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Jane Louise Watkins

An Investigation into Minichromosomal Maintenance Proteins (MCMs)
for the Diagnosis of Prostate Cancer, as a possible alternative to Prostate
Specific Antigen (PSA).

Supervisors: Dr. T A Bailey, Prof J. O'Donnell, Prof. A C Woodman

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Abstract

The current strategy for the diagnosis of prostate cancer includes serum prostate specific antigen (PSA) measurement. There is however debate into its specificity and sensitivity, so new diagnostic markers are under investigation. Minichromosomal maintenance proteins (MCMs) are potential markers for the diagnosis of neoplasia, as they are involved in cellular replication. The aim of this study is to assess MCM2, 5 and 7 as new diagnostic markers for prostate cancer, to compare the clinical usefulness of PSA and to develop a less invasive technique for diagnosis.

PSA specificity was investigated in several human cellular lines, and a clinical study was performed to assess expression in prostatic tissue and blood serum. MCM2, 5 and 7 was investigated by translational and transcriptional means in two prostate cell lines PNT1A and PC-3. In addition, a clinical study was performed to assess the expression of MCM2, 5 and 7 in prostate tissue, urine and blood

The results suggest that PSA is not prostate specific, as it is synthesised and secreted by several non-prostatic cell lines. In addition PSA testing does not conclusively indicate neoplastic tissue and serum testing only has 63% sensitivity and 60% specificity in accurately identifying prostate cancer. The *in vitro* results suggest that the PC-3 cells express less MCM2, 5 and 7 on both the protein and mRNA level compared to the PNT1A cells, suggesting that MCM2, 5 and 7 maybe performing a bigger role than just replication of DNA. The tissue results indicate that there is an increase in MCM2, 5 and 7 epithelial nuclei staining for neoplastic condition compared to BPH. While the clinical study on urine sediment indicates increased MCM2, 5 and 7 staining in prostatic neoplasia compared to BPH and the transcriptional study on MCM5 can identify neoplastic tissue from BPH as 11/12 cancerous patients expressed MCM5 compared to only 3/23 BPH patients. Finally the transcriptional study on the blood samples is inconclusive and need to be repeated

These results suggest that serum PSA testing is not ideal for the diagnosis of prostate cancer, that MCM2, 5 and 7 appear to have potential as new diagnostic markers and may aid the histopathologist to allocate Gleason score. Also the MCMs may have potential in the development of a less invasive technique through the use of urine sediment.

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Abbreviated Symbols

µl	-	microlitre
°C	-	Degrees Celsius
ABCComplex	-	Avidin-Biotin Complex
ASAP	-	As soon as possible
BPH	-	Benign prostatic hyperplasia
CD44	-	Cluster of differentiation marker 44
Cdc6	-	Cell division cycle 6
Cdc7	-	Cell division cycle 7
CDK	-	Cyclin dependent Kinase
cDNA	-	Complementary DNA
CHAPS	-	3-(3-Cholamidopropyl)dimethylammonio)-1-proanesulfonate
DAB	-	3,3-diaminobenzidine
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic acid
DRE	-	Digital rectal examination
EGF	-	Epidermal growth factor
EGFR	-	Epidermal growth factor receptor
ICC	-	Immunocytochemistry
IgG	-	Immunoglobulin G
IHC	-	Immunohistochemistry
FCS	-	Fetal Calf Serum
GF	-	growth factor
GSTP1	-	Glutathione S transferase P1 gene
MCM	-	Minichromosomal Maintenance Protein
mRNA	-	Messenger RNA
ORC	-	Origin of recognition
P53	-	53kDa tumour suppressor gene
PBS	-	Phosphate buffered saline
PC-3	-	Human prostate adenocarcinoma
PCR	-	Polymerase chain reaction
PIN	-	Prostatic intraductal neoplasia
pH	-	Logarithmic measure of hydrogen ion concentration

PNT1A	-	Normal human post pubertal prostate epithelial cells
PRB	-	Retinoblastoma tumour suppressor gene
PSA	-	Prostate specific antigen
RNA	-	Ribonucleic acid
RT	-	Room temperature
RT-PCR	-	Reverse transcription polymerase chain reaction
Taq	-	<i>Thermus aquaticus</i>
TBS	-	Tris buffered saline
TEMED	-	Tetramethylethylenediamine
TNM	-	Tumour-node metastasis
TGF α	-	Transforming growth factor alpha
TRUP	-	Trans-urethral prostatectomy
TRUS	-	Trans-rectal ultrasounded guided biopsy
TSG	-	Tumour suppressor gene
UK	-	United Kingdom

Chapter 1 – Introduction

1.1 Introduction into Cancer Biology

1.1.1 Cancer Statistics

The National Statistics Office (2005), stated that the four most common cancer types for England and Wales are breast, lung, colorectal and prostate, and that these account for 227,500 new cases, which was over half of the cases recorded for 2003 in the UK. Currently individuals have a 33% chance of developing some form of cancer within their lifetime, and a 25% chance of dying of cancer (Cancer Research UK, 2004).

Cancer is predominantly age related, occurring more frequently in the elderly, and it was stated that only 0.5% of cases registered in 2003 were found in children (National Statistic Office, 2005). Between 1950-2003 age-standardised cancer mortality for England and Wales changed little, while heart disease, strokes, and infectious diseases declined (National Statistics Office, 2005). Consequently cancer became the most common cause of death, in females has been since 1969, and in males since 1995 (National Statistics Office, 2005).

1.1.2 Basis of Cancer

Carcinogenesis can be very aggressive and nearly every tissue in the human body has the ability to become neoplastic. Cancer is caused by numerous factors including genetic predisposition and epidemiological agents, and although inheritable predisposition cannot be changed, the exposure to external carcinogens can be. As the general public becomes aware of carcinogens they actively reduce contact with them and consequently decrease the incidence of disease. It is suggested that if awareness continues with subsequent reduced exposure to causative factors, over 200,000 lives could be saved annually in the USA, governments and other authorities should therefore take more responsibility to 'highlight' carcinogenic agents (Rennie and Rusting, 1996). It is presumed that sporadic cancer occurs for 95% of human tumours, and this depends on interactions between host genetic make-up and environmental carcinogens (Balmain and Harris 2000). As we learn more about causative factors and genetic predisposition, genetic profiling of risks will hopefully become easier. Despite this huge range of

carcinogens such as sunlight, diet and tobacco with variable causative effects, this thesis will only focus on a few which cause prostate cancer, these are explained in *Section 1.3*.

1.1.2 Cancer Development

Carcinogenesis is the transformation of a normal cell to a cancer cell, and it is believed that a single abnormality can initiate cancer development. For most however, multiple chromosome abnormalities are required (Knudson, 2001). Tumours accumulate a large number of mutations over time, typically 10^{12} or more, and these are due to pre-existing changes in stem cells and mutations obtained during carcinogenesis and tumourigenesis (Tomlinson, *et al* 2002). Nowell (1976) proposed that a two-stage clonal expansion model must come into play, to change a normal cell to a neoplastic cell.

The model hypothesised:

1) Two or more events must occur within a normal cell, these are presumably mutational and could happen spontaneously or by specific carcinogens – initiation (Curtis, *et al* 2001).

2) Cells emerge with altered net cell proliferation kinetics (enhanced growth) – promotion (Curtis, *et al* 2001).

3) An initiated cell can sustain spontaneous or induced genomic events, which may lead to the appearance of a malignant cell (Curtis, *et al* 2001).

Carcinogenesis is therefore a complex process, and there are two main stages relating to the two-stage clonal expansion model, and these are dependent on the number of mutations or errors that a neoplastic cell acquires.

These stages are:

Initiation

Promotion

Initiation arises when a cell receives an internal or external signal, which causes a single gene mutation or a cluster of gene mutations. There are hundreds of known carcinogens that can act as initiators, and their signals can cause a point mutation, translocation, or amplification of the genetic material, which is rapid and irreversible (Spence and Johnson, 2001). For instance the polycyclic hydrocarbon benzo(a)pyrene was shown to be a skin tumour initiator in mice (Slaga *et al*, 1977), as well as the alkylating agent

triethylenemelamine (TEM) (Perrella and Boutwell, 1983). These polycyclic hydrocarbons or alkylating agents act directly as initiators, and in rat mammary carcinomas and mouse skin tumours cause a point mutation of the *ras* gene (Balmain and Harris 2000).

After initiation has occurred the transformed cell must undergo promotion, this process is poorly understood but very influential. Promotion factors differ to the initiators as they are not necessarily carcinogenic, and may only cause accelerated cellular replication, there by increasing the chance of further mutations. Again like the initiators there are believed to be hundreds of different types of promoting factors, and numerous studies have investigated mutagens. The earliest study by Percival Potts in 1775, showed that chimney sweeps were more susceptible to scrotal cancer, and the disease was attributed to the level of soot (Hall, 1998). A more recent study by Curtis, *et al* (2001) that shows that Colorado Plateau miners exposed to high-LET radiation for longer developed a higher lifetime risk of developing lung cancer.

Once promotion is complete cells with altered proliferation emerge. This however is not the end of cancer development, as the transformed cells may then undergo progression. Progression is a similar process to promotion, as the cells continue to collect DNA damage and their rate of proliferation continues to increase. However, unlike promotion the process occurs on a bigger scale, and is a very aggressive step, causing dedifferentiation and increased replication (Smith and Wood, 1992).

Once the cells approach 1mm cubed angiogenesis must occur, this process causes the formation of blood vessels, to ensure the transformed cells obtain a good nutrient supply to continue growth. These transformed cells grow, producing a complex set of cells in a heterogeneous population. This growth is now referred to as a tumour and performs tumourigenesis.

1.1.3 The Pathological Stages and Physiological Requirements.

When neoplastic cells move through carcinogenesis and tumourigenesis, changes occur in their pathological appearance (*Figure 1.1*). Firstly a cell receives a mutation that causes increased growth, this cell undergoes excessive proliferation producing new progeny with the same defect, but still has a similar appearance to the normal cellular area, this is termed the hyperplastic stage. Hyperplasia is then followed by metaplasia, and metaplasia occurs when a tissue type transforms to another. For instance in Barretts Oesophagus specialised epithelium of the lower oesophagus (squamous cells), change to incomplete intestinal metaplasia from the stomach (columnar epithelium) (Fléjou, 2001). The metaplastic stage is then followed by the dysplastic stage, however these stages can co-exist at the same time. For dysplasia the transformed cells then have further loss of cellular control so their orientation, cellular size, nuclear size, shape and staining alters (Freeman *et al*, 1999).

After dysplasia cells can then continue to transform, obtaining more cellular abnormalities and orientation. These cells can then enter an anaplastic stage, and receive further organisational, structural, and functional losses. If these cells then stay confined to the locality in which they are produced, and never become invasive, they are termed *in-situ* cancers or benign. However if these cells leave their site of origin and invade surrounding or distant tissue, they are termed malignant.

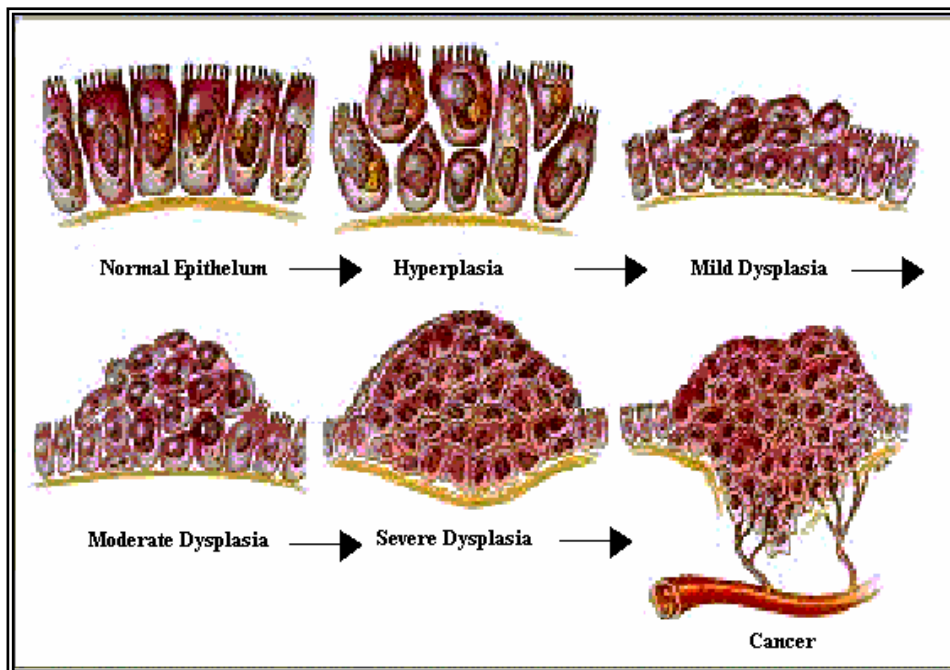


Figure 1.1. Cancer Progression. The pathological alteration from a normal cell to a malignant cell (www.intouchlive.com/myts.lung/Lung03.htm).

Benign tumours stay encapsulated within their cellular area and grow to a particular size then stop (Purves *et al*, 1997). In contrast, malignant cells invade the surrounding areas and have the capacity to settle in other organs, in a process called metastasis. Once cancerous cells become malignant, they detach from their site of origin and invade and penetrate the underlying tissues. Once penetrated, the cells are then capable of entering the blood and lymph supply to move and invade new sites.

Metastasis is a very complex process, as the transformed cells must first obtain the ability to penetrate other tissue, as well as protect themselves from the immune system. Consequently, only a few cancer cells ever achieve this ability, and Ruoslahti (1996) suggested that probably only 1 in every 10,000 cancer cells survive to invade new areas. Once metastasis occurs cancer is very hard to cure, as removal of the primary tumour no longer controls the disease.

The physiological changes which occur in cancer cell development is a multistep process, and Hanahan and Weinberg (2000) stated that cancer cell genotypes must manifest six essential alterations in cell physiology, to allow them to be collectively known as a malignant growth. These are: self-sufficiency in growth signals, evasion of programmed cell death (apoptosis), insensitivity to growth-inhibitory signals (antigrowth), limitless replication, sustained angiogenesis, and tissue invasion (Hanahan and Weinberg 2000). These are explained in more detail in *Table 1.1*.

Table 1.1. The six essential alterations required for transformation of a normal cell to a malignant cell. With a brief description of what is involved for each essential alteration (adapted from Hanahan and Weinberg 2000).

Essential Alteration	Requirements
Self-sufficiency in growth signals	Growth signals (GS) are received by normal cells before they move into proliferation. In cancer cells they are able to generate their own GS, thereby reducing their dependence on the normal microenvironment and then grow independently.
Evasion of programmed cell death (apoptosis)	For a mutated cell to expand its population, not only is proliferation important but so is programmed cell death. Therefore in cancer cells it is believed that they acquire resistance to apoptosis which enables them to grow uncontrollably.
Insensitivity to growth-inhibitory signals (antigrowth)	In a normal cellular environment antiproliferative signals work to maintain cellular quiescence and tissue homeostasis. Normally cells do this as they contain a cell cycle clock, which informs them when they are ready. In cancer cells these antiproliferative signals can be evaded as the cell cycle clock is de-ranged.
Limitless replication	Normal cells contain a certain doubling time after which growth stops and they are termed senescent. In cancer cells however they have found the ability to overcome this so they can replicate indefinitely and never enter senescence.
Sustained Angiogenesis	Nutrient supply to cells is essential for cellular function and growth, therefore normal cells are highly vascularised and reside within 100µm of capillary blood vessels. Therefore for tumour cells to grow, angiogenesis must occur so that a larger growth can be sustained.
Tissue invasion.	For most tumours over time, they generate cells which move out of the site of origin, to try and invade adjacent tissue. In order for the cell to do this, it requires alterations in the cell to cell adhesion molecules, and synthesises new proteins like proteases to digest the various cellular layers.

Once a cell has passed through the various stages of carcinogenesis, obtained the six essential alterations, and passed through the pathological stages to be termed malignant, it no longer listens to the cellular environment and therefore follows its own routine.

1.2 The Prostate Gland and Prostatic Conditions

1.2.1 The Prostate Gland - Anatomy and Histology

The prostate gland is an accessory sex gland and is made up of glandular epithelium and fibromuscular stroma surrounded by connective tissue. The gland allows the passage of urine from the bladder to the urethra, and plays an important role in fertilisation. The gland starts life pea sized and during the first few years up to puberty grows rapidly. Once puberty is reached, growth slows down and at the third decade growth stops (Rous, 1988). In most men however this is not the end of prostatic enlargement, as the prostate gland starts to re-grow in later life. This means 60% of men in their sixties, and 80% of men in their eighties, show benign prostatic hyperplasia (BPH) (Roehrborn *et al*, 1999).

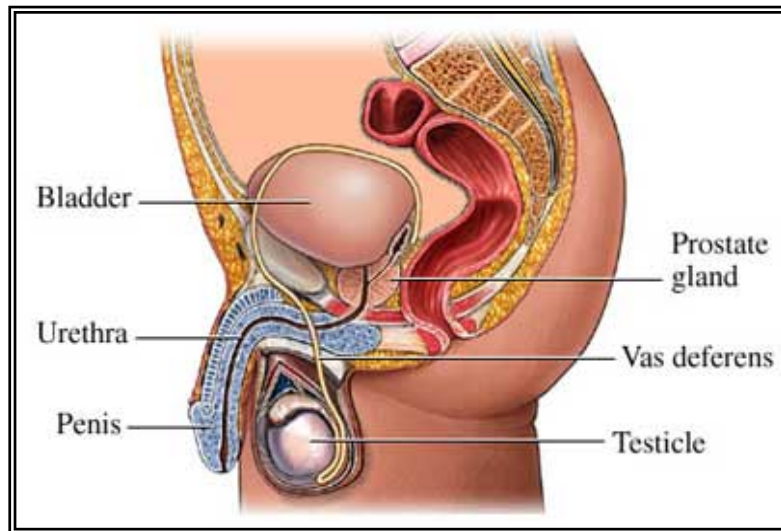


Figure 1.2 – A Diagrammatic Representation of the Male Genital Tract
<http://www.liv.ac.uk/researchintelligence/issue21/images/prostate.jpg>

The anatomy of the male genital tract is given in *Figure 1.2*; the prostate is situated in the pelvic region between the urinary bladder and urethral sphincter. The gland on average is 3-4cm long and 3-5cm wide, walnut in shape with an average adult weight of 20 grams. However, due to prostatic enlargement the gland can weigh up to 150 grams. The gland has two ejaculatory ducts situated just above the urethral sphincter and surrounds the urethra which runs vertically through the gland (Rous, 1988).

As the gland consists of different areas, the McNeal model has simplified the structure into two aspects. These are the central inner periurethral aspect which consists of the

transitional zone and periurethral zone, and the outer peripheral aspect which consists of the peripheral zone and central zone (Epstein and Wojno, 1999; *Figure 1.3B*). Of these zones it has been shown that most cancer arises in peripheral zones, and that most hyperplastic lesions arise in the transitional and periurethral zones (Kumar *et al*, 1997).

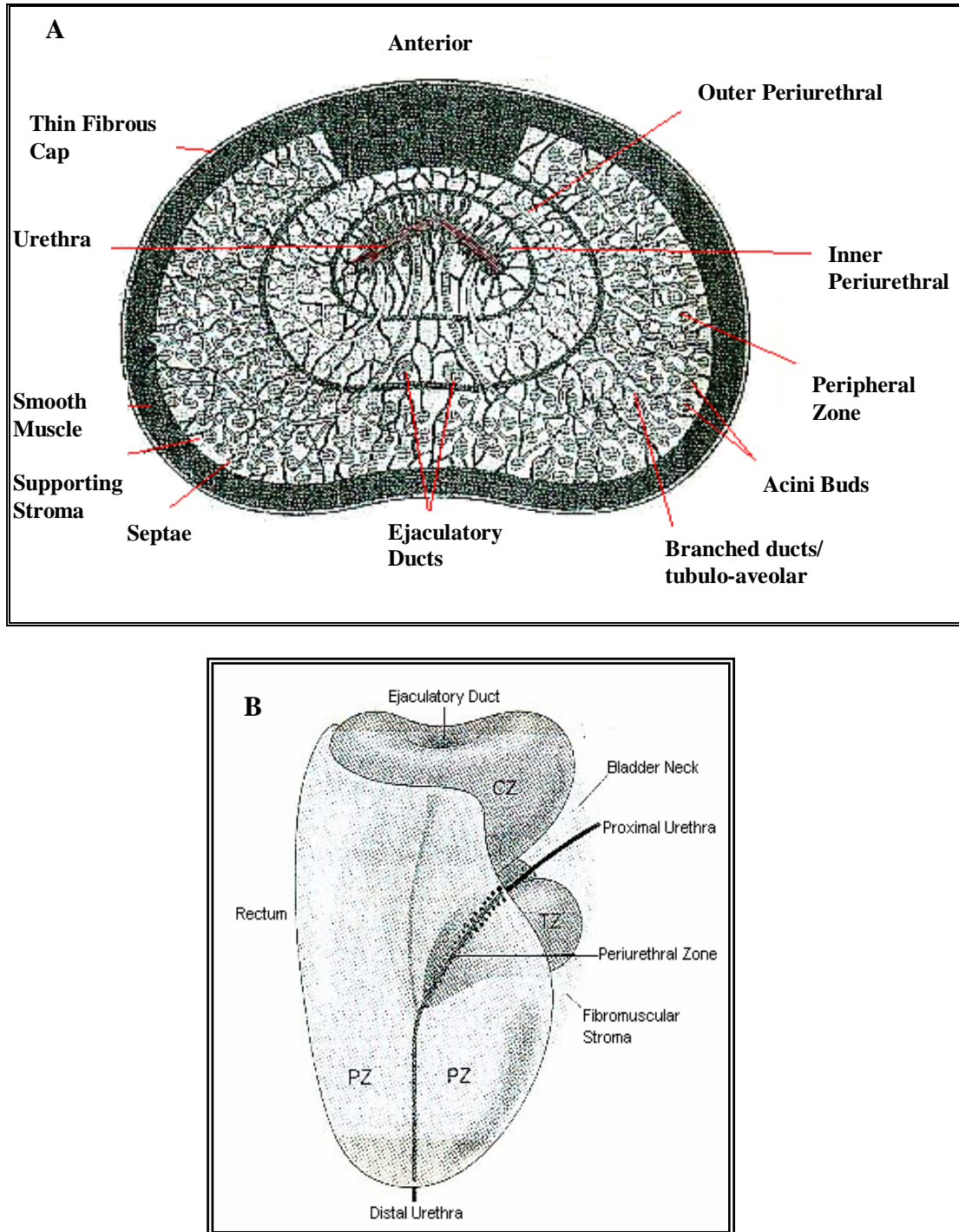


Figure 1.3. A) A diagrammatic presentation of the structure of the prostate gland from Stevens and Mosby, 1997.

B) A diagrammatic representation of the prostate gland using the McNeal model from Kumar *et al*, 1997 CZ = Central Zone, TZ = Transitional Zone, PZ = Peripheral Zone.

As mentioned before, the gland is composed of glandular epithelium and fibromuscular stroma in a complex arrangement (Epstein and Wojno, 1999) (*Figure 1.3A*). The gland is covered with numerous blood vessels that form a sheath, and surround a fibrous cap that covers the prostate, underneath is smooth muscle, septae, and stroma, which act as structural support (Stevens and Mosby, 1997). Within the stroma are roughly 30-50 branched ducts/tubulo-alveolar, which tend to be long, branched with acini 'buds'. These acini buds empty their content into the prostatic urethra via the excretory ducts.

There are three cell types found within the prostate gland and these are; epithelial cells/secretory cells, stromal cells/basal cells and neuroendocrine cells (Epstein and Wojno, 1999). The epithelial cells/secretory cells make the glandular structures (acini) and are involved in producing the secretory products. The secretion produced makes up 13-33% of the ejaculation fluid, and when the spermatozoa and secretion from the seminal vesicles pour through the ejaculatory ducts, the prostate epithelial cells add their milky secretion. This liquid produced by the prostate is at pH 6.5 and contains fibrinolysin (which aids in liquefaction of semen), zinc, amylase, prostate specific antigen (PSA), acid phosphatase, calcium and other factors (Kierszenbaum, 2002). This mixture combines with the rest of the secretory fluids to form the ejaculation fluids, which help in nourishment and transportation of the sperm, as well as making the uterine environment more favourable.

The stromal/ basal cells make up the connective and muscular part of the gland, and are located between the secretory cells, aiding in ejaculation. The neuroendocrine cells however, are distributed throughout the ducts and acini and are believed to help regulate adjacent cells in a paracrine fashion (Epstein and Wojno, 1999).

1.2.2 Diseases of the Prostate

i) Benign Prostatic Hyperplasia (BPH)

BPH is a non-cancerous growth which is encapsulated (does not invade the underlying tissue), looks like the normal cellular area, but has increased proliferation (King, 2000). Normally it occurs in the transitional zone and only 25% of adenocarcinomas actually occur in this area (Spence and Johnson, 2001). BPH can also develop in the periurethral

zone of the prostate gland, but this is rare (Kumar, *et al*, 1997). It is therefore characterised by increased proliferation of the epithelium and stromal elements of the prostate gland, causing compression of the outer margins of the gland, so the peripheral parts become atrophic (Burkitt *et al* 1996).

Although BPH is not life threatening it can cause numerous problems, and the symptoms range from urinary retention, urinary tract infection, bladder function deterioration, and in some cases renal failure (Roehrborn *et al*, 1999). BPH can cause severe narrowing of the urethra, and in some complete blockage, this requires surgical intervention to remove the excessive growth (Rous, 1988).

ii) Prostatitis

Prostatitis is an extremely common condition worldwide with 2-10% of men experiencing it during their lifetime (Krieger *et al*, 2002). Prostatitis is an inflammation of the prostate gland causing pain and obstruction, and it can be caused by non-bacterial or bacterial infections. To identify which type an individual has, the clinical features of the condition are taken into account, along with microscopic examination and urine culture before and after prostatic massage (Kumar *et al*, 1997).

The original four-part classification of prostatitis consisted of; acute bacterial prostatitis, chronic bacterial prostatitis, nonbacterial prostatitis, and prostatodynia (Drach *et al*, 1978) and this enabled inflammation to be separated into subtypes. This classification was subsequently re-defined at the Chronic Prostatitis Workshop at the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) in 1995, and the National Institute of Health (NIH) described four categories I-IV. Pelmen *et al*, (2004) states that these categories take into account the various symptoms presented, diagnostic parameters and the therapeutic options.

Category I – Acute prostatitis

This condition is relatively easy to identify as it is typically due to an infection of the prostate gland, and can be associated with a gram-negative bacterial infection (Habermacher *et al*, 2006). Normally category I prostatitis does not require surgical

diagnosis and most patients are identified by physical examination, urinalysis with microscopic assessment, and urine/blood culturing. This condition is relatively rare with only 2-5% of cases and men with the disease have symptoms such as; chills/fever, difficulty in voiding, slow urinary stream, dysuria, and urgency (Pelman, 2004, Habermacher *et al*, 2006). Patients with the disease can be easily cured using antibiotics and Pelman (2004) suggested that patients can be given high-dose antibiotic therapy for a minimum of two weeks, followed by either another antibiotic therapy or the same therapy to ensure complete removal of infection, as failure can invite chronic bacterial prostatitis.

Category II – Chronic bacterial prostatitis

This is defined as recurrent urinary tract infections and, like acute prostatitis, is rare constituting only 2-5% of cases (Habermacher *et al*, 2006). Patients with category II often present symptoms similar to category I but usually symptoms are not as severe and in some patients they might be asymptomatic. Patients with category II differ from category I as these patients seem to have persistent infection of the prostate and this can manifest with intermittent cystitis like urinary symptoms but these rarely involve appreciable discomfort or pain (Habermacher *et al*, 2006). Patients that suffer from category II require a prolonged course of antibiotics to penetrate the prostate gland, to ensure adequate removal of infection (Pelman, 2004).

Category III Chronic prostatitis/Chronic Pelvic Pain Syndrome

Category III is commonly known as prostatitis syndrome and can be sub-divided into inflammatory and non-inflammatory forms, occurring in 90-95% of cases (Habermacher *et al*, 2006). Although the condition can occur in men of any age, the disease has a peak onset in men in their thirties and it can be re-current with symptoms ranging from mild to chronic.

This inflammatory form shows symptoms similar to category II and patients show excess leukocytes in prostatic secretion (Pelman, 2004). Non-inflammatory forms however differ from inflammatory forms, as although they show similar symptoms, they do not express inflammatory cells (Pelman, 2004).

Despite numerous investigations trying to define a plausible picture into the aetiology of category III, there is still considerable confusion and the current consensus suggests that category III arises from a complex interaction of immune, endocrine, neuronal events as well as the psychological status of the individual (Schaeffer *et al*, 2002). Due to the aetiology of the disease, treatment is subject to intense investigation and currently it is recommended that a single 4-6 week course of antimicrobial medication be implemented followed by pharmacotherapy and anti-inflammatory medication (Habermacher *et al*, 2006).

Category IV – Asymptomatic inflammatory prostatitis

This is typically found by accident either during examination of expressed prostatic fluid or during prostate biopsy and it is estimated to occur in 32.2% of men with elevated PSA (Habermacher *et al*, 2006). Patients with category IV typically show inflammation of the prostate, and these patients are usually asymptomatic and do not require therapy or evaluation (Pelman, 2004).

iii) Prostatic Intraepithelial Neoplasm (PIN)

Prostatic intraepithelial neoplasia (PIN) is caused by proliferation of cells located in the ducts and acini. It is associated with basal cell layer disruption, is identified in up to 16% of men that undergo a transrectal ultrasound guided prostate biopsy and is believed to be a precursor of prostate cancer (Belledgrun *et al*, 1998, PCRI, 2006).

A grading scheme has been established for PIN, with a grading range of one to three, however most pathologists now agreed on only two grades; low-grade PIN (LGPIN) and high-grade PIN (HGPIN) and many no longer report the presence of LGPIN, as it has been shown to have little or no correlation with malignancy (Brawer 2005). The incidence of PIN in prostate biopsies averages 9% (range 4-16%) representing 115,000 new cases of PIN each year in the US, also PIN is highly predictive of adenocarcinoma and its identification warrants further biopsy to check for subsequent invasive carcinoma (Bostwick, *et al* 2004).

HGPIN is characterised by cellular proliferation within pre-existing ducts and acini, cytological changes such as cells mimicking cancer cells and nuclei and nucleolus enlargement (Bostwick, *et al* 2004). The normal proliferation pattern of the cellular epithelial also alters and Cheville *et al* (1997) described four main patterns of HGPIN. These are morphologically known as tufting, micropapillary, cribriform and flat, these show no clinical important differences between architecture and their recognition appears to be only of diagnostic utility (Bostwick *et al*, 2004).

iv) Prostate Cancer - Carcinoma

Incidence of Prostate cancer accounted for 30,142 cases in the UK for 2001, and is estimated to cause 40,000 deaths annually worldwide (Cancer Research UK, 2004, Adams *et al*, 2001). In England and Wales most cases of prostate cancer arise in individuals over the age of 65 (Quinn and Babb, 2002), and it is rare in men under fifty, becoming more common as men get older. It is currently believed that about one third of men over fifty has some form of cancer, which rises to half of men by age eighty, of these one in twenty five (4%) will die from the disease (Cancer Research UK, 2004). Prostate cancer is therefore the most common cancer in men, accounting for one in five of all new male cancers diagnosed, however even though there is a huge increase in incidence, mortality rates still remain fairly stable (Cancer Research UK, 2004).

Prostate cancer is a variable disease ranging from being highly aggressive and malignant resulting in death, to slow growing and non-invasive (Nash and Melezinek, 2000). Prostate cancer normally occurs within the glandular structures (epithelial cells), with 70% of prostate cancer arising in the peripheral zone, 15-20% in the transitional zone and 15-20% in the central zone (Spence and Johnson, 2001) (*Figure 1.3*).

As most malignant adenocarcinomas occur in the peripheral or outer part of the prostate gland, DRE can aid in identification (Rous, 1988). However as most cases of prostate cancer do not give symptoms in the early stage, it is often hard to diagnose, until it is advanced (Adam *et al*, 2001). In prostate carcinoma, transformation of the cellular architecture occurs, this means there is loss of the tall columnar pattern and atypical cells invade the stroma, causing loss of glandular structure and evidence of tumour

necrosis (Burkitt *et al*, 1996). Malignant adenocarcinoma cells therefore have irregular appearance compared to normal cellular locality (King, 2000).

Adenocarcinoma can also spread out of the area of production causing secondary tumours in some, which can become lethal. Therefore in advanced cases, invasion of the seminal vesicles, periurethral zone, soft tissue and even the bladder wall may occur (Stevens and Mosby, 1997).

1.3. Prostate Cancer: Causative Factors and Current Diagnostic Techniques

1.3.1 Causative Factors

Carcinogenesis is caused by exposure to carcinogens and these causative factors can either lead to genetic alterations or alterations in biological events, which in turn lead to cancer development (Balmain and Harris, 2000). For prostate cancer, like most cancers, age is an underlying factor, however environmental factors and family history also affect cancer development.

i) Environmental Factors

Prostate cancer can be a result of a combination of factors, including age, endogenous hormones, environment, diet and lifestyle. For instance the age of first ejaculation and the number of sexual partners increase the chance of developing prostate cancer, while the frequency of ejaculation has an inverse relationship with cancer progression (Giles *et al*, 2003). In addition, an association may also be seen with the amount of spirits consumed; however this is still inconclusive. For instance Sesso, *et al* (2001) suggests that a positive association is found between the amount of liquor consumed and prostate cancer. While Schoonen, *et al* (2005) suggests that there is no clear association with the consumption of beer or liquor, and even suggests that red wine may have a beneficial role in reducing prostate cancer.

Diet can also affect cancer development, for instance a high intake of red meat or saturated fats causes a lot of different malignancies such as; colon, rectum, ovary, breast and prostate cancer (Boyle and Zarida, 1993). Therefore there is an increased risk of

cancer development with the western style diet, which consists of large intake of animal products (Key *et al*, 2003). Amount of food consumed is also an issue, as individuals who are obese have an increase risk of cancer development, especially colon, breast, endometrium, oesophagus and kidneys (Bianchini *et al*, 2002).

Some foods however actually have anti-cancerous properties, for instance a reduced cancer risk is associated with the increased intake of vitamin E, selenium and lycopene (Key *et al*, 2002). Dark green leafy and deep yellow vegetables have protective roles against human disease, such as prostate and stomach cancer (Van Duyn and Pivonka, 2000), and a reduction in prostate cancer development is associated with cereal intake (Kodama and Kodama, 1990).

Other environmental carcinogens include infectious organisms, for instance RNA and DNA viruses cause cancer, and the transforming activity of several viruses has been well documented in cell cultures and animals (Reiss and Khalili, 2003). Such as HTLV-1 and Epstein-Barr virus that can cause numerous types of lymphomas and 15% of deaths (Spence and Johnson, 2001), and human papillomaviruse type 16 and 18 which accounts for 70-80% of genital and anal cancer, affecting more women than men (Trichopoulos and Hunter, 1996).

ii) Family History

Genes and genetic susceptibility have a high level of influence on cancer development and an individual is twice as likely to develop prostate cancer if the father has the disease (Morris and Scher, 2002). Prostate cancer can also occur in adolescence, but this is very rare with only 5 cases reported in 10 year olds and 21 cases reported in 10-21 years olds (Epstein and Wojno, 1999).

iii) Geographical Location and Race

Geographical location affects the frequency of prostate cancer, with the highest incidence in males found in the USA and the lowest in India and Japan (Bishop, 1999). Race also affects the risk of prostate cancer, with Caucasians having a lower incidence, compared to Afro-Americans (Van der Cruijesen-Koeter *et al*, 2001). Geographical

location, family genetics and race all seem to affect the incidence of prostate cancer. However geographical location may have more of an effect than the others, as a study by Cook *et al* (1999) indicated that Chinese, Japanese and Filipino who migrate to the USA have a higher incidence of developing prostate cancer, than those born in China, Japan and the Philippines. This therefore indicates that due to the western 'style of life' prostate cancer incidence increases for all races.

