes, derived from kidney tubular epithelium, also carry TF (Lwaleed 1999), and their potential as diagnostic or prognostic markers of malignancy is well described. Abdulkadir et al have implicated urinary TF expression as being correlated with PSA levels in prostate carcinoma (Abdulkadir 2000), and the procoagulant activity of prostatic tissue in an experimental model of prostate cancer in rats was highest in metastatic disease. (Adamson 1994).

1.9.7 Summary

Prostasomes are relatively neglected entities, certainly in urological circles. For an overview of the field from a non-urological perspective, see the Proceedings of the First International Congress on Prostasomes (Ronquist 2002). They are best known in domains of human and veterinary fertility. However, given their readiness to fuse with cells, their content of potentially influential molecules and anti-oxidant properties, it can be argued that prostasomes are more than just vehicles for the disposal of unwanted

cellular material. This may or may not be the case for the exosomes produced by prostate cell lines *in vitro* and caution should be exercised in assuming homology. There are also seminosomes, liberated in the testis and presumably contaminating prostasome preparations (except in vasectomised individuals). Indeed experimental comparisons between prostatic exosomes and those released by other epithelia would help differentiate the specific prostasomal properties from more general attributes.

Circumstantial evidence exists that prostasomes might have some influence on proliferative events in the prostate. Antibodies that react with prostasomes, whatever the original immunogen, are intriguing both for their very existence and the opportunities for patho-physiological mischief they suggest. It is timely for Academic Urology to address the patho-physiology of prostasomes, in particular more prostate-specific issues such as prostatic carcinoma, prostatitis and benign prostatic hyperplasia.

1.9.8 Thesis objectives

1. To reliably and consistently purify prostasomes from human semen.
2. To confirm purification and to review prostasomal ultra-structure using electron microscopy.
3. To further confirm prostasome purification by means of biochemical characterisation.
4. To assess the presence of antibodies to prostasomes in patients with prostate cancer, and determine the associations, if any, between antibodies to prostasomes and the aetiology and progression of prostate cancer.

Chapter 2

General Materials and Methods

2.1 Reagents and chemicals

Unless otherwise stated all chemicals and reagents were analytical or molecular biology grade from Sigma Chemical Co. (Poole, U.K.)

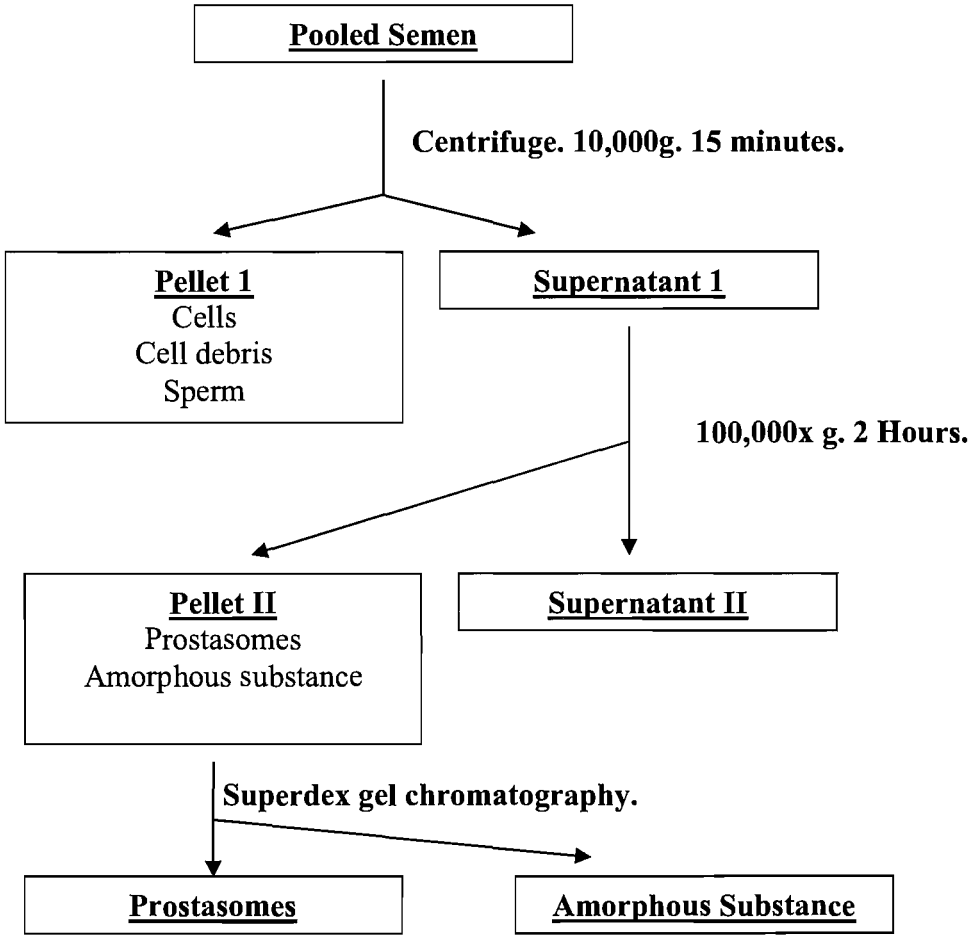
Plastic ware for tissue culture and general laboratory use was obtained from Becton Dickinson (Oxford, U.K.). All gel electrophoresis apparatus was from Bio-Rad (Hemel Hempstead, U.K.).

This chapter deals with general methodologies used. Specific methodologies will be described in the relevant chapters. All experiments were performed personally unless otherwise stated.

2.2 Prostasome preparation

Prostasomes were isolated from human semen by means of differential centrifugation and gel chromatography using Superdex 200. Using a technique modified from that originally described by (Ronquist 1985), Figure 2.1 outlines the general scheme for prostasome preparation.

Figure 2.1; Prostasome preparation. Schematic summary.



2.2.1 Semen source, collection and storage

Semen samples were obtained from men having undergone bi-lateral vasectomy for the purpose of voluntary sterilisation. Semen analysis is routinely performed on all patients at 10-14 weeks post vasectomy to determine the success of the procedure. Patients must not abandon other contraceptive methods until they have produced at least two semen samples negative for motile spermatozoa. This provides an ideal window of opportunity for semen collection. Samples were collected following informed consent (Appendix 2&3) from patients producing samples for routine post vasectomy semen analysis at Southampton General Hospital, Southampton, Hampshire, UK, and The Wessex Nuffield Hospital, Chandlers Ford Hampshire UK. Following completion of semen analysis by the hospital laboratory staff, individual semen samples were frozen at –70 degrees and stored for use in batches for the preparation of prostasomes. Initially prostasomes were isolated from fresh semen, and compared to prostasomes prepared from frozen semen.

Unless stated otherwise the source of semen was from vasectomised men. For some experiments semen was collected and stored in the same manner as above, but the source of the semen was different. Alternative sources included men producing samples for the investigation of infertility. In other experiments semen was collected from normal healthy male volunteers with no history of vasectomy, infertility, testicular or prostatic disease.

2.2.2 Differential centrifugation

Batches of frozen semen were thawed and pooled. Five or six samples were typically used giving a pooled semen volume of approximately 12-15mls, as the average ejaculate volume is usually 2.5mls –3.5mls. (Guyton 1991). The thawed liquid semen was initially centrifuged in Eppendorf tubes at 10,000 x g for 15 minutes at room temperature to produce "pellet 1", and "supernatant 1" (Figure 2.1). This initial spin was to remove any cells, cell debris or remaining spermatozoa. The clear resulting supernatant 1, also termed seminal plasma, was recovered and centrifuged further using a Beckman Ultra-Centrifuge with a 50.2 Ti rotor for 100,000 x g for 2 hours at 4 degrees centigrade. The centrifuge speed was calculated using manufacturers hand book (33,160 RPM = 100,000g). The

small pellet obtained (pellet 2) contained the prostasomes and amorphous material. (Ronquist 1985). The protein containing amorphous material is believed to originate from the seminal vesicles. (Brody 1981 & Stegmayr 1982). It can be sedimented at 104,000 x g and possess anti-fertility activity. (Pinsker 1968, Dukelow 1969 & Reddy 1979). Pellet 2 was re-suspended in 2mls of PBS. The supernatant resulting from ultra-centrifugation (supernatant 2) was in some cases kept for further evaluation, or discarded.

2.2.3 Superdex gel filtration.

In gel filtration molecules in solution are separated according to differences in their sizes as they pass through a column packed with chromatographic medium (Porath J 1959) which is a gel. In this case we used Superdex 200 a gel based on highly cross-linked porous agrose beads to which dextran has been covalently bonded. A glass column 60cm high with a diameter of 2.6cm was set up in a cold laboratory maintained continuously at 4 degrees centigrade. The column was carefully packed with Superdex 200 (Sigma, Poole, UK). Pellet 2 suspension was loaded onto the Superdex 200 column equilibrated in de-gased PBS with a flow rate of 0.5ml/min. An automated fraction collector was set to advance every 5 minutes thus 2.5ml fractions were collected. The UV absorbance of the fractions was measured at 280nm using a spectrophotometer. The spectrophotometer was zeroed using PBS. The purified prostasomes were initially subjected to further investigation both fresh and after a freeze thaw cycle. Later batches were frozen in convenient aliquots at –70 degrees centigrade for use at a later date.

2.3 Aminopeptidase assay

Prostasomal CD13 (Aminopeptidase: 150Kdaltons) a zinc dependent proteolytic enzyme has been found to be a useful marker of prostasomes. (Laurell 1982). Thus an aminopeptidase assay was used to confirm the presence of aminopeptidase activity in the prostasome containing fraction produced from the Superdex 200 gel column.

Ultracentrifuged prostasomes were resuspended in 2mls of PBS and applied to the Superdex 200 column at flow rate of 0.5mls per minute. The fractions were assayed for aminopeptidase activity using two assays. The substrates used were, L-Alanine-p-

Nitroanilide and L-Leucine-p-Nitroanilide (Sigma). Solutions of each were made at 0.5mg/ml in 0.2mol TRIS Buffer at pH 8.0. 30microlitres of test fraction were added to 1ml of substrate in a test tube. After 10 minutes incubation at room temperature the reaction was terminated with 200microl acetic acid. The UV absorbance was recorded at 410 nm using a Unicam Heyios E Spectrometer.

2.4 Activated partial thromboplastin time (APTT) assay to detect procoagulant activity in purified seminal prostasomes

Prostasomes were prepared from vasectomised semen. The fractions produced from the Superdex column were screened for procoagulant activity using a commercially available hospital activated partial thromboplastin time (APTT) assay. (Sysmex CA-50 Coagulometer). 50mcl of fraction was added to an equal volume of normal human plasma (NHP) and incubated for 1 minute at 37^0c. Samples were divided equally to allow testing in duplicate. 50mcl of actin (Dade Behring, Newark, USA) was added for 1 minute followed by the addition of 50 mcl of calcium chloride (Dade Behring, Newark USA). The activated partial thromboplastin time was recorded.

2.5 Bradford dye assay: Estimation of prostasomal protein concentration

The protein concentration of our pooled purified prostasome re-suspension was measured each time a batch of prostasomes was produced. The Bradford dye assay was used. (Bradford 1976). Under appropriate conditions, the acidic COO- and basic NH3 + groups of proteins interact with coloured dyes to form coloured precipitates. The Bradford assay uses Coomassie Brilliant Blue G-250 dye which binds to protein causing a shift in the absorbance maximum of the dye from 465nm to 595nm. This is a simple method of detecting protein concentration.

Stock solutions of BSA of known concentration were prepared. In duplicate an equal volume of prostasome solution was tested. 1ml of Bradford dye was added to each solution and left at room temperature for 30 minutes. The absorbance was measured at 595nm.

Using the mean absorbance of the known BSA solutions a curve was plotted and the prostasomal protein concentration calculated.

2.6 Gel Electrophoresis

2-D gel electrophoresis was performed on purified seminal prostasomes and other non-prostasome containing fractions from the column and the supernatant II.

2.6.1 Reagents

Those in brackets were made in advance.