1.3.2 Current Diagnostic Techniques

Diagnosis of prostate cancer, is carried out by measuring prostate specific antigen (PSA), and DRE. These techniques provide valuable information, but there is uncertainty into their specificity and sensitivity. If an individual is identified as having either elevated PSA (greater than 4ng/ml), and/or an abnormally shaped prostate by DRE, and/or haematuria (blood in the urine), they will then be referred for further investigation. On further assessment if any/all symptoms persist, a Transrectal ultrasound (TRUS) guided biopsy will be performed. It is believed, that even with combined use of DRE, PSA, and biopsy, about 40% of men considered to have localised tumours will already have extracapsular disease upon examination (Clements *et al*, 1999).

i) Prostate Specific Antigen (PSA)

PSA is a 33KDa glycoprotein and a member of the Kallikrein family, which are serine proteases. Serine proteases are enzymes which catalyse the hydrolysis of peptide bonds, and have roles in processes such as digestion, clotting, immune response, hormone activation, and cell migration. The kallikrein family is made up of three glycoproteins: human glandular kallikrein 1, human glandular kallikrein 2, and human glandular kallikrein 3 (PSA) (Diamandis, 1998). Hara *et al* (1971) were the first to extract PSA from human seminal fluid, and subsequently the protein from the tissue itself was identified as PSA (Wang *et al*, 1979). PSA is produced by the prostatic secretory epithelium and is one of the most abundant proteins in seminal plasma, found at levels of 0.2-5.0mg/ml, which is a million-fold higher than normal serum levels of 0.1-4ng/ml (Nash and Melezinek, 2000).

PSA measurement was first approved for use as a diagnostic marker in 1986 by the Food and Drug Administration (FDA), and in 1994 implemented for early diagnosis and management of prostate cancer (Diamandis 1998). PSA is measured in serum by a direct sandwich ELISA using monoclonal antibodies and this is a very quick and efficient technique. The assay identifies total PSA within serum and the concentration is referred to an age standardised PSA range (*Table 1.2*). There are currently several types of assay equipment, these can take between 45 minutes and 4 hours to produce results and have sensitivity between 0.02ng/ml and 0.2ng/ml (Prostate Cancer Research Campaign 2004).

PSA testing enhances the detection of prostate cancer, but there are still doubts as to its specificity, especially in the range of 4-10ng/ml where the specificity in distinguishing prostate cancer from BPH is only 25-30% (Adam *et al*, 2001). In addition, 20-30% of men believed to have 'biologically important prostate cancer' have PSA levels less than 4ng/ml (Goldstein and Messing, 1998), while it is estimated that 27% of men over the age of fifty five and 56% of men over the age of seventy five investigated for adenocarcinoma are subsequently found to have no abnormality (Draisma, *et al* 2003). These findings demonstrate that PSA testing can potentially miss some adenocarcinoma patients (false negative) and BPH patients (false positive). Consequently many men undergoing biopsy because of elevated PSA have no cancer, resulting in unwarranted stress and anxiety, as well as increased risk of infections and bleeds (Hanks and Scardino, 1996). For instance, it is already known that prostatitis, prostatic massage, needle biopsy, trauma, BPH and urinary retention, all elevate PSA levels (Belledgrun *et al*, 1998).

It is now also believed that PSA is not organ specific, with PSA detected at low levels in the endometrium, breast tissue, adrenal and renal carcinomas (Van der Crujjsen-Koeter *et al*, 2001). Despite this, PSA is still currently used for diagnosis, because there are no other methods for non-invasive monitoring of prostate cancer.

Due to the problems with interpreting PSA measurements, numerous methods have been tried to improve detection, and currently the most widely used method is age

specific PSA testing. Other methods include PSA density, PSA velocity, and PSA complexes.

Age-Specific PSA

The age specific range was developed to increase the number of undiagnosed patients younger than sixty years, and to decrease the number of over-diagnosed patients detected over the age of sixty (Belledgrun *et al*, 1998). As an individual gets older the prostate gland increases in size, so the amount of PSA produced also increases (*Table 1.2*).

Table 1.2. Age specific PSA range for serum (Oesterling, *et al* 1992).

Age	Serum PSA ng/ml
40-49	0-2.5
50-59	0-3.5
60-69	0-4.5
70-79	0-6.5

Although the use of the age specific range has improved diagnosis, it does have its drawbacks. For instance it has increased the use of biopsy in younger men with only minimal increase in cancer detection, and reduced diagnosis of cancer in older men due to the higher cut-off value (Morris and Scher, 2002).

PSA Density

This is performed by measuring the PSA level and dividing it by the size of the prostate (which is measured by TRUS). The value is then compared to a standard range and if the value exceeds the reference range, the patient is believed to have prostate cancer. PSA density, is not very reliable as it cannot take into account variations between race, and assumes all men have the same PSA levels (Morris and Scher, 2002).

PSA Velocity

This involves a yearly PSA test, this means sudden rises in the PSA concentration can be noticed, and patients assessed further for neoplastic disease (Goldstein and Messing, 1998). A linear increase in yearly PSA is expected, while an exponential relationship of PSA with an annual increase of 0.75ng/ml/year is suggestive of prostate cancer (Van der

Crujisen-Koeter *et al*, 2001). This test has limitations, as it depends on the initial PSA level and the age of the patient, therefore a PSA co-efficient variation of 8.94% represents a 30% change in PSA levels daily (Nixon *et al*, 1997). Measuring velocity has therefore been shown to be no more accurate than measuring PSA alone (Nixon *et al*, 1997).

PSA Complexes

PSA is known to form complexes when exposed to a number of protease inhibitors (Haese *et al*, 2003). For instance in serum, 80-90% of PSA is bound to protease inhibitors such as alpha 1 antichymotrypsin (ACT) or alpha 2 macroglobulin (A2M), and only a small percentage (10-20%) is free/unbound PSA (Chan and Sokoll, 1999). There is a strong correlation between the ratio of free to complex PSA and the likelihood of prostate cancer. For example if the ratio of free to total PSA reduces, then an individual is more likely to have prostate cancer (Stenman *et al*, 1991). This ratio is however affected by inflammation and can fail to differentiate between cancerous and non-cancerous conditions (Jung *et al*, 1998).

ii) Digital Rectal Examination (DRE)

DRE is an invasive procedure involving the doctor inserting a finger into the anus and feeling the prostate for abnormalities in shape or structure. If an abnormality is found the patient is admitted for a TRUS guided biopsy. DRE alone misses approximately 40% of prostate cancers (Goldstein and Messing 1998) but it is believed to have additional value in detecting clinically significant cancer especially with a low PSA range (<4ng/ml) (Van der Crujisen-Koeter *et al*, 2001).

iii) Transrectal Ultrasound (TRUS) Guided Biopsy

Once tests show that a patient either has an elevated PSA, abnormal prostate or haematuria (or a combination of these), the gland is assessed by TRUS guided biopsy. This allows the problem to be properly identified and the ideal course of treatment to be implemented. TRUS involves inserting a probe with a mounted needle into the patient's rectum and this is guided to the problematic area by sound wave emissions, which produce an ultrasound picture of the internal architecture of the gland. TRUS can detect

cancer as a hypoechoic lesion, but this is not specific to prostate cancer and can appear as a result of inflammation (Littrup and Bailey, 2000). Once the probe has reached the problem site, 5-10 cores are taken which are approximately 3-4mm in length and 1-2mm in diameter, these are then sent for histological analysis. This procedure takes 5-10 minutes and the patient is awake throughout, once complete the patient's urine flow is assessed and if there are no problems, the patient is sent home to await results.

1.4 Current treatment and Grading and Staging of Prostate Cancer

1.4.1 Treatment

The type of treatment implemented is dependent on a number of factors and these are:-

- Histology
- Health of the patient
- Age of the patient
- Medication
- Benefits in carrying out a procedure.

The treatment decided upon, must increase the 10-15 years survival rate, which is the time needed for the tissue to metastasise. There are therefore a number of routes that can be followed and these depend on the diagnosis of the patient. These routes are illustrated in *Table 1.3*.

Table 1.3. The course of treatment implemented depending on the patients' condition, identified during diagnosis. Diagnosis involves the patient having ever/or an elevated PSA, haematuria, abnormal DRE for a TRUS to be performed adapted using American Cancer Society/NCCN (2004).

Diagnosis	Life Expectancy Symptoms	Staging Work-up	Chance of Recurrence	Condition	Treatment Options
DRE PSA Biopsy (TRUS/ LNB/Radical)* Gleason Score CBC Imagine Test (RBS/CT/MRI)*		BPH		After diagnosis urine output improved, low PSA no haematuria After diagnosis urine output still low, high PSA, haematuria	<ul style="list-style-type: none"> Urine output improved and growth not cancerous - sent home. Not all obstruction removed TURP performed
	Life expect less than 5 years/ no symptoms	No other Test	Low Chance	After diagnosis low volume cancerous lumps identified still localised to the prostate gland. T1-T2a and Gleason score 2-6 and PSA below 10	<ul style="list-style-type: none"> TURP performed to remove cancer cells, followed by external beam radiation or brachytherapy or chemotherapy. Watchful waiting if patient is very old and/or on medication which would interfere with treatments. Hormone therapy used if most of the cancer cells removed by TRUS so only few cells remaining. Also implemented if patient too old and/or on medication so unable to have TURP.
		Bone Scan if Tumour T1-T2 and <ul style="list-style-type: none"> PSA above 20 Gleason 8 or higher Tumour growing out of prostate gland (T3, T4) 	Intermediate Chance	After diagnosis medium volume cancerous lumps identified still localised to the prostate gland. T2b-T2c or Gleason 7 or PSA 10-20	<ul style="list-style-type: none"> Radical retro pubic prostatectomy or TURP followed by external beam radiation, chemotherapy. Watchful waiting – if the patient is very old and/or on medication which would interfere with treatment. Radiation therapy – used if radical retro pubic prostatectomy can't be performed to kill cancer cells, remove or shrink the tumour
	Life expect at least 5 years/ symptoms	Symptoms present	High Chance	After diagnosis large volume cancerous lumps identified may not be localised to the prostate gland. T3a or Gleason 8-10 or PSA above 20	<ul style="list-style-type: none"> Hormone therapy used as removal of the gland no longer controls the disease; this maybe followed by external beam radiation or chemotherapy. Radiation therapy used to kill cancer cells, remove or shrink the tumour Radical retro pubic prostatectomy
		CT or MRI of Pelvis if tumour T1-T2 and <ul style="list-style-type: none"> Lymph node spread suspected Tumour is growing out of prostate gland (T3, 4). 	Very High Chance	Large volume cancerous lump which is no longer localised to the prostate gland T3b-T4 Any T, but lymph node spread Any T, any N but distant spread present (M).	<ul style="list-style-type: none"> Hormone therapy used as removal of the gland no longer controls the disease; this maybe followed by external beam radiation or chemotherapy. Radiation therapy used to kill cancer cells, used to shrink the tumour. Watchful waiting – if the patient is very old and/or the medication which would interfere with treatment

* LNB – Lymph Node Biopsy, Radical – Radical Retro-Pubic Prostatectomy, CBC- Complete Blood Count, to determine if patient has the correct number of various blood cells types. RBS – Radionuclide Bone Scan, inject radioactive material this attaches to diseased bone cells. CT – Computed Tomography, X-ray beams which create a series of picture, visualise the gland and surrounding area, MRI- Magnetic Resonance imaging – Magnetic fields used to create picture and visualise the gland.

These treatment options all aid in reducing/ removing prostate cancer and the procedures are described in more detail below.

a) TURP (Trans-urethral prostatectomy) is the most widely used treatment for prostate problems (used 80-90% of the time) and involves the removal of excess tissue, which maybe benign and causing blockage to the flow of urine. This procedure involves a thin tube being inserted up the penis to the prostate gland, where sections of the gland are pinched off. Once the TURP is complete the chips are assessed for cancer by a histopathologist. If only BPH is found and the urine flow has improved, PSA has reduced and the haematuria gone, the patient is sent home and has yearly check ups.

b) Radical Retro-Pubic Prostatectomy is performed in 10-15% of prostate cancer patients, and provides the most effective treatment for localised disease. This treatment involves the surgeon making an incision in the lower part of the abdomen about 4-5 inches long. Through this hole the two lobes of the prostate, seminal vesicles and any lymph nodes are removed, then the bladder neck is rejoined to the urethra. Radical prostatectomy can cause many side effects from pain, impotence, incontinence, and sometimes death (Quinn and Babb, 2002). Once complete the patient may have external beam radiation to ensure all cancerous cells are destroyed.

c) External beam Radiation therapy involves exposing the patient to reactive oxygen species and X-rays. These cause DNA damage, breaking the DNA strands and causing cellular death, (King, 2001).

d) Internal beam Radiation therapy - Brachytherapy is similar to radiation therapy but instead of focusing an external beam of radiation onto the affected area externally, radioactive seeds are embedded into the centre of the cancerous site to destroy the cells from within.

e) Hormone therapy involves using androgens like testosterone to try and reduce the growth. Although historically this type of treatment was only applied to advanced and metastatic cancer to try and shrink the tumour (Spence and Johnson, 2000) recently this

type of therapy has been applied also to treat low volume tumours (T1/T2a). The types of hormone therapy available are: Orchiectomy (castration), Luteinising hormone-releasing hormone (LHRH) analogs, Luteinising hormone-releasing hormone (LHRH) antagonist and antiandrogens.

f) Watchful waiting is not really a therapy but may be implemented if a patient is very old, very ill or on medication.

g) Chemotherapy is rarely used in prostate cancer. This involves taking oral substances which cause cellular death, these however are not only lethal to prostate cancer cells but will target all cancer types. In prostate cancer, chemotherapy is used in small amounts, (Bishop, 1999) due to non-specificity. Radiation therapy and chemotherapy may be used in combination with each other, or a surgical procedure to target all cancer cells within the body.

Once a patient has been successfully treated for prostate cancer, and the PSA level drops, urine flow improves and the cancerous lump has either been removed or significantly reduced, they will be sent home. Typically, they then return every 3 months for the first 12 months, then every 6 months for the next 12 months, thereafter they return once a year, if no recurrence occurs.

Although there are numerous treatments, one-third of prostate cancer patients are not diagnosed until a late stage, by which time no surgical care is possible. The only option then is hormone therapy (Belledgrun *et al.*, 1998).

1.4.2 Grading and Staging of Prostate Cancer

Grading and staging of prostate cancer enables the investigator to assess how far the disease has developed, and what optimal treatments can be implemented. The Gleason score is used to grade prostate cancer and is determined by the architecture of the cellular area under a low powered microscope (Bishop, 1999). This score is calculated by analysis of two histological areas, which are allocated a pattern between, one to five depending on how differentiated the areas are (*Figure 1.4*) (Spence and Johnson, 2001).

These two patterns are then added together to give a score (between 2-10), this then placing the tissue within one of three descriptive grades (*Table 1.4*).

Table 1.4. Table showing the three separate areas in which the histological grades can be placed.

Low Grade	Score between 2-4 well differentiated
Intermediate Grade	Scores between 5-7 moderately differentiated
High Grade	Scores between 8-10 poorly differentiated

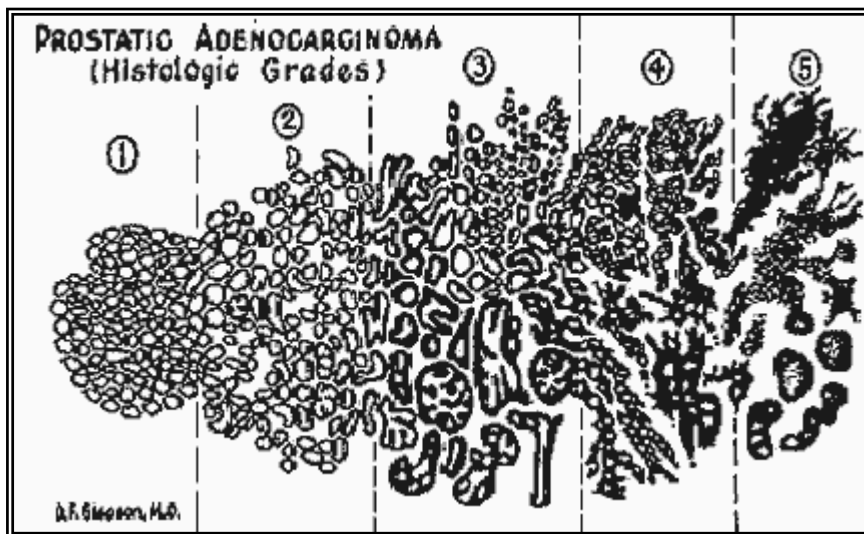


Figure 1.4. Diagram showing the grading of prostate adenocarcinoma. From well-differentiated (pattern 1) to poorly differentiate (pattern 5).
<http://www.phoenix5.org/Infolink/GleasonGrading.html>

As well as the Gleason score, the **Tumour, Node, Metastasis** classification (TNM) is also used to stage prostate cancer (Spence and Johnson, 2001). This classification system describes the extent of the primary tumour (T), the absence/presence of cancer to the nearby lymph nodes (N) and the absence/presence of cancer to distant sites (M). This system takes into account the appearance of the abnormal cells, their cellular area and the size of the tumour. The T stage classification system for prostate cancer can be divided into two stages, the *clinical stage* and the *pathological stage*.

The clinical stage is based on DRE and TRUS, and is used in making treatment decisions (American Cancer Society/NCCN, 2004). While the pathological stage is

based on surgical removal (radical prostatectomy) and examination of the entire prostate gland, therefore both seminal vesicles and in some cases the nearby lymph nodes are removed, this makes the pathological staging more accurate in predicting patient survival (American Cancer Society/NCCN, 2004).

Figure 1.5 Shows the four T stages for describing prostate cancer as well as the N and M stage.

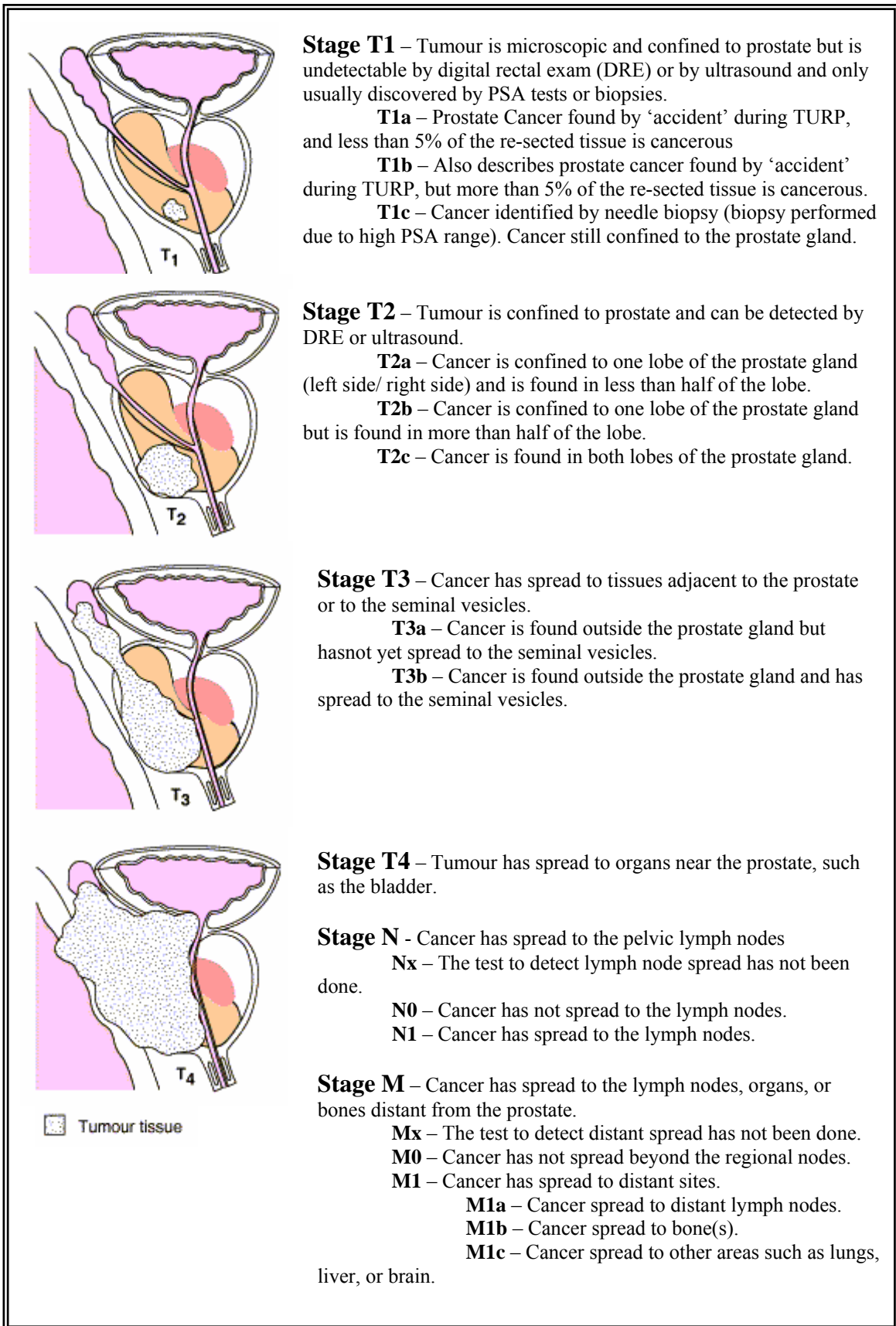


Figure 1.5. Diagrammatic representation of the TNM (Tumour, Nodes, Metastases) system, which gauges the severity of cancer on an escalating scale and the description of different tumour stages. Adapted using:- American Cancer Society/NCCN, 2004), www.prostateline.com/.../9898_12069_4_5_0.aspx, <http://www.phoenix5.org/Basics/stagingfda.html>.

Being able to allocate stage and grade to prostate cancer helps to determine what treatment will be best to cure or control the disease.

1.5 Minichromosomal Maintenance Proteins (MCMs)

Due to the current debate into the usefulness of PSA, alternative marker/s for the diagnosis of prostate cancer are being investigated. For this study minichromosomal maintenance proteins (MCMs) are under analysis, as these molecules are involved in the cellular cycle. Recent work on MCMs suggests that they are potential markers for the diagnosis of prostate cancer, as they are expressed in replicating cells as well as cells with a potential to proliferate (Bailis and Forsburg, 2004). This means that the MCMs are ideal markers for pre-cancer/cancer diagnosis and possible targets for anticancer drugs (Shreeram *et al*, 2002).

1.5.1 Function of the Minichromosomal Maintenance Proteins (MCMs)

Minichromosomal maintenance proteins (MCMs) were first identified in *Saccharomyces cerevisiae* by two laboratories independently (Maine *et al*, 1984, Henessy *et al*, 1990). Of the sub-units identified, MCM2-7 is highly conserved in eukaryotes and essential for DNA replication (Johnson *et al*, 2003). These MCMs are crucial for the precise replication of genetic material, are expressed during particular phases of the cellular cycle, and are not expressed in quiescent, differentiated or senescent cells (Davidson *et al*, 2003, Padmanabhan *et al*, 2004) (*Figure 1.6*).

The cellular cycle allows the production of genetic entities and is made up of essential phases and proteins these are illustrated in *Figure 1.6*. There are two main phases within the cell cycle, the Interphase and Mitotic phase (M phase). The Interphase is essential for mitosis to occur and requires numerous biological and physiological functions, this phase is therefore subdivided into the G1 phase, the S phase (S = synthesis), and the G2 phase. The Mitotic phase or M phase is an essential process where nuclear division and cytokinesis occurs producing two identical daughter cells.

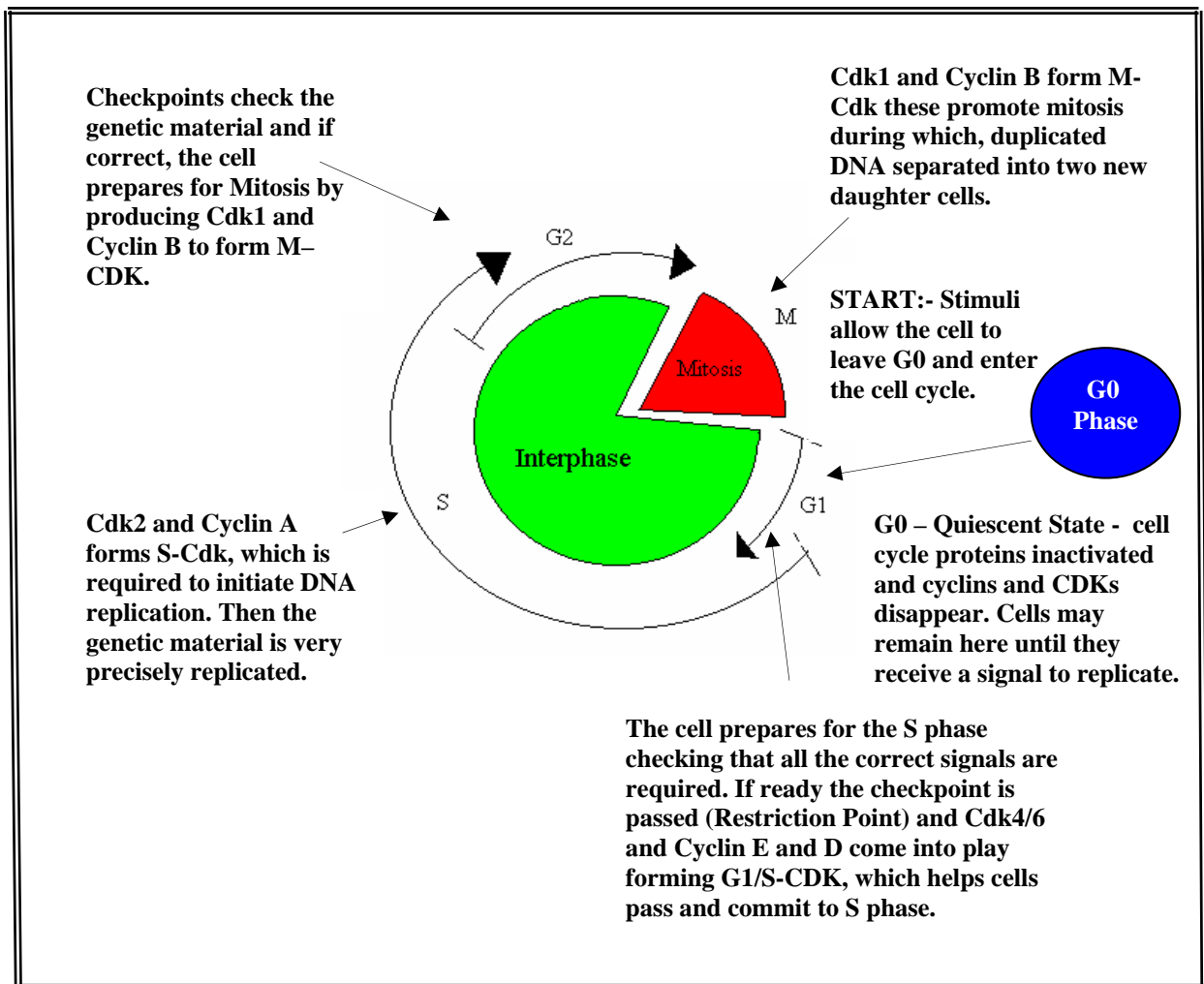


Figure 1.6. A diagrammatic representation of the stages in the cell cycle and the corresponding cyclins and cyclin dependent kinases (picture adapted from Cotran, *et al*, 1999).

Although MCMs are essential for the cellular cycle they only come into play at the start of the G1 phase and then start to deplete during the S phase. Therefore in this section only the MCM part of the cellular cycle will be discussed, and more information into the other areas can be found in the papers by Laskey and Fairman (1988), Pardee (1989), Koshland (1989). Once the MCMs come into play they are involved in the formation of the pre-replication complex and the initiation complex. These complexes are essential for licensing of replication and for the production of genetic material.

a) The Pre-Replication Complex

The pre-replication complex was first proposed in 1989 by Laskey and Blow and is an essential mechanism for the precise replication of genetic material. The pre-replication complex is made up of a variety of sub-units and the first components recruited are

called the origin recognition complexes (ORCs). These ORCs bind to the chromatin at late mitosis/early G1 phase and only become dissociated at the end of the S phase/start of the G2 phase, when DNA replication is in process and re-replication must be prevented. In eukaryotes when a cell receives a signal to replicate, the ORCs bind to the chromatin in an ATP dependent manner at specialised DNA binding sites (Dillin and Rine 1998). These ORCs remain bound to the binding sites throughout the cell cycle and act as landing pads for the recruitment of other essential replication proteins (Bell and Stillman, 1992) (*Figure 1.7A*). In eukaryotes thousands of binding sites are located along the chromatin, ensuring the entire genome is copied quickly and efficiently (Blow and Hodgson, 2002).

After the ORC complexes are loaded several other loading factors are recruited, such as Cdc6 and Cdt1 (*Figure 1.7B*). These molecules bind to the ORC and then aid in the migration and recruitment of MCMs. For the MCMs to then associate to the pre-replication complex they are firstly transported to the nucleus and then signalled by G1-CDK and G1/S-CDK. These cyclins and cyclin dependent kinases enable the association of the MCMs by the phosphorylation of Retinoblastoma (Rb), which in its unphosphorylated form is in contact with the carboxyl terminal of MCM4 and MCM7 (Laskey *et al*, 1996). Once phosphorylated, MCM7 and 4 promote the assembly of the pre-replication complex (Laskey *et al*, 1996). Therefore the six MCMs bind to the chromatin in two staggered trimers, as MCM4, 6 and 7 bind very rapidly to the chromatin, while MCM2, 3 and 5 bind at a later stage (Coue *et al*, 1998).

When all six MCMs are bound they form a 450KDa hetero-hexamer complex (Kimura *et al*, 1996) (*Figure 1.7D*) and the pre-replication complex is acted upon by S-CDK, G1/S-CDK and the transcription factor E2F to license DNA replication. Once instructed to replicate, it used to be believed that the role of the MCMs was complete. However Labib and Diffley (2001), believe that there is a growing body of evidence to show that the MCM complexes unwind DNA and therefore have DNA helicase activity. Therefore the pre-replication complex is not the end of the MCMs role, and a new complex called the initiation complex comes into play.

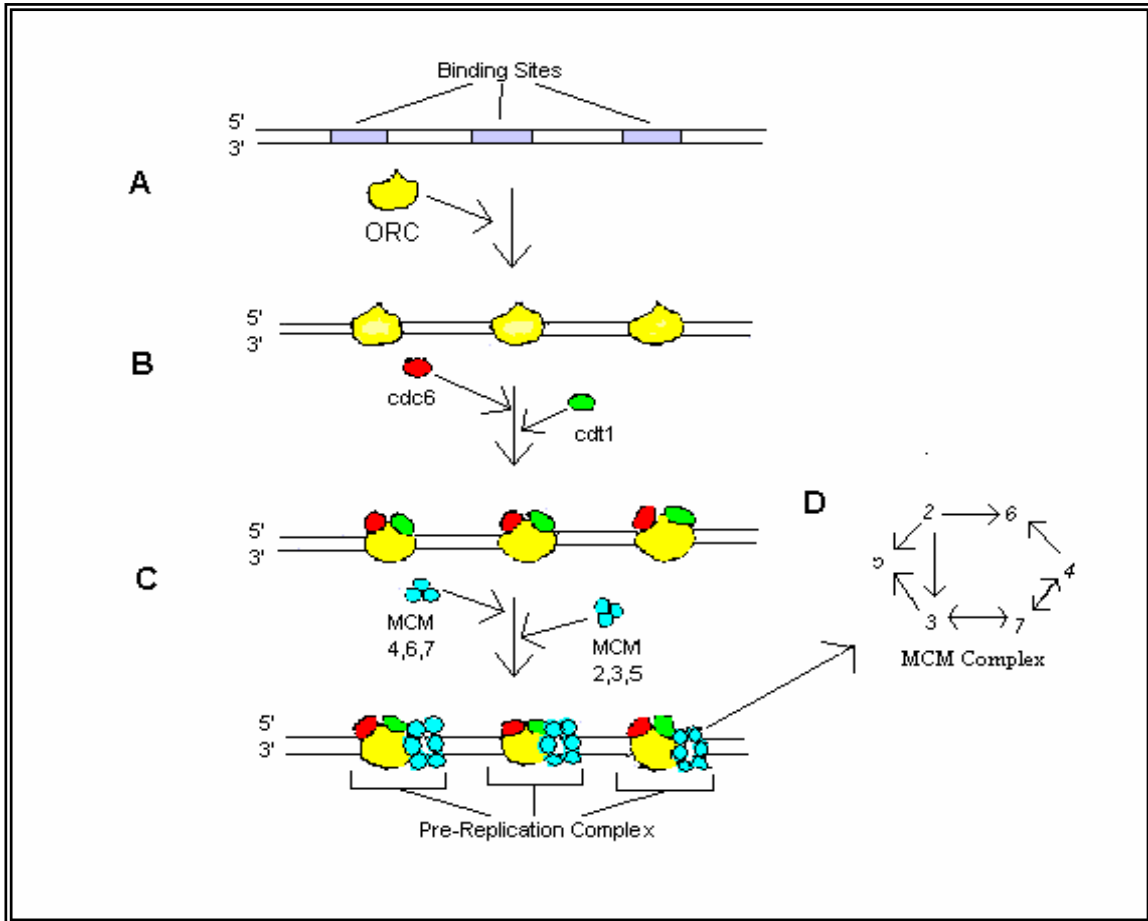


Figure 1.7. Shows the steps involved in the formation of the pre-replication complex. A – The ORCs bind to binding sites in an ATP dependent manner. B – Cdc6 and CDT1 bind to the ORC and aid in the recruitment of the MCMs. C - The MCMs are signalled to bind to form the pre-replication complex. D – The MCMs form a heterohexameric ring consisting which has a rough cylindrical shape with a wide central channel.

Adapted using:- Masuda, *et al* (2003), Diffley (2002), www.biochem.umd.edu/biochem/kahn/molmachines/ORC/ORC.html.

b) The Initiation Complex

After licensing of replication the pre-replication complex is broken-down and a new complex called the Initiation complex is produced. This complex is produced by S-CDK, G1/S-CDK and Cdc7 acting on the pre-replication complex and causing the phosphorylation of MCMs, loading of Cdc45, and the removal of Cdc6, Cdt1 and later on the ORC *Figure 1.8*.

It is important that the MCMs are phosphorylated for helicase activity to occur, and it was found that *in vivo* an active complex containing one copy of each of the 6 MCM sub-units can be detected (Schwacha and Bell, 2001), however these failed to contain

helicase activity. Therefore Schwacha and Bell (2001) suggested the possibility of two sub-complexes of MCMs: MCM2, 3 and 5 and MCM 4, 6 and 7 arranged in a two-staggered trimer. Due to the discovery of the trimers it is believed that the MCM sub-complexes perform different roles, with MCM4, 6, 7 providing helicase activity and MCM2, 3, 5 providing a regulatory function.

The phosphorylation of MCM2 and MCM3 causes the removal of potential inhibitors from MCM4, 6 and 7 (Sato *et al*, 2000). Therefore the pre-replication complex is broken down as Cdc6 and Cdt1 are removed. Once Cdc6 and Cdt1 proteins are removed, MCM2, 3 and 4 are continually phosphorylated by Cdc7 whilst Cdc45 binds to the chromatin, causing the release of MCM2, 3 and 5 (Leatherwood, 1998) (Fletcher *et al*, 2003) (*Figure 1.8*).

More MCM4, 6 and 7 now bind, and a 600KDa MCM complex is formed. These complexes move away from the site of origin and anchor themselves along the DNA strand (*Figure 1.8*) (Laskey and Madine, 2003). Ritzi *et al* (1998) shows by experimentation with human cells, that the MCMs are separated by at least 0.5-1 kb of DNA from the ORC. Once this separation is complete, the ORC are then able to leave the site of origin and the initiation complex is formed. Numerous possible mechanisms have been put forward to explain how the MCMs function in the unwinding of genetic material, these are shown in *Table 1.5*.

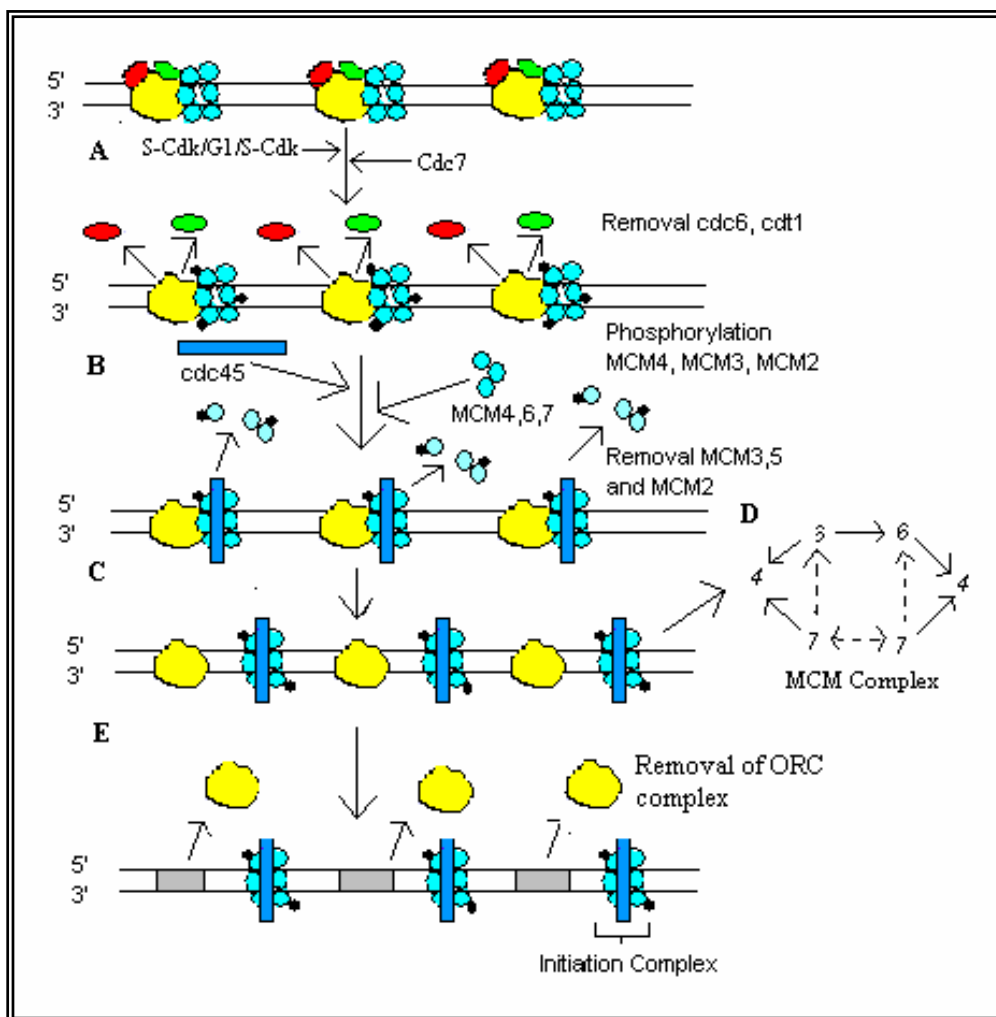


Figure 1.8. Show the formation of the initiation complex.

A - S-CDK, G1/S-CDK and Cdc7 acts on the pre-replication complexes causing the removal of Cdc6, Cdt1 and phosphorylation of the MCMs.

B - Cdc45 then binds to the MCMs causing the removal of MCM2, 3 and 5 and the addition of MCM4, 6 and 7.

C - The Initiation Complex then translocates along the DNA strand.

D - A 600kDa MCM Complex is formed

E - The ORC complexes are removed and the MCM complexes move away from the site of origin.

Adapted from: Masuda, *et al* 2002, Yabuta, *et al* 2003.

Table 1.5. Shows the types of models suggested for DNA unwinding.

Model	Mechanism	References
Torsion Model	When the helicase activity binds to both DNA tails and rips the duplex.	Patel and Picha, 2000,
Helix Destabilising Model	Where helicase binds to the directly melt duplex of the DNA.	Patel and Picha, 2000,
Wedge Model	Where helicase binds to one strand and drives the DNA apart.	Patel and Picha, 2000,
Pump in Ring Model	Where single stranded DNA is pulled through the central cavity unwinding the DNA.	Kaplan, <i>et al</i> 2003 Laskey, Madine, 2003

Of the proposed mechanisms the ‘pump in model’ is becoming increasingly evident (Figure 1.9). For this, hypo-phosphorylation of MCM4 allows the MCM complexes to express helicase activity. DNA unwinding then occurs, and the MCMs twist the DNA strands in opposite directions, so small segments of DNA are transported back to the site of origin. Cdc45 then acts on the single strands producing complementary strands (Figure 1.9). These complementary strands are produced because Cdc45 has physical interaction with DNA polymerase α , so Cdc45 is directly involved in the loading of polymerase (Mimura and Takisawa, 1998).

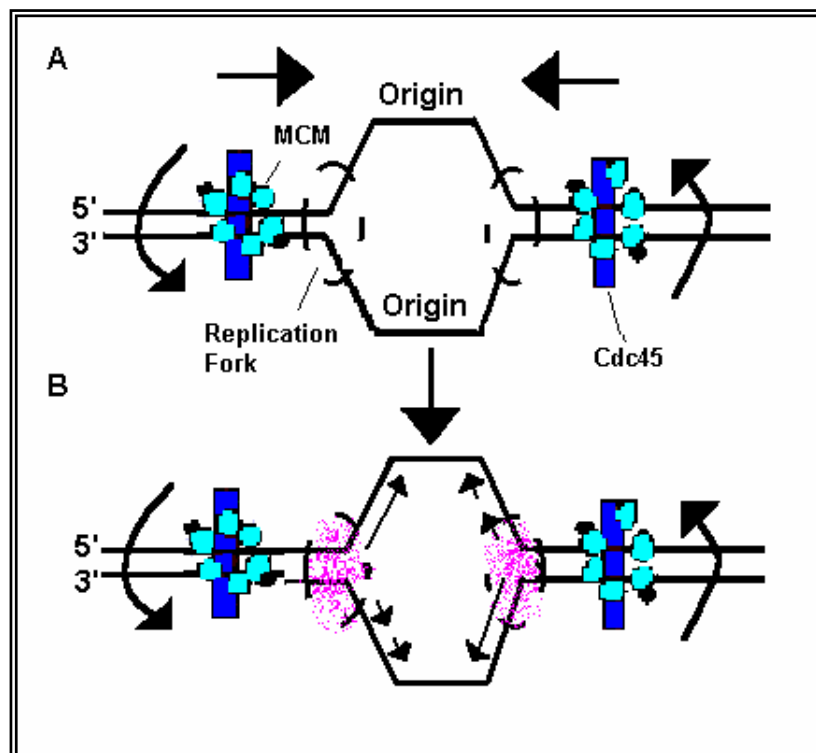


Figure 1.9. Show the ‘Pump in Model’ mechanism involved in unwinding and synthesis of new complementary strands of DNA.

A – MCMs twist in opposite directions to separate DNA strands, separated strands move back to site of origin.

B – Cdc45 activates DNA polymerase which produces the new complementary strands at the replication fork.

Mechanism illustrated is the Pump in model. Adapted using Laskey and Madine 2003, Kaplan, *et al* 2003.

c) Genomic Stability

Although the MCMs play an important role in replication, this is not their only role as it is estimated that the MCMs are 40-100 fold more abundant than the origins require (Bailis and Forsburg, 2004). Also, the majority of the MCMs are not co-localised to the site of new DNA synthesis (Krude *et al*, 1996). Numerous articles are now postulating

that MCMs may be implicated in other roles, for example it has been suggested that they are involved in genome stability, transcription, DNA repair (Pingy, 2003), as well as playing a role in chromosomal dynamics and integrity (Bailis, Forsburg 2004). For instance Liang, *et al* (1999) has shown that a modest reduction in MCMs confers chromosome instability and DNA damage.

1.5.2 Role of the Minichromosomal Maintenance Proteins (MCMs) in Diagnosis

Due to the role of the MCMs during DNA replication, they have been investigated as potential diagnostic markers for cancer. For instance, MCM2, 5 and 7 have been investigated in numerous cancerous tissues from prostate to lung, and have been shown to be expressed in restricted proliferation compartments (<2% basal layer) for normal tissue. In cancerous conditions however they are no longer localised to the 'normal' proliferative areas and seem to be increasingly expressed in the majority of the cells (nuclei epithelial cells) showing diagnostic potential (Padmanabhan *et al*, 2004, Meng *et al*, 2001, Freeman *et al*, 1999).

1.6 Project Aims and Objectives

An increased understanding of molecular pathogenesis may aid in identifying possible new methods for early diagnosis of prostate cancer and may provide further understanding into the differences between benign and malignant disease.

Although MCM2, 5 and 7 have already been investigated in prostate cancer separately, no one has performed a conclusive study analysing all three markers collectively, to determine their expressional alterations in neoplastic conditions and to assess these as potential diagnostic biomarkers.

Aims

The aim of this study is to investigate MCM's as possible molecular markers of prostate disease, and to assess their potential as diagnostic biomarkers to improve current diagnostic strategies. In addition, the current diagnostic marker PSA is analysed to assess its ability to diagnose prostate cancer effectively and to observe how specific it is to the prostate gland.

Objectives

The objectives are:

- To analyse PSA expression in a number of human cell lines to optimise detection techniques at a protein level and to establish if PSA is specific to prostatic cells.
- To analyse MCM expression in prostatic human cell lines, to optimise detection techniques at both RNA and protein levels and to assess expressional differences between malignant and non-malignant cells.
- To assess MCM/PSA expression in human prostatic tissue using the optimised techniques from cell culture, to distinguish differences between cancers of different Gleason score and BPH To then utilise this information to aid development of a non-surgical technique that may aid in diagnosis.
- To investigate the expression of MCM/PSA in clinical samples such as urine and blood, to investigate whether a difference can be detected between malignant and non-malignant conditions and to assess if this information can provide a possible starting platform for monitoring prostate cancer using the MCM's, by non-surgical means.

Chapter 2 – Prostate Specific Antigen (PSA) Cellular Expression and Secretion in Human *In Vitro* Models.

2.1 Introduction

The assumption that PSA expression/secretion is exclusively prostatic in origin is now being questioned and due to this dispute, the work described in this chapter was undertaken. The work performed was carried out to examine whether PSA was expressed in non prostatic human cell lines *in vitro*, and then to consider the implication of this, for PSA to be regarded as a reliable prostatic diagnostic marker.

Due to the uncertainty into the usefulness of PSA this marker is under heavy debate, as some believe PSA is the best bio-marker for prostate cancer, and that screening should be applied at age 40 with annual testing (Catalona and Loeb, 2005), while others believe that the relationship between prostate cancer and PSA is of limited value (Stamey, *et al*, 2002).

Not only this, but some believe PSA is produced mainly by the prostatic epithelium, with only low concentrations in endometrium, breast tissue, adrenal, renal and female serum (Van der Cruijssen-Koeter *et al*, 2001), while others show that the prostate does not exclusively produce PSA, with PSA detected in breast cancer tissue and ovarian cancer tissue (Kucera *et al*, 1997). In addition, a number of other human cell lines have been shown to express and secrete PSA such as; WI-38 cells (normal human embryonic lung), and KB cells (epidermoid carcinoma) (Waheed and Etten, 2001).

The work for this chapter mainly focuses on six human cell lines, of which five were carcinomas and two were of prostatic origin.

2.2 Material and Methods

Human cell lines were cultured as stated in *Appendix A Section A.2.2* to assess PSA expression. Cellular PSA was assessed by immunocytochemistry (ICC), western blotting and an ELISA, while secreted PSA was assessed by western blotting, dot blot and an ELISA.

2.2.1 Cellular PSA

Initial screening was performed on thirteen human cell lines cultured in our laboratories, by ICC and the methodology for this procedure is shown in *Appendix A Section A.4 and Section 2.2.1.i*.

2.2.1.i Immunocytochemistry (ICC)

The primary antibodies used were either Mouse Monoclonal antibodies to PSA Clone ER-PR8 (Stratech, Cambridge UK) or Rabbit Polyclonal antibodies to PSA RB-9065-P (Stratech, Cambridge UK). These primary antibodies were applied to the slides in a 1:25 dilution in a 1% (v/v) secondary serum (either rabbit or goat) in 1M TBS pH 7.5, and incubated in a humidity chamber overnight at 4°C. For all slides appropriate negative controls were produced by removal of the primary antibodies.

Once the initial screening by ICC was completed, six human cell lines were chosen to assess their cellular PSA levels further, therefore a protein extraction and western blot was performed.

2.2.1.ii Protein extraction

A protein extraction was performed on six human cell lines and the reason for their choice is shown in *Table 2.1*. The protein extracted was then quantified and PSA expression was assessed.

The protocols for protein extractions and protein quantification are shown in *Appendix A Section A.5 and A.6*.

Table 2.1. The six cell lines studied and why.

Cell Line	Cell Line Derived from:	Reason for Choosing Cell Line.
PNT1A	Normal Prostate Epithelium	To assess PSA expression between normal and cancerous prostatic epithelium cells, to compare normal expression with other non-prostatic epithelium cells.
PC-3	Prostate Adenocarcinoma	To assess PSA expression between normal and cancerous prostatic epithelium cells, to compare cancerous expression with other non-prostatic epithelium cells
RT112	Bladder Epithelium Carcinoma	To assess other non-prostatic epithelial carcinoma cell lines to observe if they express PSA and to compare expressional variations of PSA in cells from organs found in close proximity to the prostate.
CaCO2	Colon Adenocarcinoma Grade 11	
PE/CA-PJ41	Oral Squamous Cell Carcinoma	To assess other non-prostatic epithelial carcinoma cell lines to observe if they express PSA, and to compare expressional variations of PSA in cells found in areas away from the prostate.
OE21	Oesophageal Squamous Carcinoma	

2.2.1.iii Western blot

A western blot was performed on the six human cell lines to assess which cells produced PSA and to compare differences in the amount present.

The methodology for performing a western blotting is shown in *Appendix A Section A.7*.

2.2.2 Secreted PSA

To test secreted PSA the six cell lines were seeded into 12-well plates at 2×10^4 cells/well and a cell count was performed every 24 hours on 2 wells (*for cell count refer. to Appendix A Section A.3*), until a logarithmic growth curve was established. Once the growth curve was produced, the average seeding density at which the cells started to enter the log phase was chosen (1×10^5), and the cells were again seeded at this density into a 12-well plate. The cells were then left to grow for 24 hours in normal medium, washed in PBS, and supplied with new medium that contained no FCS. These cells

were then grown for a minimum of 24 hours and a maximum of 72 hours. Every 24 hours 2 wells had their medium aspirated (this was inhibited by 50µl of protease inhibitor cocktail MP Biomedicals Inc, Ikirch France) and these wells were then trypsinised and a cell count was performed.

Once the medium was collected, the samples were concentrated using a 10KDa concentrator (Millipore), to remove any protein of a lower molecular weight than 10KDa. The apparatus was set up as shown in the manufacturer guide then 0.5ml of the sample was loaded into the sample reservoir and centrifuged for 30 minutes at 14000 xg at RT. After centrifugation the sample reservoir was emptied into a new retention vial and centrifuged at 1,000 xg for 3 minutes at RT. The concentrated protein was then collected and stored at -80°C until required.

2.2.2.i Western blot and Dot blot

To assess the secreted PSA from the six human cell lines a western blot and dot blot were performed on the concentrated secreted protein, protocols are shown in *Appendix A Section A.7 and A.8*.

2.2.2.ii PSA ELISA

A direct PSA sandwich ELISA PS092F (Calbiotech, CA USA) was then performed on the cellular/secreted PSA to assess which cell produced PSA and to compare the amounts.

The protocol for the ELISA is shown in *Appendix A Section A.9*.

2.3 Results

2.3.1 Cellular PSA Expression

ICC (*Section A.4 and Section 2.2.1.i*) was performed using monoclonal and polyclonal antibodies to PSA, on thirteen human cell lines.

Table 2.2 and Figures 2.1-2.10 and Appendix C Figure c.1 shows the ICC results for monoclonal and polyclonal antibodies to PSA. The legend for each figure includes a description of the results.

Table 2.2. – Table showing the ICC results obtained when thirteen human cell lines were tested for PSA expression using Monoclonal and Polyclonal Antibodies.

Cell Line Names	Derived From	PSA Staining Visible	Monoclonal/ Polyclonal Antibody	Locality of Staining
PNT1A	Normal Prostate Epithelium	Yes	P	CN
PC-3	Prostate Adenocarcinoma	Yes	B	CN
ZR 75 1	Breast Epithelium Carcinoma	Yes	B	CN
RT 112	Bladder Epithelium Carcinoma	Yes	B	CN
T 24	Bladder Epithelium Carcinoma	Yes	P	C
HT 29	Colon Adenocarcinom Grade 1	Yes	P	C
CaCO 2	Colon Adenocarcinoma Grade 11	Yes	B	C
HET1A	Oesophageal Cells	Yes	B	CN
PE/CA- PJ 41	Oral Squamous Cell Carcinoma	Yes	B	C
SK MES	Lung Squamous Carcinoma	Yes	P	C
OE 33	Oesophageal Adenocarcinoma	No	-	-
OE21	Oesophageal Squamous Carcinoma	No	-	-
NCL H23	Lung Adenocarcinoma	No	-	-

Key

B = PSA detected in cell line by both Polyclonal and Monoclonal Antibodies to PSA,

P = PSA detected in cell line only by Polyclonal Antibodies to PSA,

M = PSA detected in cell line only by Monoclonal Antibodies to PSA,

CN = Both Cytoplasmic and Nuclear PSA staining visible in cell line,

C = Only Cytoplasmic PSA staining visible in cell line,

N = Only Nuclear PSA staining visible in cell line.

- = No staining Visible

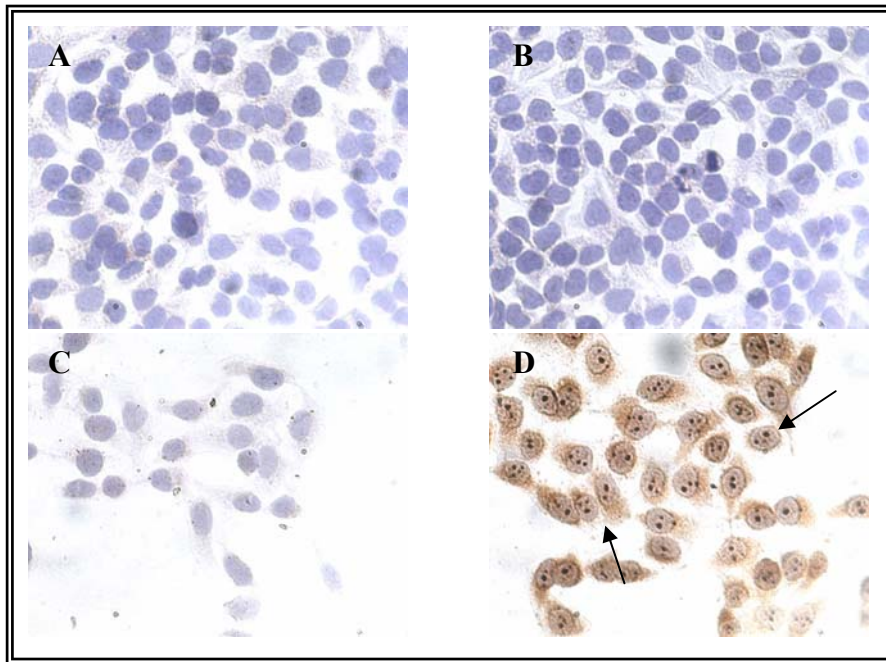


Figure 2.1. – ICC results for PNT1A using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised), nuclear and cytoplasmic staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, No PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

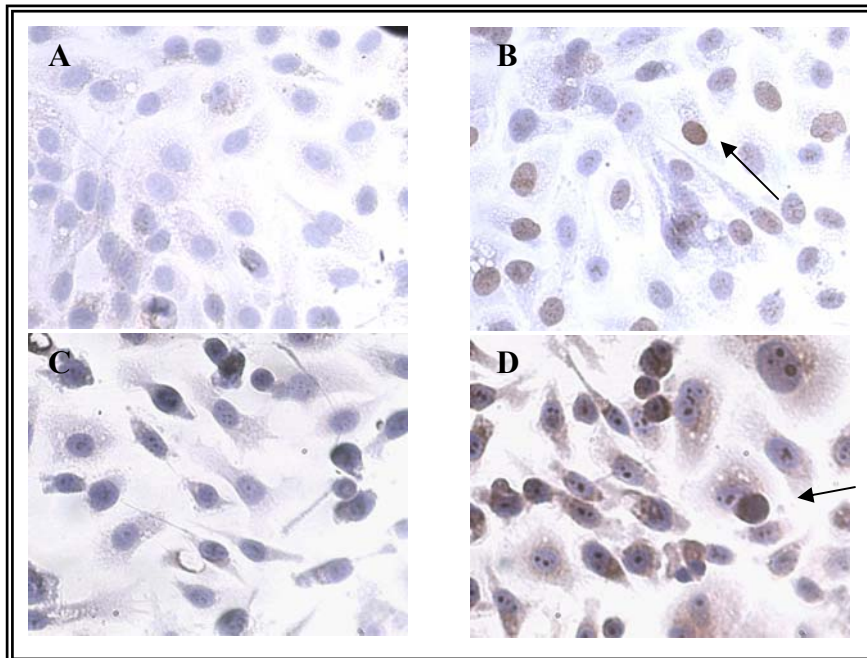


Figure 2.2. - ICC results for PC-3 using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised), nuclear and cytoplasmic staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

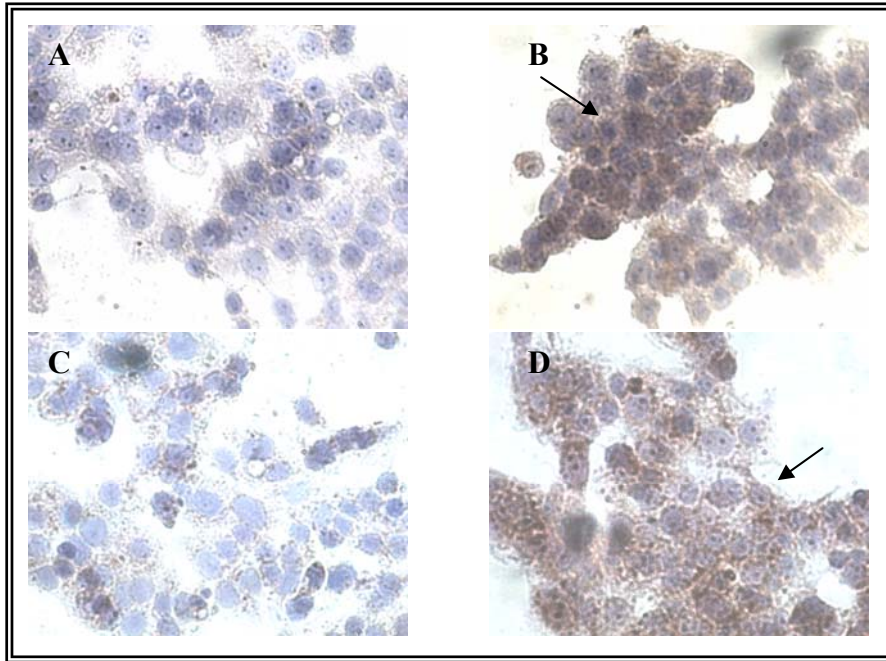


Figure 2.3. – ICC results for ZR-75-1 using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised), cytoplasmic and nuclear staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

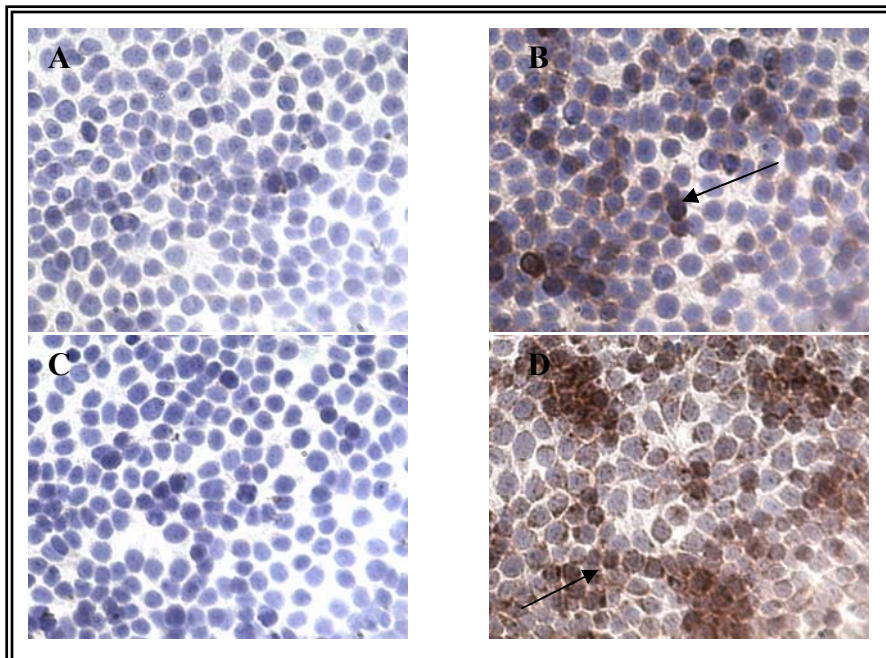


Figure 2.4. – ICC results for RT112 using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised), cytoplasmic and nuclear staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

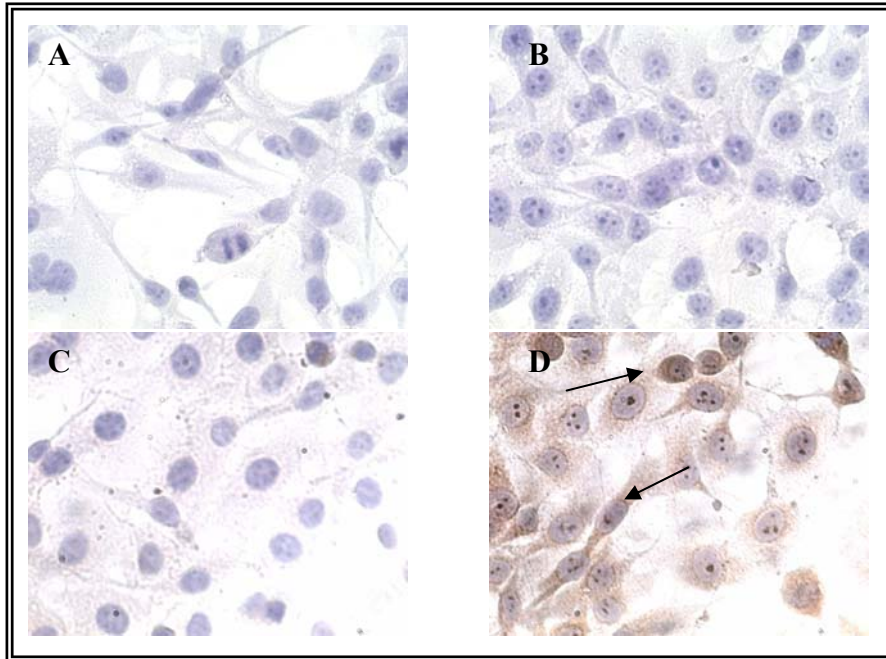


Figure 2.5. – ICC results for T24 using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised) cytoplasmic staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, No PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

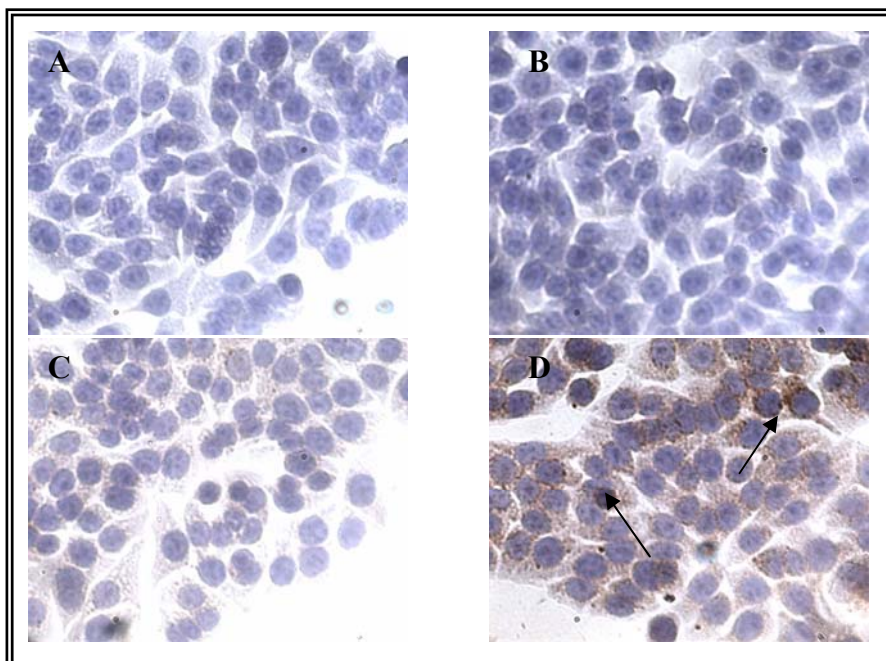


Figure 2.6. - ICC results for HT29 using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised) cytoplasmic staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, No PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

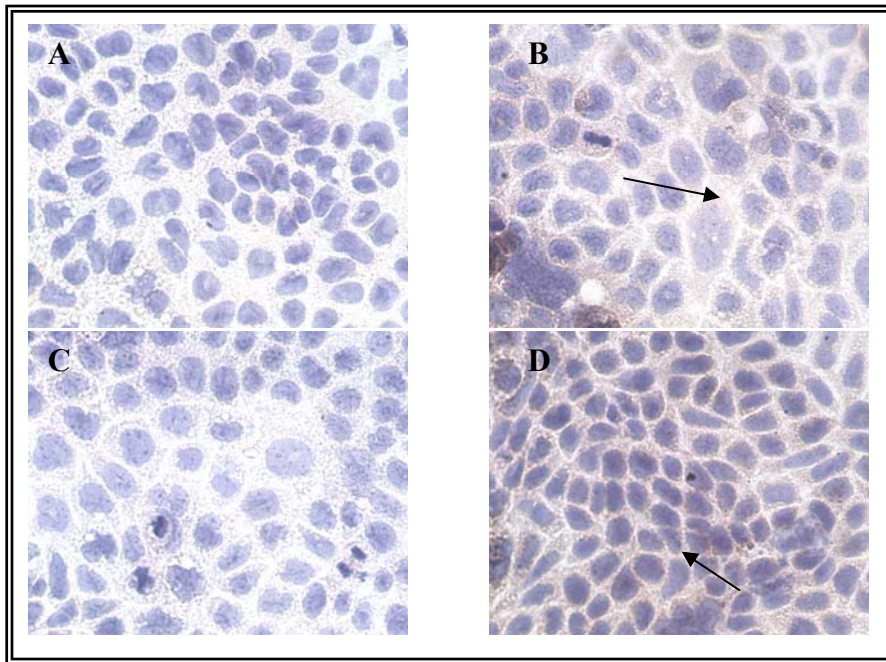


Figure 2.7. – ICC results for CaCO₂ using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised) cytoplasmic staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

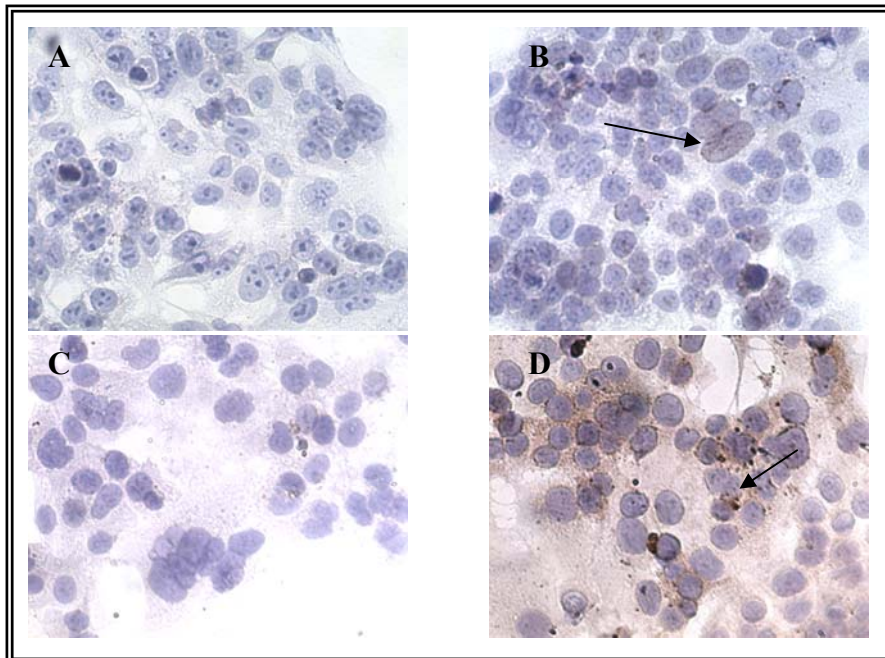


Figure 2.8. – ICC results for HET1A using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised), cytoplasmic and nuclear staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

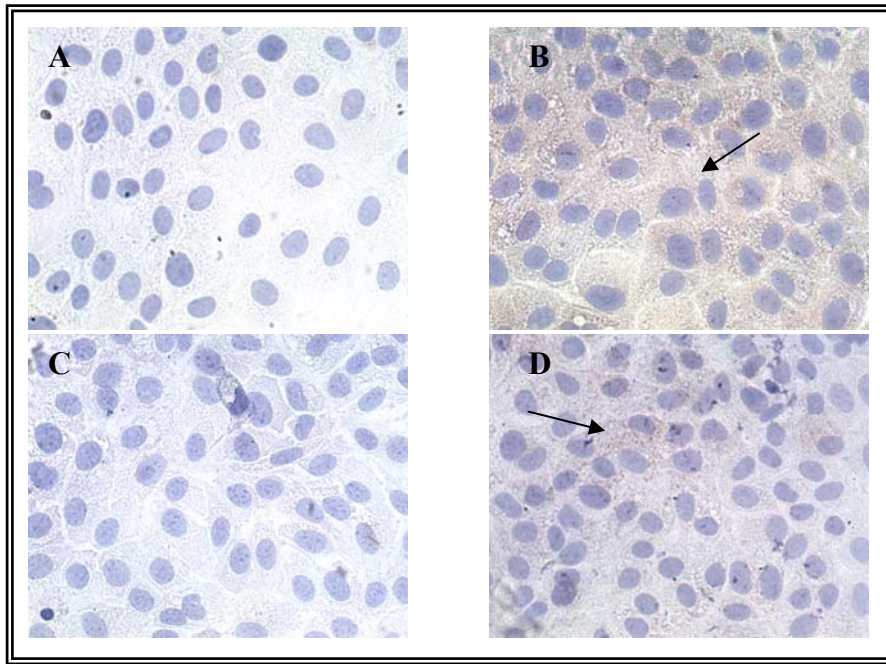


Figure 2.9. – ICC results for PE/CA-PJ 41 using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised), cytoplasmic and nuclear staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

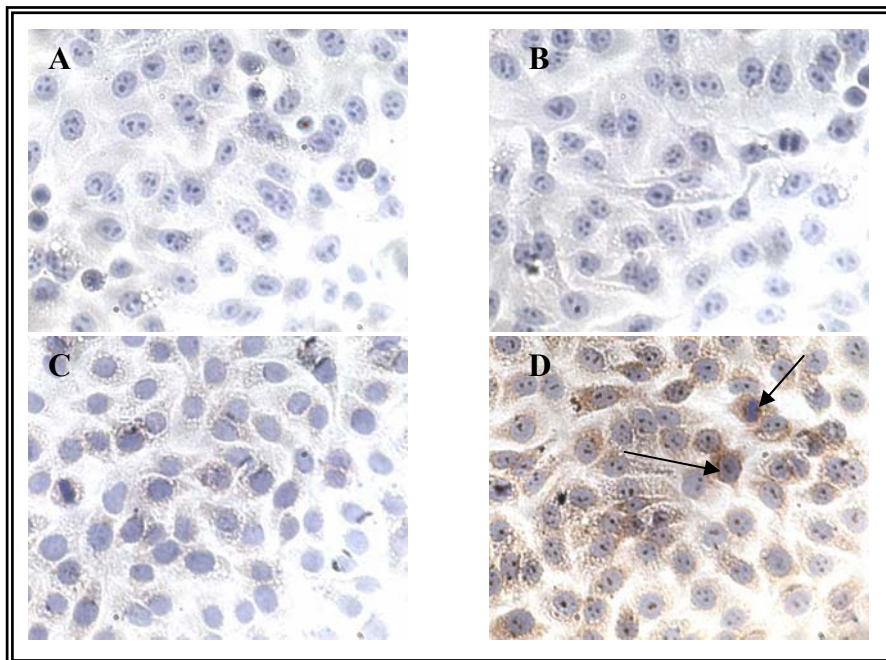


Figure 2.10. - ICC results for SK MES using antibodies to PSA. Arrows indicate examples of positive cells (brown precipitate visualised) cytoplasmic staining apparent. A = Negative Control, No Monoclonal Antibodies to PSA applied, B = Monoclonal Antibodies to PSA applied, No PSA expression detected C = Negative Control, No Polyclonal Antibodies to PSA applied, D = Polyclonal Antibodies to PSA applied, PSA expression detected. Magnification x400

PSA immunoreactivity (*Table 2.2, Figures 2.1 – 2.10, Appendix C, Figure c.I*) is detected in ten out of the thirteen human cell lines. Of these ten, PSA is detected in six cell lines using both the monoclonal and polyclonal antibodies to PSA (PC-3, ZR-75-1, RT112, CaCO₂, PE/CA-PJ41, HET1A) while the other four only express PSA when the polyclonal antibodies are used (PNT1A, T24, HT29, and SK-MES). Also, the cellular distribution of immunoreactivity varies between the cell lines, with PNT1A, PC-3, ZR-75-1, RT112 and Het1A cells expressing both cytoplasmic and nuclei PSA, while the T24, HT29, CaCO₂, PE/CA-PJ41 and SK-MES cells only show cytoplasmic expression of PSA.