1. *Running Gel.*

- 6.8mls deionised water.
- 3.0ml 1.875M Tris-HCL at pH 8.8. (22.7g TRIS + 80mls deionised water pH to 8.8 with concentrated HCL then make up to 100mls with deionised water).
- 5ml Acrylamide stock (Sigma)

Mix Gently then add

- 150 microLitres 10% SDS (1g SDS in 10mls deionised water).
- 7.5 microlitres TMED.
- 50 microlitres 10% ammonium persulphate. (made fresh)

2. *Stacking gel.*

- 3.6mls deionised water.
- O.5mls 1.25M Tris HCL, pH 6.8. (7.6g TRIS in 40 mls deionised water, pH to 6.8 with concentrated HCL then made up to 50 mls with deionised water)
- 0.8ml acrylamide stock (sigma)

Mix Gently then add

- 50 microL 10% SDS
- 5 microL TMED
- 17 microL 10% ammonium persulphate.

3. *Sample loading buffer;*

- 11.6ml Glycerol
- 5ml 1.25M Tris HCL pH 6.8.
- 2.5ml 0.4% w/v bromophenol blue.
- 5ml 2-mercaptoethanol
- 2g SDS. (10 mls of ready mixed 20% SDS)
- make up to a final volume of 50 mls with deionised water.

2.6.2 Method

The SDS polyacrylamide gel electrophoresis (PAGE) apparatus (Bio-Rad mini-protean system) was set up as described in the manufacturer's manual. The stacking gel and running gel were poured into the frame and allowed to set, with the teeth of a carefully placed comb forming the wells in the stacking gel. The prepared prostasomes samples were loaded at up to 10µl per well. Seeblue protein standards (250, 98, 64, 50, 36, 30, 16, 6 and 4 kDa; Novex/Invitrogen, U.K.) were run alongside the protein samples to allow estimation of protein size and confirm protein transfer. Gels were electrophoresed at 200V for approximately 45 minutes in a tank of running buffer (25 mM Tris-HCl, 190 mM glycine, 0.1% SDS).

Chapter 3

Prostasomal Ultra-structure

3.1 Aims

To visualise prostasomes in their native form (in whole semen samples) and to confirm their presence in the purified state. All processing, sectioning and transmission electron microscopy was undertaken personally. In addition an attempt was made to immuno-label prostasome-bound tissue factor.

3.2 Methods

3.2.1 TEM preparation schedule. Purified prostasomes.

Following ultracentrifugation of seminal plasma as described in chapter 2, the supernatant was decanted leaving a small pellet (pellet II, figure 2.1). The pellet was fixed using 3% glutaraldehdye & 4% formaldehyde in 0.1M PIPES buffer at pH 7.2 for one hour at room temperature. The pellet was carefully removed and cut into 1mm blocks. The subsequent processing was according to a standard protocol. (Page 1994). The blocks were twice rinsed in 0.1M PIPES buffer at pH 7.2 for 10 minutes. The rinse buffer was decanted and in a fume cupboard post fixed in 1% osmium tetroxide in 0.1M PIPES buffer at pH 7.2 for 1 hour. Following 2 further 10 minute buffer rinses the blocks were dehydrated in graded ethanol, 30%, 50%, 70% and 95% each for 10 minutes respectively, then twice for 20 minutes in absolute ethanol. The ethanol was decanted and acetonitrile added, after 10 minutes the acetonitrile was decanted and the samples submerged in a 50:50 mix of acetonitrile:SPURR (Spurr 1969) resin overnight. The following morning the samples were transferred into 100% resin for 6 hours. Finally the samples were polymerised in fresh resin in an oven at 60^0c for 24 hours.

The polymerised blocks were removed from the plastic eppendorf tubes, and ultra thin sections of 90nm (Silver) were cut using a Reichert –Jung Ultratome with a glass knife. (Glass knives cut personally). Before proceeding representative sections were stained with toludine blue and examined under the light microscope to check the content. Sections were mounted on TEM slides and stained first with urinyl acetate for 15 minute in the dark and then with lead citrate in the presence of sodium hydroxide pellets for 5 minutes.

The slides were viewed and photographed using a Hitachi H7000 transmission electron microscope (Hitachi Scientific Instrument, Nissei Sangyo LTD, Japan).

3.2.2 TEM preparation schedule. Whole semen and non prostasome containing Superdex fractions

The TEM preparation schedule for liquid samples had to be modified and is described below (Page 1994). Fresh semen was obtained from vasectomised men and pooled. The samples were fixed in primary fixative. (3% glutaraldehdye & 4% formaldehyde in 0.1M PIPES buffer at pH 7.2) for 1 hour. Fixed semen was embedded in 5% sodium alginate by addition of the fixed suspension to a test tube containing 1ml of 5% sodium alginate and centrifugation at 1250x g for 5 minutes.

Fixative was decanted leaving semen embedded in alginate. A solution containing 0.1M PIPES and 0.05M $CaCl_2$ was added and left to set for 10 minutes. The pellet was carefully displaced with a needle or pipette and left to set further for 15 minutes. Subsequent processing was as described in 3.2.1 starting at the post fixative osmium tetroxide step.

3.2.3 TEM preparation schedule for tissue factor labelling

In an attempt to label prostasome-bound tissue factor, rabbit monoclonal antibody to human tissue factor was used (American Diagnostica Inc, Greenwich, CT, USA) with gold labelled anti-rabbit antibody as the secondary antibody. (Bio Cell, Wales,UK). We had a limited supply of primary anti tissue factor antibody allowing only one run which was performed before we had optimised prostasome purification and thus was performed on vasectomised semen samples. Unfortunately processing was not successful on this occasion due to the liquid nature of the samples which dissolved.

3.3 Results.

In whole semen scattered vesicles both with and without granular content and in the size range 100 –400nm were present (fig 3.1). We presume these to be prostasomes. The bi-layer membrane architecture is clearly visible on high magnification. (fig 3.2 & 3.3).

When the pellet (pellet 2) of ultra-centrifuged seminal plasma was examined under low power there were densely packed prostasomes again showing a bi-layered membrane (fig. 3.4).

Figure 3.1

TEM of prostasomes in whole semen x 40 000.

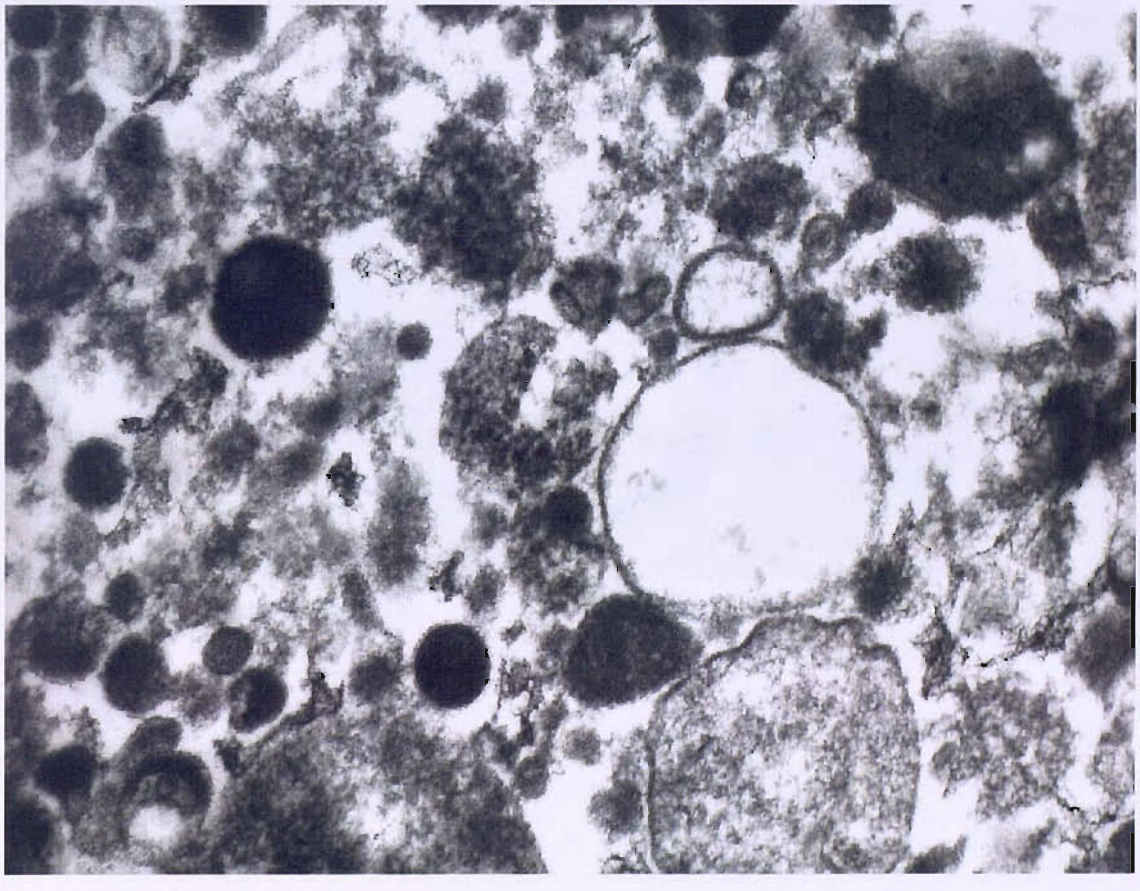


Figure 3.2

High power TEM of light prostasomes x 80 000.

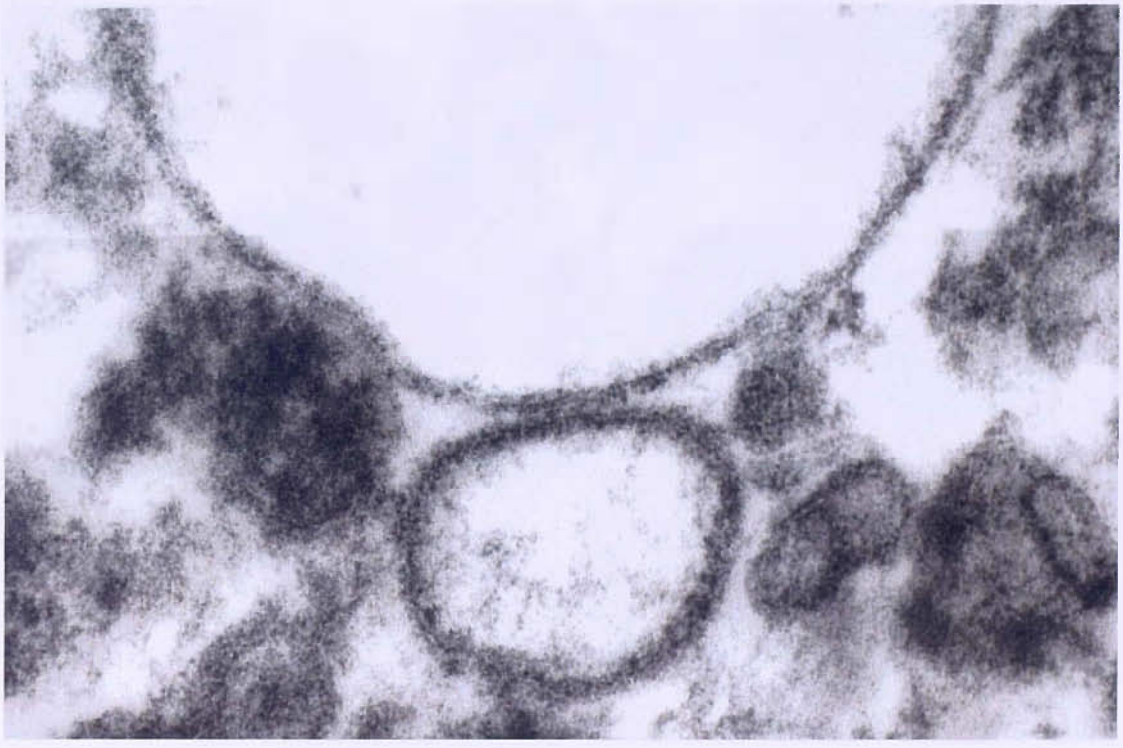


Figure 3.3

High power TEM of dark prostasomes x 80 000.