Western blot

Western blotting for six human cell lines was then performed, to assess if PSA expression varies between cancerous and non-cancerous prostatic conditions and to compare PSA expression in non-prostatic cells to prostatic cells. The six cell lines chosen are:- PNT1A, PC-3, OE21, RT112, CACO₂, PE/CA-PJ41 (Ref. *Table 2.1* for the reasons these cell lines were chosen) and all, with the exception of OE21, are shown to express PSA via ICC.

Figure 2.11 illustrates the results of the western blot, using polyclonal antibodies to PSA.

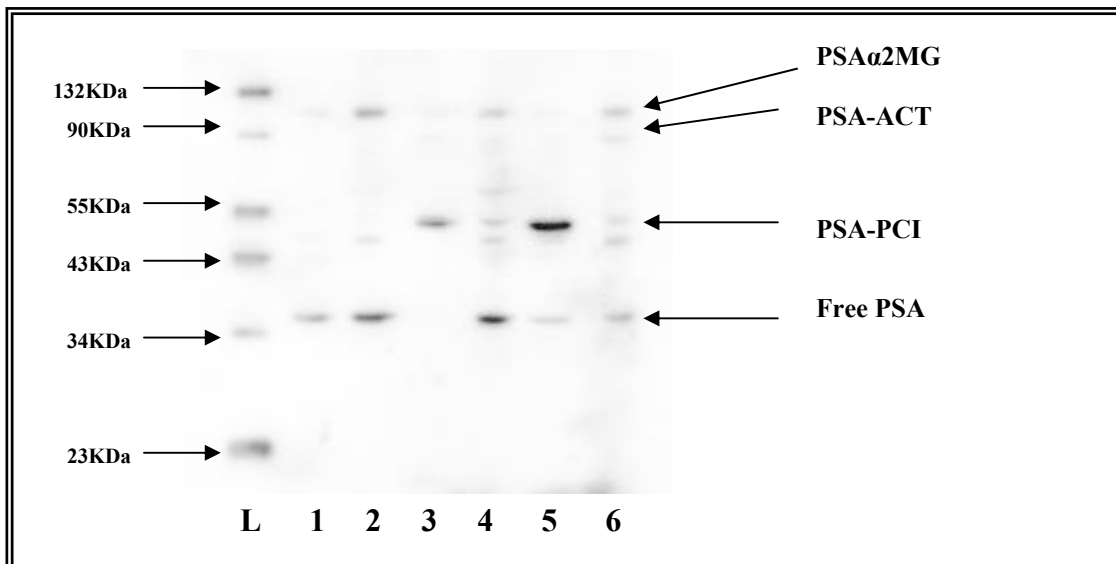


Figure 2.11. PSA results obtained for the six human cell lines when using Polyclonal Antibodies (PSA RB-9065-P).

The arrows on the left-hand side, indicate the different complexes of PSA and these are:

**PSA α 2MG = PSA complexed with α 2-macroglobulin,
 PSA-ACT = PSA complexed with α 1-antichymotrypsin,
 PSA-PCI = PSA complexed with protein inhibitor C,
 Free PSA = PSA alone.**

While the six cell lines are indicated by the numbers and these are:

1 = PNT1A, 2 = PC-3, 3 = OE21, 4 = CaCO₂, 5 = PE/CA-PJ41, 6 = RT112.

L = Ladder (All lanes had 4mg/ml of Protein loaded).

The western blot indicates that all six human lysates express some form of PSA. With all but OE21 expressing the free form of PSA (34KDa), with PNT1A, PC-3, OE21, CaCO₂, PE/CA-PJ41 and RT112 expressing PSA α 2-macroglobulin (α 2MG) (115KDa), with PC-3, OE21, CaCO₂, RT112 expressing PSA α 1-antichymotrypsin (α 1ACT) (90KDa), and OE21, CaCO₂, PE/CA-PJ41 and RT112 express PSA-Protein-C-inhibitor (PCI) (56KDa).

2.3.2 PSA Secretion

Before the PSA secretion experiment was performed, a proliferation experiment was carried out to determine when exponential growth occurred. By performing a proliferation experiment it is possible to produce a cellular growth curve for the cell lines. From this, a cellular density can be selected where most of the cells start to move from the lag phase to the logarithmic phase.

Figure 2.12 and Appendix C Table c.1-c.2 shows the proliferation results for the six human cell lines.

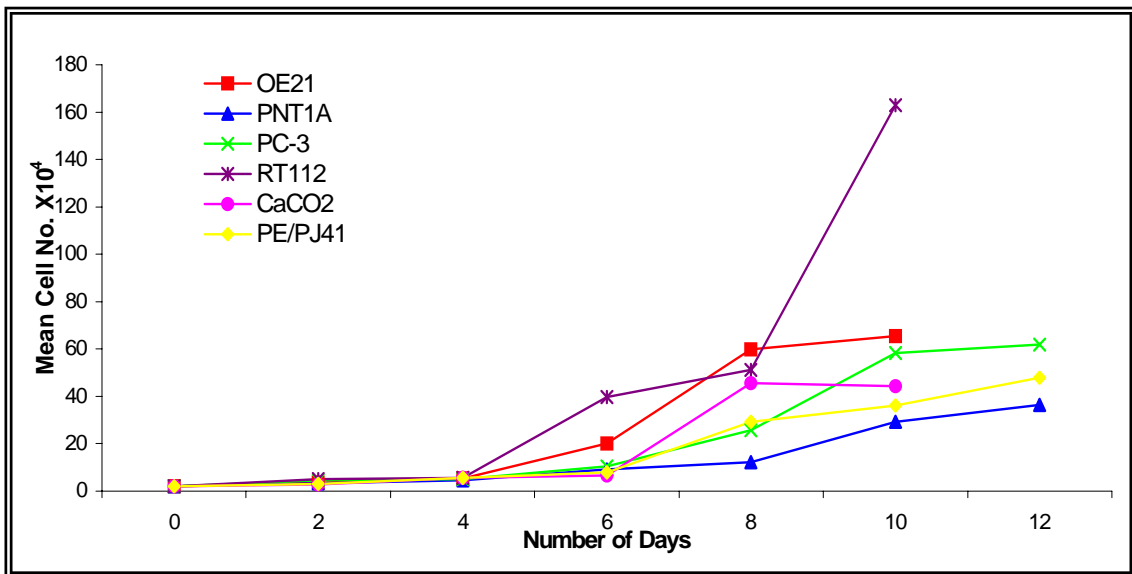


Figure 2.12. A line graph of the mean cellular proliferation rate for the six human cell lines seeded into a 12 well plate at a starting density of 2×10^4 and supplemented with normal media. These cells were fed and counted every other day.

The results show that logarithmic growth starts for most cells between 4-8 days, therefore for the PSA secretion experiment, a starting density of 1×10^5 cells/well is chosen.

For the PSA secretion experiment (*Section 2.2.4*), medium aspirated from the wells was tested for secreted PSA using western blotting. No signal however was observed by western blotting, so a dot blot was performed. *Table 2.3 and Figure 2.13* shows the dot blot results obtained, when the medium is tested for PSA secretions using polyclonal antibodies to PSA.

Table 2.3. Dot blot analysis results, showing PSA expression after different lengths of time, for the six human cell lines. + = Positive result: PSA secreted into medium, - = Negative result: PSA not secreted into medium, Time = length of time FCS free medium left with actively growing cells.

Time (hours)	RT112	OE21	PNT1A	PC-3	CaCO2	PE/CA-PJ 41
24	+	+	+	-	+	+
48	+	+	+	+	+	+
72	+	+	-	+	+	+

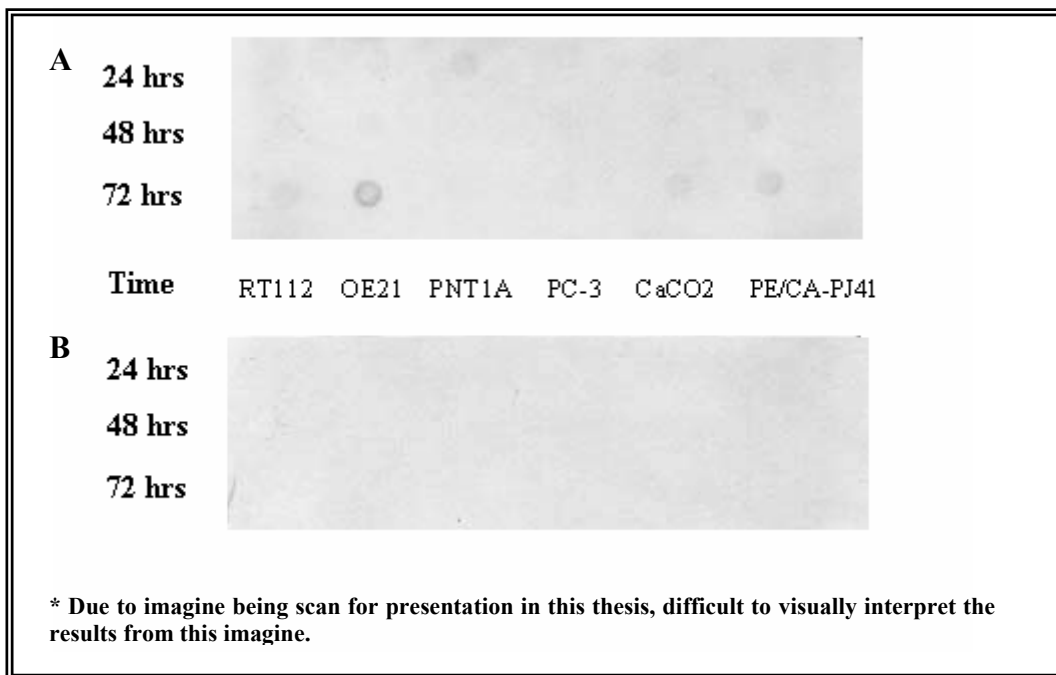


Figure 2.13. A Dot blot showing PSA expression in medium after different lengths of time, for the six human cell lines using polyclonal antibodies to PSA. A = Shows the positive results (dots indicate PSA present in medium), B = Negative control. Time = length of time FCS free medium left with actively growing cells.

The dot blot shows that all six human cell lines secrete PSA. Of these RT112, PE/CA-PJ41, OE21 and CaCO2 secrete PSA throughout the 24–72 hours of growth in FCS free medium, PC-3 starts to secrete PSA after 48 hours growth and PNT1A stops secreting PSA after 72 hours growth, in FCS free medium. These results imply that both prostatic and non-prostatic cell lines secrete PSA.

PSA ELISA

An ELISA was performed (*Appendix A.9*) to assess if the amount of PSA produced by the six human cell lines could be quantified. Cellular lysates extracted from the six human cell lines and the medium aspirated from the six human cell lines after 24, 48 and 72 hours growth in FCS free medium were tested.

No results are obtained (*Appendix C Table c.3*), as the absorbance readings are below the standard detection limit of the assay, please see *Discussion 2.4* for reasoning.

2.4 Discussion

The results presented in this chapter indicate that a number of prostatic and non-prostatic cell lines *in vitro* express PSA at the protein level, with initial screening using ICC, detecting PSA (*Figure 2.1-2.10, Table 2.2*) in ten of the thirteen human cell lines tested, while western blotting indicating (*Figure 2.11*) that all six cell lines express PSA, and the dot blot results suggesting that all six cell lines secrete PSA (*Figure 2.13 and Table 2.3*). These cell lines tested were derived from all different localities of the human body, therefore the results from this chapter further emphasise that PSA is not prostate specific. These findings correlate with other studies that show PSA is produced in comparative levels by human cell lines, such as human epidermoid carcinoma (KB) cells, embryonic lung (WI-38) cells (Waheed and Etten, 2001) and breast cancer (BT-474, T-47D, MFM-223, MCF-223, MCF-7, ZR-75-1, MDA-MD-435) cells (Magklara, *et al* 2000).

There is however a strong possibility that the PSA expression detected in the human cell lines is not representative of the situation *in vivo*, due to the cell being transformed to grow *in vitro*. *In vitro* cell cultures have been achieved for various cell types to assess cellular interactions, biochemical, and physiological changes, which may be present *in vivo* (Roguet and Schaefer 1997), but they are transformed (which can be done virally or occur spontaneously) to allow indefinite propagation, and so may have alterations in their expression of various proteins. For instance transformed foci of chicken embryo fibroblasts infected with Rous Sarcoma virus have been found to be morphologically different from their uninfected counterparts, and show loss of anchorage dependence (Drayton and Peters 2002). It is therefore plausible that PSA expression within the cell lines *in vitro* may be modified due to the cells being transformed, and not truly representation of the cells *in vivo*. However, as numerous other cellular and tissue studies agree with these findings, it is plausible that the cell types represented by these lines do express PSA *in vivo*. One study by Diamandis (1995) found that PSA immunoreactivity could be detected in breast, colon, ovarian, parotid, kidney, and lung tumours (*in vivo*).

The ICC results show that for six human cell lines PSA can be detected when using both polyclonal and monoclonal antibodies, while for four human cell lines PSA can only be detected when using polyclonal antibodies (refer to *Table 2.2*). Polyclonal antibodies are derived from different B cell lines that detect a specific protein, this means a mixture of IgG molecules are present that can identify multiple epitopes of a specific protein. Monoclonal antibodies are derived from clones of a specific B cell line, therefore only one specific type of IgG is present and only a single epitope is detected.

Due to the differences between the types of antibodies used in this study, there are a number of possible reasons for the differences in PSA detection, and these are:

- 1) The epitope for the monoclonal PSA antibody is masked (in complex with another molecule) in the cell lines,
- 2) The epitope for the PSA molecule is not present in the cell lines,
- 3) The PSA form which contains the epitope, is not present in the cell lines.

These possible reasons are all as likely as each other, therefore until the western blot results are discussed, it is impossible to speculate which is more plausible.

The ICC results also show that the locality of PSA immunoreactivity varies between the human cell lines (refer back to *Table 2.2*). These changes in staining locality could be due to the role of PSA within the cells, for instance PSA is a serine protease and serine proteases are enzymes which catalyse the hydrolysis of peptide bonds, therefore they are known to perform different functions, within different cells. Although PSA is involved in the liquefaction of seminal coagulum in the prostate gland, it may also have other functions within these cells (Diamandis 1995). For instance, it may act as a cellular matrix protein (Takahashi *et al* 1999), play a role as a growth factor modulator or translational / posttranscriptional protein regulator (Malatesta, *et al* 2000), it may hydrolyse insulin chains and interleukin-2 (Watt, *et al* 1986), digest insulin-like growth factor-binding proteins (Cohen, *et al* 1992), activate latent transforming growth factors (Killian, *et al* 1993) or inactivate protein C inhibitors (España, *et al* 1991). For instance Hsieh and Wu (1999) found that PSA was expressed in the nuclei of LNCaP cells and raised the possibility that PSA may have an auto-genius mode of control, to regulate PSA gene expression.

A PSA western blot was then performed on six of the human cell lines tested for PSA expression via ICC (reasons for six cell lines chosen shown in *Table 2.1*), the results show (*Figure 2.11*) that all six human cell lines express some form of PSA. These results are different from what is postulated, as even the cell line which showed no immunoreactivity for PSA via ICC, expressed complexes of PSA (OE21, *Figure 2.11 and Appendix C Figure c.1*). There is however a possible explanation for the differences seen between the western blotting and ICC results. Firstly it is possible that ICC using the monoclonal antibodies to PSA, can only identify an epitope present on free PSA (34KDa), as this free PSA is not present for OE21 by western blotting (*Figure 2.11*). This means that as western blotting identifies PNT1A as expressing the free form of PSA (*Figure 2.11*), but ICC using monoclonal PSA antibodies does not (*Figure 2.1*). PSA must have either been expressed at undetectable levels for this technique or the PSA epitope must have been either masked or missing. Diamandis (1998) stated that an antigenic site is masked upon binding of PSA to $\alpha 1$ antichymotrypsin (ACT), making this site a useful marker for free PSA assays.

The polyclonal and monoclonal antibodies must therefore detect different epitopes of PSA and it has been shown by epitope-mapping studies, that there are five to six major PSA epitopes and five are named A-E (McCormack *et al*, 1995, Pettersson *et al*, 1995). Of these epitopes, A, B and C are reactive for both free PSA and PSA complexes. Epitope E is found to be reactive on free PSA and is blocked if PSA complexes with ACT, while epitope D is reactive for free PSA at a seven times higher affinity than PSA-ACT (McCormack *et al* 1995 and Pettersson *et al* 1995). These epitopes are also divided into two categories: linear, where amino acid residues are adjacent in the primary sequence (continuous), and non-linear, where amino acid residues are separate in the primary structure, but brought into proximity when the protein is in its native form (discontinuous) (Corey *et al*, 1997). Therefore due to the 3D structure of PSA, ICC detects non-linear epitopes (discontinuous), while western blotting and treatment with SDS destroys the 3D structure, so linear epitopes can be detected (continuous) (Corey *et al* 1997). This however is not necessary universal as linear and non-linear epitopes can be potentially detected by ICC. As well as it being possible that linear/non-

linear epitopes can be hidden in ICC by PSA 3D structure or by complex formation with inhibitors, which maybe unmasked and detected by western blotting.

This data suggests that the monoclonal antibody to PSA as determined by ICC might be detecting epitope D or E, as these are present on free PSA, but are masked if PSA is in complex. This suggests that PNT1A is negative as the epitope is hidden, while OE21 is negative because the free PSA molecule is absent.

The polyclonal antibody however detects numerous linear and non-linear epitopes of free/complex PSA, as ICC and western blotting both produce results. There is however conflicting results for the cell line OE21 when using the polyclonal antibody, as it is negative for ICC, but positive for western blotting with complexes of PSA detected (*Figure 2.11*). The results suggest that either linear epitope hidden by ICC must have been unmasked by western blotting, allowing the polyclonal antibody to detect the PSA molecule, or that due to concentration of protein for western blotting, PSA is now expressed at a detectable level. The first suggestion maybe plausible, as PSA binding sites can be masked by binding to protein inhibitors, which can cause a conformational change inactivating or blocking the PSA epitope/s. From the epitope mapping study (McCormack *et al* 1995), there is evidence to suggest epitope D or E as the binding site, as complexes with PSA inhibitors can hide these sites.

The western blotting results further support the notion that PSA is not prostate specific and these finding correlate with a number of studies. Such as Waheed and Etten (2001) as they found by western blotting, that PSA is secreted by non-prostatic cell lines, such as KB cells (human epidermoid carcinoma) and WI-38 cells (normal human embryonic lung). Takahashi *et al* (1999) that when RT-PCR was performed on twelve-urogenital cancer cell lines, positive signals were amplified in all. Also, PSA was expressed in prostate, salivary gland, pancreas and the uterus and PSA mRNA was detected in tumours in the salivary gland, bile ducts, pancreas, bladder and thyroid (Ishikawa *et al*, 1998).

Although numerous human cell lines expressed some form of PSA, when the two prostatic cell lines are compared to each other a variation in expression of PSA is found.

For instance, the PC-3 cells are shown to express PSA by both polyclonal and monoclonal antibodies using ICC, in contrast PNT1A cells are only found to express PSA by polyclonal antibodies (*Figure 2.2-2.3*) and western blotting indicates that the PC-3 cells seems to express slightly more free PSA (34KDa), slightly more PSA-ACT (PSA to α_1 -antichymotrypsin, 90KDa) and slightly more PSA- α 2MG (PSA to α_2 -macroglobulin, 115KDa) than the PNT1A cell (*Figure 2.11*). These results suggest that although other non-prostatic cell lines do express PSA, when comparing the two prostatic cell lines a slight difference in cellular PSA expression can be detected. These findings correlate with other studies such as Stenman *et al* (1991); that found that the proportion of PSA-ACT is higher for patients with prostatic cancer. Zackrisson *et al* (2003); that found that serum PSA is higher for patients with prostate cancer, BPH and prostatitis and Catalona *et al* (2000); that found that a higher percentage of free PSA was associated with favourable histopathologic findings of neoplasia in prostatectomy specimens.

Another prostatic adenocarcinoma cell line is available, but was not included in this study due to its expense. This cell line was originally isolated from a lymph node metastatic lesion of human prostate cancer and is known as LNCaP (ATCC, CRL-1740). This cell line differs from the PC-3 (androgen-independent cell line) cells as it is an androgen dependent cell line. This means that while the PC-3 cells showed a slight increase in their cellular PSA expression compared to the PNT1A cells, if the LNCaP cells were included, theoretically they may have shown a greater variation (higher expression of PSA) compared to the PNT1A cells. This is because the effect of androgens on prostate cancer includes the stimulation of PSA production (Sun *et al*, 2001). It has also been shown that LNCaP cells express more PSA than another type of androgen independent cells (DU45) (Song *et al*, 2003).

Secreted PSA was also analysed to assess if PSA was secreted in detectable levels by other human cell lines (*Section 2.2 4*) and a western blot was then performed, but no

results are obtained. There are a number of possible reasons as to why no signal is observed for western blotting. For example the technique may have been inefficient as the protein may not have been transferred during blotting, or the exposure time may not have been long enough to illuminate and cause banding on the film. However as the molecular weight ladder appeared and the technique is optimal for cellular PSA expression, it seems more feasible that either the cell lines did not secrete enough PSA to be detected, or the epitopes are not present or masked.

A dot blot was then performed on the secreted PSA and the results (*Table 3.3 Figure 3.13*) indicate that PSA is secreted by all of the six human cell lines but only at very low levels, with no increased expression of PSA detected for prostatic cells (PNT1A or PC-3) compared to the non prostatic cells. These results indicate that the expression of PSA may have been too low for western blotting to detect, as Larson (1992) stated that the sensitivity of dot blotting is several folds greater than that of a western blotting. An experiment comparing western blotting to dot blotting for beta galactosidase shows, that 1pg could be detected by a dot blot, while 10pg could only be detected by a western blot (Larson 1992).

A PSA ELISA was also performed on the secreted PSA, but again no results are obtained (*Appendix C Table c.3*). There are a number of reasons why the ELISA may not have produced results. Firstly, it is possible that because the ELISA is designed for serum PSA, and the medium samples collected are serum free, the normal binding ability of the wells may have been reduced, compared to the standards (Albumen in serum would bind to the wells and therefore increase standard reading). This may have caused the ELISA readings to be negligible. Secondly it is possible that the amount of PSA secreted by the six human cell lines is too low to be detected via an ELISA (ELISA sensitivity is considerably lower than dot blotting). This seems more feasible than the first statement, as medium not grown with cells shows an absorbency reading at relatively the same levels as the medium aspirated from cells grown for 24, 48 and 72 hours (*Appendix C Table c.3*).

Although it seems more plausible that secreted PSA is too low to be detected by the PSA ELISA, two experiments need to be performed to conclusively prove this. Firstly the PSA ELISA needs to be performed using medium containing FCS collected from cells over 24-48 hours. By collecting medium contain FCS this may hopefully increase the binding ability, so that the secreted PSA from the cells can be compared to the standard. Secondly the PSA ELISA needs to be performed using medium collected and concentrated, from T75 flasks. Hopefully by increasing the cellular number more PSA should be secreted into the medium and the ELISA may be able to detect any cellular difference in PSA secretions.

Although only weak results are obtained for the cellular medium samples demonstrated by dot blotting (*Figure 3.13*), these results do agree with current findings. Western blotting on W1-38 (normal human embryonic lying), KB (epidermoid carcinoma) and PC-3SF12 (prostate carcinomas) cells show that PSA is expressed from cellular and medium samples (Waheed and Etten, 2001). While a PSA assay on placental explants cultured *in vitro* shows, a PSA content of 5.47 ± 0.07 $\mu\text{g}/\text{mg}$ protein of which $1.52 \pm 0.06\mu\text{g}/\text{mg}$ was in the free form in medium (Malatesta, *et al* 2000). It was also noticed, that human placental tissue obtained at delivery from full term pregnancies also synthesised and secreted PSA (Malatesta, *et al* 2000).

It is interesting to also consider what may have been found if the LNCaP cells were included in this study. For these, it is possible that a greater increase in PSA expression may have been detected. This is because previous studies such as Sun, *et al* (1998) found that PSA is readily released into the incubation medium by LNCaP cells (on androgen stimulation). Also, Mannello (1998) found that LNCaP cells produce/secrete PSA in culture medium and that extracts from 10^7 LNCaP cells show a high total PSA content ($26.20 + 1.89\text{ng}/10^7\text{cells}$). It is difficult to conclude from this study alone if there is a perceivable difference in secreted PSA expression between non prostatic and prostatic cells, so further analysis including the LNCaP cell line would be recommended.

In conclusion, the work in this chapter provides further evidence to suggest that PSA expression is not prostate specific as PSA expression and secretion by non-prostatic human cell lines was demonstrated. These findings highlight problems with PSA as a diagnostic marker of prostate cancer, as other cellular localities may affect PSA serum readings. Therefore this chapter emphasises a potential problem regarding the usefulness of PSA, for the diagnosis of prostate cancer.

Chapter 3 – Minichromosomal Maintenance Proteins (MCMs) Expression in Human Prostatic *In Vitro* Study.

3.1 Introduction

The role of MCMs in DNA replication has already been reviewed in section 1.5. The aim of this chapter was to examine MCM2, 5 and 7 expression in two human prostatic cell lines, to establish whether there is a difference in expression between cancerous (PC-3) and normal prostatic (PNT1A) cells, with the intention of providing an insight into what might be expected in clinical samples (tissue, urine and blood) and to establish a better understanding of the function of MCMs in prostatic cells.

The potential clinical usefulness of investigating MCMs has been illustrated by a number of studies. For instance, MCM2 has been investigated by IHC in gastrointestinal stromal tumours showing that an increase in MCM2 expression correlates to poor prognosis (Huang, *et al* 2006), Meng, *et al*, (2001) found that MCM2 expression is consistently higher in malignant prostate tissue, Padmanabhan, *et al*, (2004) found that MCM7 expression increases with prostatic neoplasia, while Freeman *et al*, (1999) showed a quantitative difference in the level of MCM5 expression between neoplastic and ‘normal’ tissue, with an increase in MCM5 expression observed in carcinomas of the bladder and colon, which are not detectable in equivalent ‘normal’ tissue. Although the MCMs are promising for diagnosing neoplasia, they do have limitations, as they are not tissue specific so their use as biomarkers may share some of the same problems as existing tumour markers, such as PSA.

MCMs have already been studied in various cell lines. For example MCM7 has been assessed in human colorectal carcinoma cells (HCT116), showing that over-expression of the MCM7 protein supports DNA replication of Epstein Barr virus oriP and rapid tumour formation (Yoshida and Inoue, 2003), MCM2 loading requirement for the formation of the pre-replication complex has been assessed in cervical cancer cells and ‘normal’ rat embryo fibroblast cell line (HeLa and REF52 cells) (Cook *et al*, 2004), and MCM2/MCM5 role as essential replication components have been assessed in mouse

embryonic fibroblast cell lines (NIH/3T3 cells), showing that unlike replicating cells, in quiescent cells Cdc6 and MCMs proteins are no longer chromatin-bound (Madine *et al*, 2000). However, no one has yet studied MCM2, 5 and 7 simultaneously in ‘normal’ and cancerous cell lines, so this was the focus of studies reported in this chapter, while the investigation of MCM expression in clinical samples from patients with prostate abnormalities will be carried out separately (Chapters 4 and 5).

To enable a detailed assessment of the potential differences in MCM expression between PC-3 and PNT1A cells, experiments were carried out at various proliferative states (controlled via level of confluence in the culture vessel) and cellular phases (controlled by synchronising cells using serum withdrawal). Both transcriptional and translational methods were employed (mRNA and protein analysis respectively).

3.2 Materials and Methods

3.2.1. Cell Culturing

The cell lines PNT1A (Normal Prostate Epithelium) and PC-3 (Prostate Adenocarcinoma) were cultured as described in *Appendix A Section A.2.2*, to perform translational and transcriptional studies at different proliferative states (confluence) and cellular intervals (synchronisation).

3.2.2 Proliferative States (Confluence)

The proliferative state of the two cell lines was assessed using ICC and reverse transcription-polymerase chain reaction (RT-PCR). Two slide flasks and two T75 flasks for each cell line were grown to a desired confluence (desired confluence chosen were approximately 30%, 50%, 75%, 100%, >100% and >100% confluence was assessed by culturing cells for an extra two days after 100% confluent). Confluence was assessed using Chark-Ley point method, protocol shown in *Section 3.2.2.i*. The slide flasks were then fixed and ICC was performed as shown in *Section 3.2.2.ii* and *Appendix A Section A.4*, while the T75 flasks were trypsinised and pelleted for RNA extraction. Before the contents of T75 flasks were pelleted for RNA extraction, a cell count (to assess percent confluence) and viability test was performed, protocol shown in *Appendix A Section A.3*.

3.2.2.i Chark-ley Point

The Chark-Ley point analysis involved the use of an eyepiece, which contains 25 random dots. This eyepiece was used with a 40X objective, so that one spot covered a whole cell. The numbers of cells then covered by the 25 spots were then counted for 4 fields of view, and the confluence of the flask is calculated. This technique enabled the investigator to obtain a rough estimate of confluence.

3.2.2.ii Immunocytochemistry (ICC)

ICC was performed on PNT1A and PC-3 according to protocol *Appendix A Section A.4*. This was performed to determine the expressional difference of MCM2, 5 and 7 between the two cell lines, at different proliferative states and cellular intervals.

The primary antibodies used were Mouse Monoclonal Antibodies to Minichromosomal Maintenance Proteins 2, 5 and 7 (MCM2 Ab-1 Clone CRCT2.1, MCM5 AB-1 Clone CRCT5.1 and CDC47/MCM7 AB-2 Clone 47DC141 or DCS141) (Stratech, Cambridge UK) and Mouse Monoclonal Antibody to Ki67 Clone MIB-1 (Dako Cytomation, Cambridge UK). These antibodies were used at a dilution of 1:50 for MCM 2, 5, 7 and KI67 in 1% rabbit serum in 1M TBS (v/v) pH7.5, all these were incubated in a humidity chamber overnight at 4°C. For all slides, appropriate negative controls were produced by removal of the primary antibodies.

Criteria for Assessing Cellular Staining

Once ICC was performed, the level of staining on each slide was assessed. The cells were therefore allocated a number, ranging from 0+ to 3+ and this was dependent on the amount of staining visualised. The grading system used is explained in *Table 3.1*.

Table 3.1. Criteria for assessment of MCM2, 5, 7 and PSA. Adapted from: www.aruplab.com/home/anatomic_pathology.jsp (2003).

Grade	% Cells Staining	Interpretation	Microscopic finding
0+	0-4%	None or little Staining	No Staining seen or very few cells stain
1+	5-25%	Weak Staining	<25% but >4% stain. Staining nuclei or nuclei and cytoplasmic.
2+	26-75%	Moderate Staining	<75% but > 25% stain. Staining nuclei or nuclei and cytoplasmic.
3+	>75%	High Staining	Near enough 100% stain. Staining nuclei or nuclei and cytoplasmic.

3.2.2.iii Polymerase Chain Reaction (PCR)

RNA was then extracted from the pellet according to the protocol in *Appendix A Section A.10.2*, and RT-PCR was performed using primers for MCM2, 5, 7 and β -Actin, as shown in *Appendix A Section A.11*. Although sequencing was not performed on the PCR products produced by these experiments, an earlier study in the same laboratory (Degan, 2004) did sequence the PCR products obtained from these primers to verify their specificity.

3.2.3 Cellular Intervals (Synchronisation)

Cellular synchronisation was performed for the two cell lines using eight T75 flasks and eight slide flasks. Cells in the eight flasks were grown until roughly 70% confluent (As assessed by Chark-Ley point: *Section 3.2.1.i*), their medium was removed and the cells were washed twice in cold PBS. The cells were then grown in FCS free medium for 48 hours to synchronise the cells, and cause mitotic inhibition (Kues, *et al* 2000). After 48 hours the contents of the two T75 flasks per cell line were trypsinised, and pelleted for RNA extraction, while two slide flasks produced at the same time were fixed for ICC. The rest of the flasks were washed with cold PBS, and fresh medium containing FCS was added. The remaining flasks were then grown for 8 hours (48/8), 24 hours (48/24) and 30 hours (48/30) in normal medium, so that the maximum number of cells per cell cycle could be detected. From this, it was noticed that the maximum number of cells obtained in the late G1 phase was after 0 hours (48hour in FCS free medium). The maximum number in the early G1 phase was after 8 hours in normal medium (48/8), the

maximum number of cells in the S-phase was after 24 hours in normal medium (48/24) (Davis, *et al* 2001), whilst the maximum number of cells in the G2/M phase was obtained after 30 hours in normal medium (48/30) (Kues, *et al* 2000). After each interval, the contents of two T75 flasks were trypsinized and pelleted for RNA extraction, while the two slide flasks were fixed for ICC.

3.2.3.i Immunocytochemistry (ICC)

ICC was performed on the slide flask from the two cell lines at different cellular intervals, using the protocol shown in *Section 3.2.2.ii Appendix A Section A.4*.

3.2.3.ii Polymerase chain reaction (PCR)

RT-PCR was performed on the RNA extracted from the two cell lines at different cellular intervals, using the primers MCM2, 5, 7 and β -Actin (Protocol for RNA extraction *Appendix A Section A.10.2*, Protocol for RT-PCR *Appendix A Section A.11*).

3.3 Results

3.3.1 – MCM2, MCM5 and MCM7 Expression for ‘Normal’/Cancerous Prostatic Cell Lines at Different Proliferative States.

Immunocytochemistry

MCM2, 5 and 7 were assessed at different proliferative states (desired confluence) for ‘normal/cancerous’ prostatic cell lines to compare expressional differences (*Section 3.2.2*). The results are shown in *Figure 3.1* and *Appendix D Table d.1 Figure d.1 – d.5*.