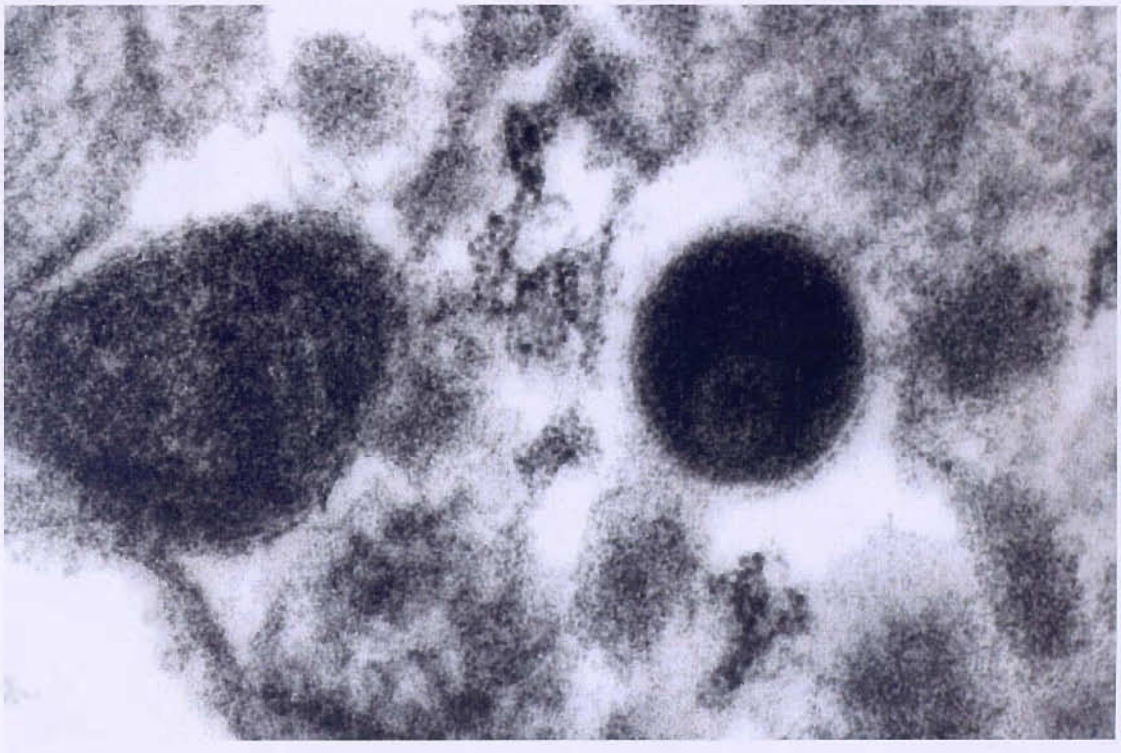
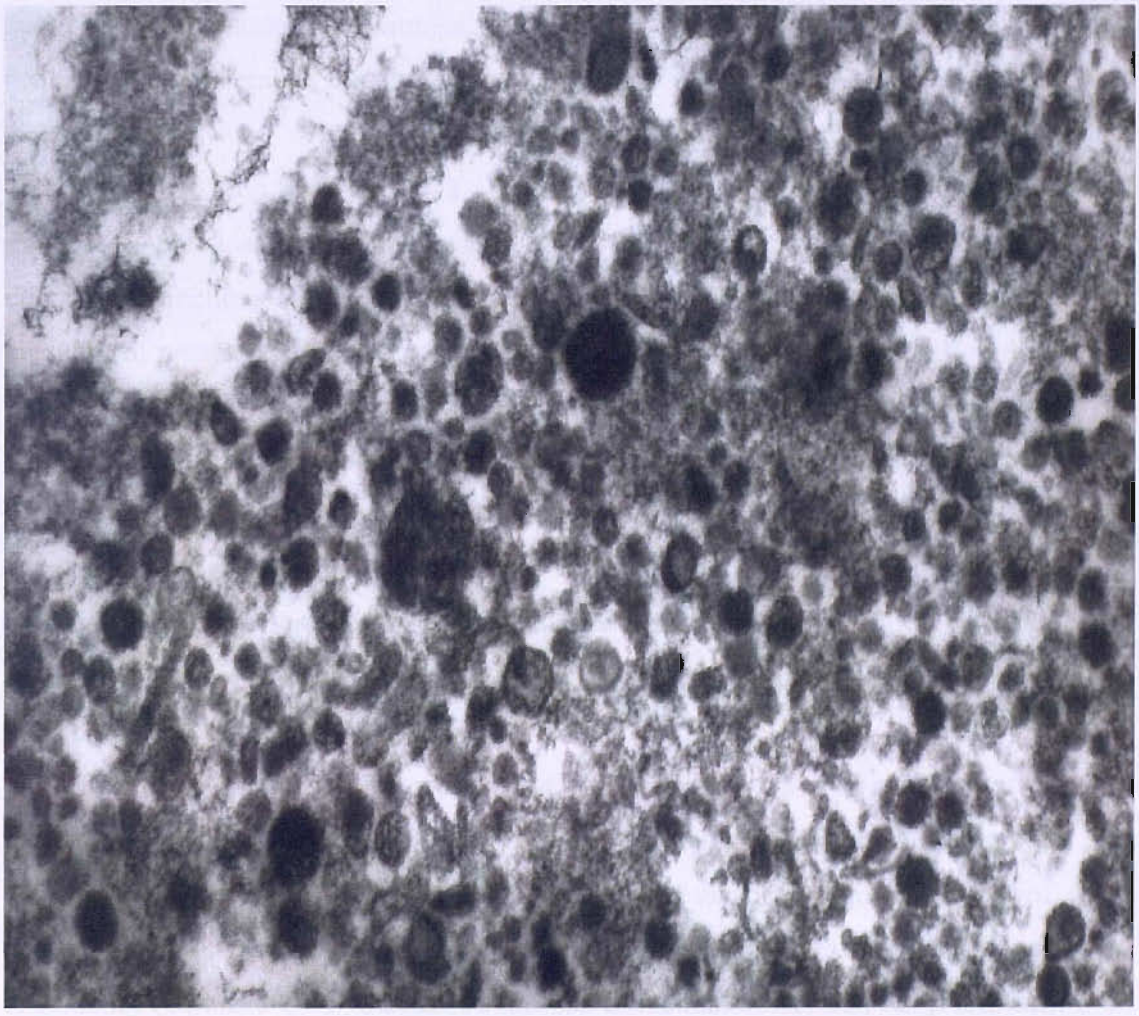


Figure 3.4

TEM of purified seminal prostasomes x 40 000.



3.4 Discussion

The scanty distribution of prostasomes in native semen may be a true reflection of their density in whole semen, or artifactual due to processing. The results of TEM on native prostasomes has not been previously described in the literature. Our preparation schedule may have decreased the apparent concentration of prostasomes. Preparing liquid samples for TEM is more difficult than solid samples. In our method whole semen was mixed with fixative then centrifuged to embedded it into an alginate drop at the bottom of an epindorf tube. The alginate/specimen gel was then set. It is possible that the centrifugation excluded prostasomes, although one would hope with a short gentle spin the sample would embed into the alginate en masse.

TEM in combination with the APTT and aminopeptidase assay confirmed that our prostasome purification technique was sound. Other (biochemically non prostasome containing) fractions of the Superdex column were examined using TEM to exclude the presence of prostasomes. However as previously mentioned TEM of clear liquid is difficult and TEM of clear non prostasome containing Superdex fractions revealed no particulate mater, only the alginate was seen. We can not exclude small particulate matter in these fractions as they might not have been embedded into the alginate during preparation and thus lost in the supernatant. Given the biochemical evidence it is reasonable to conclude that prostasomes only occur in the previously mentioned fractions from the Superdex column.

Unfortunately immunolabelling prostasome bound tissue factor with anti tissue factor antibodies did not succeed due to technical difficulties and could not be repeated for reasons of cost and time. Previous studies suggest that the anti coagulant activity of semen is due to tissue factor that is prostasome-bound. (Fernandez 1997). However there is evidence (Lwaleed 2003) that active seminal tissue factor may be both prostasome bound and free.

Chapter 4

Purification & Biochemical Properties of Prostasomes

4.1. Aims

To reliably and consistently purify prostasomes from human semen for use in later research. To confirm the presence of purified prostasomes by means of aminopeptidase assay and APTT assay. To quantify the protein concentration of purified prostasomes and to study the effects of freeze thawing at various stages of the purification process as it was most practical to freeze semen and purified prostasomes to allow more efficient batch wise processing.

4.2. Introduction

4.2.1 CD13 (Aminopeptidase)

Prostasomal CD13 (Aminopeptidase: 150Kdaltons) a zinc dependent proteolytic enzyme has been found to be a useful marker of prostasomes. (Laurell 1982). Thus an aminopeptidase asay was used to detect the prostasome containing fraction from gel filtration of ultra-centrifuged seminal plasma.

4.2.2 Prostasome bound tissue factor

The pro-coagulant activity of seminal serum when added to human blood plasma was first described in 1942. (Huggins 1942). In 1997 Fernandez et al demonstrated that this effect was due to prostasome bound tissue factor. We therefore used an APTT assay to detect pro-coagulant activity in purified seminal prostasomes to further detect the prostasome containing fractions produced from gel filtration.

4.3 Methods

Prostasomes were prepared as described in chapter 2. Electron microscopy, aminopeptidase assay, APPT assay, Bradford assay and gel filtration were all performed as previously described.

4.3.1 Effects of freezing

Prostasomes were produced from;

a) Fresh semen

b) Frozen semen

The results of the aminopeptidase, APTT and Bradford dye assays were compared to investigate the effects of a freeze thaw cycle. In addition prepared prostasomes were frozen and the results of aminopeptidase assay, Bradford assay and gel electrophoresis compared.

4.3.2 Gel filtration

2-D gel electrophoresis was performed on purified seminal prostasomes, other non-prostasome containing fractions from the column and the supernatant II.

4.4 Results

Prostasomes were reliably prepared from vasectomised semen. The tests to confirm their presence are reported below. Multiple batches of independently purified prostasomes were created from different samples of pooled semen. Results were virtually identical between different batches of purified prostasomes. All charts thus represent the results of a typical batch of prostasomes.

4.4.1 Ultra-centrifugation

Supernatant 1 was a clear straw coloured liquid. Ultra-centrifugation of seminal plasma (supernatant 1) produced a small solid pellet (pellet 2) that was quite difficult to re-suspend. The volume of PBS used to re-suspend pellet 2 was kept to a minimum to prevent diluting the prostasomes.

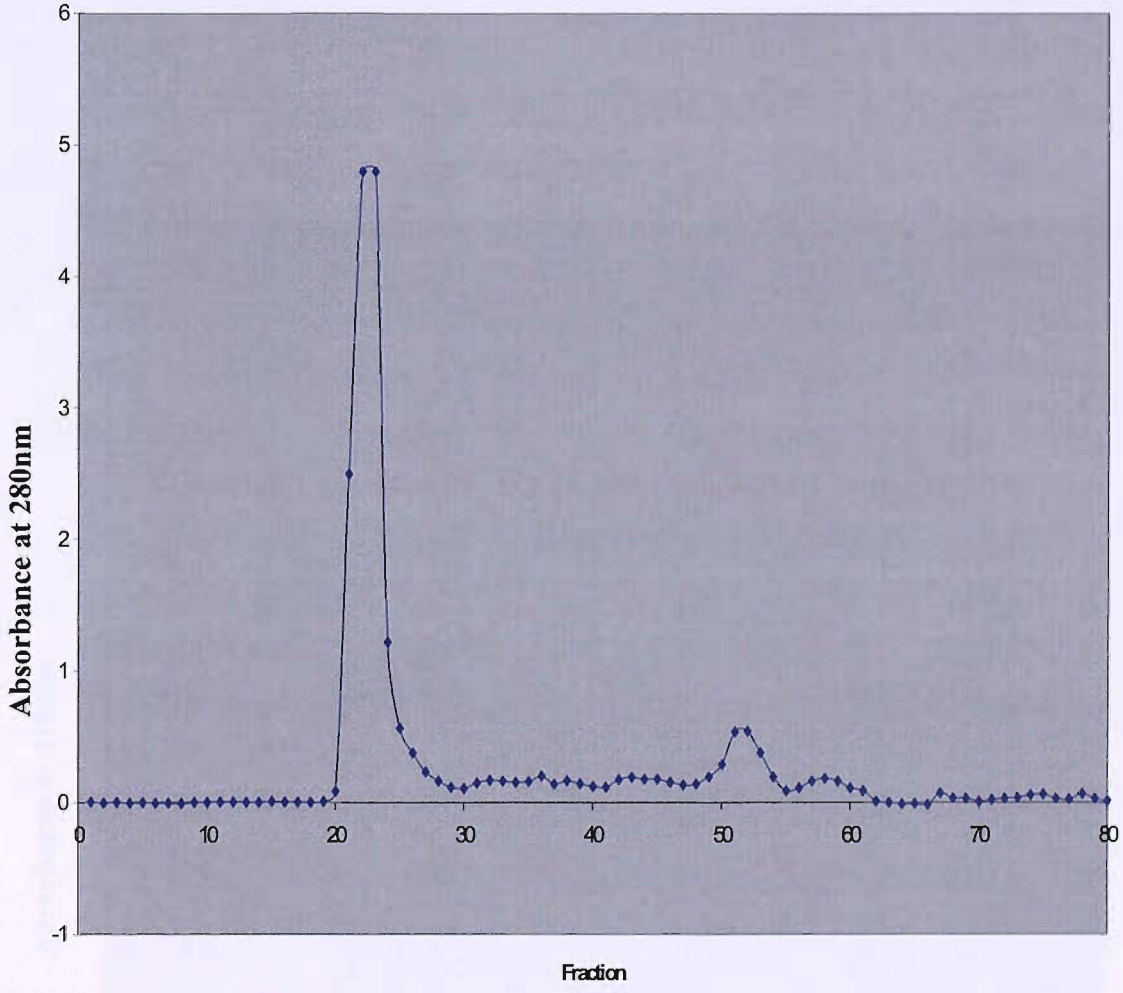
4.4.2 Gel filtration

Consistently fractions 21-24 from the Superdex 200 gel column filtrate were turbid and contained the peak UV absorbance. (Figure 4.1). All other fractions were clear. A second much smaller peak of UV absorbance was noted, occurring in fractions 52-53.

4.4.3 Electron microscopy

Electron microscopy of fractions 21-24 confirmed the presence of prostasomes. They were absent in the other fractions examined. (Chapter 3).

Figure 4.1 Typical UV absorbance of the Superdex 200 fractions at 280nm



4.4.4 Aminopeptidase assay

The aminopeptidase activity was maximal in the fractions with maximal UV absorbance. The results were similar for both substrates used, L-Leucine-p-Nitroanilide (figure 4.2) and L-Alanine-p-Nitroanilide (figure 4.3). Figure 4.4 shows maximal aminopeptidase activity occurring after approximately 20 minutes.

Figure 4.2 Typical aminopeptidase activity (L-Leucine-p-Nitroanilide) of the Superdex 200 fractions produced from resuspended pellet II

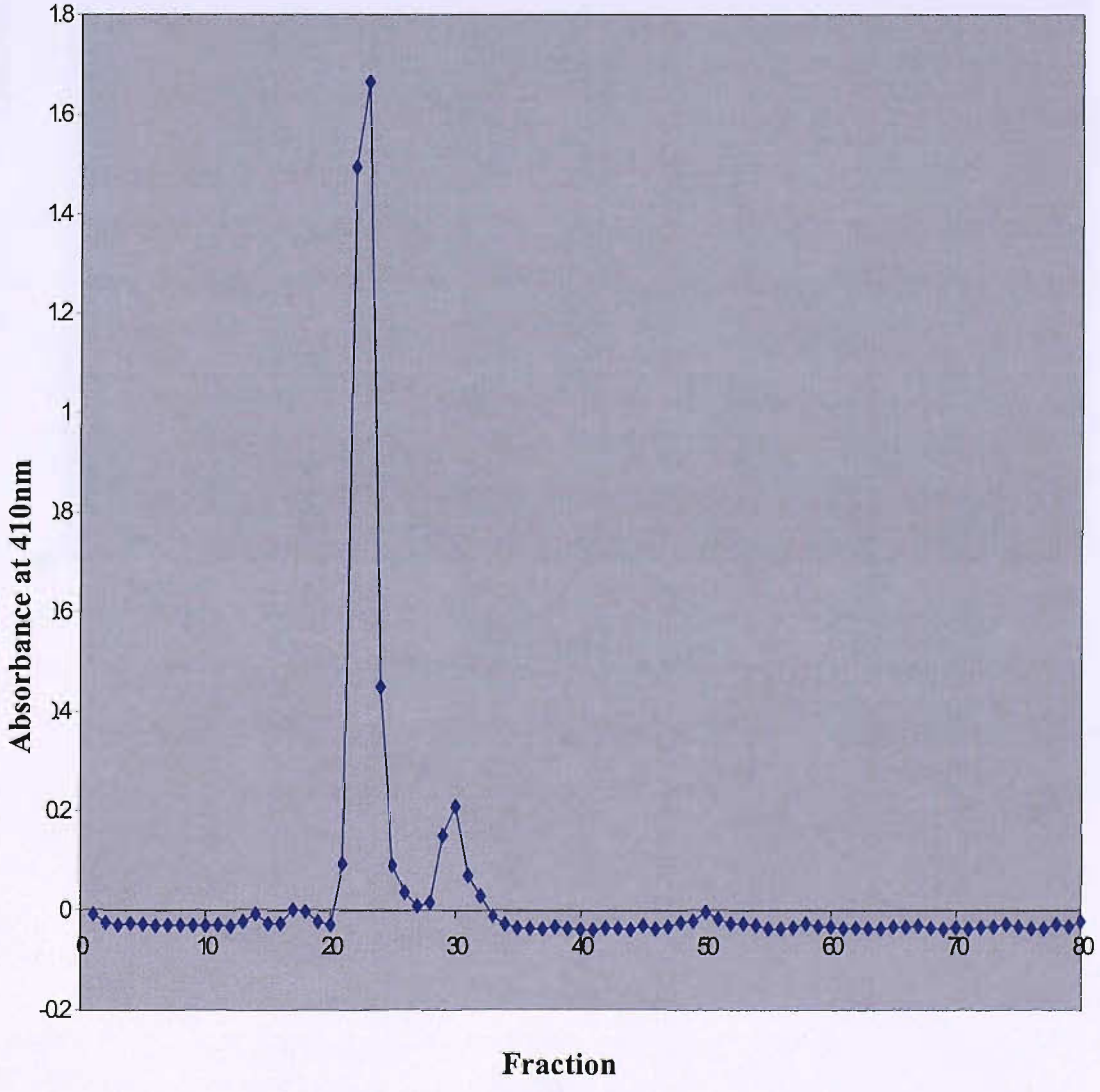


Figure 4.3 Typical aminopeptidase activity (L-Alanine p-Nitroanilide Substrate) of the Superdex 200 fractions produced from resuspended pellet II.

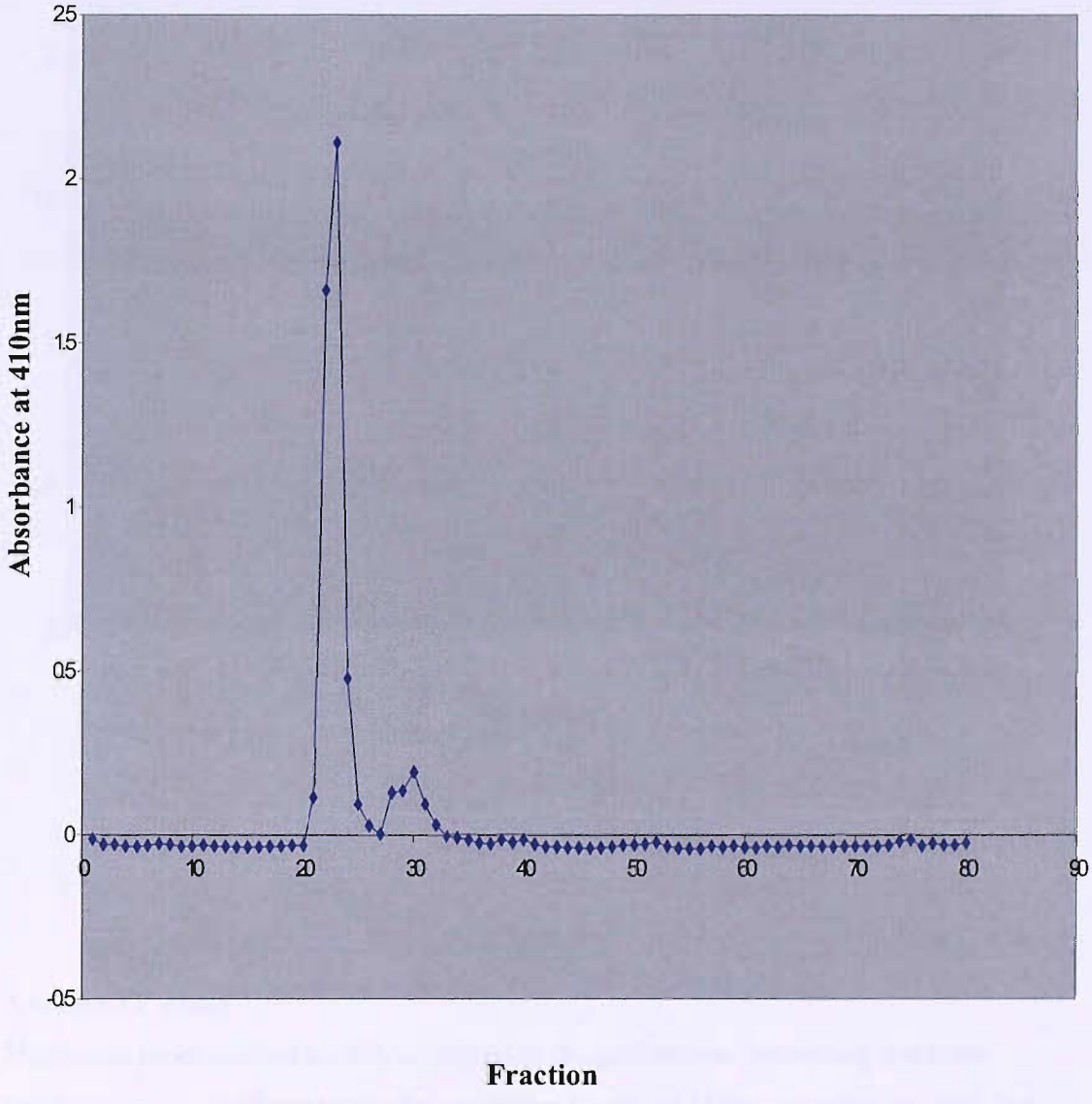
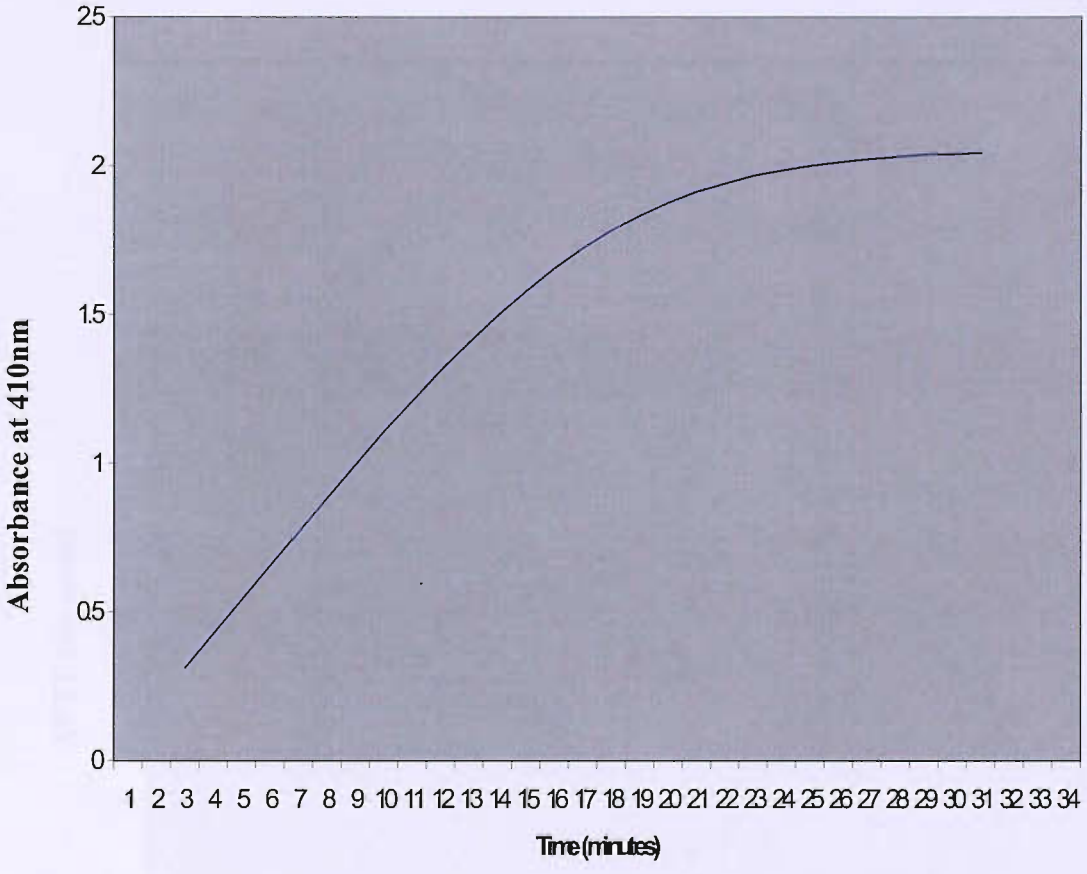