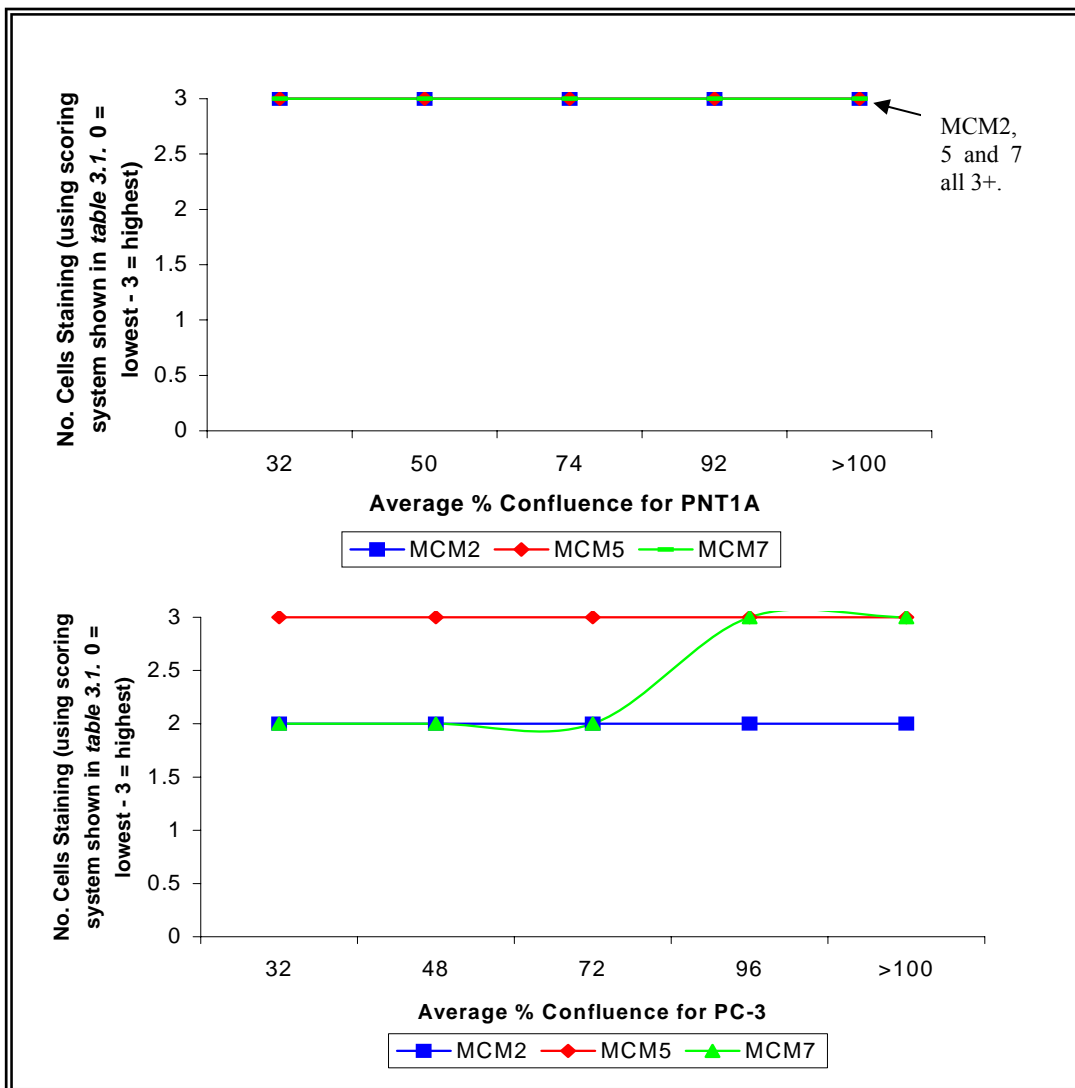


Figure 3.1. A line graph of the ICC results showing the mean number of cells staining (Chark-Ley point) using antibodies to MCM2, 5, and 7 (scoring system used shown in *table 3.1*) for the cell lines: PNT1A and PC-3 at different mean confluence. Sample number is n-2.

Figure 3.1, shows that the immunoreactivity for MCM2 and 7 differs between the two prostatic cell lines throughout confluence (proliferative state). For instance MCM2, 5 and 7 immunoreactivity remains high (3+) throughout the proliferative states, for PNT1A, while for PC-3 the immunoreactivity is high (3+) for MCM5 throughout, moderate (2+) for MCM2 throughout, and moderate (2+) which changes to high (3+) for MCM7 as proliferative state increases.

The staining locality also varies between the two cell lines for MCM2 and 7 (*Refer Appendix D Figures d.1-d.5*), as only nuclear staining for the PNT1A cells occurs, while nuclei and cytoplasmic staining occurs for PC-3. MCM5 however show nuclei and cytoplasmic staining, for both cell lines throughout.

To determine if the two cell lines are actively proliferating at the same rates, the viability and proliferation rates of the cells is assessed. Antibodies to Ki67 were used to assess proliferative rates, while trypan blue was used to assess cellular viability (*Section 3.2.5 and Appendix A Section A.3*).

Raw data for cellular viability and proliferative rates are shown in *Appendix D tables d.2-d.3*. ICC results for Ki67 staining for the PNT1A and PC-3 cells are shown in *Appendix D Figures d.6 and d.7*. *Figure 3.2* shows the average cellular viability and average proliferation rate for PNT1A and PC-3 .

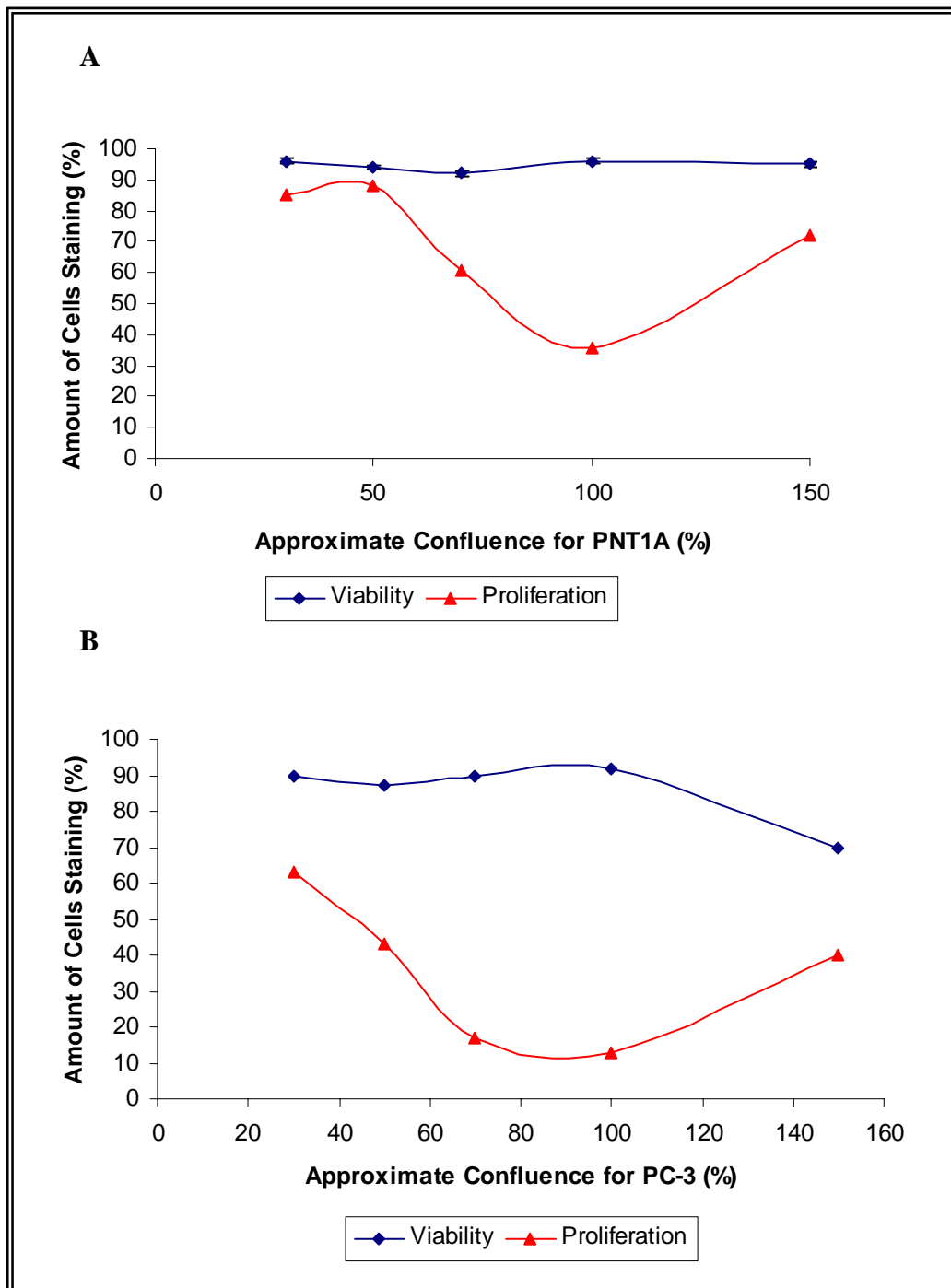


Figure 3.2. A - Mean proliferation (%) (Ki67 staining - immunocytochemistry) and mean viability (%) (Trypan blue staining – Cell Count) against percent confluence for PNT1A.

B - Mean proliferation (%) (Ki67 staining - immunocytochemistry) and mean viability (%) (Trypan blue staining – Cell Count) against percent confluence for PC-3. Sample number n=2

Figure 3.2A and B show that the cellular viability for PC-3 and PNT1A is comparable, as both have a similar number of viable cells and both show a similar pattern of

expression, at different confluence. There does however seem to be a difference in proliferation rates between PNT1A and PC-3, as although they have comparable patterns of proliferation, there seems to be less cellular proliferation for PC-3 cells, compared to PNT1A cells. These results may help explain the differences seen between the ICC results for MCM2, 5 and 7 (*Figure 3.1*).

RT-PCR

RT-PCR was performed at different levels of confluence to compare transcription of MCM2, 5 and 7 in PNT1A and PC-3 cells (*Appendix A Section A.11*). Before this was carried out the optimal technique for RNA extraction was chosen, and this is shown in *Appendix D Figure d.8*. Also, RT-PCR was performed for MCM2, 5 and 7 at different cycle numbers, so that a cycle number could be chosen, when PCR is still exponentially amplifying the DNA region of interest. This means that there are no limiting factors to confound the semi-quantification. These results are shown in *Appendix D Tables d.4 – d.7 and Figures d.9-d.10*, and the optimal cycle number chosen for MCM2 and 5 is 25 cycles, and 35 cycles for MCM7.

RT-PCR for MCM2, 5 and 7 was then performed for each confluence level using the cycle numbers chosen, on PNT1A and PC-3. This was performed on two T75 flasks extracted at each confluence and RT-PCR was repeated three times on each extraction. The raw data showing the relative brightness unit (rbu) obtained for each confluence is tabulated in *Appendix D Table d.8-d.10*, and *Appendix D Table d.11-d.13* shows the average rbu obtained, for MCM2, MCM5 and MCM7 for the confluence levels, and the two cell lines. *Figure 3.3- 3.5* shows line graphs of the average rbu obtained for RT-PCR on MCM2, 5 and 7, for PNT1A and PC-3, *Figure 3.6-3.8* shows the gel pictures for PNT1A and PC-3 when RT-PCR was performed for MCM2, 5 and 7, at the different confluence levels. *Appendix D Figure d.11* shows the RT-PCR gel pictures for negative controls (No RT) and positive controls (β -Actin), for each confluence level and for each cell line.

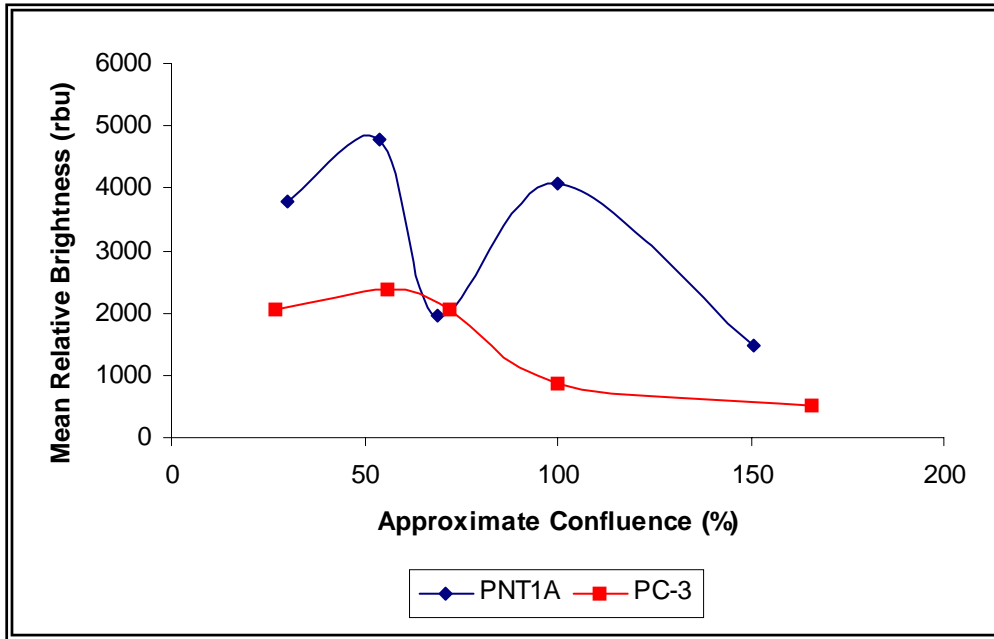


Figure 3.3. RT-PCR results obtained when average Relative Brightness (rbu) (*Appendix D table d.11*) is plotted against percent confluence for PNT1A and PC-3 for MCM2. Samples Size for each cell line and confluence = 2.

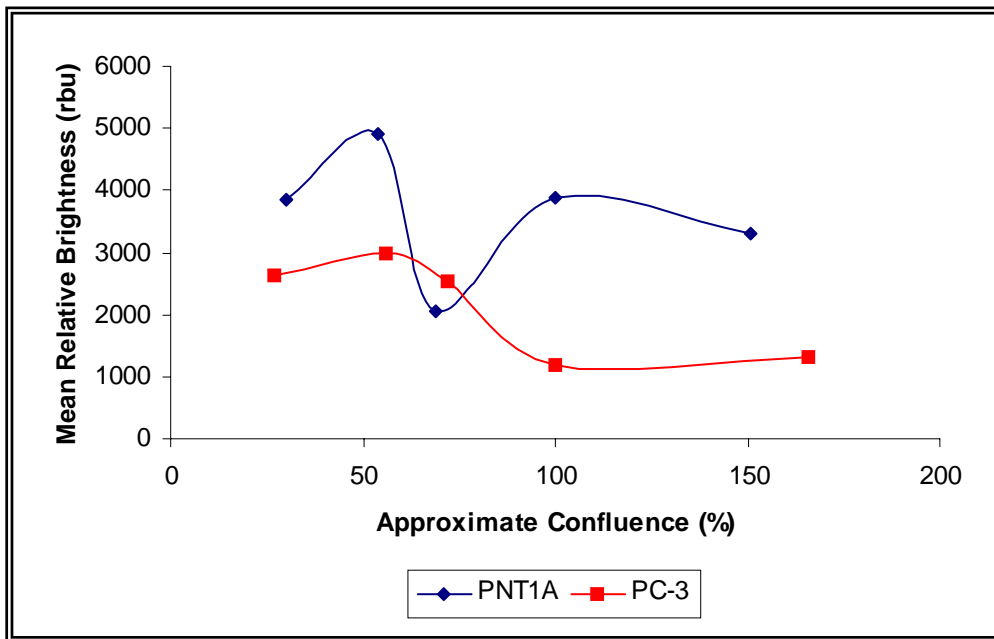


Figure 3.4. RT-PCR results obtained when average Relative Brightness (rbu) (*Appendix D table d.12*) is plotted against percent confluence for PNT1A and PC-3 for MCM5. Samples Size for each cell line and confluence = 2.

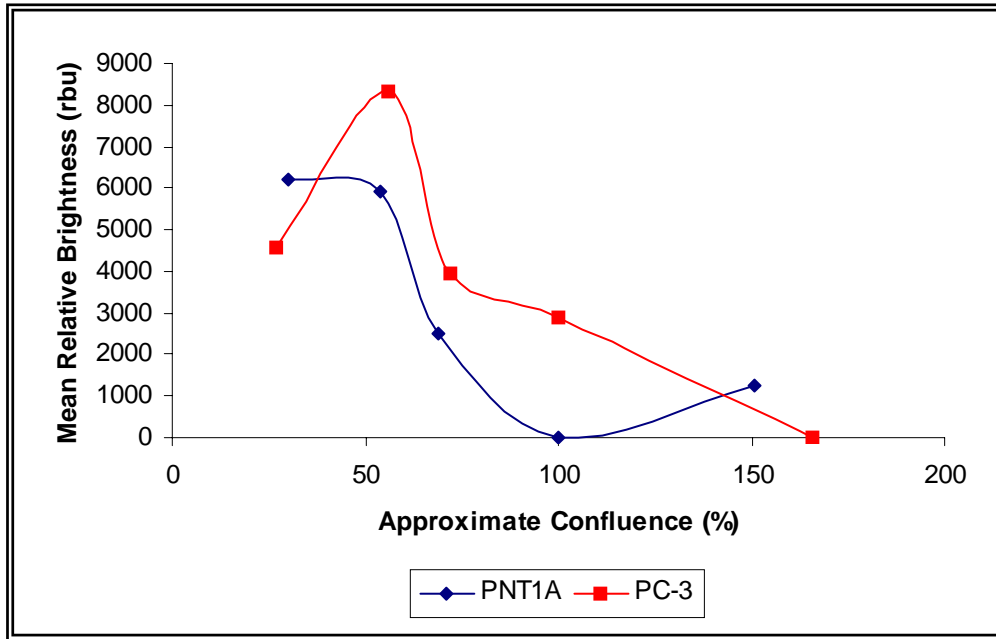


Figure 3.5. RT-PCR results obtained when average Relative Brightness (rbu) (*Appendix D table d.13*) is plotted against percent confluence for PNT1A and PC-3 for MCM7. Samples Size for each cell line and confluence = 2.

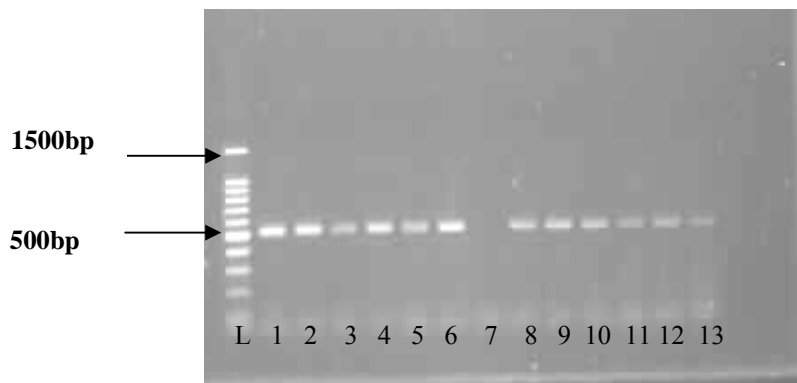


Figure 3.6. Ethidium bromide stained gel of the RT-PCR results for MCM2 (500bp band visible) at different confluence for 200µg/µl of RNA from PNT1A and PC-3 cell. L = Ladder, 1 = PNT1A cells at 35% confluence, 2 = PNT1A cells at 54% confluence, 3 = PNT1A cells at 69% confluence, 4 = PNT1A cells at 100% confluence, 5 = PNT1A cells at 139% confluence, 6 = PNT1A cells at 162% confluence, 7 = Negative Control 8 = PC-3 cells at 34% confluence, 9 = PC-3 cells at 58% confluence, 10 = PC-3 cells at 77% confluence, 11 = PC-3 cells at 100% confluence, 12 = PC-3 cells at 136% confluence, 13 = PC-3 cells at 197% confluence.

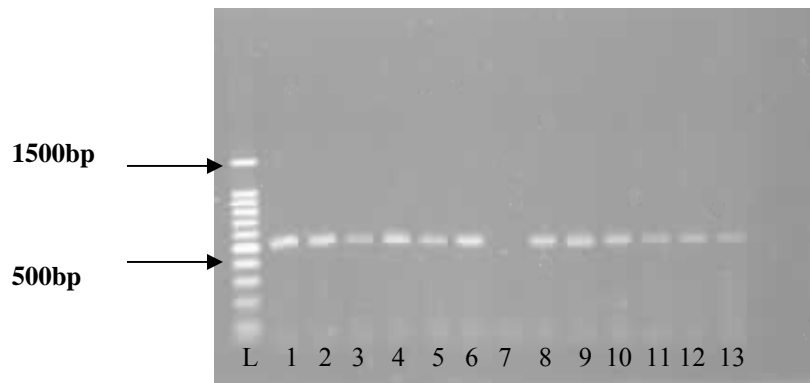


Figure 3.7. Ethidium bromide stained gel of the RT-PCR results for MCM5 (500bp band visible) at different confluence for 200 μ g/ μ l of RNA from PNT1A and PC-3. Ladder, 1 = PNT1A cells at 35% confluence, 2 = PNT1A cells at 54% confluence, 3 = PNT1A cells at 69% confluence, 4 = PNT1A cells at 100% confluence, 5 = PNT1A cells at 139% confluence, 6 = PNT1A cells at 162% confluence, 7 = Negative Control 8 = PC-3 cells at 34% confluence, 9 = PC-3 cells at 58% confluence, 10 = PC-3 cells at 77% confluence, 11 = PC-3 cells at 100% confluence, 12 = PC-3 cells at 136% confluence, 13 = PC-3 cells at 197% confluence.

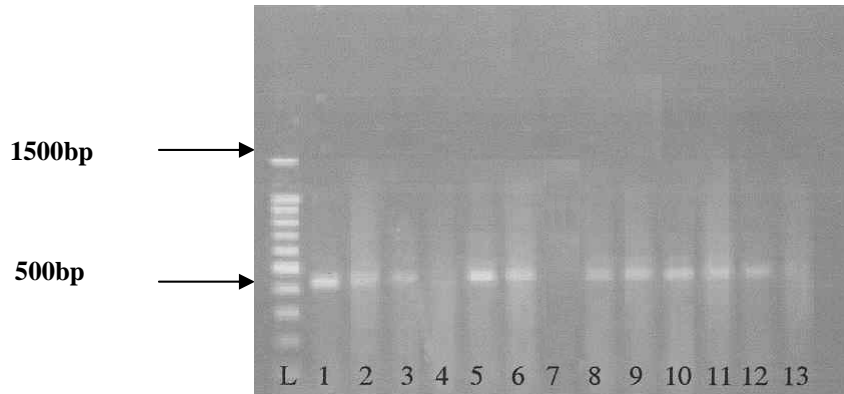


Figure 3.8. Ethidium bromide stained gel of the RT-PCR results for MCM7 (407bp band visible) at different confluence for 200 μ g/ μ l of RNA from PNT1A and PC-3 cells. Ladder, 1 = PNT1A cells at 35% confluence, 2 = PNT1A cells at 54% confluence, 3 = PNT1A cells at 69% confluence, 4 = PNT1A cells at 100% confluence, 5 = PNT1A cells at 139% confluence, 6 = PNT1A cells at 162% confluence, 7 = Negative Control 8 = PC-3 cells at 34% confluence, 9 = PC-3 cells at 58% confluence, 10 = PC-3 cells at 77% confluence, 11 = PC-3 cells at 100% confluence, 12 = PC-3 cells at 136% confluence, 13 = PC-3 cells at 197% confluence.

Figures 3.3-3.8, show that there is a difference in the amount of MCM2, 5 and 7 expressed in PNT1A and PC-3 cells. For instance the PNT1A cells show a cyclic pattern of expression for MCM2, 5 and 7, with increasing confluence. MCM2 and 5 show similar cyclic patterns and equivalent expressional levels with the highest rbu occurring at around 50% and 100% confluence. While MCM7 shows an inverse relationship to MCM2 and 5, as although MCM7 shows a cyclic pattern of expression, the highest rbu occurs at around 25%, whilst the lowest is at around 100%.

In contrast the PC-3 cells do not appear to display a cyclic pattern of expression, and show reduced expression of MCM2, 5 and 7 as the confluence increases. MCM2 and 5 have similar expression patterns and levels, and MCM7 shows a similar expression pattern to MCM2 and 5, but at a much higher amplification level.

The rbu which indicates the level of DNA expression also varies between the two cell lines, as a higher rbu occurs for MCM2 and 5 in the PNT1A cells for all confluence levels except 70% when the PC-3 cells seem to express a similar if not higher level. For MCM7, the PNT1A cells show higher expressions at around 25% and <100%, while the PC-3 cells shows a higher expression at 50-100%.

3.3.2 – MCM2, MCM5 and MCM7 Expression for ‘Normal’/Cancerous Prostatic Cell Lines at Different Phases of the Cell Cycle (Synchronisation).

Immunocytochemistry

Synchronisation was carried out to see how MCM2, 5 and 7 vary as the cell passes through different stages of the cellular cycle (*Cellular Synchronisation Section 3.2.3*).

The ICC results are shown in *Figure 3.9* and *Appendix D Table d.14* and *Figures d.12-d.19*.

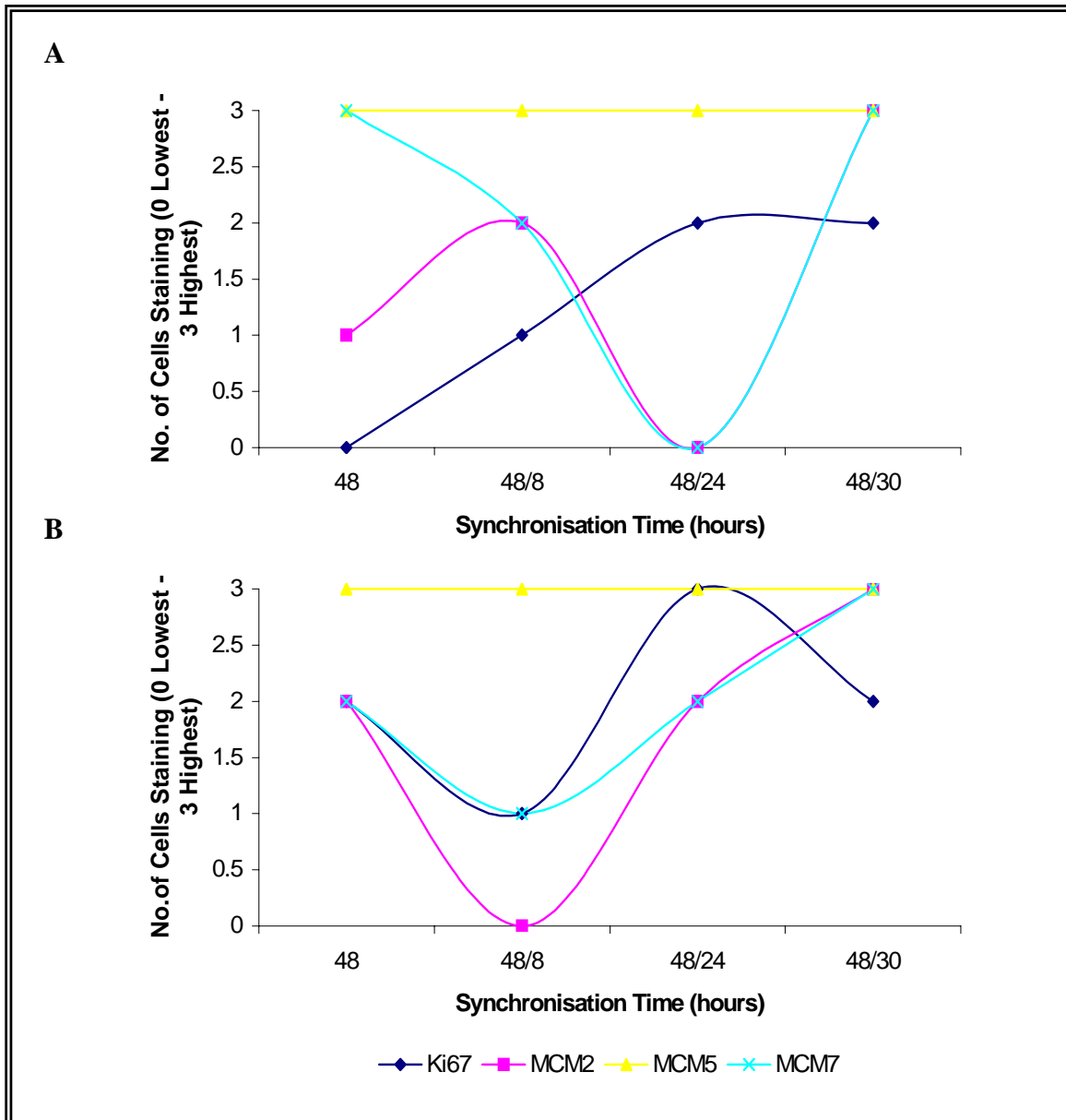


Figure 3.9. A line graphs of the ICC results showing the mean number of cells staining for antibodies to MCM2, MCM5, and MCM7 for the A) PNT1A B) PC-3 cells at different synchronisation times. Sample number is n-2.

Figure 3.9 shows that the immunoreactivity of PNT1A and PC-3, for antibodies to Ki67, MCM2, 5 and 7. When comparing the two cell lines, Ki67 staining increases with the synchronisation time for PNT1A cells. Whilst for the PC-3 cells a cyclic pattern of expression is seen, with the highest immunoreactivity detected after 48 hours growth with FCS free medium (48 hours) and after 48 hours growth with FCS free medium and then 24 hours growth in normal medium (48/24 hours).

MCM2 immunoreactivity (*Figure 3.9*) also differs between the two cell lines, as although they both show a cyclic pattern of expression, the cycles are slightly out of phase. For instance, MCM2 for the PNT1A cells shows, moderate (2+) staining for cells grown for 48 hours in FCS free medium and then 8 hours in normal medium (48/8 hours), no staining (0+) for cells grown for 48 hours in FCS free medium and then 24 hours in normal medium (48/24hours), and high (3+) staining for cells grown for 48 hours in FCS free medium and then 30 hours in normal medium (48/30hours). Whilst for the PC-3 cells, MCM2 shows moderate (2+) staining for cells grown for 48 hours in FCS free medium (48hours), no (0+) staining for cells grown for 48 hours in FCS free medium and then 8 hours in normal medium (48/8hours), and an increase in staining to high (3+) staining, for cells grown for 48 hours in FCS free medium and then 30 hours in normal medium (48/30hours).

MCM5 immunoreactivity (*Figure 3.9*) is identical for the two cell lines with high (3+) staining occurring throughout the synchronisation times. Whilst the immunoreactivity for MCM7 (*Figure 3.9*) differs between the two cell lines. With a drop in staining ability occurring in PNT1A to none (0+), after 48 hours growth in FCS free medium and then 24 hours in normal medium (48/24hours), and then an increase in staining after 48 hours growth in FCS free medium and then 30 hours in normal medium (48/30hours). The PC-3 however, displays a cyclic pattern of immunoreactivity to MCM7, with 48 hours growth in FCS free medium (48hours) and 48 hours growth in FCS free medium and 24 hours in normal medium (48/24hours) showing moderate (2+) and high (3+) staining.

Staining locality for MCM2 and 7 also differs between the cell lines, as only nuclear staining occurs for PNT1A cells, while nuclear and cytoplasmic staining occurs for the PC-3 cells. Staining for MCM5 is however the same in the two cell lines, with both nuclear and cytoplasmic staining apparent.

RT-PCR

RT-PCR for MCM2, 5 and 7 was performed for each synchronisation time and on each cell line, using the same cycle numbers chosen for RT-PCR on the proliferative states.

This was performed on two T75 flasks extracted at each synchronisation time and RT-PCR was repeated at least twice on the extractions. The raw data showing rbu obtained for each synchronisation time, is tabulated in *Appendix D Table d.15-d.17* and *Appendix D Table d.18-d.20* shows the average rbu obtained for MCM2, 5 and 7, against different synchronisation times and cell lines. *Figure 3.10- 3.12* show line graphs of the average rbu obtained for RT-PCR on MCM2, 5 and 7, for PNT1A and PC-3 and *Figure 3.13- 3.15* show the gel pictures for PNT1A and PC-3, when RT-PCR was performed for MCM2, 5 and 7, at different synchronisation times. *Appendix D Figure d.20-d.21* shows the RT-PCR gel pictures for negative controls (No RT) and positive controls (β -Actin), for synchronisation time for PNT1A and PC-3 cells.

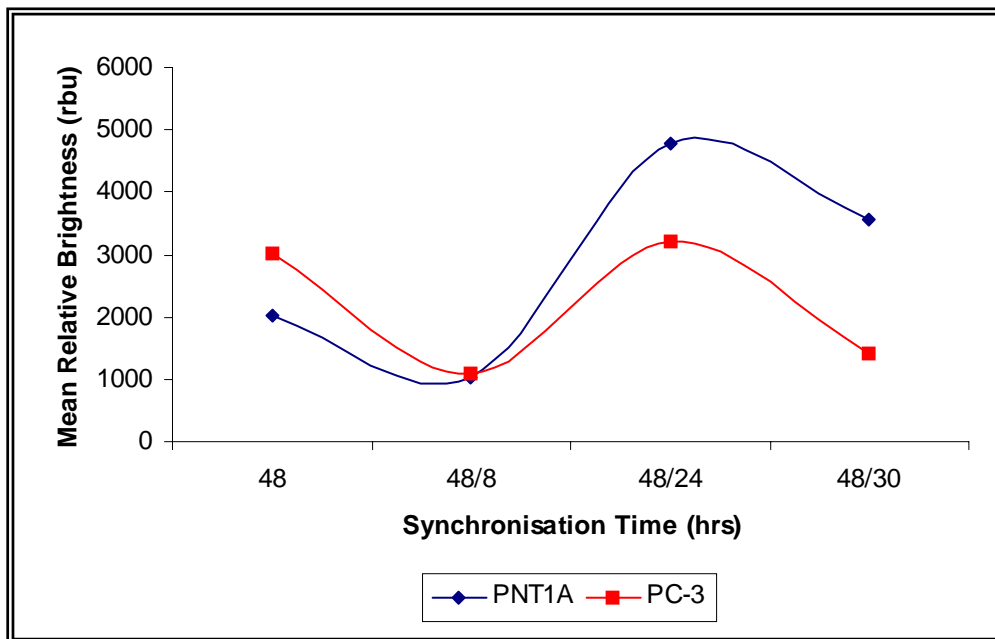


Figure 3.10. A line graph of the RT-PCR results obtained when average Relative Brightness (rbu) (*Appendix D Table d.18*) is plotted against mean synchronisation time for PNT1A and PC-3 for MCM2. Sample Size $n = 2$.

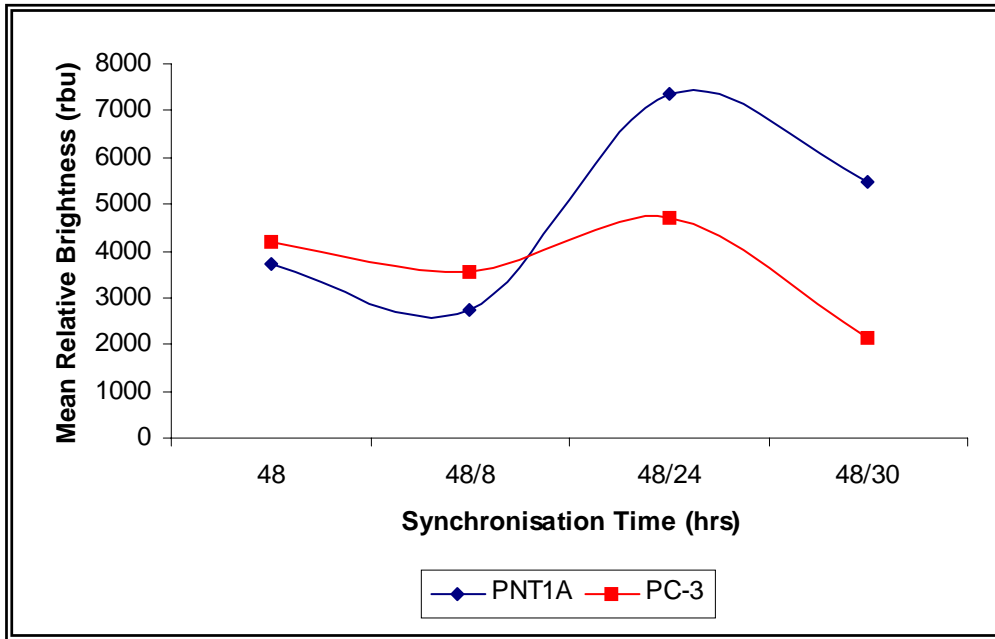


Figure 3.11. A line graph of the RT-PCR results obtained when average Relative Brightness (rbu) (*Appendix D Table d.19*) is plotted against mean synchronisation time for PNT1A and PC-3 for MCM5. Sample Size n = 2.