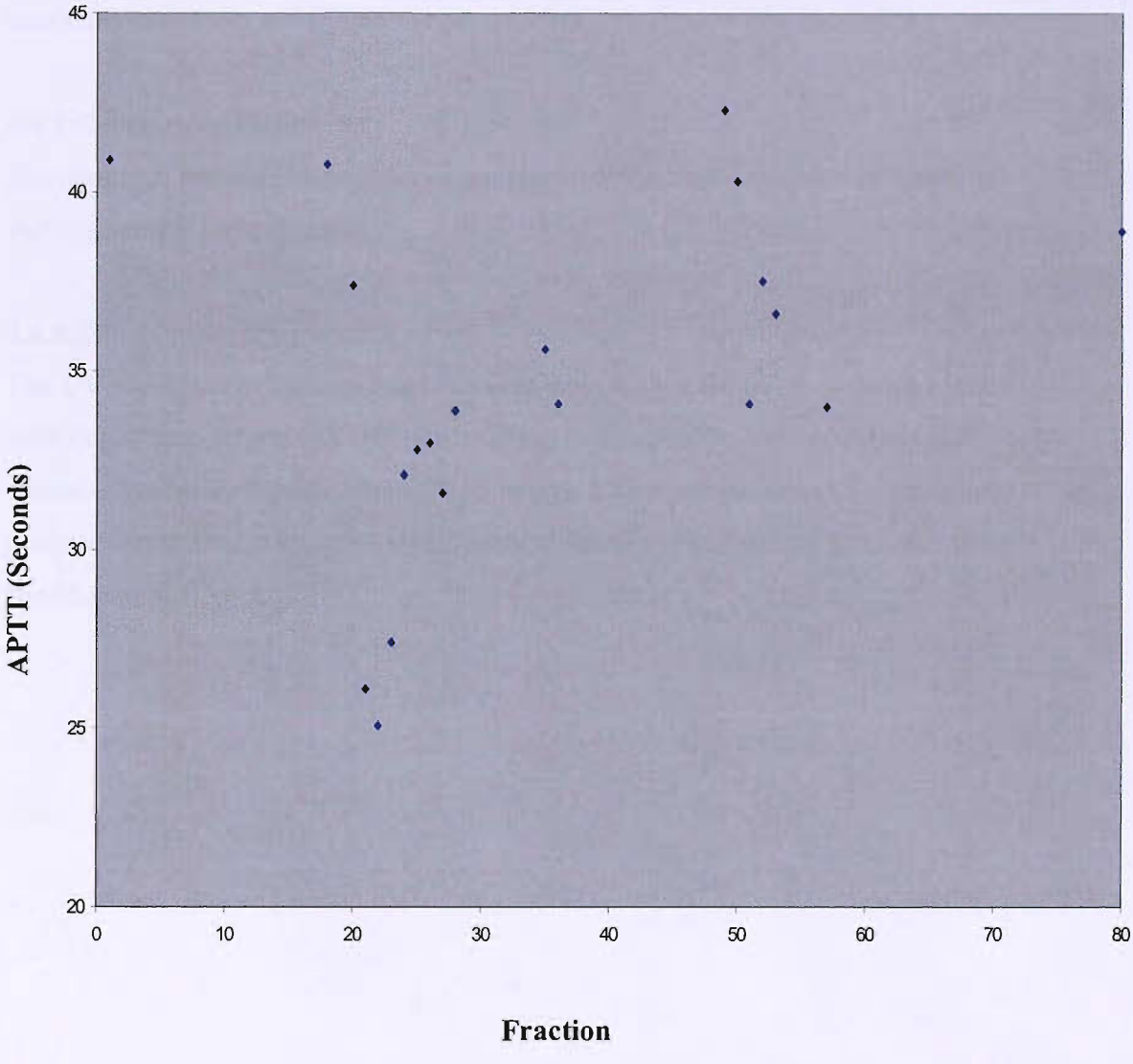
Figure 4.4 Prostasomal aminopeptidase activity. (L-Leucine-p-Nitroanilide).



4.4.5 APTT assay

Maximum procoagulant activity occurred in the prostasome containing fractions produced from gel filtration of ultracentrifuged seminal plasma as indicated by a low APTT. (Figure 4.5).

Figure 4.5 Procoagulant activity of the superdex 200 fractions as measured by APTT assay



4.4.6 Bradford assay

Using the Bradford assay the protein concentration of our prostasome solution was consistently estimated to be close to 0.3mg/ml. This result was the same for prostasomes obtained using fresh and frozen semen.

4.4.7 Gel electrophoresis

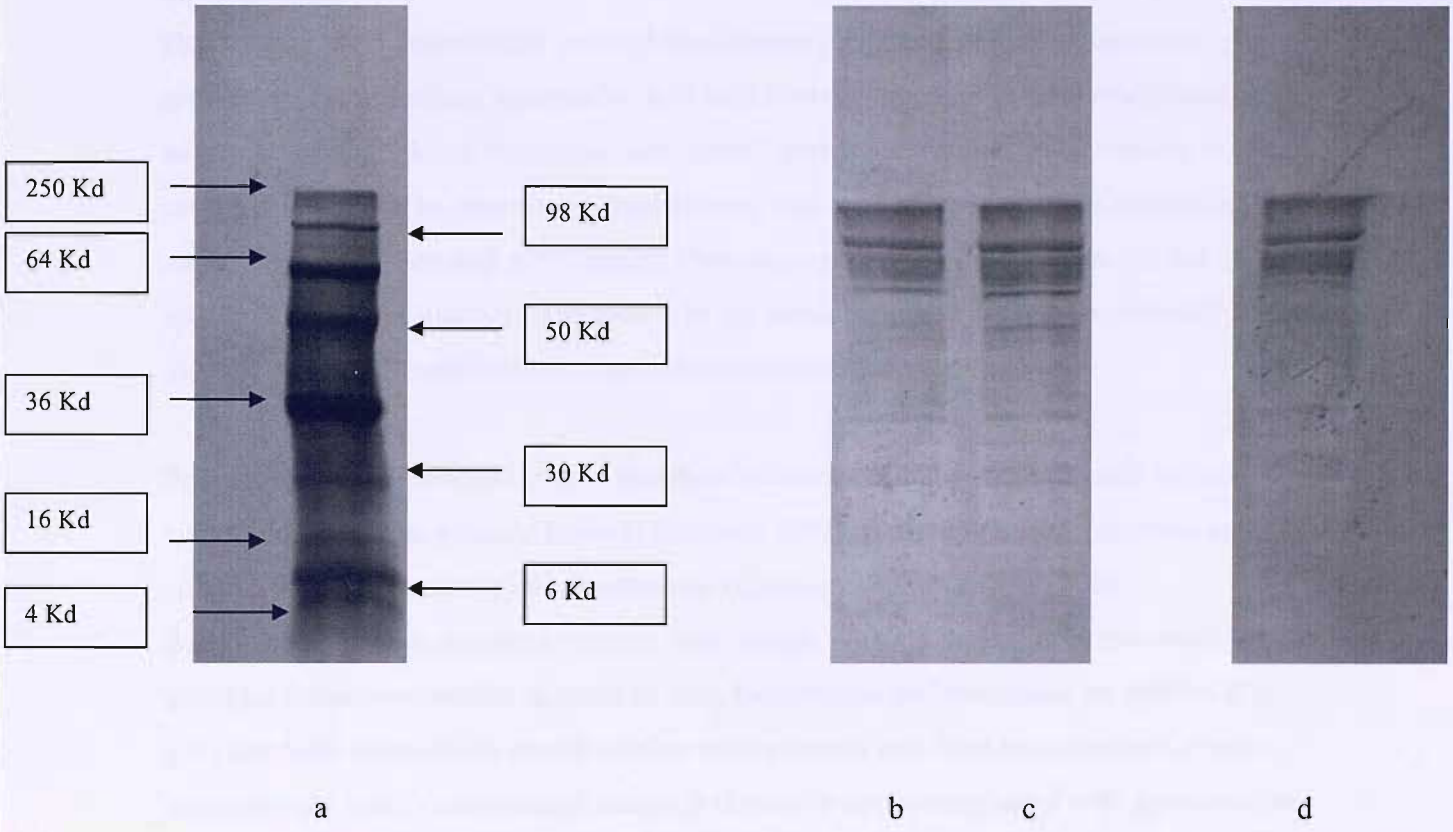
The results of protein electrophoresis are shown in figure 4.6. At least 20 bands of varying density were apparent.

4.4.8 Effects of freezing semen

The UV absorbance characteristics obtained from the gel filtration fractions of ultra-centrifuged seminal plasma was similar using fresh semen or thawed semen as were the results of the aminopeptidase and APTT assays. Gel electrophoresis of prostasomes produced from fresh semen revealed identical bands to prostasomes produced from frozen semen. (Figure 4.6)

Figure 4.6: 2-D SDS PAGE gel electrophoresis of prostasomes

a; Protein standard. b; Using fresh pooled semen and freshly prepared prostasomes. c; Using thawed frozen semen and freshly prepared prostasomes. d; Using thawed frozen semen and thawed frozen prostasome preparation.



4.4.9 Effects of freezing prostasomes

Aminopeptidase activity was virtually identical in freshly produced and thawed prostasome samples. The APTT assay was only undertaken using thawed prostasome samples. Freezing and thawing prostasomes did not produce any obvious new bands on gel electrophoresis.

4.5 Discussion

Prostasomes were successfully isolated from semen obtained from post vasectomy specimens. Frozen semen appeared to be a satisfactory source for prostasome production as prostasomes produced from fresh and thawed semen had similar characteristics in the assays performed. The presence of prostasomes was confirmed by electron microscopy, amino peptidase assay and APTT assay. Freezing and thawing prostasomes did not appear to alter their function. The results of the aminopeptidase assay were virtually identical when different batches of prostasomes were analysed.

Prostasomes can be isolated from a variety of sources, normal semen, infertile semen, vasectomised semen, prostate tissue (Fernandez 1997), prostatic massage, prostate cell culture and prostate cancer bone metastasis. (Carlsson 2003). Carlsson 2003 demonstrated that prostasomes isolated from semen, prostate bone cancer metastasis and prostatic tissue were similar in terms of size, biochemical and functional properties. It is not clear from the methods stated whether native semen was from vasectomised or non vasectomised men. Vasectomised semen is obviously not contaminated with spermatozoa and other debris and secretion from the testis and epididymis and is arguably therefore a preferred source of prostasomes when studying the role of prostasomes in prostatic disease. Thus we decided to use vasectomised semen specimens. Whether or not prostasomes in non-vasectomised semen have the same properties as those from vasectomised semen is a question that remains unanswered.

The most marked procoagulant activity as detected by a low APTT was seen in the prostasome containing fractions from the Superdex 200 column. Variable procoagulant

activity was seen in the other fractions with activity matching the second peak seen on spectrophotometry.

Tissue factor is the main physiological initiator of normal blood coagulation. (Guha 1986, Kang 1988, Bach 1988). Prostasome bound tissue factor is believed to be responsible for the procoagulant activities in human semen. (Fernandez 1997). Semen liquefaction is required for successful delivery and transit of semen in the female genital tract. (Pauber 1976, Mandal 1986). Procoagulant activity is also believed to prevent bleeding and spread of haematogenous infection at sites of abrasion within the female genital tract. (Fernandez 1997).

The procoagulant activity seen in the prostasome containing fractions is arguably due to prostasome bound tissue factor. The variable level of procoagulant activity in the other fractions may be due to free tissue factor or other coagulation triggering factors. The presence of free tissue factor in the supernatant of ultracentrifuged seminal plasma has been described by (Lwaleed 2003). Our findings support this. Indeed Lwaleed et al found that seminal tissue factor expression was inversely related to fertility, possibly due to abnormal semen liquefaction and capacitance (Lwaleed 2004 & 2006).

Histological preparation of prostate tissue shows tissue factor antigen on normal cells but it is absent on malignant cells. (Callander 1992).

Chapter 5

Anti-Prostasome Antibodies in Prostate Cancer

5.1 Introduction

Prostate cancer cells generate prostasomes (Nilsson 1999) as do prostate cancer metastasis (Carlsson 2003). Antibodies to prostasomes have been detected in the serum of patients with prostate cancer. A pilot study by Nilsson et al using an enzyme linked immunosorbent assay (ELISA) demonstrated markedly raised serum levels of antibodies to prostasomes in 13 patients with prostate cancer (Nilsson 2001). The patients in this study were a heterogeneous group of prostate cancer patients with serum PSA between 50-500mcg/l. The healthy control subjects (n=39, men and women) with normal PSA values had background levels only and there was no overlap between control and cancer group. The metastatic status of the patients in this study was not recorded although with PSA values greater than 50 extra-prostatic and metastatic disease is likely (Partin 1993).

Nilsson postulates that only in metastatic or locally invasive prostate cancer should prostasomes escape into the vascular and lymphatic systems thus inducing antibody production thus Nilsson suggested that anti prostasome antibodies (APA) could be a new marker for prostate cancer and that APA might be specific to metastatic disease. If true, this would have great clinical ramifications not only as a prognostic marker but also on the natural history of the disease. Serum prostate specific antigen (PSA) is currently the best tumour marker available for prostate cancer. However it does have its limitations, it is relatively non specific test for prostate cancer and further investigations are required to confirm the diagnosis and metastatic status.

If true, the role of APA as a new marker for metastatic prostate cancer would be of great potential clinical significance.

5.2 Aims

The aim of this pilot study was to assess the presence of antibodies to prostasomes in patients with prostate cancer, and determine the associations, if any, between antibodies to prostasomes and the aetiology and progression of this prevalent disease.

5.3 Specific methods

5.3.1 Prostasome preparation

Prostasomes were prepared from pooled vasectomised semen as described in chapter 2.

5.3.2 Sample collections

Ethical committee approval was granted for the study by the Southampton and South West Hampshire Local Research Ethics Committee, UK (submission number 045/03/w). Informed consent was sought from all subjects. Blood specimens were obtained from healthy volunteers (7 males, mean age 32 years, range 29-35) and patients with prostate cancer (22 males, mean age 74, range 59-86). The cancer patients were a heterogeneous group with a PSA range between <0.06-520 ng/ml and Gleason score range of 3-9 (Table 5.1). Blood samples were spun at 9,000g for 10 minutes at room temperature. Serum specimens were immediately obtained and stored at -72°C for batch-wise ELISA analysis.

5.3.3 Serum APA

Serum APA were assayed using an ELISA as described by Nilsson et al 2001 and Carlsson et al 2004. In principal, 96 well microplates (Nunc Polysorp Immunoplates) were coated with prostasomes in sodium carbonate coating buffer (pH 9.6) and incubated for 2 hours at 37°C then washed with sodium carbonate buffer. To block non-specific binding 3% bovine serum albumin was added into each well and plates incubated at 37°C for 1 hour. This was followed by 3 washes with PBS in 0.1% TWEEN. Test serum (1:50 diluted in PBS) was added and plates incubated for a further 90 minutes at 37°C. (Negative controls were performed by adding PBS without test serum). Plates were then washed and an anti-human IgG peroxidase conjugated antibody (1:30,000 dilution; Sigma, Poole, UK) added. Plates were then incubated in the dark for 30 minutes at room temperature, then washed twice with PBS/TWEEN and then once with PBS alone. Orthophenylene Diamine Dihydrochloride (Sigma Fast TM, Poole, UK) was added for 15 minutes and the reaction stopped with 0.5% sulphuric acid. The absorbance values were then read at 490nm. Wells were duplicated; the ELISA was performed twice, with

independently pooled and purified prostasomes. Results were consistent between prostasome batches.

5.3.4 Serum PSA

Prostate specific antigen was measured in fresh serum using the IMx^{R} PSA assay system according to the manufacturer's instructions (Abbott Laboratories, USA). Prostate cancer patients PSA levels at diagnosis were obtained from their medical notes.

5.3.5 Statistical analysis

Results were entered in a database and analyzed by the *STATGRAPHICS*^{TM} statistical software system. Data were not normally distributed, so summary statistics are expressed as medians and inter-quartile ranges (IQR). The median value is the 50th centile. The 25th and 75th percentiles of the data specify the values covered by the IQR. Differences between two groups were assessed by Mann-Whitney U-Test. The PSA values were log-transformed and correlations were determined using Spearman's Rank Correlation Test. $P<0.05$ was considered to be statistically significant.

Table 5.1: Patient characteristics and ELISA absorbency values.

Patient	Age	Gleason Score	Disease Duration (Months)	PSA	PSA at diagnosis	Proven metastasis	Hormonal Treatment	Surgery	Radiotherapy	ELISA mean absorbance
1	74	7	96	8.1	-	N	Y	N	Y	0.183
2	82	8	1	180	180	N	N	N	N	0.2745
3	76	7	12	<0.06	78	N	Y	N	N	0.706
4	74	9	8	<0.06	64	N	Y	N	N	0.9355
5	62	6	13	130	300	N	Y	N	N	0.152
6	81	5	3	19	18	N	N	N	N	0.173
7	73	3	60	13	14	N	N	N	N	0.172
8	80	9	60	67	470	N	Y	N	N	0.237
9	64	6	6	9.7	9.7	N	N	N	N	0.227
10	75	4	36	12	14	N	N	N	N	0.172
11	77	9	29	8.2	540	Y	Y	N	N	0.3395
12	69	7	2	520	2200	Y	Y	N	N	0.1005
13	80	7	54	0.34	49.2	N	Y	TUIP	N	0.3385
14	56	6	22	0.69	9.3	N	Y	N	Y	0.318
15	73	9	6	2.7	51	Y	Y	N	N	0.14
16	81	7	26	0.67	30	N	Y	N	N	0.118
17	70	7	25	<0.06	20	N	Y	N	Y	0.1205
18	62	4	36	1.8	1.6	N	N	TURP	N	0.343
19	86	-	7	2.6	380	N	Y	N	N	0.4785
20	78	8	48	0.26	5.1	N	Y	N	N	0.576
21	84	7	120	2.3	13	N	Y	TURP	N	0.2985
22	65	6	2	5.4	5.4	N	N	N	N	0.134

ELISA = Enzyme linked immunosorbent assay. PSA = Prostate specific antigen. TUIP = Transurethral incision of the prostate. TURP = Transurethral resection of the prostate.

5.4 Results

5.4.1 Antiprostasome antibody levels

Details of the patients with prostate cancer used in this study are shown in table 5.1. All subjects tested had variable levels of binding of IgG antibodies to prostasomes. (Figure 5.1). There were a number of individuals with markedly raised levels of anti-prostasome antibodies, among these a healthy male control subject. The absorbance interval values for the prostate cancer patients were 0.10 – 0.93 (median 0.23, IQR 0.15-0.34) and those for the control group were 0.14 – 0.81 (median 0.21, IQR 0.16-0.42). Statistically there was no significant difference in anti-prostasome antibody occurrence between the control group and prostate cancer patient group. (p=0.7379). Background readings from control wells were minimal.

5.4.2 Correlation of APA and PSA

In patients with prostate cancer, anti-prostasome antibodies were inversely and significantly correlated to log-PSA levels (r = -0.5; p= 0.04, Figure 5.2). Four prostate cancer patients with the most markedly raised antibody levels were all being successfully treated with hormonal androgen ablation with very low or undetectable PSA levels. When PSA at diagnosis is considered there is no correlation. (r = - 0.05 p= 0.83, Figure 5.3).

Figure 5.1: Serum anti-prostasome antibodies in normal controls and patients with prostate cancer. Results are shown as median and inter-quartile ranges (IQR). The bottom and top of the "box" represents the 25th and 75th centile, while the line within the box represents the median value. Binding of APA to prostasomes is measured by the UV absorbance at 490nm

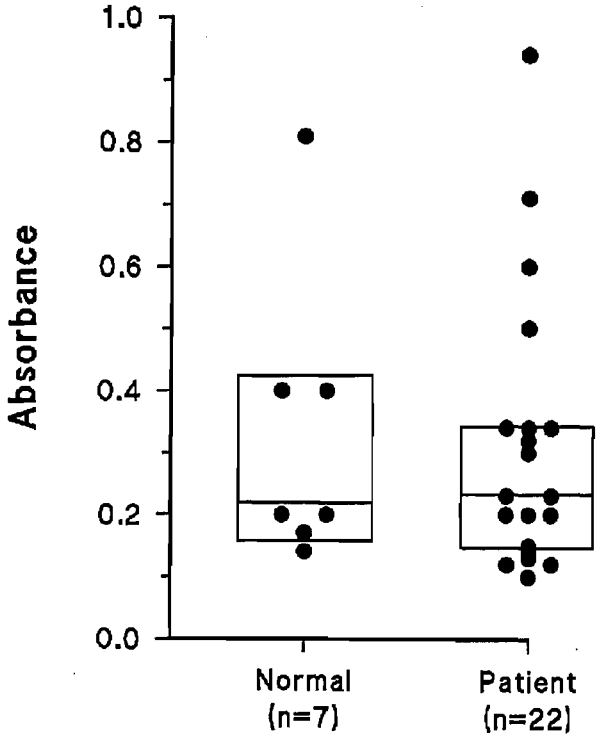


Figure 5.2: Correlation between anti-prostasome antibody and log-prostate specific antigen. Binding of APA to prostasomes is measured by the UV absorbance at 490nm