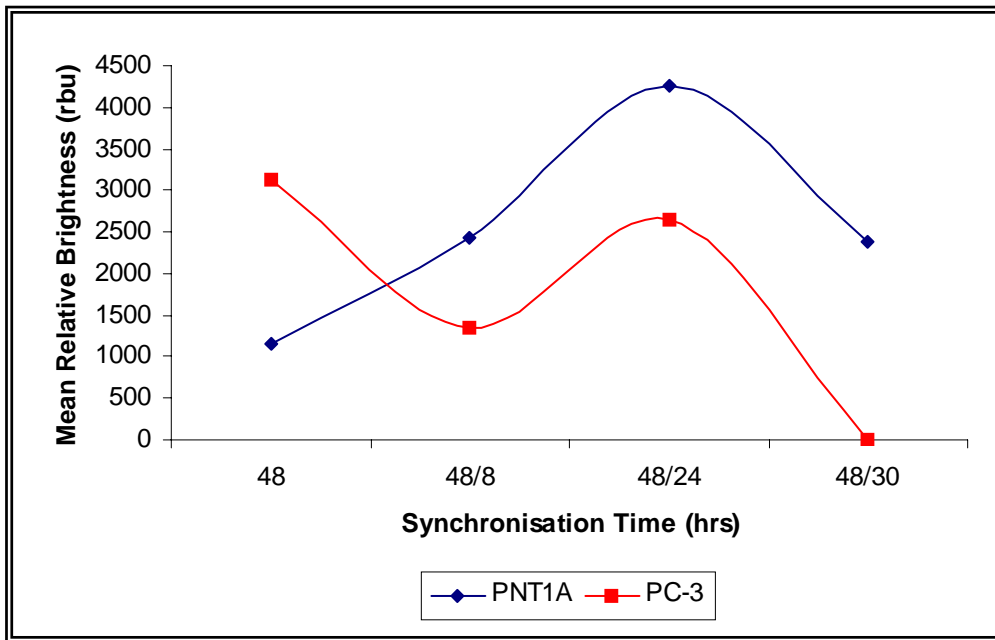


Figure 3.12. A line graph of the RT-PCR results obtained when average Relative Brightness (rbu) (*Appendix D Table d.20*) is plotted against mean synchronisation time for PNT1A and PC-3 for MCM7. Sample Size n = 2.

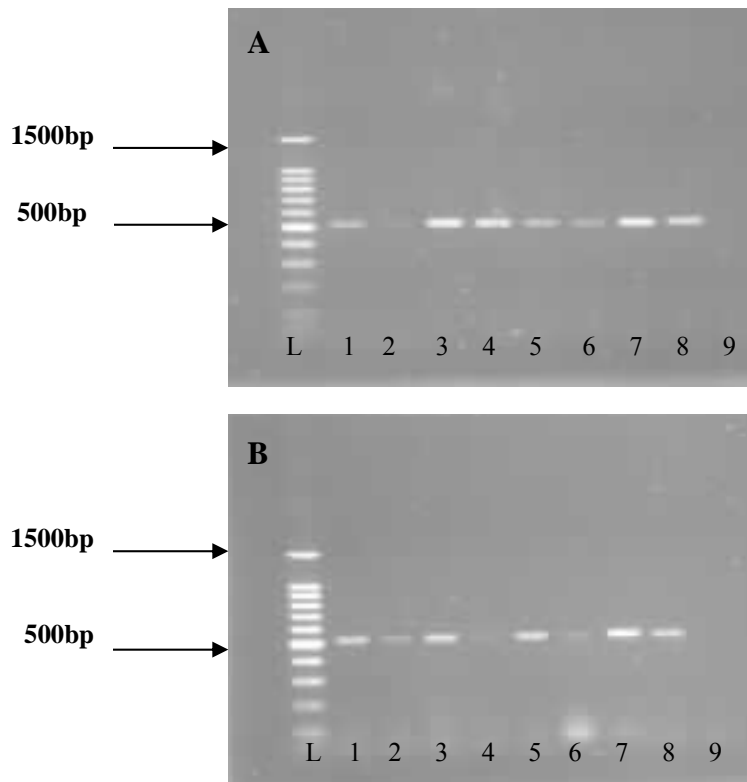


Figure 3.13A. Ethidium bromide stained gel for RT-PCR for MCM2 (500bp bands visible) using 200 μ g/ μ l of RNA extracted from PNT1A cell lines at different synchronisation times:- L = Ladder, 1 = Cells extracted after 48hours FCS free media, 2 = Cells extracted after 48hours FCS free media then 8hours complete media, 3 = Cells extracted after 48 FCS free media then 24hours complete media, 4 = Cells extracted after 48hours FCS free media then 30hours complete media, 5 = Cells extracted after 48hours FCS free media, 6 = Cells extracted after 48hours FCS free media then 8hours complete media , 7 = Cells extracted after 48hours FCS free media then 24hours complete media , 8 = Cells extracted after 48hours FCS free media then 30hours complete media, 9 = Negative control .

Figure 3.13B. Ethidium bromide stained gel for RT-PCR for MCM2 (500bp bands visible) using 200 μ g/ μ l of RNA extracted from PC-3 cell lines at different synchronisation times:- L = Ladder, 1 = Cells extracted after 48hours FCS free media, 2 = Cells extracted after 48hours FCS free media then 8hours complete media, 3 = Cells extracted after 48 FCS free media then 24hours complete media, 4 = Cells extracted after 48hours FCS free media then 30hours complete media, 5 = Cells extracted after 48hours FCS free media, 6 = Cells extracted after 48hours FCS free media then 8hours complete media , 7 = Cells extracted after 48hours FCS free media then 24hours complete media , 8 = Cells extracted after 48hours FCS free media then 30hours complete media, 9 = Negative control .

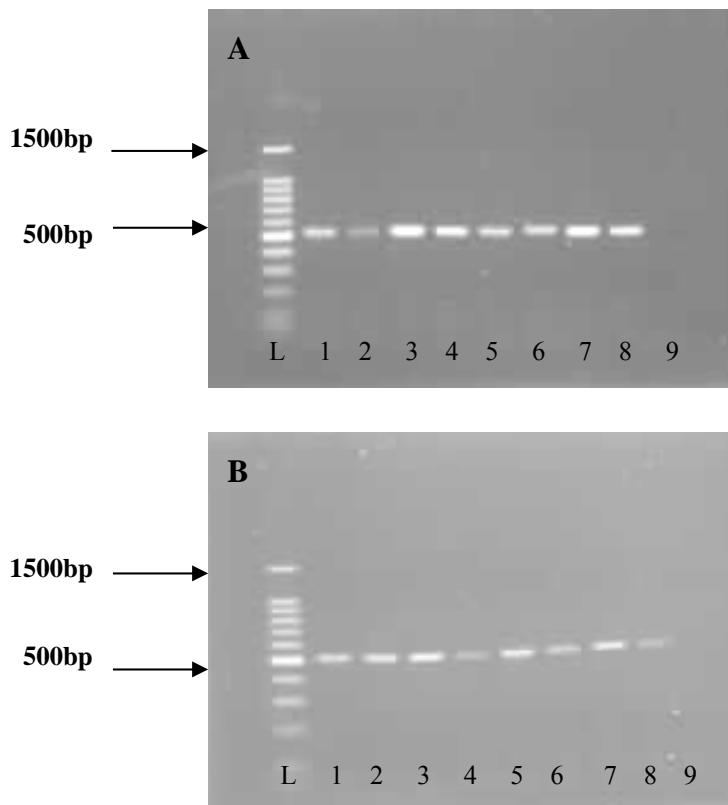


Figure 3.14A. Ethidium bromide stained gel for RT-PCR for MCM5 (500bp bands visible) using 200 μ g/ μ l of RNA extracted from PNT1A cell lines at different synchronisation times :- L = Ladder, 1 = Cells extracted after 48hours FCS free media, 2 = Cells extracted after 48hours FCS free media then 8hours complete media, 3 = Cells extracted after 48 FCS free media then 24hours complete media, 4 = Cells extracted after 48hours FCS free media then 30hours complete media, 5 = Cells extracted after 48hours FCS free media, 6 = Cells extracted after 48hours FCS free media then 8hours complete media , 7 = Cells extracted after 48hours FCS free media then 24hours complete media , 8 = Cells extracted after 48hours FCS free media then 30hours complete media, 9 = Negative control .

Figure 3.14B. Ethidium bromide stained gel for RT-PCR for MCM5 (500bp bands visible) using 200 μ g/ μ l of RNA extracted from PC-3 cell lines at different synchronisation times:- L = Ladder, 1 = Cells extracted after 48hours FCS free media, 2 = Cells extracted after 48hours FCS free media then 8hours complete media, 3 = Cells extracted after 48 FCS free media then 24hours complete media, 4 = Cells extracted after 48hours FCS free media then 30hours complete media, 5 = Cells extracted after 48hours FCS free media, 6 = Cells extracted after 48hours FCS free media then 8hours complete media , 7 = Cells extracted after 48hours FCS free media then 24hours complete media , 8 = Cells extracted after 48hours FCS free media then 30hours complete media, 9 = Negative control .

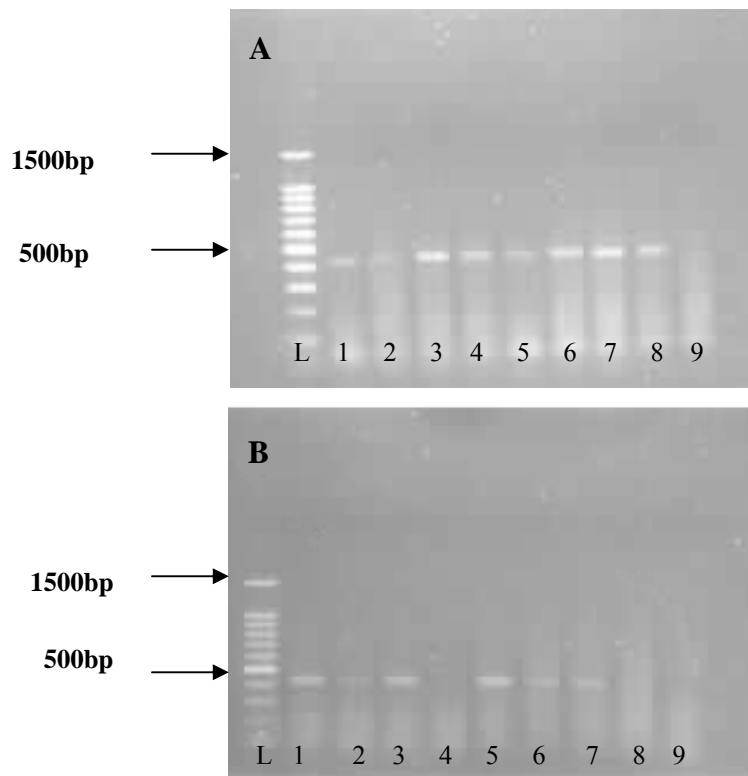


Figure 3.15A. Ethidium bromide stained gel for RT-PCR results for MCM7 (407bp bands visible) using 200 $\mu\text{g}/\mu\text{l}$ of RNA extracted from PNT1A cell lines at different synchronisation times :- L = Ladder, 1 = Cells extracted after 48hours FCS free media, 2 = Cells extracted after 48hours FCS free media then 8hours complete media, 3 = Cells extracted after 48 FCS free media then 24hours complete media, 4 = Cells extracted after 48hours FCS free media then 30hours complete media, 5 = Cells extracted after 48hours FCS free media, 6 = Cells extracted after 48hours FCS free media then 8hours complete media , 7 = Cells extracted after 48hours FCS free media then 24hours complete media , 8 = Cells extracted after 48hours FCS free media then 30hours complete media, 9 = Negative control .

Figure 3.15B. Ethidium bromide stained gel for RT-PCR results for MCM7 (407bp bands visible) using 200 $\mu\text{g}/\mu\text{l}$ of RNA extracted from PC-3 cell lines at different synchronisation times :- L = Ladder, 1 = Cells extracted after 48hours FCS free media, 2 = Cells extracted after 48hours FCS free media then 8hours complete media, 3 = Cells extracted after 48 FCS free media then 24hours complete media, 4 = Cells extracted after 48hours FCS free media then 30hours complete media, 5 = Cells extracted after 48hours FCS free media, 6 = Cells extracted after 48hours FCS free media then 8hours complete media , 7 = Cells extracted after 48hours FCS free media then 24hours complete media , 8 = Cells extracted after 48hours FCS free media then 30hours complete media, 9 = Negative control .

The synchronisation results show (*Figure 3.10-3.15*) that there is no clear difference in the RNA expressional patterns of MCM2, 5 and 7 between the two cell lines, with both cell types following a similar cyclic pattern. There is however a difference in the expressional amounts of MCM2, 5 and 7 between the two cell lines and this is explained below.

MCM2 (*Figure 3.10*) expression is higher for the PC-3 cells after 48 hours growth in FCS free medium (48hours) compared to the PNT1A cells. After 48 hours growth in FCS free medium and then 8 hours growth in normal medium (48/8hours) however, both cell lines show similar expressional amounts. Then after 48 hours growth in FCS free medium and then 24/30 hours grown in normal medium (48/24hours- 48/30hours) it becomes apparent that the expressional amount of MCM2 in the PC-3 cells drops, below that produced by the PNT1A cells.

MCM5 (*Figure 3.11*) expression also varies at different synchronisation times, as although there are similar levels of expression for both cells lines after 48 hours growth in FCS free medium (48hours) and then 8hours in normal medium (48/8hours). After 48 hours growth in FCS free medium and 24/30 hours growth in normal medium (48/24hours-48/30hours) the expression of MCM5 drops for the PC-3 cells, compared to PNT1A cells.

Finally, MCM7 (*Figure 3.12*) rbu also varies between the two cell lines, with PC-3 cells showing less expression detected after 48 hours growth in FCS free medium and then 8-30 hours growth in normal medium (48/8hours - 48/30hours), compared to the PNT1A cells.

3.4 Discussion

The ICC results from the various proliferative states show that there are expressional variations between the two cell lines at different confluence states (*Figure 3.1*). For instance, PNT1A cells show high (>75%) staining for MCM2, 5 and 7 compared to the PC-3 cells. These differences may be related to the proliferative activity of the cells,

therefore to accurately interpret the data the viability (trypan blue) and proliferative state (Ki67) was also considered.

Trypan blue assesses viability, while antibodies to Ki67 help analyse proliferative state: Trypan blue is able to enter cells which are no longer viable, as the cells lose their functional membrane and allow the dye to enter; Ki67 is only believed to be expressed in proliferating cells located in the nucleolus, and is expressed only during the cell cycle (MacCallum and Hall 1999). The function of Ki67 within the cell is still unclear, but there is some indirect evidence to indicate interaction with nucleic acid, and it is absent in non-proliferating cells (MacCallum and Hall 1999).

The results suggest (*Figure 3.2*) that the cellular viability (trypan blue) in the two cell lines cannot explain the expressional difference of MCM2, 5 and 7 as both cell lines show similar cellular death rates. However, proliferative rates (Ki67 staining) do vary between the two cell lines, with less proliferation detected in the PC-3 cells, compared to the PNT1A. These findings may help to explain the differences in proliferative activity between the two cell lines, and there are three possible explanations.

Firstly, it is plausible to assume, that as Ki67 staining reduces in the PC-3 cells indicating that these cells are replicating at a lower rate than the PNT1A cells, the expression of MCMs in these cells should also reduce. This (*Figure 3.1*) reduction in expression only occurs for MCM2 in the PC-3 cells (2+), as MCM5 is high throughout same as the PNT1A cells (3+), and MCM7 shows low expression to start and then increases as confluence increases (2+ to 3+), becoming the same as PNT1A cells. These results suggest that increased expression of MCM5 and 7 at higher confluence occurs in the PC-3 cells, compared to PNT1A cells. These findings confer with previous tissue studies, as MCMs in human tissue increase in neoplastic conditions, and a positive correlation has been found between MCM5 expression and the increase in progression from normal prostate to adenocarcinoma (Freeman, *et al* 1999).

The second explanation supports the suggestion that Ki67 is not accurate at indicating proliferative state. For instance, Freeman, *et al* (1999) stated that MCMs identify a

greater number of cells in the cell cycle than Ki67, while Kato, *et al* (2003) stated that MCM2 is more reliable than Ki67 in assessing growth of normal and tumour cells. Stoeber, *et al* (2001) showed that MCM7 is present in cells about to license replication and Going, *et al* (2005) described MCM5 as a marker of dysplasia. If this is the case and the Ki67 staining is ignored, this would mean that MCM5 expression does not alter between the two cell lines, MCM2 expression is less in adenocarcinoma and MCM7 is low to start and increases as confluence increases, in adenocarcinoma.

These findings now suggest a whole new paradox. Instead of an increased expression of MCMs in neoplastic conditions, which occurs for most neoplastic tissue, the MCMs seem to be either expressed at relatively the same level, or at a slightly lower level than normal. There is however a possible explanation for these findings and it can be speculated that MCMs may perform more roles in the cell cycle than just DNA replication. For instance, MCMs are estimated to be 40-100 fold more abundant in normal cells than required (Bell and Dutta 2002), and the loss of MCMs could lead to DNA damage (Bailis and Forsburg 2004). If this is then consider, reduced expression of MCM2 and 7 (at the start) in neoplastic cells, maybe due to loss of regulation and stability in prostatic adenocarcinoma.

The final explanation suggests that the PC-3 cells, may require either more time to start replicating (longer lag period), or they perform at a lower rate of proliferation than the PNT1A cells. Therefore, while the PNT1A cells have an active turnover which remains high even at high confluence levels, the PC-3 cells only start to show active proliferation at about 50% confluence when MCM7 expression is also high (3+).

Although there seems to be an alteration in expression of MCM2 and 7 between the two prostatic cell lines, and any of the three reasons mentioned above are plausible explanations of the findings, it is also important to consider the effects of *in vitro* cell culture on these cells and therefore their cellular behaviour. For instance, the PNT1A cell lines were transformed *in vitro* by SV40 to enable them to sustain cellular replication. This essentially means that the normal replication pathway of these cells may have been distorted by the SV40 virus and therefore their cellular expression of the

MCMs may also have altered in an unknown manner. Deppert *et al*, (1987) showed that rats cells transformed with SV40 express metabolically stable p53, instead of metabolically unstable p53, as in their untransformed counterparts.

The PC-3 cells were not virally transformed, but arose spontaneously as they are of neoplastic origin, so transformation has already occurred to allow them to evade replicative senescence. Although this means these cell are less likely to have cellular alterations as no external manipulation has been required to culture them, they may still express altered protein levels (such as the MCMs). This may be due to their removal from their normal cellular environment and the normal extracellular signals that they might have received *in vivo*. For instance Freshney (1987) stated it is important to consider that differences may occur in cellular behaviour *in vitro*, due to dissociation of the cells from a 3D geometry to a 2D substrate.

Whatever the reason for the differences between the two cell lines, it is important to notice that MCM2 expression for PC-3 cells does not change with confluence, remaining lower than PNT1A cells throughout. Although these alterations in MCM expression maybe due to the PNT1A cells being virally transformed, there is a possibility that these findings are truly representative of the cells and may be of importance. For instance MCM2 is involved in the licensing of replication (Blow and Hodgson 2002), in regulating the activity of MCM4, 6 and 7 by abolishing helicase activity (Yabuta, *et al* 2003), and providing stability to the DNA strand to stop errors occurring. If this reduction in MCM2 expression is true and not due to cellular immortalisation, then the cancerous cells may be deficient in one or all of these functions.

Not only does the amount of staining vary between the two cell lines, but the locality of immunoreactivity also varies, with MCM2 and 7 localised only in the nuclei for PNT1A cells, whilst for the PC-3 cells, nuclei and cytoplasmic staining is apparent. MCMs are located in the nuclei from late mitosis to the onset of the S phase, but are found in the cytoplasm during the G2 phase, therefore elimination of these proteins from the nuclei is to prevent re-replication (Hennessy *et al* 1990). It can therefore be postulated that the

PC-3 cells are actively producing too much MCM2 and 7 therefore these molecules are being transported to the cytoplasm to be broken down.

Again it is important to realise the staining locality maybe changed due to the cells being grown *in vitro*, however if this is not the case and the staining is truly representative of these cells, then it is possible that the nuclear localisation signal for MCM2 may be altered due to the cells becoming neoplastic. For instance MCM2 transports itself and MCM7 into the cytoplasm, when it would normally not do so. Kimura, *et al* (1996) stated, that MCM2 and 3 have nuclear localisation signals and that MCM2 forms a complex with MCM4, 6 and 7 to pull them into the nucleus, while MCM3 binds to 5 to pull it into the nucleus. MCM5 however is located in the nuclei and cytoplasm for both cell lines, so maybe this is over expressed in both due to a deranged nuclear localisation signal(s).

A transcriptional analysis was then performed at different proliferative states for MCM2, 5 and 7. These results show (*Figure 3.3-3.5*) that expression of MCM2, 5 and 7 varies between the two cell lines, with MCM2 and 5 showing a cyclic pattern of expression for PNT1A cells, whilst reduced expression occurs for PC-3 cells as confluence increases. MCM7 however starts high for both cell lines and then drops as confluence increases.

The cyclic pattern of expression which is seen for the PNT1A cells is expected, as both MCM2 and 5 are required for; licensing of DNA replication, the inhibition of the initiation complex, and to provide stability to the DNA. It is therefore possible to speculate that the reduced expression of MCM2 and 5, which occurs in the PC-3 cells, maybe due to; loss of DNA stability, loss of requirement to license DNA replication, or loss in providing inhibition to DNA replication. Again however it is important to consider that like the translational results, the alteration in the transcriptional results maybe due to *in vitro* cell culture and not truly representative of the mRNA expression that would be obtained *in vivo*.

If however these expressional alterations for MCM2 and 5 between the two cell lines are considered 'true', it is perhaps plausible that loss of DNA stability may be occurring in the PC-3 cells, as this would mean that the MCMs are no longer required to maintain the integrity of the replicated strands, allowing the DNA to be prone to errors. However in neoplastic cells, errors do/have already occurred, so stability is not as essential. Loss of MCM function during the S phase generates chromosome instability and DNA damage, and MCMs may be specifically targeted by oncogene activation (Bailis and Forsburg 2004).

Not only does there seem to be differences in expression of MCM2 and 5 between the cell lines, but also between MCM7, with a slightly higher surge of MCM7 occurring in PC-3 cells at the beginning of confluence compared to the PNT1A cells. MCM7 binds to Rb, a key regulator in progression of the cell cycle, therefore when Rb is phosphorylated MCM7 is released (Bailis and Forsburg 2004). This may imply that in the PC-3 cells, more G1/S-cyclin-CDK is produced, so more MCM7 is released than the PNT1A cells. This however does not necessarily allow for more replication to occur than the PNT1A cells, as it is possible that the excess protein is being transported to the cytoplasm (cytoplasmic staining for PC-3 cells seen for MCM7). There is also another explanation to the high surge in MCM7 expression, as in neuroblastoma cells a transcription factor (MYCN – myelocytomatosis viral related oncogene) binds to MCM7 and enhances its expression (Shohet, *et al* 2002), therefore it is possible that in the PC-3 cells there may be a similar transcription factor, which is activated and enhancing MCM7 expression compared to normal.

Synchronisation of the cells was achieved by serum deprivation and this was performed as stated in *Section 3.2.3*. Again like the confluence results it is important to consider the effects of *in vitro* growth on both the translational and transcriptional results obtained from synchronisation, as *in vitro* conditions can potentially cause increase/decrease in the MCMs, this means the finding may not be truly representative of these cells *in vivo*. The following discussion however considers the findings to be 'true' for both cell lines and gives reason for the differences seen.

The ICC results from the various synchronisation states show (*Figure 3.9*), that a difference in expression of MCM2 and 7 occurs between PNT1A and PC-3. For instance the PNT1A cells show a cyclic pattern of expression with a drop in immunoreactivity to zero (48/24hours –S phase), when most of the cells are already in the S phase and MCM required for replication should already be in place, and then an increase in immunoreactivity (48/30hours- G2/M phase), indicating loading of the MCMs and therefore preparing the cells for another round of replication. The replication licensing system becomes active at the beginning of G1/end M phase (MCMs loaded), and once the replication system has been licensed, no further MCMs are required, so they are inactivated as the cells enter the S, G2 and M phase (Blow and Hodgson 2002). The MCMs are removed from DNA as it is replicating and this is the primary reason why replication occurs once per cell cycle (Blow and Prokhorova 1999).

For the PC-3 cells however, a drop in MCM2 and 7 immunoreactivity at the G1 phase occurs (48/8hours), when normally MCMs are required here, so this may possibly indicate that either the MCMs are no longer required for replication, or that the MCMs involved in DNA stability are reduced. From these it is more feasible that the MCMs involved in stability are reduced, as stated previously, MCMs are 40-100 fold more abundant than required (Bell and Dutta 2002). MCM5 expression however does not differ between either of the cell lines during the synchronisation times, remaining high throughout. It is therefore possible that both cell lines over-express MCM5, due to the fact they are immortalised.

The locality of expression during the synchronisation times is similar to that seen for the confluence results, with cytoplasmic and nuclei immunoreactivity detected for the PC-3 cells for MCM2, 5 and 7, and only nuclei and cytoplasmic staining occurring for the PNT1A cells for MCM5. These findings can therefore be related to the above confluence reasons.

A transcriptional analysis at different synchronisation times was also carried out in the two cell lines (*Figure 3.10 – 3.12*). This however did not show intensive differences like that for proliferative state, but did show alterations in the amounts expressed, with

reduced rbu for MCM2, 5 and 7 being detected at most synchronisation times, for the PC-3 cells compared to the PNT1A cells. This reduction in expression of MCM2, 5 and 7 in cancer cells, further enhances the possibility that the MCMs may be involved in DNA stability.

Like the confluence results it is important to consider the implication of *in vitro* cell culture on the MCM results obtained by synchronisation. For instance, as mentioned previously, the PNT1A cells are SV40 transformed so have been manipulated to replicate indefinitely. This may have had an effect on expression of MCMs, as these proteins are required for proliferation and the PNT1A cells have been immortalised to allow continuous replication. This means that while increased MCM expression may occur for the cells *in vitro*, *in vivo* this would not necessarily be expected. The timing of MCM expression within the different proliferative states and synchronisation times are? unlikely to be altered in the PNT1A cells, as although the SV40 virus ensures continuous replication, it is unlikely to manipulate the cell to cause incorrect timing of expression.

For the PC-3 cells viral transformation was not performed as the cells are already transformed *in vivo*. This essentially means that PC-3 cells are less likely to have alterations in their MCM expression, so the data obtained from these cells lines is potentially more comparable to what might be expected *in vivo*. However, it is still important to consider the difference between *in vivo* and *in vitro* cellular environments and its effect on cellular behaviour due to loss of the normal 3D microenvironment *in vitro*.

When the data from the two cell lines is compared,, this study shows that for the PC-3 (cancer-derived) cells the translational expression of MCM2 and 7 is reduced, as well as the transcriptional expression of MCM2, 5 and 7 compared to the 'normal' PNT1A cells. If these results are representative of the cellular situation *in vivo*, they may contribute to the evidence for additional roles for MCMs: For instance it is becoming more apparent that the MCMs are not only important for replication of DNA, but may also play roles in stability and in stopping genetic damage. It has been questioned why

there is such an excess of MCM complexes if they are simply acting as helicases (Laskey, Madine 2003); and it has been shown that loss of MCM function can cause DNA damage and genomic instability (Bailis and Forsburg 2004).

Chapter 4 – Analysis of Minichromosomal Maintenance Proteins (MCMs) in Clinical Samples.

4.1 Introduction

Distinct cellular characteristics must be observed to facilitate diagnosis/prognosis of prostate cancer, so there is an urgent need for biomarkers that are specific and sensitive at identifying morphological differences which correlate with disease status. Currently due to unsatisfactory diagnostic techniques, some patients identified as having prostatic problems such as difficulty urinating, already have extrapolated disease upon examination (Clement et al 1999), so biomarkers that provide further information into disease prevalence are of clinical value.

Due to the current difficulty in distinguishing malignancy by microscopic evaluation and histological staining alone, small foci of disease can be missed and intermediate Gleason scores difficult to distinguish. Histological biomarkers are therefore under investigation to assess whether they can provide additional assistance. Currently, markers such as cytoplasmic keratin 34[beta]E12 or nuclear p63 are being investigated for use in identifying BPH, while α -Methylacyl CoA Racemase P504S (AMACR), hedgehog signalling pathway (Hh) and MCMs are under investigation for use in distinguishing between BPH and prostate cancer. Recent studies suggesting the value of these markers are outlined below.

Cytoplasmic keratin 34[beta]E12 and p63 are basal specific markers. Due to the loss of basal cells in malignancy they can be used to help distinguish BPH from adenocarcinoma (Fleshman and MacLennan, 2005). These markers are therefore used as negative markers for cancer, in the sense that when positive staining is apparent, foci of cancer are believed to be absent.

AMACR is a mitochondrial and peroxisomal enzyme (Mubiru et al, 2003) which plays an important role in the β -oxidation of branched-chain fatty acids and fatty acid derivatives,

as it catalyses several (2R)-methyl-branched-chain fatty acyl-CoAs to their (s)-stereoisomers (Ferdinandusse, *et al* 2000). During tumourigenesis this marker has been shown to be over-expressed in a number of cancers and pre-cursor lesions, particularly in the prostate and colon (Ananthanarayanan *et al*, 2005), indicating that AMACR is a potential marker for early prostate cancer detection.

Hh is conserved in vertebrates and invertebrates governing embryonic development and homeostasis (Daya-Grosjean & Couve-Privat, 2004). The secreted glycoprotein of the Hh pathway plays a number of essential roles, and during prostate development high Hh is required, while in developed adults only low levels of the pathway are detected (McMahon *et al*, 2003 Sheng *et al*, 2004). In prostate cancer development over-expression of members of these pathways may therefore occur (Karhadkar *et al*, 2005 Sheng *et al*, 2004) and a study performed by Sheng *et al* (2004) found that the hedgehog target gene (PTCHI) and the hedgehog interacting protein (HIP) were identified in 50% of prostate cancer patients with Gleason score 8-10 and in 22% of patients with Gleason score 3-6.

Numerous antibody cocktails using a number of negative and positive markers for prostate cancer are also under investigation, for instance three-antibody cocktails containing AMACR(P504S)/34[beta]E12 and p63 are being used to detect small foci of prostate adenocarcinoma in tissue (Jiang *et al*, 2005). Iczkowski (2006) found that 76% of atypical small acinar can be resolved by AMACR(P504S)/34[beta]E12 and Jung *et al*, (2005) found that 95% of prostate adenocarcinoma expressed AMACR(P504S) and were negative for basal cell staining (34[beta]E12, p63), while for BPH positive basal cell markers were present (100%) and no AMACR(P504S) expression was detected.

Although the biomarkers outlined above seem promising, for this chapter MCM2, 5 and 7 were analysed to assess their potential for prostate cancer diagnosis. These markers have already been investigated in numerous clinical conditions with very promising results in terms of correlation of expression with disease status (Ref *Section 3.1*), therefore the aim of this chapter is to investigate whether MCM2, 5 and 7 can be assessed collectively as

multiple markers for distinguishing prostate cancer from BPH histologically, and to provide further insight as to whether these biomarkers should be analysed as potential clinical diagnostic markers (urine/blood). In this chapter, PSA is also analysed histologically, to observe how non-neoplastic prostatic tissue varies in PSA expression compared to neoplastic prostatic tissue.

4.2 Materials and Methods

To investigate MCM2, 5, 7 and PSA expression in prostate cancer, 51 tissue sections from consenting patients and 23 sections from archives were examined (Raw data *Appendix E Table e.1-e.2*). These sections varied from Gleason Score 4-10 to benign prostatic hyperplasia (BPH) and nuclei expression of MCM2, 5 and 7 was assessed, while cellular and nuclei expression of PSA was assessed for the glandular epithelial cells, of the prostate gland. The staining criterion used is shown in *Table 4.1* and a consultant histopathologist correlated tissue staining with Gleason Score.

4.2.1 Clinical Sample Collection

Ethical approval was obtained (copies of the relevant information are found in *Appendix E*) and patients identified as needing a TURP or a Radical Prostatectomy were invited to take part in the trial at Northampton General Hospital during their initial consultation. Then on arrival at Pre-assessment (TRUP/ Radical Prostatectomy) they were asked if they agreed to participate and if in agreement, written consent was obtained (there is at least 24 hours between initial invitation and consent).

On the day of the operation a notice of consent was sent to histopathology with the patient biopsy, to inform them that any waste biopsy tissue could be sectioned for this trial (TRUS patients only had urine sample collected). The biopsy tissue was then fixed in formalin and embedded in paraffin and 3.5µm sections were cut by histopathology at Northampton General Hospital. All samples collected (urine, blood and tissue) from the same patient were allocated identical ID numbers and this number was logged in a Logbook, with the patients details. As well as sections being cut from consenting patents,

archive tissue was collected to increase sample number (copies of relevant ethical approval shown in *Appendix E*).

4.2.2 Paraffin embedded cells

To optimise immunohistochemistry for antibodies to MCM2 Ab-1 Clone CRCT2.1, MCM5 Ab-1 Clone CRCT5.1, CDC47/MCM7 Ab-2 Clone 47CD141 or DCS141 and PSA RB-9065-P, the cell lines PNT1A and PC-3 were cultured until they reached 75% confluence. These were then washed in cold PBS and trypsinised to dissociate the cells from the flask as in *Appendix A Section A.2*. Once the cells were pelleted, 2mls of a 10% buffered formalin (w/v) (Triangle Biomedical Science Ltd, Lancashire) was added to fix the cells and the suspension was transferred to a gamma irradiated tube. These were then centrifuged at 105 xg for 5 minutes and the pellet was washed twice with a PBS solution, to remove any of the remaining formalin.

The cellular pellets were then embedded in 1% agarose (w/v) by transferring the agarose quickly to the pellets. These were then vortexed to ensure a good distribution occurred (agarose had been boiled and then incubated in a water bath at 50°C to stop it from setting) and then transferred to well plates, which were placed on ice to allow the agarose to set. After 30 minutes the agarose cellular pellets were placed in a cassette and transported in formalin to Northampton General Hospital, here the agarose embedded pellets were then processed, embedded in paraffin and 3.5µm sections were cut. These sections were then used to optimise antigen retrieval for immunohistochemistry and the optimisation results are shown in *Appendix E Figure e.1* (Antigen retrieval time decided upon for immunohistochemistry was 25 minutes).

4.2.3 Immunohistochemistry

Immunohistochemistry was then performed on the biopsy tissue, as described in *Appendix A Section A.4* and the antibodies used were Mouse Monoclonal antibodies to MCM2 Ab-1 Clone CRCT2.1, MCM5 Ab-1 Clone CRCT5.1, CDC47/MCM7 Ab-2 Clone 47DC141 or DCS141 and Rabbit Polyclonal antibodies to PSA RB-9065-P (Stratech, Cambridge UK). The dilution factors used were 1:50 for MCM2 and MCM7,

1:50 for PSA and 1:200 for MCM5. All these primary antibodies were incubated on the slides in a humidity chamber at 4°C overnight and negative controls were produced by removal of the primary antibodies.

Criterion for Assessing Cellular Staining

A staining system was then used to assess the percentage of cells that showed staining and this is given in *Table 4.1*. For all tissue sections only epithelial cell staining was assessed, as this is the area which becomes neoplastic in the prostate gland. Assessment was undertaken on the nuclei expression of MCM2, 5 and 7, as this staining indicates the participation of the MCMs in the cell cycle, while both nuclei and cytoplasmic staining for PSA was assessed, as PSA does not take part in the cell cycle and is a serine protease.

Table 4.1. Shows the staining criterion used for immunohistochemistry on antibodies to MCM2, MCM5, MCM7 and PSA for the tissue section. Adapted from: www.aruplab.com/home/anatomic_pathology.jsp (2003).

Grade	% Cells Staining	Interpretation	Microscopic finding
0-	0%	No Staining	No Nuclei Staining of epithelial cells seen
0+	1-4%	little Staining	Few nuclei Staining*
1+	5-25%	Weak Staining	Less than 25% but more than 4% of the epithelial nuclei staining. *
2+	26-75%	Moderate Staining	Less than 75% but more than 25% of the epithelial nuclei staining.*
3+	>75%	High Staining	Near enough 100% of the epithelial nuclei stain*

* Staining was only assessed for the nuclei of epithelial cells for antibodies to MCM2, MCM5 and MCM7, however cytoplasmic and nuclear expression of the epithelial cells was assessed for antibodies to PSA

4.2.4 Statistical Analysis

An analysis of variance (ANOVA) was then performed on the staining criterion received for MCM2, MCM5, MCM7 and PSA, to identify if a significant difference in staining occurs between the different prostatic conditions. A Bonferroni test was also performed for MCM2, MCM5, MCM7 and PSA to assess the homogeneity (similarity) of the different prostate conditions. If a *P-value* of <0.05 is obtained, this indicated that the results were considered to be significantly different.

4.3 Results

4.3.1 MCM2, MCM5, MCM7 and PSA Expression in Clinical Samples

Immunohistochemistry

Table 4.2 summarises the immunohistochemistry results obtained for prostate sections, which ranged from BPH and Gleason Score 4-10 (raw data Appendix E Table e.1 and e.2, while Figure 4.1-4.8 demonstrates some of the staining seen visually for different prostate conditions.