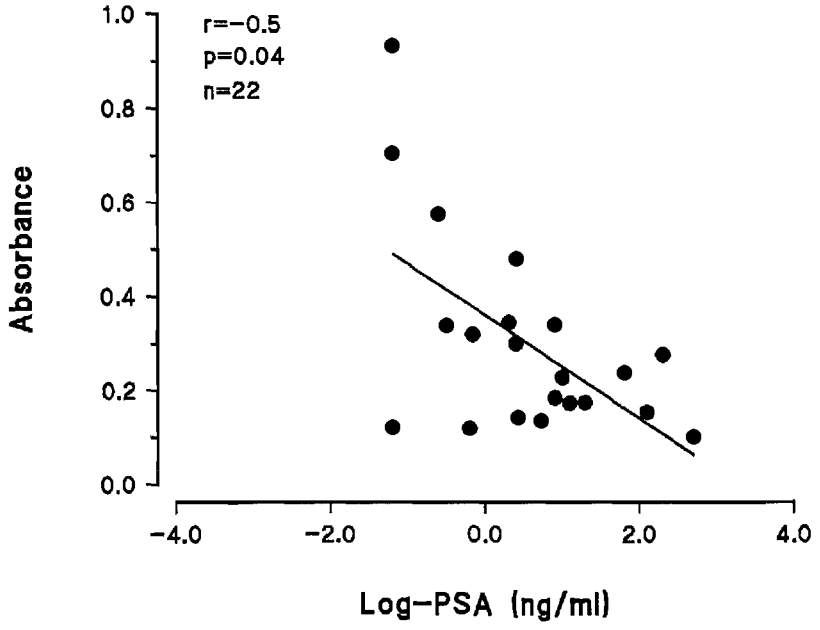
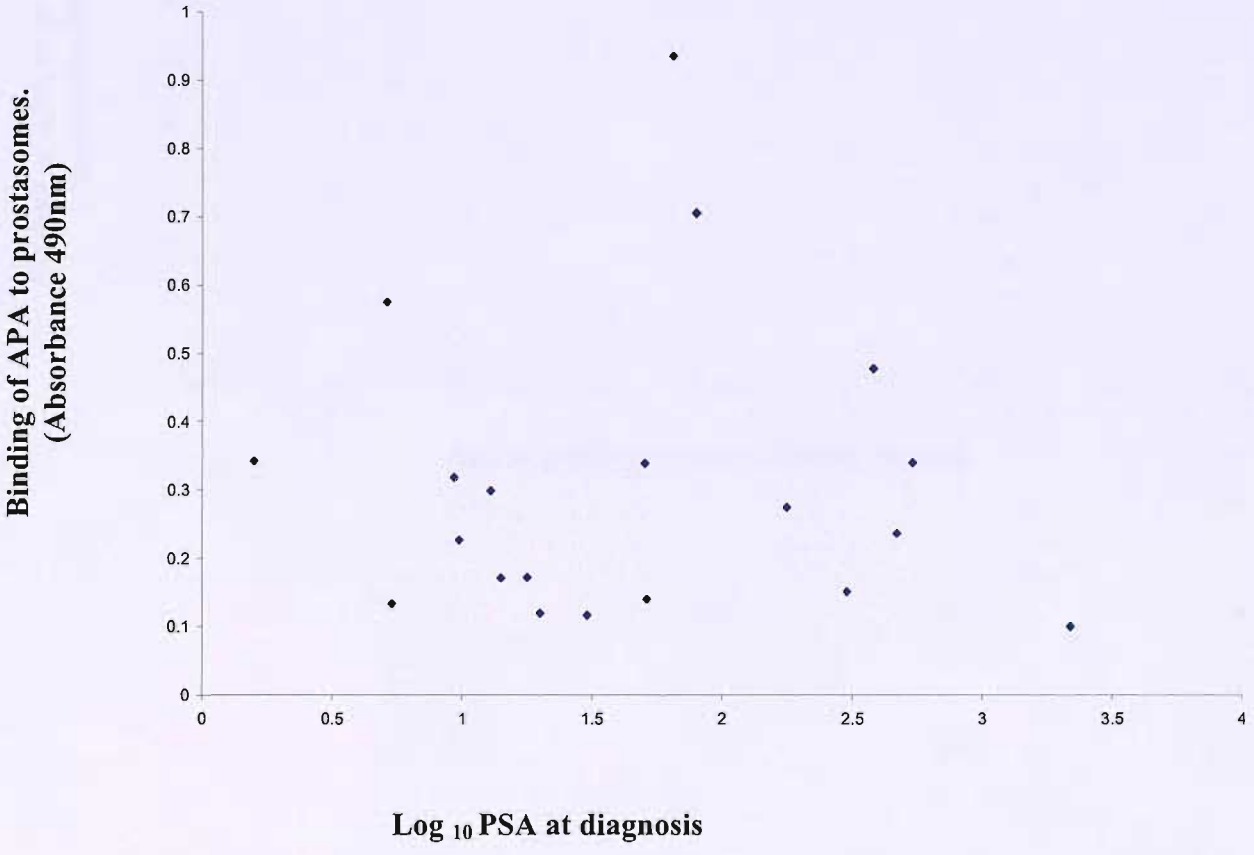


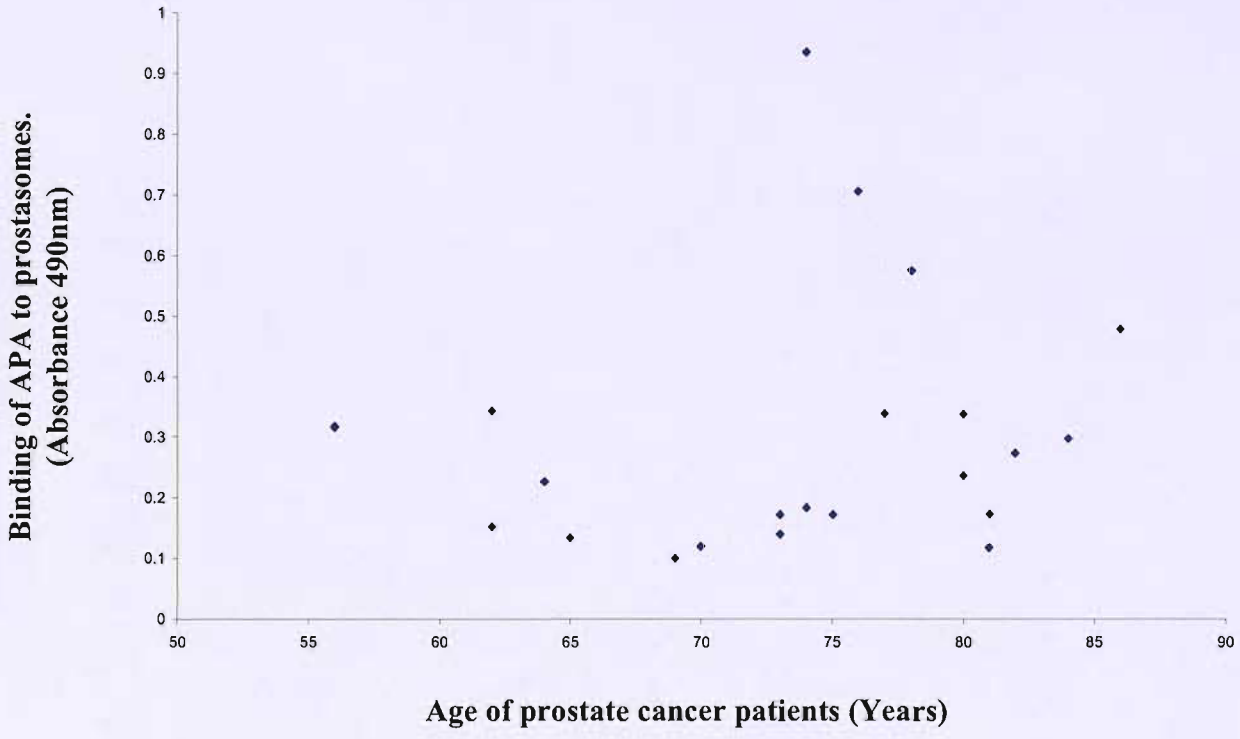
Figure 5.3: Correlation between anti-prostasome antibody and log-prostate specific antigen at diagnosis



5.4.3 The relationship between APA and age

There was no correlation between age and APA levels. Correlation coefficient (r) = 0.1882 (Pearson), p=0.4. Figure 5.4.

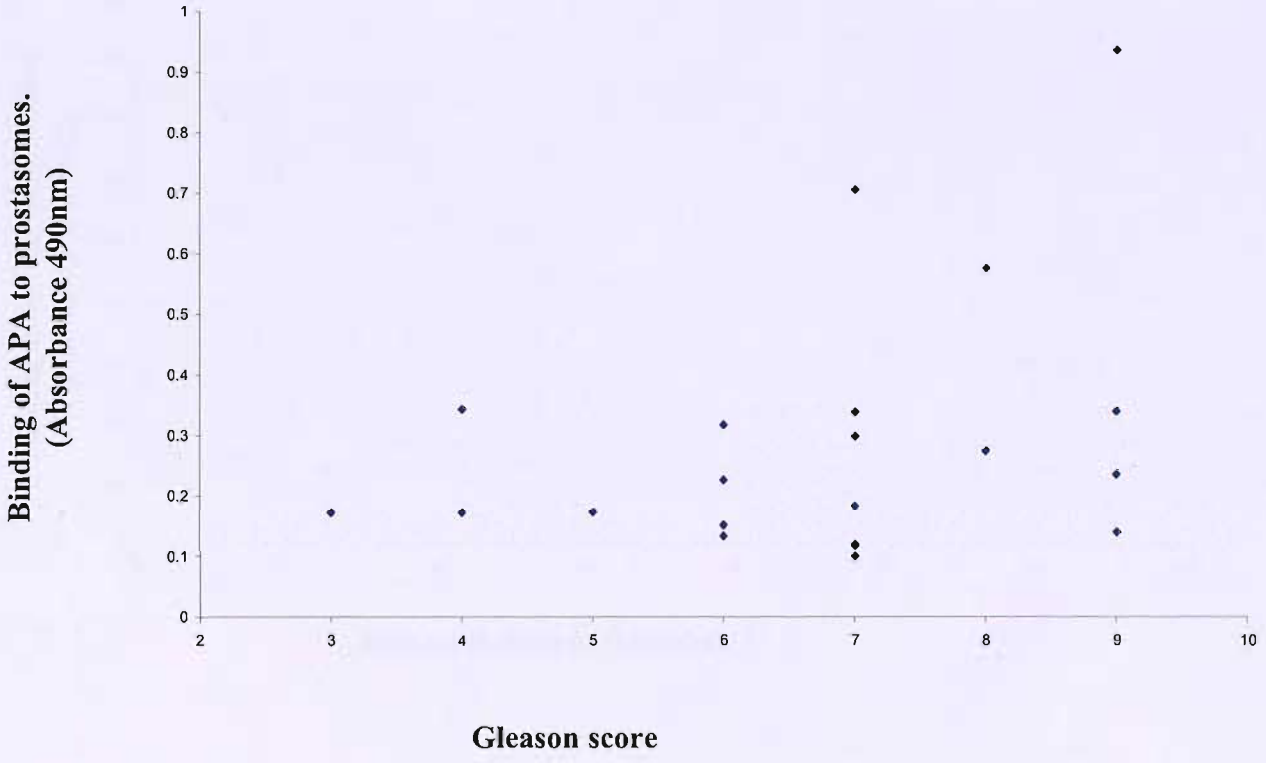
Figure 5.4: Correlation between APA and patient age



5.4.4 APA and Gleason score

There was a trend for anti-prostasome antibody levels being associated with poorly differentiated high Gleason grade prostate cancer. However the correlation was not significant. Correlation coefficient (r) = 0.3418, P= 0.1294. Figure 5.5.

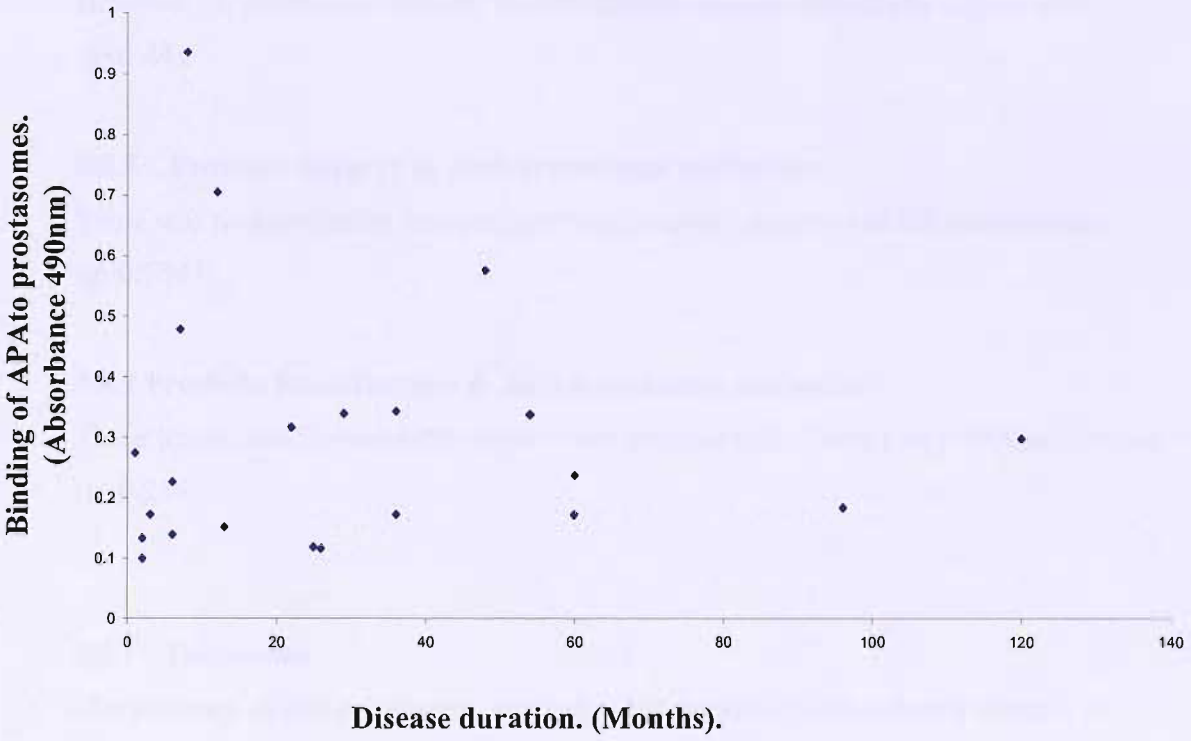
Figure 5.5: Correlation between APA and Gleason score



5.4.5 APA and disease duration

There was no significant correlation between duration of disease and APA levels. ($r = -0.06822$ $p = 0.7629$). Figure 5.6.

Figure 5.6: APA and disease duration.



5.4.6 Metastasis & anti-prostasome antibodies

Patients with metastatic disease had lower levels of APA (n=4; median=0.15; IQR=0.12-0.25) compared to those without (n=18: median=0.3; IQR=0.2-0.34) however the difference was not significant (p= 0.095) in part due to the low number of patients with confirmed metastasis.

5.4.7 The effect of hormonal androgen ablation treatment on APA levels

Patients being successfully treated with hormonal androgen ablation with low or undetectable PSA levels, had higher APA levels (n=15; median=0.3; IQR=0.14-0.48) compared to those not on hormonal treatment (n=7; median=0.17; IQR=0.17-0.27). However the difference between the two groups was not statistically significant. (p=0.44).

5.4.8 Prostatic Surgery & Anti-prostasome antibodies

There was no association between previous prostatic surgery and APA occurrence. (p=0.5344).

5.4.9 Prostatic Radiotherapy & Anti-prostasome antibodies

There was no association between previous prostate radiotherapy and APA occurrence. (p=0.236).

5.5 Discussion

The presence of anti-prostasome antibodies has previously been shown to occur in patients with metastatic prostate cancer (Nilsson 2001, Larsson 2006). As a result it has been suggested that APA could be a marker of prostate cancer and perhaps a specific marker of metastatic prostate tumours (Nilsson 2001, Larsson 2006). Nilsson et al found raised levels of APA in 13 patients with prostate cancer and serum PSA between 50-500mcg/l (Nilsson 2001). The healthy control subjects (n=39) with normal PSA values had background levels only and there was no overlap between control and cancer groups. The metastatic status of these patients was not recorded, however with PSA values of greater than 50 extra-prostatic and metastatic disease was likely. Nilsson et al postulated that only in metastatic or locally invasive prostate cancer should prostasomes escape into the vascular and lymphatic systems thus inducing antibody production. (Nilsson 2001).

Due to the potential clinical significance of such a finding the present study was undertaken. However, the current results are not in agreement with previously published

reports on serum APA (Nilsson 2001). This study found quantifiable levels of serum APA in both healthy controls and prostate cancer patients. This discrepancy might be due to the different source of prostasomes used as the primary antigen for the ELISA assay. In Nilsson's study prostasomes were prepared from "prostatic tissue" while in this study prostasomes were prepared from vasectomized semen specimens as described above. The source of prostatic tissue in Nilsson's study was not clearly stated. One can assume however, that human prostatic tissue was either obtained from radical prostatectomy specimens, transurethral prostate resection (TURP) specimens or trans-rectal prostatic biopsy specimens. Carlsson et al 2003, reported the preparation of prostasomes from human prostate obtained from radical cysto-prostatectomy for transitional cell carcinoma of the bladder. This would seem the most likely source, as the prostate should not have any malignant disease within it, not have diathermy artefact (caused by TURP) and be a good size specimen (needle biopsies are very small). Inevitably, prostate derived from radical cystoprostatectomy would have evidence of benign enlargement. The source of our prostasomes for use in this ELISA was from semen of healthy young men who had recently had vasectomies. We assumed that these prostasomes were in no way structurally altered by prostatic disease of any kind.

The fact that Nilsson's group chose prostatic tissue as a source of prostasomes for this experiment is intriguing and raises the possibility that prostasomes obtained from different sources might be different and in particular express different proteins that are antigenic to APA. There might be differences in prostasome behaviour and protein expression between individuals. Indeed prostasomal proteins might vary according to prostatic disease state or stage of physiological development (Utleg 2004).

As mentioned above, in our hands, APA occur in varying degrees in healthy controls and in patients with prostate cancer alike. The APA were not related to age or time since diagnosis and we observed no association between APA and other markers of prostatic disease progression such as metastatic status or Gleason histological score. Anti-prostasome antibodies were also inversely and significantly correlated with serum PSA ($r=-0.5$; $n=22$; $P=0.04$; Figure 3). Furthermore, our finding of markedly raised serum

APA in patients with hormone sensitive prostate cancer being successfully treated with androgen ablative hormonal therapies, with a very low or undetectable PSA levels, rather contradicts the evidence that prostasome secretion is an androgen dependant process. (Stegmayr 1980 & Ronquist 1984). However from our results, one cannot determine at what stage of disease progression APA appear or whether prostasomal secretion from primary or metastatic tumour can occur independently of androgen stimulation.

In our control group, one subject, a healthy 28-year-old male with no history of prostatic disease had markedly raised levels of serum anti-prostasome antibodies. Anti sperm antibodies (ASA) are known to be associated with infertility in men and women and prostasomes have been shown to be antigenic for ASA. (Allegrucci 2001, Carlsson 2004 a & b). This does raise the possibility that the elevated antibody titre in this subject might be related to his infertility status. Indeed Carlsson (2004) has demonstrated the use of an ELISA based on anti-prostasome antibodies as a new test for immunoinfertile patients with raised ASA. Unfortunately, we have not been able to test the fertility profile of our control subject with raised anti-prostasome antibodies for ethical reasons.

Recent work by Carlsson 2000, suggests that prostasomes have a growth inhibitory effect on prostatic cancer cell lines in culture with the effect being most marked with DU 145 cells. We have work in progress specifically looking at the effects of prostasomes on various aspects of tumour development *in vitro*. (Delves & Stewart et al 2005). One might expect that if prostasomes protect sperm in the female genital tract (Fernandez 1997) they might also modulate the effects of host immune response during malignant transformation of prostatic epithelium. A recent review by Ronquist & Nilsson 2004, suggested that having assisted sperm survival and the acrosome reaction, prostasomes immunosuppressive and anti-complementary activity is actually harmful to the prostatic epithelium, favouring neoplastic malignant transformation.

Due to the large number of prostasome membrane proteins further investigation would be useful to determine whether prostasomal protein phenotype is disease specific and to further characterise the antigenic target(s) against which serum anti-prostasomal

antibodies are directed. Schrimph et al 1999 identified Dipetidyl Peptidase IV (CD26) as the antigen of a monoclonal antiprostasome antibody developed in mice, but experimental immunogenicity in a different species is far removed from autoimmunity.

A further interesting aspect of APA, is that for them to occur prostasomes must be leaking out of their normal luminal domain. Prostate cancer cells generate prostasome like granules (Nilsson 1999) as do prostate cancer metastases (Carlsson 2003, Sahlen 2004). Once in the intercellular matrix there are the possibilities for their associated proteins to affect the course of whatever pathological process caused their presence there. Indeed the possibility of immuno modulation with APA has been suggested. (Nilsson 2001, Morris 2000 & Catalona 1980).

In summary, both normal healthy individuals and patients with prostate cancer have quantifiable levels of anti-prostasome antibodies in their serum. This finding does not seem to agree with previously published reports. Thus the role of anti-prostasome antibodies as a diagnostic and/or prognostic marker for prostate cancer is currently doubtful. However, the immune reactions to prostasomes might be of rather considerable interest and work in our laboratories is continuing to further identify the role of prostasomes in the biology of prostatic disease and in the context of fertility.

Appendix

1. Patient Consent Form.

2. Patient Information Sheet.

Department Of Urology Research,
Mail Point 67, E-Level, West Wing,
Southampton General Hospital,
Tremona Road,
Southampton,
SO16 6YD.
Tel 02380 796838

Patient Identification Number for this trial:

CONSENT FORM
Title of Project:
Studies on prostasomes in prostate disease.

Name of Researcher: Mr Alistair Stewart. MRCS. Urology Research Fellow.

Please initial box

1. I confirm that I have read and understand the information sheet dated 03/03 (version 2) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of my medical notes may be looked at by the urology research team. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient, 1 for researcher

Version 2. 03/03. LREC No 045/03/w.

Department Of Urology Research
Mail Point 67, E-Level, West Wing,
Southampton General Hospital,
Tremona Road,
Southampton,
SO16 6YD.

Tel 02380 796838

Patient Information Sheet.

Study Title.
Studies on prostasomes in prostate disease.

Introduction.
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of this study?
Prostasomes are particles formed by the prostate gland. Not much is known about their exact function. We believe they may play a role in diseases of the prostate and would like to investigate this further.

Why have I been chosen and what will happen to me if I take part?

You will have been asked to take part in this study because you belong to one of the groups below.

a) You have recently had a vasectomy. We would like to use your semen sample after the routine sperm count analysis. We will use it to obtain normal prostasomes, then discard it.

Or

b) You are attending urology outpatients with a prostate problem. We would like to take a sample of your blood to test for antibodies to prostasomes.

Or

c) You are attending urology outpatients with a non prostate related problem. We would like a sample of your blood as a control (comparison).

We only require a semen or blood sample on this one occasion. No extra hospital visits are required as a result of this study.

Version 2. 03/03. LREC No 045/03/w

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not effect the standard of care you receive.

What do I have to do?
If you are willing to take part in this research we require you to sign a consent form then donate either a blood or semen sample.

What are the possible benefits of taking part?
There is no intended clinical benefit for patients taking part in this research at present. The information we get from this study will help us to understand the role of prostasomes in prostate disease. In the future this might lead to clinical benefits.

Will my taking part in this study be kept confidential?
In accordance with the data protection act 1998, all information which is collected about you during the course of this research will be kept strictly confidential and will have your name and address removed so that you cannot be recognised from it. Your GP will not be informed about your participation in this research.

What will happen to the results of the research?
We aim to publish the results of this research in a medical journal. Copies can be obtained from us in the future if required. You will not be identified in any publication.

Who is organising and funding the research?
This research is being organised by the Urology Research Team at Southampton University Hospitals NHS Trust. No payment is being made to patient or doctors.

Who has reviewed the study?
This study has been reviewed by the Southampton & SouthWest Hampshire Local

Research Ethics Committee.

Contact for further information:
If you have any questions about this study please contact:

Mr Alistair Stewart. BM.MRCS. Urology Research Fellow.
Tel: 02380 796838.

Thank you for taking part in this study! Please keep this sheet and a copy of the consent form for your information.

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