Table 4.2. Expression of MCM2, MCM5, MCM7 and PSA in different prostatic conditions, as determined by immunohistochemistry. Sample number n=74

MCM2 Grade						MCM5 Grade					
Score	0-	0+	1+	2+	3+	Score	0-	0+	1+	2+	3+
BPH	30	2				BPH	20	12			
G-4		1				G-4			1		
G-5		4				G-5			3	1	
G-6	3	4	2			G-6		1	3	5	
G-7		2	3			G-7			2	3	
G8	1	4	2			G8			3	4	
G-9		1	9			G-9			1	8	1
G-10		1	3	2		G-10			1	4	1

MCM7 Grade						PSA Grade					
Score	0-	0+	1+	2+	3+	Score	0-	0+	1+	2+	3+
BPH	8	24				BPH				1	31
G-4			1			G-4					1
G-5		1	3			G-5					4
G-6		1	6	2		G-6					9
G-7			5			G-7					5
G8			6	1		G8			1	1	5
G-9			3	6	1	G-9					10
G-10				4	2	G-10			1	4	1

Grade - 0- = 0%, 0+ = 1-4%, 1+ - 5-25%, 2+ = 26-75%, 3+ = >75% (Percentage of cells staining)
 Score - BPH = Benign prostatic hyperplasia, G = Gleason Score
 Samples Number - BPH n=32, G-4 n=1, G-5 n=4, G-6 n=9, G-7 n=5, G-8 n=7, G-9 n=10, G-10 n=6.

The immunoreactivity appears to differ between different stages of disease progression (BPH-Gleason Score 4-10) for antibodies to MCM2, 5 and 7, with low immunoreactivity occurring for BPH and high immunoreactivity occurring for cancerous conditions, which amplifies as disease progression increases. Of the three MCMs under analysis, it was observed that MCM2 produces the weakest results, with relatively low differences in staining ability, when BPH is compared to neoplastic conditions, whilst MCM5 and MCM7 produced the highest differences in staining ability when BPH is compared to cancerous conditions. Of these, MCM7 seems to produce more useful results than MCM5, as the staining locality is fixed to the nuclei of the prostatic cells, which makes it easier to assess. MCM5 however shows cytoplasmic staining as well as nuclear and this makes assessing the staining criterion of the epithelial cells that bit more difficult.

The second observation noticed from *Table 4.2* is that most cases show high PSA staining (3+) when BPH is compared to different Gleason scores. There is however a drop in PSA staining ability at high Gleason scores for a few cases, where high staining become moderate and low (Gleason Score 8 and 10).

The results illustrate that as Gleason score increases in prostate cancer, the amount of nuclear epithelial staining of MCM5 and 7 also increases, which means a difference in nuclear epithelial cell staining can be seen between neoplastic and non-neoplastic conditions. MCM2 and PSA however do not seem to show a major differences in staining ability when BPH is compared to most Gleason scores, therefore these marker are not ideal for prostate cancer diagnosis.

A statistical analysis was then performed on the raw data obtained from the immunohistochemistry staining results, to identify if a significant difference in staining ability occurs between BPH to Gleason score 4-10. *Figures 4.9 – 4.12* Show mean staining for the different Gleason scores and BPH for antibodies to MCM2, 5, 7 and PSA and their 95% confidence limit, whilst *Tables 4.3-4.4* Show the analysis of variance results (ANOVA) and Bonferroni results, for antibodies to MCM2, 5, 7 and PSA.

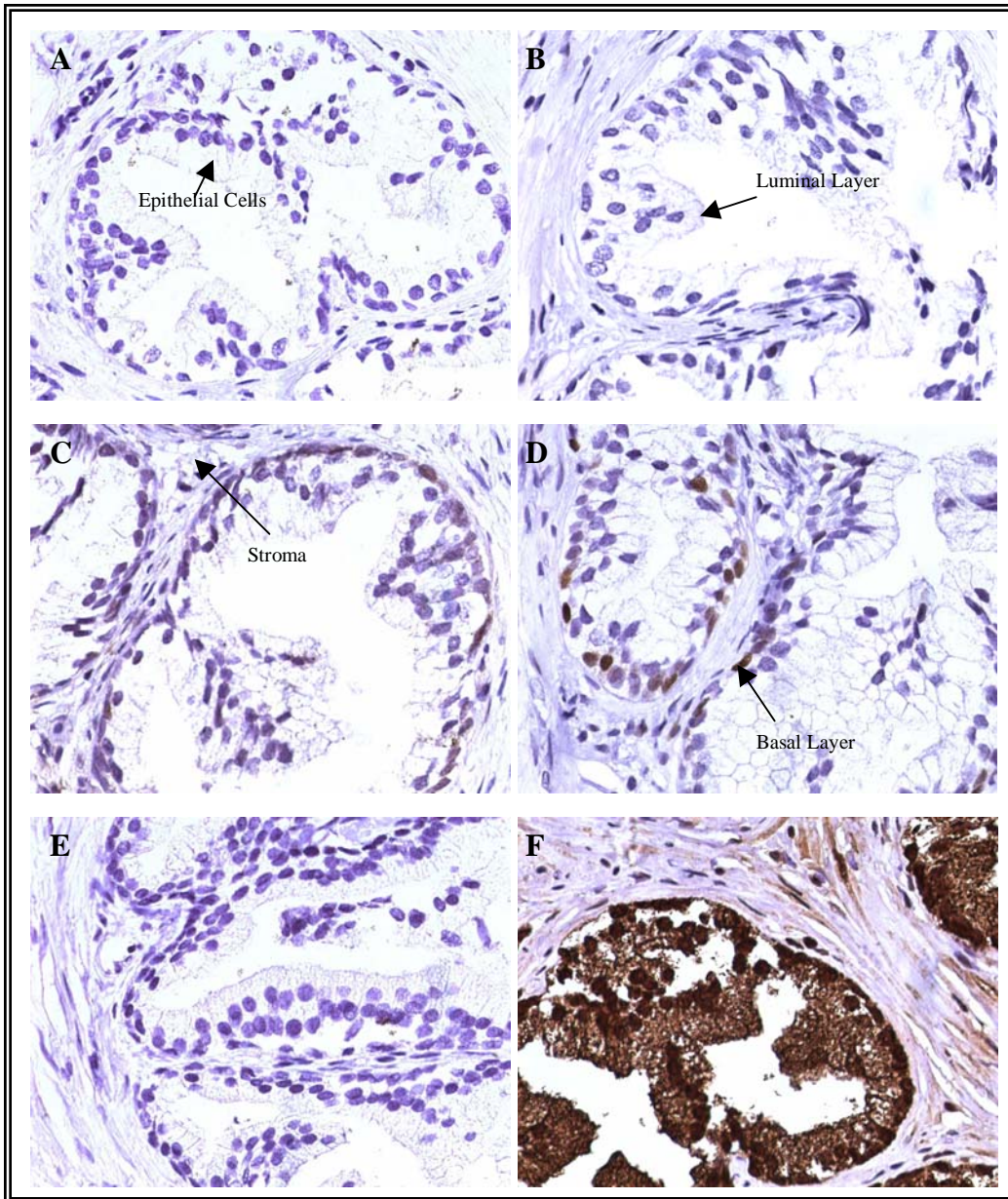


Figure 4.1. Immunohistochemistry for a hyperplastic gland stained with B= antibody to MCM2, C = antibody to MCM5, D = antibody to MCM7, F= antibody to PSA, A = MCM2, MCM5, MCM7 negative control, E= PSA negative control. Appearance of brown precipitate indicates positive cellular staining. B-D shows that relatively little epithelial staining occurs for hyperplastic conditions with mainly basal cells staining with antibody to MCM2 (-0), MCM5 (+0) and MCM7 (0+). F shows that high epithelial/basal and some stromal staining occurs for hyperplastic conditions with antibody to PSA (3+). Magnificant X400.

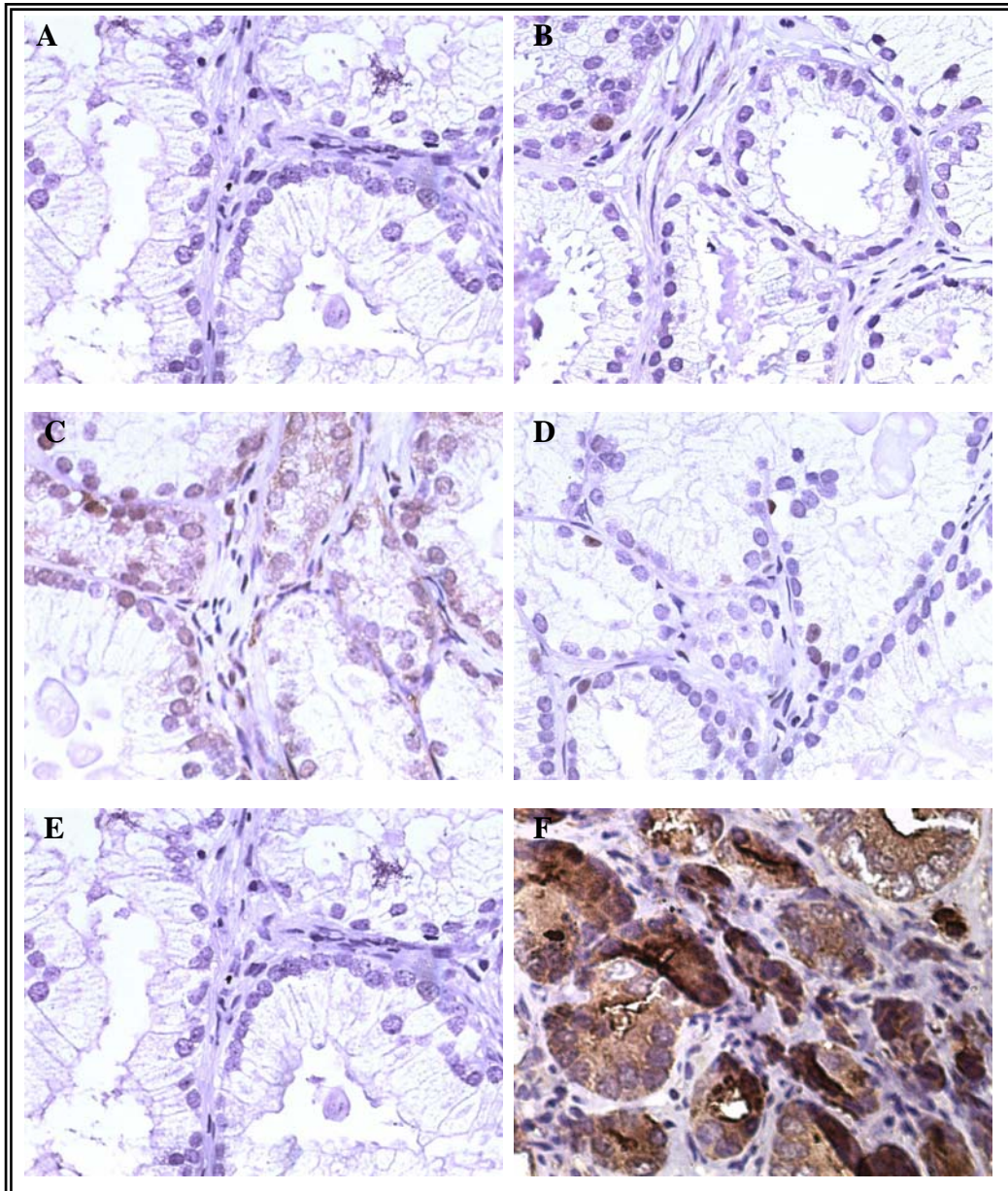


Figure 4.2. Immunohistochemistry for Gleason Score 4 (Gleason Pattern 2+2) stained with B= antibody to MCM2, C = antibody to MCM5, D = antibody to MCM7, F = antibody to PSA, A = MCM2, MCM5 and MCM7 negative control, E = PSA negative control.

Appearance of brown precipitate indicates positive cellular staining.

Low grade tumour consists of large, open uniform glands with numerous crytalloids located in the centre of the glands. The glands are still single and separate.

B shows nuclear epithelial cell staining for MCM2 which was critical assessed as a O+, while C, D shows cytoplasmic and nuclear epithelial cell staining with the nuclear staining correlated and given a 1+.

F shows that high epithelial/basal cell staining occurring with antibody to PSA and this was given a 3+.

Magnificant X400.

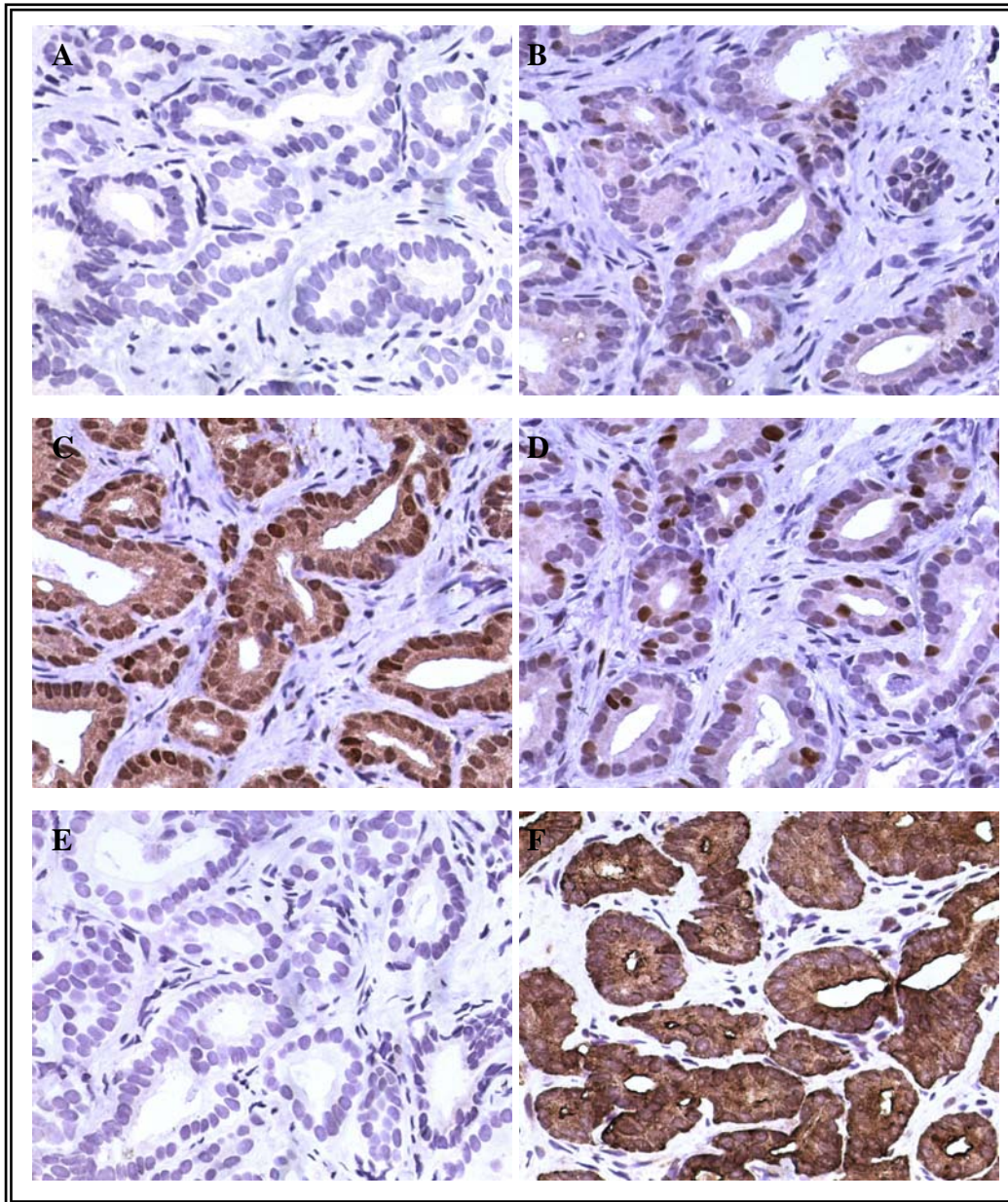


Figure 4.3. Immunohistochemistry for Gleason Score 6 (Gleason Pattern 3+3) stained with B= antibody to MCM2, C = antibody to MCM5, D = antibody to MCM7, F = antibody to PSA, A = MCM2, MCM5 and MCM7 negative control, E = PSA negative control.

Appearance of brown precipitate indicates positive cellular staining.

The tumour has infiltrated in/among non-neoplastic tissue, with the glands having variation in their size and shape. The glands are still single and separate but show loss of basal cells and are more loosely arranged and not so uniform, as they would be for pattern 1 to 2.

B and D shows nuclear epithelial cell staining for MCM2 and MCM7 which was critical assessed as a 1+, while C shows cytoplasmic and nuclear epithelial cell staining however only the nuclear staining was correlated and give a 2+.

F shows that high epithelial/basal cell staining occurring with antibody to PSA and this was given a 3+.

Magnificant X400.

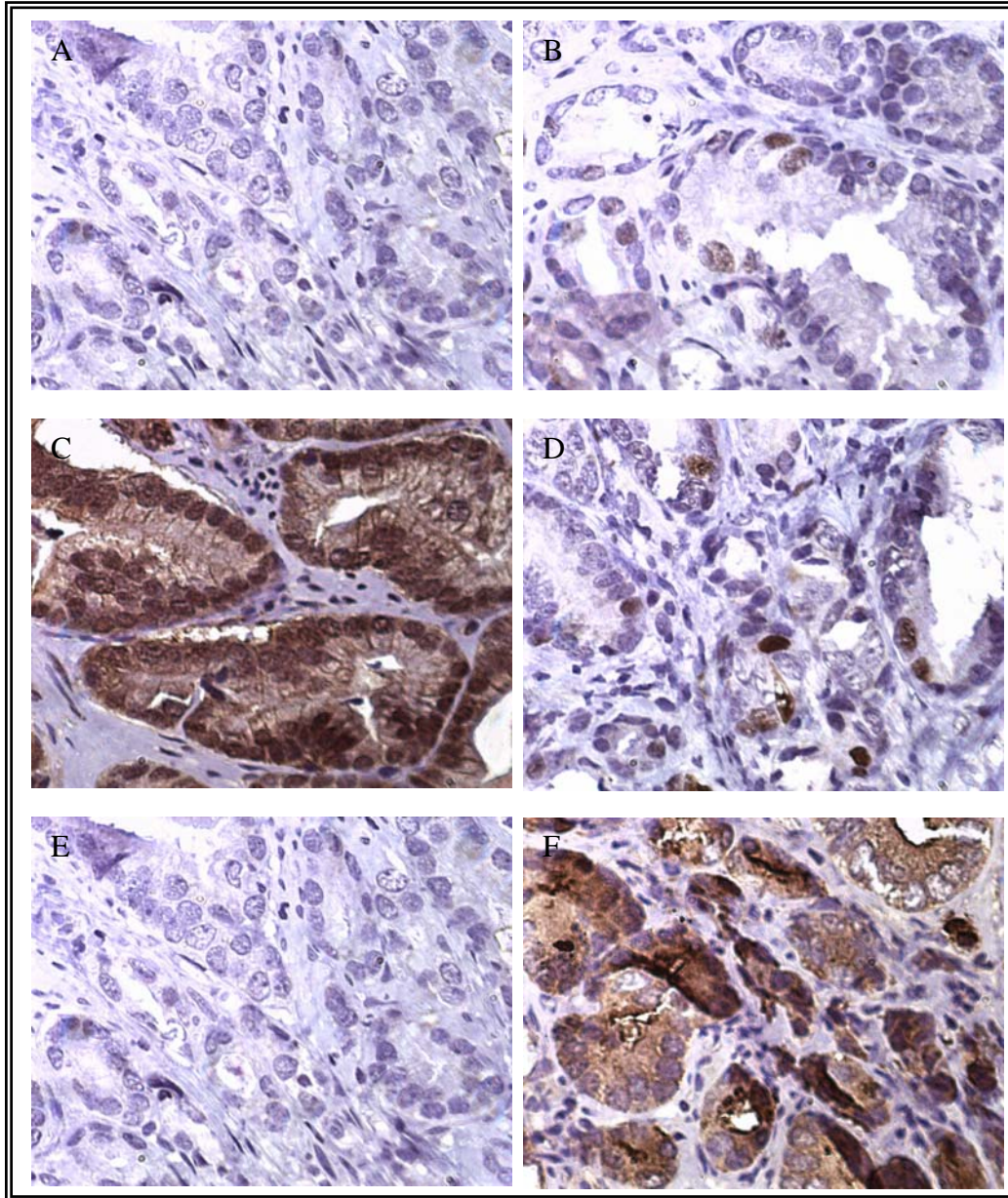


Figure 4.4. Immunohistochemistry for Gleason Score 7 (Gleason Pattern 3+4) stained with B= antibody to MCM2, C = antibody to MCM5, D = antibody to MCM7, F = antibody to PSA, A = MCM2, MCM5 and MCM7 negative control, E = PSA negative control.

Appearance of brown precipitate indicates positive cellular staining.

The tumour has infiltrated in/among non-neoplastic tissue, with the glands having variation in their size and shape. The glands are still mainly single and separate but show loss of basal cells and some glands are beginning to fuse.

B, and D shows nuclear epithelial cell staining for MCM2 and MCM7 which was critical assessed as a 1+, while C shows cytoplasmic and nuclear epithelial cell staining however only the nuclear staining was correlated and give a 2+.

F shows that high epithelial/basal cell staining occurring with antibody to PSA and this was given a 3+.

Magnificant X400.

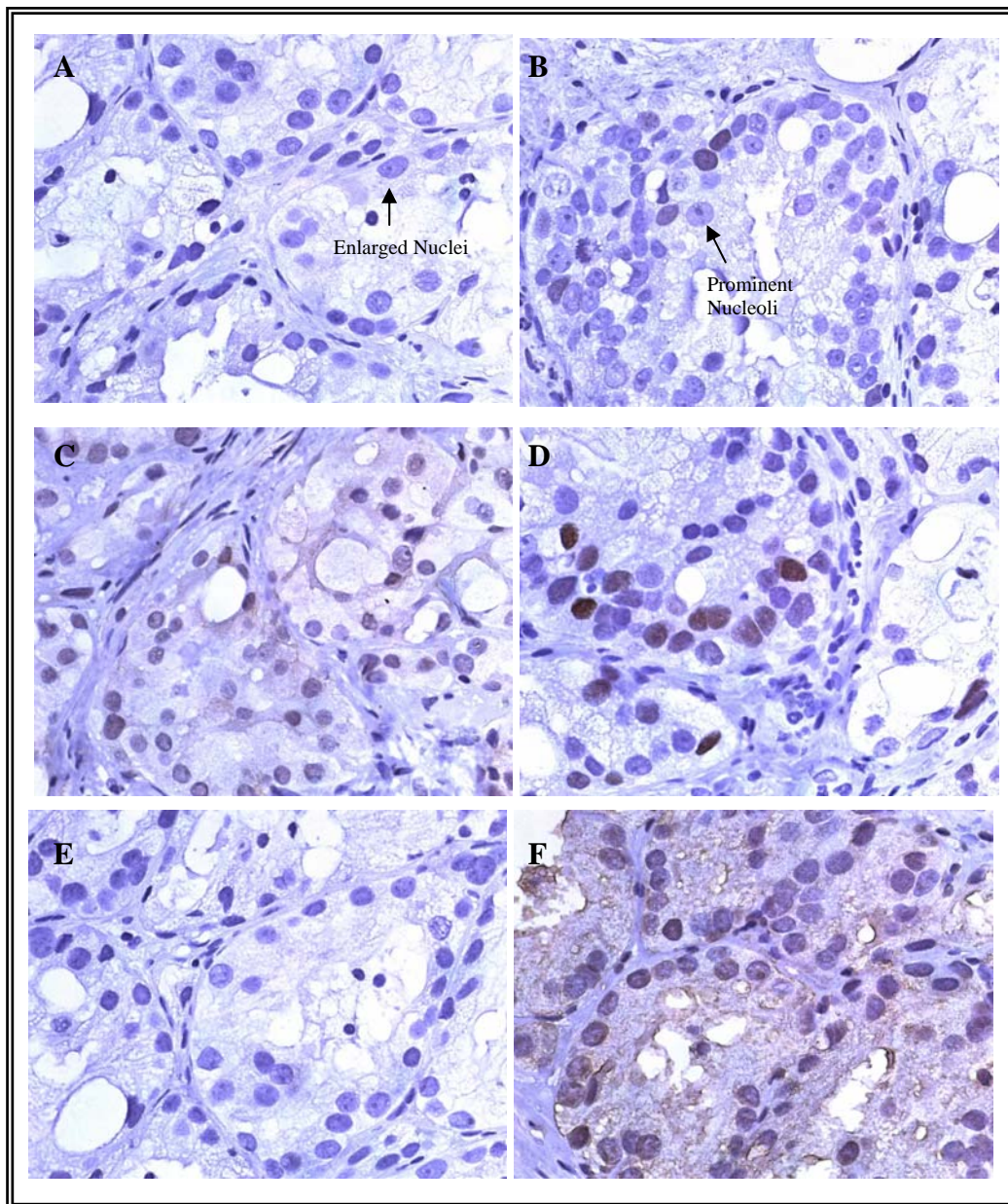


Figure 4.5. Immunohistochemistry for Gleason Score 8 (Gleason Pattern 4+4) stained with B= antibody to MCM2, C = antibody to MCM5, D = antibody to MCM7, F = antibody to PSA, A = MCM2, MCM5 and MCM7 negative control, E = PSA negative control.

Appearance of brown precipitate indicates positive cellular staining.

The prostate glands are no longer single and separate and start to fuse together. The glands have also lost their basal cell layer and the epithelial nuclei enlarge with some showing prominent nucleoli.

B-D shows nuclear epithelial cell staining for MCM2, MCM5 and MCM7 these antibodies were critical assessed as a 0+ for MCM2 and a 1+ for MCM5 and MCM7.

F shows that weak epithelial/basal cell staining occurring with antibody to PSA and this was given a 1+.

Magnificant X400.

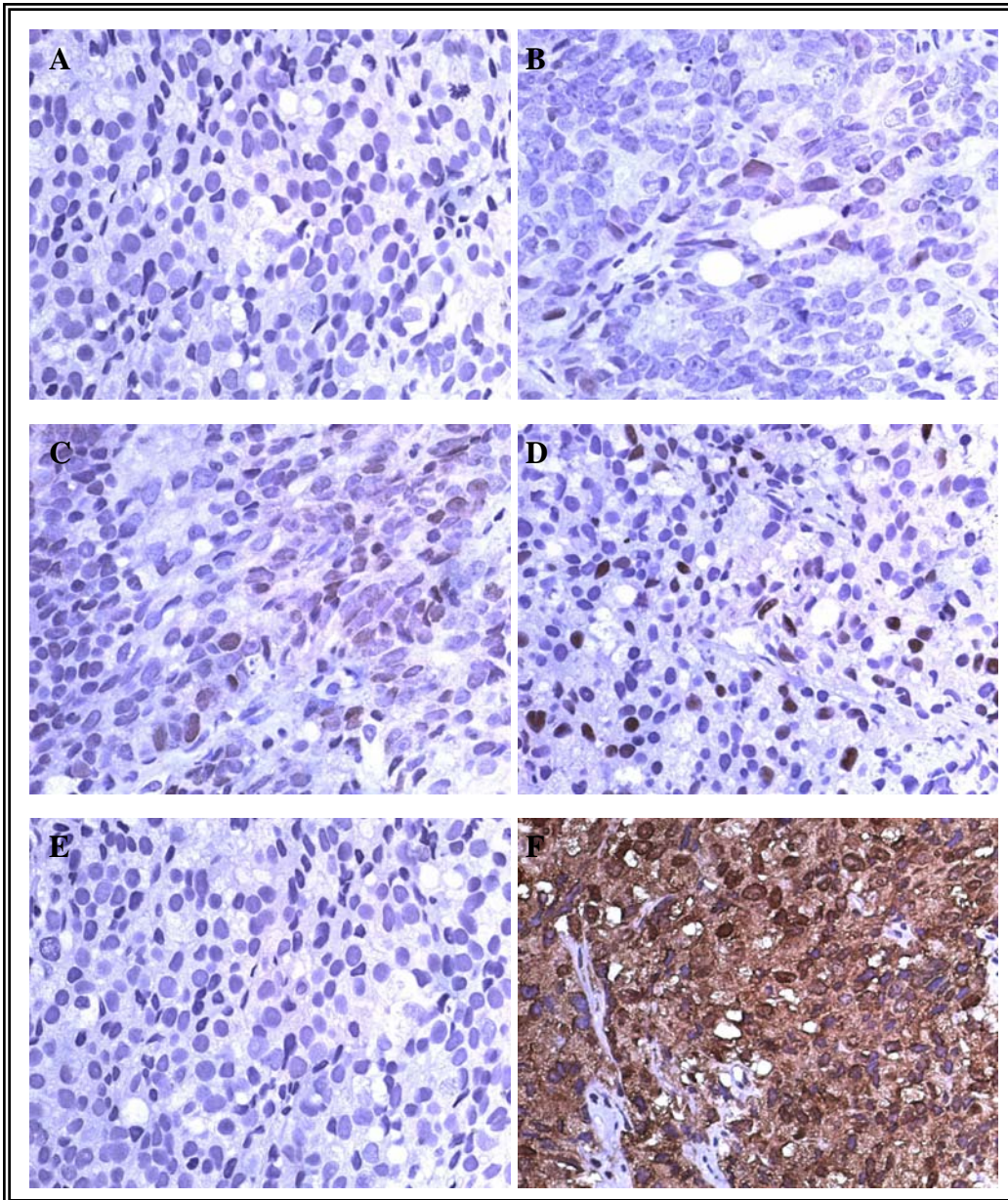


Figure 4.6. Immunohistochemistry for Gleason Score 8 (Gleason Pattern 3+5) stained with B= antibody to MCM2, C = antibody to MCM5, D = antibody to MCM7, F = antibody to PSA, A = MCM2, MCM5 and MCM7 negative control, E = PSA negative control.

Appearance of brown precipitate indicates positive cellular staining.

The prostate glands are no longer single and separate and are composed of fused glands with ragged edges. The glands have also lost their basal cell layer and the epithelial nuclei enlarge with some showing prominent nucleoli.

B-D shows nuclear epithelial cell staining for MCM2, MCM5 and MCM7 these antibodies were critical assessed as a low 1+ for MCM2, a low 2+ for MCM5 and a median 2+ MCM7.

F shows that high epithelial/basal cell staining occurring with antibody to PSA and this was given a 3+.

Magnificant X400.

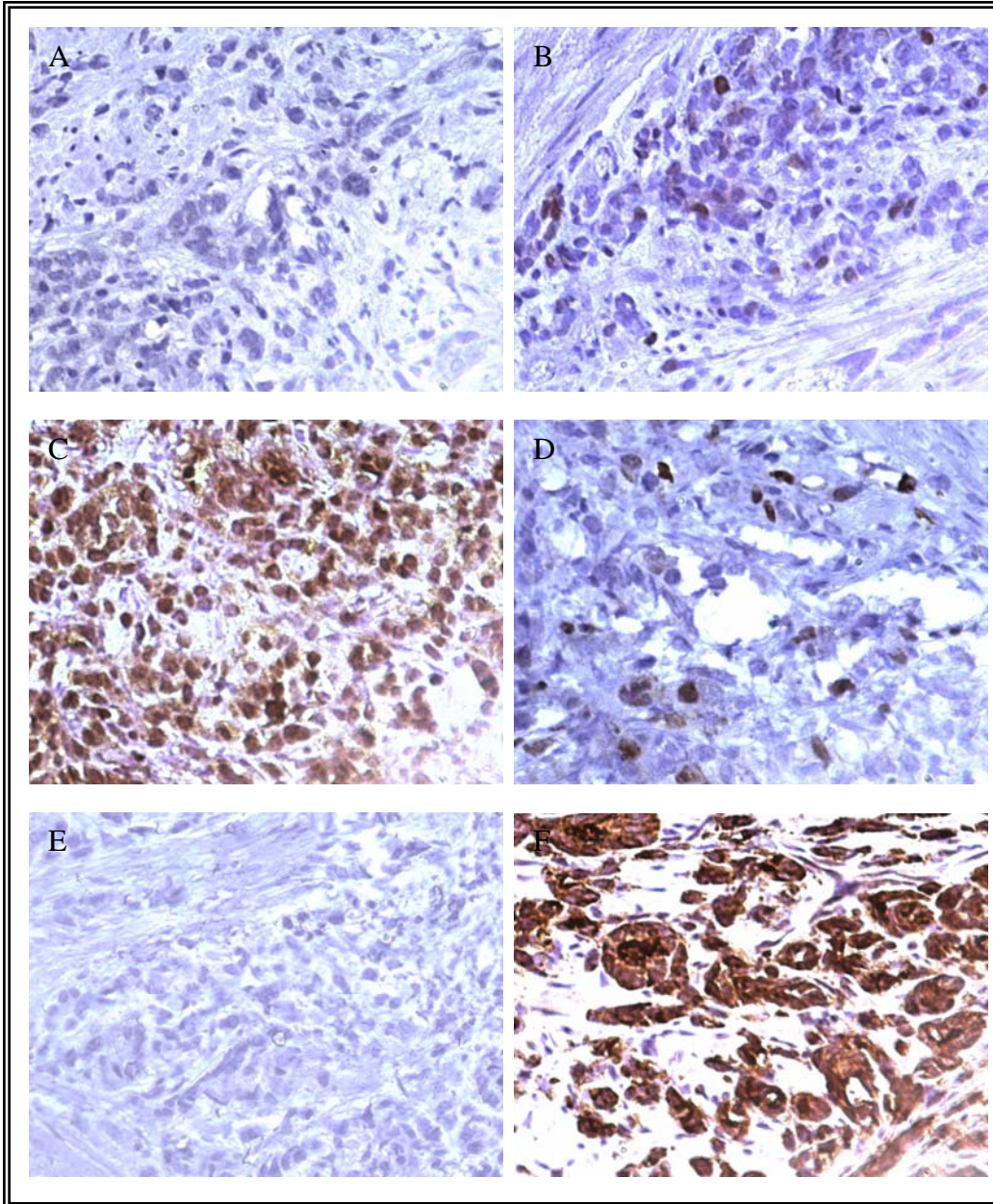


Figure 4.7. Immunohistochemistry for Gleason Score 9 (Gleason Pattern 4+5) stained with B= antibody to MCM2, C = antibody to MCM5, D = antibody to MCM7, F = antibody to PSA, A = MCM2, MCM5 and MCM7 negative control, E = PSA negative control.

Appearance of brown precipitate indicates positive cellular staining.

The prostate gland is no longer showing glandular differentiation made up of solid masses of cells. The basal layer can no longer be distinguished from the epithelial cells and may not even be present.

B-D shows nuclear epithelial cell staining for MCM2, MCM5 and MCM7 these antibodies were critical assessed as a low 1+ for MCM2, a low 2+ for MCM5 and a median 2+ MCM7.F shows that high epithelial/basal cell staining occurring with antibody to PSA and this was given a 3+.

Magnificant X400.

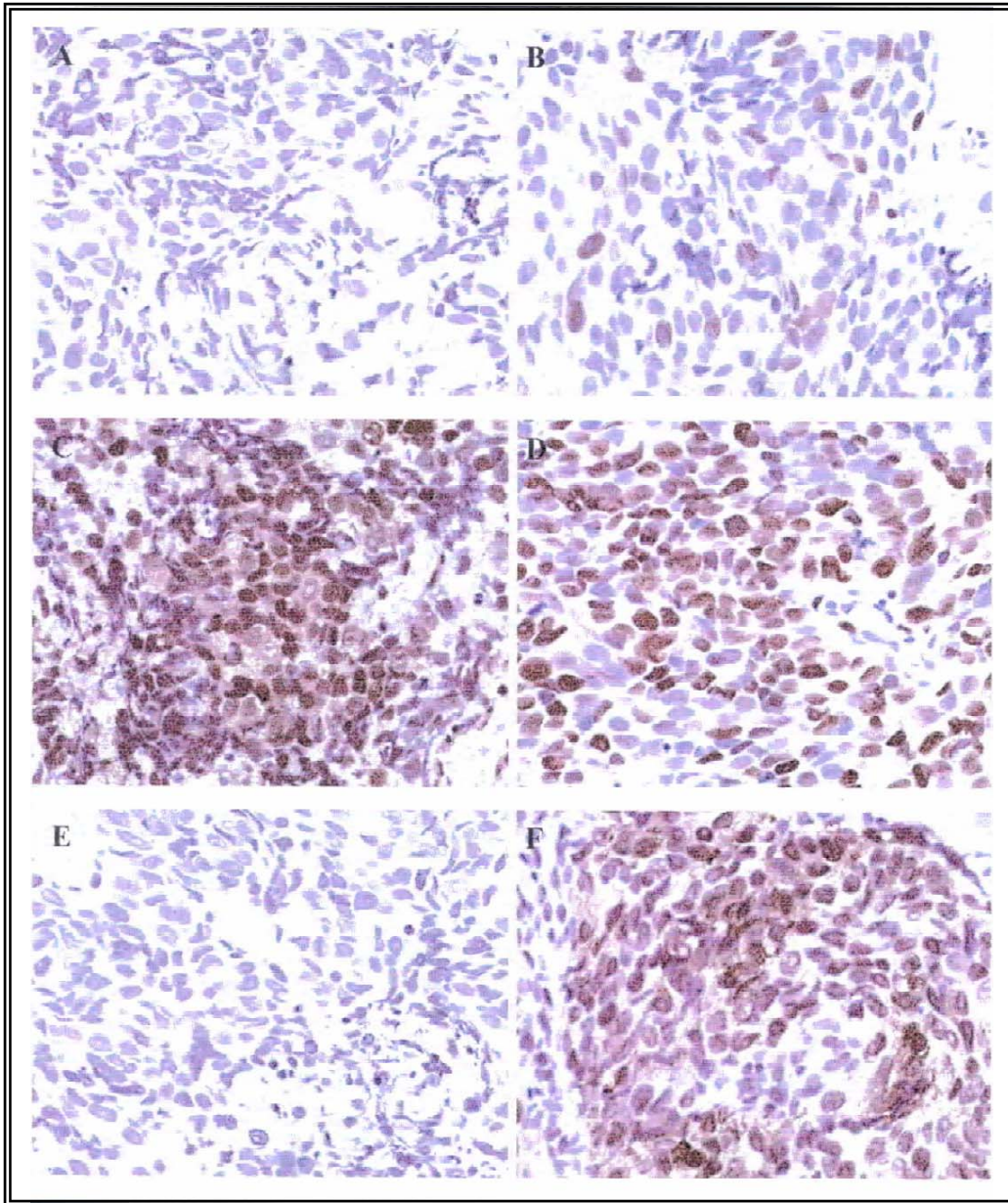


Figure 4.8. Immunohistochemistry for Gleason Score 10 (Gleason Pattern 5+5) stained with B = antibody to MCM2, C= antibody to MCM5, D = antibody to MCM7, F = antibody to PSA, A = MCM2, MCM5 and MCM7 negative control, E = PSA negative control.

Appearance of brown precipitate indicates positive cellular staining.

The prostate gland shows no glandular differentiation and is made up of a solid mass of cells. The basal cell layer is no longer distinguishable from the epithelial cell layer and may not even be present. Also the epithelial nuclei enlarge with some showing prominent nucleoli.

B-D shows nuclear epithelial cells staining for MCM2, MCM5 and MCM7 these antibodies were critical assessed as a high 1+ for MCM2, a high 2+ for MCM5 and a high 2+ for MCM7.

F shows that epithelial/ basal cell staining occurring with antibody to PSA and this was given a high 2+.

Magnification X400.

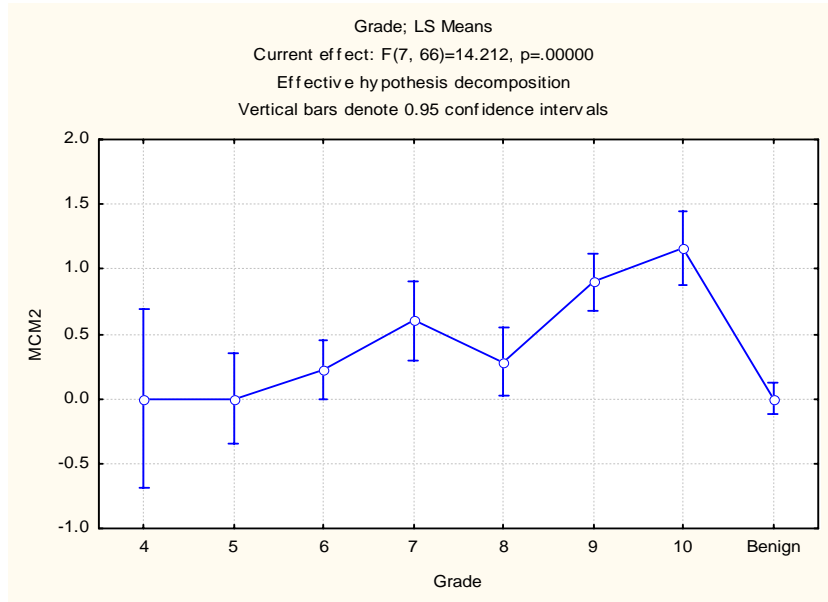


Figure 4.9 A line graph of the IHC results showing mean number of cells staining for antibodies to MCM2 against gleason score 4-10 and BPH (Benign). (raw data shown in Appendix E table e.1 and e.2). Error bar shows the 95% confidence limit for each graph.

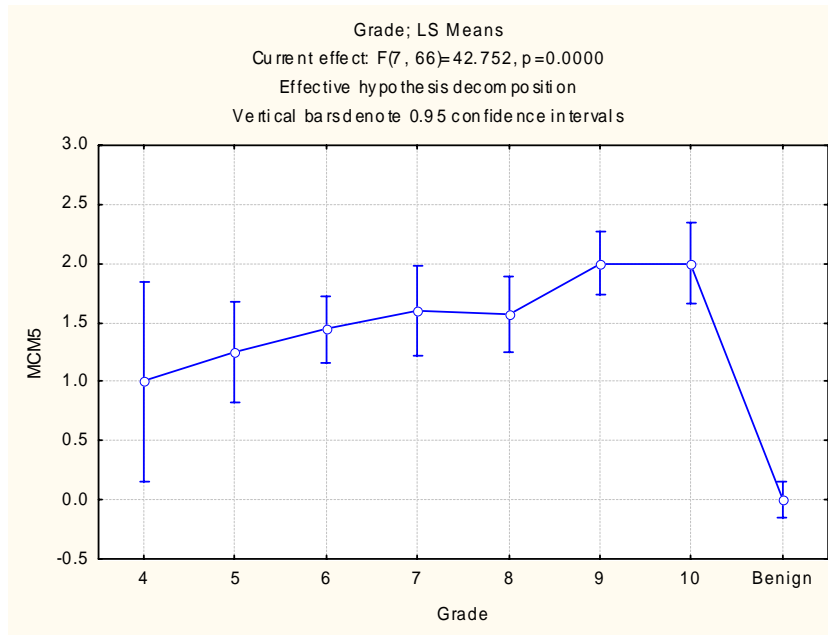


Figure 4.10 A line graph of the IHC results showing mean number of cells staining for antibodies to MCM5 against gleason score 4-10 and BPH (Benign). (raw data shown in Appendix E table e.1 and e.2). Error bar shows the 95% confidence limit for each graph.

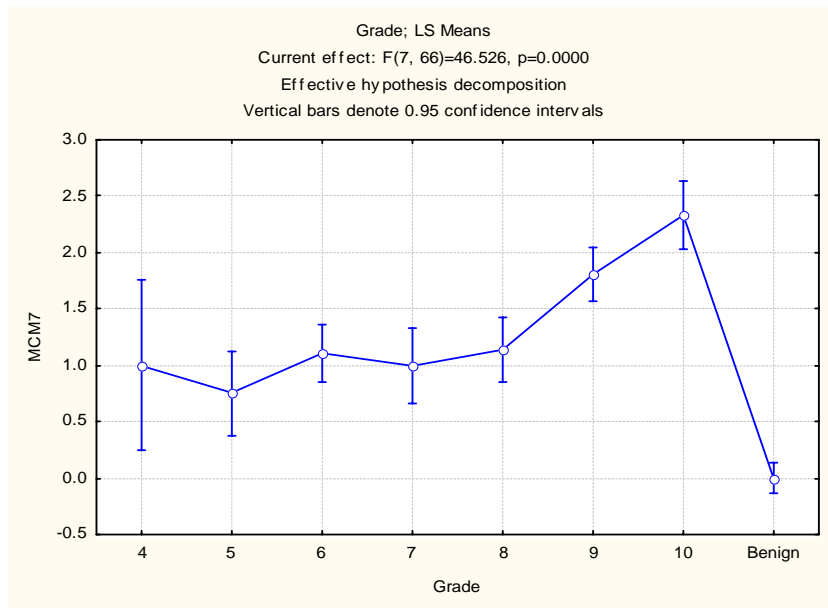


Figure 4.11 A line graph of the IHC results showing mean number of cells staining for antibodies to MCM7 against gleason score 4-10 and BPH (Benign). (raw data shown in Appendix E table e.1 and e.2). Error bar shows the 95% confidence limit for each graph.

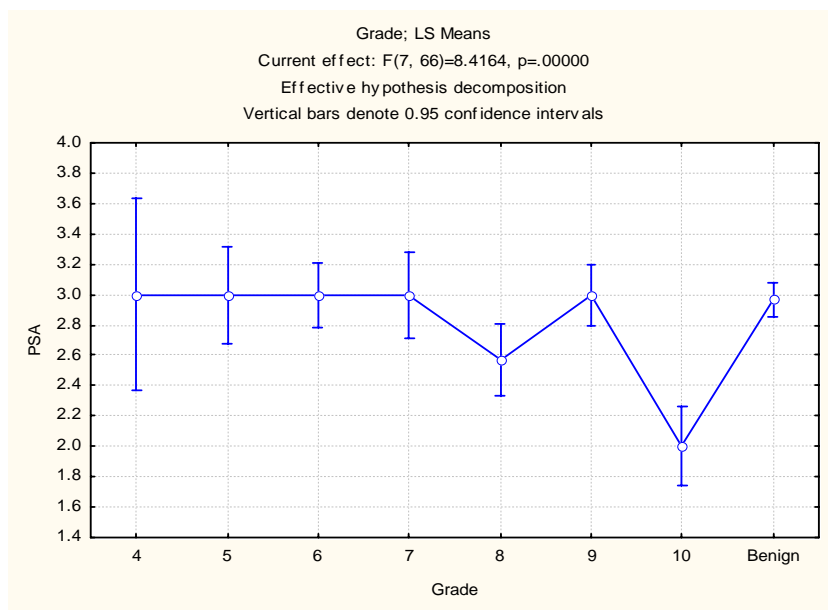


Figure 4.12 A line graph of the IHC results showing mean number of cells staining for antibodies to MCM7 against gleason score 4-10 and BPH (Benign). (raw data shown in Appendix E table e.1 and e.2). Error bar shows the 95% confidence limit for each graph.

Table 4.3 One way ANOVA results for IHC for antibodies to MCM2, MCM5, MCM7 and PSA against different prostatic conditions (BPH-Gleason Score 10) (Raw data *Appendix E Table e.1 and e-2*). If P-value less than <0.05 is obtained it means a significant difference is seen between the scores.

MCM2			Analysis of variance (ANOVA)	
	N	Degrees of Freedom	F	P
Intercept	1	1	41.96602	<0.0001
Score	8	7	14.21157	<0.0001
Total	74	66		

MCM5			Analysis of variance (ANOVA)	
	N	Degrees of Freedom	F	P
Intercept	1	1	327.4762	<0.0001
Score	8	7	42.7522	<0.0001
Total	74	66		

MCM7			Analysis of variance (ANOVA)	
	N	Degrees of Freedom	F	P
Intercept	1	1	291.9163	<0.0001
Score	8	7	46.5262	<0.0001
Total	74	66		

PSA			Analysis of variance (ANOVA)	
	N	Degrees of Freedom	F	P
Intercept	1	1	2506.373	<0.0001
Score	8	7	8.416	<0.0001
Total	74	66		

Table 4.4 Shows the bonferroni test on the IHC results for antibodies to MCM2, MCM5, MCM7 and PSA, all conditions that are homogenous for these antibodies are shown by ****.

Score**	Homogenous Groups*										
	MCM2			MCM5		MCM7				PSA	
	1	2	3	1	2	1	2	3	4	1	2
Benign	****			****		****				****	****
4	****	****	****	****	****	****	****	****		****	
5	****	****			****		****			****	
6	****	****			****		****			****	
7		****	****		****		****			****	
8	****	****			****		****			****	****
9			****		****			****	****	****	
10			****		****				****		****

Bonferroni test

* = Indicates Homogenous groups, variables MCM2, MCM5, MCM7 and PSA

** Score: - BPH (Benign Prostatic Hyperplasia): G-4 (Gleason Score 4): G-5 (Gleason Score 5): G-6 (Gleason Score 6): G-7 (Gleason Score 7) : G-8 (Gleason Score 8): G-9 (Gleason Score 9): G-10 (Gleason Score 10)

The ANOVA revealed that MCM2 (p-value=.0000), MCM5 (p-value=.0000), MCM7 (p-value=.0000) and PSA (p-value =.0000) all have significant differences in their immunoreactivity between BPH and Gleason score 4-10, with the bonferroni test distinguishing which conditions are significantly different.

The bonferroni results revealed that for MCM2 (*Figure 4.9 Table 4.4*) there is no significant difference in protein level between BPH and Gleason score 4-8 but a significant difference can be seen between BPH and Gleason score 9 and 10. For MCM5 (*Figure 4.10 Table 4.4*) no significant difference is demonstrated between BPH and Gleason score 4, but a significant difference is shown when Gleason scores 5-10 are compared to BPH. For MCM7 (*Figure 4.11 Table 4.4*), no significant difference was demonstrated between BPH and Gleason score 4, but significance difference occurs between Gleason score 5-8 and 9-10 compared to BPH. In each case, the MCMs show an increase in epithelial nuclei staining as Gleason score increases. Finally, the statistical results reveal that for PSA (*Figure 4.12 Table 4.4*) there is no significant difference in level between BPH and Gleason score 4-9, but a difference is demonstrated between BPH and Gleason score 10, with a drop in staining of PSA at high Gleason score compared to BPH.

4.4 Discussion

The results do not show a conclusive relationship between the staining ability of the prostatic tissue to PSA, with similar staining occurring for BPH to moderate Gleason score (*Table 4.2 and Figure 4.1-4.5*). However, there does appear to be a slight reduction in PSA staining at high Gleason score. This reduction could be due to the tissue becoming poorly differentiated, and therefore unable to produce PSA (*Figure 4.5*). These results emphasise that PSA can not histologically distinguish differences between prostatic conditions. This study therefore agrees with that of Goldstein and Messing (1998) which was unable to detect a large percentage of autopsy cancers via PSA staining, as well as Carter and Isaacs (2004) who also suggest that PSA is not an ideal marker for aggressive cancer, since high-grade cancers actually produce less PSA, than low-grade cancer.

Currently, elevated serum PSA is associated with prostate adenocarcinoma and PSA staining is believed to reflect cancer volume rather than cancer aggressiveness. The staining results from this study do not seem to support this, as less staining is seen in high-grade cancer, compared to low/intermediate-grade cancer and BPH, where the volume of cancer in these cases has clearly increased. These results therefore contradict Kuriyama, *et al* (1981) who suggested that PSA IHC could identify cells of prostatic origin, particularly at metastatic sites. Since the tissue results do not show increasing immunoreactivity with prostate neoplasia, and even show reduced staining as Gleason score increases, it is difficult to perceive how serum PSA can be used to distinguish prostatic adenocarcinomas from BPH. These findings therefore contradict a number of clinical studies, such as Brawer and Lange (1986), which found a correlation between serum PSA level and tumour volume/stage, as well as finding variations in PSA level in localised, as well as advanced disease

Staining for PSA also appeared less uniform compared to MCM staining, as the stromal layer, as well as the epithelial cells and the basal cells show staining. This protein therefore seems to be less specific than the MCMs in terms of not being able to discriminate proliferative areas from non-proliferative areas.

Analysis of the MCM results from this study suggest (*Table 4.2 Figure 4.1-4.5*) that as the prostatic condition changes from BPH to neoplastic, there is an increase in MCM2, 5 and 7 levels in epithelial nuclei. These findings correlate with a number of other studies, such as Freeman, *et al* (1999) who found an increase in the percentage of staining for MCM2 and 5 in neoplastic conditions of cervix, skin, larynx, oesophagus, lung, bladder, colon and prostate, with Davidson, *et al* (2003) who found MCM2 and 5 increases in expression in vulval intraepithelial neoplasia, and with Scott, *et al* (2003) who found MCM2 expression increases in colorectal cancer.

This study also shows that staining of BPH for MCM2, 5 and 7 is mainly confined to the basal layer of the gland, with <4% of the epithelial nuclei staining (*Figure 4.1*). These findings were expected, as Bonk, *et al* (1994) stated that the basal layer is the actively proliferating compartment of the prostate gland in normal tissue, while Carroll,

et al (1993) stated that only 1-4% of the normal epithelial cells are cells in cycle (S-phase). Therefore, since BPH is an encapsulated non-cancerous growth which looks like the normal cellular area, but has increased proliferation (King, 2000), it would be plausible to assume that the well-differentiated cells would not express many MCM proteins, so a staining level similar to that of normal epithelium is expected. Also expected is the appearance of low MCM2, 5 and 7 staining in the stromal elements, this is because the stromal elements have low proliferative activity (Berges, *et al* 1995).

The statistical results (*Figure 4.9-4.12 Table 4.3-4.4*) revealed that a significant difference occurs between neoplastic tissue and BPH, for MCM2, 5 and 7. These results reveal that MCM2 immunoreactivity cannot distinguish low/intermediate-grade prostatic cancer from BPH, but can distinguish high-grade prostatic cancer from BPH, as a significant difference is recorded (Tissue changes from a 0-/± BPH to a 1+ for Gleason Score 9/10). MCM5 can distinguish intermediate/high-grade neoplastic conditions from non-neoplastic conditions, as a significant difference is recorded, and that MCM7 can distinguish intermediate/high grade neoplastic conditions to non-neoplastic conditions, since as the Gleason Score increases, so does the immunoreactivity to MCM7. Although low-grade prostatic cancer (Gleason Score 4) is not significantly different to BPH for MCM5 and 7, it can be postulated that this is due to sample size (Gleason score 4, n=1), and it may be possible to observe a difference between BPH and low-grade neoplasia, if sample size is increased. Also, a significant difference is seen for PSA, but instead of an increase in staining ability with adenocarcinoma, a reduction occurs, with Gleason score 10 being significantly lower in expression, than lower-scores and BPH.

These results indicate that PSA has no significant difference in staining ability between non-neoplastic and prostate adenocarcinoma, and is unable to detect differences between malignant and non-malignant tissue. Also MCM2 may not be a reliable indicator of cellular proliferation for the prostate gland, as the staining intensity is not sufficient to distinguish low/intermediate-grade malignancy from BPH and even at high Gleason score the staining ability never exceeds 2+ (26-57%). This data does however

correlate with Meng, *et al* (2001) that found for adenocarcinoma staining of MCM2 ranges between 1-41%.

Other studies have suggested MCM2 provides information on the prognosis and treatment of cancer. For instance, when IHC was performed for MCM2 in transitional cell carcinoma of the bladder, it was shown to bear some prognostic relevance (Kruger, *et al* 2003). It has also been shown by IHC that MCM2 may be a predictor of disease free-survival for non-small cell lung cancer, as tumours which showed less than 25% MCM2 staining, had a significantly longer survival rate than patients with staining greater than 25% (Ramnath, *et al* 2001). Prognostic relevance of MCM2 by IHC has been assessed for kidney and renal cell carcinoma (Rodins, *et al* 2002), oesophageal squamous cell carcinoma (Kayto, *et al* 2003) and for prostate cancer (Meng, *et al* 2001) with all of these studies indicating that a reduced expression of MCM2 indicates a longer disease free survival rate. It is therefore plausible that MCM2 expression may be correlated to the disease free survival rate of the tissue and not the frequency of malignancy, therefore MCM2 may provide valuable prognostic information to clinicians.

MCM5 and 7 appear to be more reliable indicators of malignant disease, as they show increased epithelial nuclei staining with proliferative state. These findings are in agreement with Freeman, *et al* (1999), who found that prostate adenocarcinoma expresses 40% more nuclear staining for MCM5 than BPH. Also Padmanabhan, *et al* (2004) found that in prostate cancer a higher proportion of cells were positive for MCM7, as the proliferative index (PI) of the tissue increased (Normal tissue has the lowest PI; cancerous tissue has the highest PI).

The results from this study are in agreement with previous studies, as MCM7 is the best marker for malignant adenocarcinoma, as MCM2 is unable to distinguish low-grade cancer, MCM5 produced high background staining and is unable to discriminate between Gleason scores, and PSA is not able to discriminate between BPH and neoplasia. In contrast, MCM7 produced obvious differences between BPH to Gleason Scores, a difference in staining between intermediate and high grades (Gleason Scores

5-8 to Gleason Scores 9-10), high specificity and sensitivity and the staining is fixed to the nuclei of the cells, which made it easier to assess.

It is important to also consider the limitations of these markers. Firstly, it is essential to realise that although the MCMs are indicators of replication, they are not prostate specific markers and are shown to be up-regulated in all types of malignancies. Although this should not cause any problems when these markers are used in a histological sense as a pathologist is able to distinguish tissue types, this may become problematic when considering the design and implementation of a non invasive diagnostic assay. For example, results obtained from voided urine or blood will not only contain proliferating cells which are shed from the prostate, but other proliferating cells such as urothelial cells (from voided urine). Nonetheless they may provide a non-invasive indication of neoplasia but this warrants further investigation.

The semi-quantitative nature of IHC analysis and the assessment scheme used to correlate staining ability also have limitations. Firstly, it is important to realise that false positive and false negative results can be obtained when performing this staining technique, although to ensure that errors do not occur appropriate negative and positive controls should be in place. These hopefully provide some kind of reference and allow for comparison between experiments. For instance if the intensity in one run on a positive control is higher than that of another run, then it is plausible to assume that either the intensity on the experimental slides should also be greater, or that the staining on the positive control is due to some sort of over-expression and therefore the experimental slides for this run should be lighter when comparing to other runs. Taylor, *et al* (2006) states that biological stains are difficult to control in terms of intensity of colour from cell to cell and more so from section to section, therefore for this study the intensity of the slides were not assessed as too many variables are present.

In the assessment scheme itself, the histopathologist allocates a score 'by eye' ranging between 0- to 3+. The score allocated is therefore very subjective and may vary depending on the assessor. Unfortunately it is very difficult to overcome this problem, therefore it is essential that a well trained histopathologist allocates the score for all the

slides to try and limit the possible errors. Alternatively a spectral imaging microscope could be used to assess the staining ability and a software package applied, which could quantitatively assess the staining and therefore calculate intra-observer error removing this from the analysis.

This chapter concludes that MCM5 and 7 could offer potential as new diagnostic markers for prostate cancer. The ability of MCM2 as a diagnostic marker is still under question, as it is unable to discriminate low Gleason scores from non-malignant tissue. Other studies suggest MCM2 to be of more prognostic value, and combined analysis of all three MCMs may therefore be useful, as MCM5 and 7 could diagnose and grade the tissue, while MCM2 could aid in indicating the best course of treatment. This study indicates that MCM2, 5 and 7 could be used alongside H&E staining to aid in distinguishing the severity of disease and therefore the optimal treatment pathway. These results therefore correlate with Adam, *et al* (2001) who suggested that analysis of individual markers often provides disappointing results, and that there is a need for the development of multiple diagnostic/prognostic markers. This study therefore suggests that a multi-marker approach may be the most likely way by which MCMs may have a clinical application for improving prostate diagnosis.

Chapter 5 – Comparison of Prostate Specific Antigen (PSA) to Minichromosomal Maintenance Proteins (MCMs), for the Diagnosis of Prostate Cancer by Less Invasive Techniques using Clinical Samples.

5.1 Introduction

Due to numerous problems in the usefulness of serum PSA already highlighted in this thesis, there is need for potential new biomarkers for prostate cancer detection. The search for alternative biomarkers that can identify prostate cancer with a greater ability than PSA includes the investigation of telomerase, hypermethylation of the Gluthione S-transferase P1 gene (GSTP1), uPM3™ and the MCMs.

Telomerase is a ribonucleo-protein essential for cell survival by the maintenance and repair of the telomeres (Blackburn, 2001, Shay & Wright, 1996). This enzyme protects the ends of the chromosomes from shortening and is an essential component of cells. In normal cells, telomerase activity is detected at variable levels depending on the tissue type and proliferative state, as it is depleted as cells age (Autexier & Greider 1996, Gelmini, *et al* 2005). In contrast in cancer cells which are immortalised, telomerase activity is maintained therefore chromosome shorting does not occur (Gelmini, *et al* 2005). Telomerase activity can therefore be used as a positive biomarker for cancer and in prostate cancer patients telomerase activity has been detected in urine samples after prostatic massage, with 58% sensitivity and 100% specificity (Meid, Gygi, *et al* 2001). However, this level of sensitivity would mean that 42% of individuals with disease will test negative by this method, so additional tests would still be essential.

GSTP1 are a family of enzymes involved in DNA protection from electrophilic metabolites of carcinogens and reactive oxygen species by conjugating chemically reactive electrophiles to glutathione (Coles & Ketterer 1990). Silencing of the GSTP1 genes frequently occurs in prostate cancer, due to hypermethylation of the normal unmethylated

CpG island promoter region (Merlo, *et al* 2005). Studies have therefore shown, by quantitative real-time methylation-specific PCR, that increasing methylation of the GSTP1 gene occurs in prostate cancer patients and it was shown by urine sediment analysis that this test had 30% sensitivity and 97% specificity in identifying prostate cancer from BPH (Jeronimo, *et al* 2002).

UPM3™ is a urine test which relies on the fact that prostate cancer has increased expression of a non coding ribonucleic acid (RNA) differential display code 3 (DD3) (Freedland, *et al* 2005). Although the *in vivo* function of DD3 remains elusive it has been exploited, as low levels are detected in BPH while high levels are detected in prostate cancer. Fradit (2004) found that uPM3™ has a positive predictive value of 75% compared to 38% when total PSA (tPSA) was measured and a negative predictive value of 84% compared to 80% for tPSA, suggesting that the uPM3 urine test may be an important test for detecting early prostate cancer.

The aim of this chapter was to investigate the potential of MCMs as diagnostic markers for the early detection of prostate cancer, against the current clinical diagnostic test serum PSA. This was performed as the IHC results from Chapter 4 show that an increase in epithelial nuclei or nuclear expression of MCM2, 5 and 7 occur in adenocarcinoma. Therefore, the MCMs were assessed in urine and blood samples by a number of transcriptional and translational methods to assess if alterations in MCM expression can be detected by a less invasive route than biopsy and IHC. This might also solve some of the experimental limitations of IHC already discussed in Chapter 4. This type of study has in part already been performed as Stoeber, *et al* (1999, 2002) found that in urine samples a greater level of expression of MCM5 occurred in urothelial cancer patients compared to normal, by an immunoassay.

This part of the study was performed to investigate whether an expressional difference could be detected between neoplastic and non-neoplastic conditions through a less invasive route, to identify if MCM testing is more sensitive and specific than the current diagnostic

marker PSA, and to assess whether a less-invasive technique could be implemented in a hospital environment.

5.2 Materials and Methods

5.2.1 Clinical Sample Collection

Copies of the relevant information for ethical approval are shown in *Appendix E*. Patients identified as needing a TURP, TRUS or Radical Prostatectomy were invited to take part in the trial at Northampton General Hospital, during initial consultation. Then on arrival at Pre-assessment (TRUP/ Radical Prostatectomy) or on the day of their Operation (TRUS) they were asked if they agreed to participate. If in agreement, written consent was taken and a 5ml blood sample was obtained in a plain tube from the TURP and Radical Prostatectomy patients, while a fully voided urine sample was collected from the TRUS patients (there was at least 24 hours between initial invitation and consent).

On the day of the operation a fully voided urine sample was obtained from the consenting TURP and Radical Prostatectomy patients. All samples collected (urine, blood and tissue) from the same patient were allocated the same ID numbers for the trial and this number was logged in a Logbook with the patients details.

The blood samples were then stored in the Biochemistry Department at Northampton General Hospital (-20°C), until they were required for processing, while the urine samples were taken back to Cranfield University, Silsoe and processed within 48hours of collection.

Ethical approval for the healthy volunteers was also obtained from Northamptonshire Local Research/Ethics Committee and a standard e-mail was sent out to staff and students at Cranfield University at Silsoe (male and females, >18 years of age). If they agreed to participate, a reply e-mail was received and the volunteers where then supplied with a healthy volunteer's information sheet and allowed at least 24 hours to decide if they wished to participate. If in agreement, written consent was obtained, then a 5ml blood samples was collected in a plain tube as well as a fully voided urine sample. Again samples obtained

from the same volunteer were allocated correlating identical ID numbers and the volunteers were asked to tick which age range they fell into (the ID numbers were however not traceable back to the volunteer). The blood samples were then stored at -20°C, while the urine samples were processed.

5.2.2. Urine Sample Processing

The urine samples obtained from TRUS, TURP, Radical Prostatectomy patients as well as healthy volunteers were collected in sterile pots, which contained 1ml of concentrated protease inhibitor cocktail (*Appendix B formulation*), these were stored at 4°C until processing (the samples were processed within 48hours of collection).

The samples were then transferred to 50ml centrifuge tubes and spun at 800 xg for 15 minutes at 4°C. The supernatant was then discarded and the pellet was transferred to a fresh 15ml centrifuge tube and re-suspended in 10ml of PBS buffer/protease inhibitor cocktail (v/v). The samples were then re-spun and the supernatant discarded, if however the pellet contained a high abundance of cellular waste the wash steps were performed, until almost all the cellular waste was removed. Finally, the pellet was re-suspended in 2mls of PBS buffer and centrifuged between 800-1200 xg for 15 minutes at 4°C (speed dependent on size of urine pellet obtained). Once completed the supernatant was removed and pellets snap frozen and stored at -80°C until required.

The urine samples were then separated into two groups, where half were used for RNA extraction and the other half were cytopun and used for immunocytochemistry. For RNA extraction, please ref. to *Appendix A Section A.10.2*.

5.2.3 Urine Cytospin

Before the cell could be cytopun onto glass slides the slides were coated with a Poly- L- Lysine solution. This was done by immersing clean glass slides in a 0.01% Poly-L- Lysine (v/v) solution for five minutes and then allowing the slides to air-dry overnight.

The next day the slides were wrapped in foil and stored until used. The processed urine pellets were then re-suspended in 1ml of PBS and a cell count was performed. If >68,000 cells/ml were counted the pellet was re-suspended in an adequate amount of PBS to ensure a clear monolayer of cells would be obtained. It is important to note that the prostate cells were not separated from other urothelial cells when cytopun onto the glass slides, this means the monolayer produced contains a mixture of cells from different urothelial localities.

The optimal amount of cellular suspension was then placed in the cyto-chamber and the cytopspin was run at 800 xg for 30 minutes. Once complete the chamber was removed and excess solution gently blotted of. The slide was then allowed to air-dry, fixed by immersion in cold methanol for 10 minutes and left at 4°C for 1 hour. The slides were then stained by immunocytochemistry with antibodies to MCM2, MCM5, MCM7 and PSA and the working dilutions/procedure is shown in *Section 5.2.4*.

5.2.4 Immunocytochemistry

Immunohistochemistry was then performed on the cytopun slides as stated in *Appendix A Section A.4* and the antibodies used were Mouse Monoclonal antibodies to MCM2 Ab-1 Clone CRCT2.1, MCM5 Ab-1 Clone CRCT5.1, CDC47/MCM7 Ab-2 Clone 47CD141 or DCS141 and Rabbit Polyclonal antibodies to PSA RB-9065-P (Stratech, Cambridge UK). The dilution factors used were 1:50 for MCM2 and MCM7, 1:50 for PSA and 1:200 for MCM5. All these primary antibodies were incubated on the slides in a humidity chamber at 4°C overnight and negative controls were produced by removal of the primary antibodies (important to remember that the prostate cells were not separated from the urothelial cells).

A staining system was then used to assess the percentage of cells that stained and this is given in *Table 3.1*, and a statistical analysis was performed on the results as shown in *Section 5.2.6*.

5.2.5 Blood sample Processing

As the blood samples were collected in plain tubes and stored at -20°C, which is undesirable for the collection of RNA due to the blood containing high levels of RNases and polymerase inhibitors, which degrade and reduce the RNA quality. A number of different techniques were performed to try and obtain optimal yield. Firstly however, for all techniques the blood samples were boiled to thaw them and then transferred to a 15ml tube for processing, and the processing techniques tried are:-

- i) Tri-Reagent**
- ii) Red Cell lysis Buffer (Promega, UK ltd, Southampton, UK)**
- iii) RBC Lysis Solution (Gentra Systems, Mineapolis USA)**

At the end of all the RNA processes the concentration of the mRNA was then measured, by testing an aliquot of the total RNA solution and measuring the absorbance at 260nm (A_{260}).

i.1) Tri-Reagent - total RNA (tRNA)

To 5mls of blood an equal volume of Tri-Reagent was added and this was left for 5 minutes at RT to lyse the sample. Then 1ml of Chloroform (0.2ml/1ml of Tri-Reagent) was added to the mixture and this was gently mixed, by inverting the sample for 20 seconds. The sample was then left to stand for 15 minutes at RT, before being centrifuged at 12,000 xg at 4°C for 15 minutes.

After centrifugation the resulting lysate obtained was separated into three phases: the top a clear aqueous solution which contains the RNA, the middle a white precipitate containing the DNA (interphase), and at the bottom a brown/pink organic phase containing the protein.

The RNA layer (aqueous phase) was then removed by careful pipetting and then transferred to a fresh 15ml centrifuge tube, where 1/10 of the total volume of isopropanol (250µl) required (0.5ml/ml of Tri-Reagent used) for precipitation of the RNA was added and mixed by inversion. This was then left to stand for 5 minutes at RT before being centrifuged at 12,000 xg at 4°C for 10 minutes. The supernatant was transferred to a fresh 15ml centrifuge tube and the remaining isopropanol (2.25ml) required was added and left for 5 minutes at

RT, before centrifugation at 12,000 xg at 4°C for 10 minutes. After centrifugation a white precipitate appeared at the base of the tube (however in some extractions the white precipitate could not be seen) and the supernatant was removed. The white pellet was then washed in 5ml of 75% (v/v) ethanol (1ml/ml of Tri-Reagent used), vortexed and then centrifuged at 12,000 xg at 4°C for 5 minutes, removing any further impurities.

After centrifugation the supernatant was again discarded and the tube was placed in a fume cupboard to allow the RNA pellet to dry (however the pellet was not allowed to completely dry out). The pellet was then dissolved in 0.2ml of nuclease free water.

The sample was then purified further using the following methods: -

RNeasy® Mini Column (Section 5.2.5.i.2),

The Micro-Fast Track™2.0 Kit (Section 5.2.5.i.3 and Section A.10.1).

SV Total RNA isolation system (Promega UK Ltd, Southampton UK) (Section 5.2.5.ii.2)

i.2) Purification of RNA using RNeasy® Mini Column- tRNA

Purification of samples using the RNeasy® Mini Column varies slightly to the protocol given in Section 2.5.2. Firstly, for every 100µl of sample 350µl of RTL buffer was added and mixed thoroughly. Once mixed 250µl of 100% ethanol for every 100µl of sample was added and again mixed. This solution was then transferred 700µl at a time, to a RNeasy mini column and centrifuged at 8,000 xg for 15 seconds. This was repeated until all the solution was transferred. Once completed the RNeasy mini column was transferred to a new 2ml collection tube and 500µl of RPE buffer was added to the spin column, this was then centrifuged at 8,000 xg for 15 seconds. This step was then repeated but centrifuged at 8,000 xg for 2 minutes at RT.

For elution, a 1.5ml-collection tube replaced the 2ml-collection tube and 30µl of nuclease free water was added to the RNeasy mini column. This was then centrifuged at 8,000 xg for 1 minute at RT and the flow through was collected (this contained the RNA). Once the

sample was purified *Section 2.5.3* DNase treatment was performed and the concentration was calculated by measuring the absorbance at $A_{260\text{nm}}$.

i.3) Isolation of mRNA using the Micro-Fast Track™2.0 Kit- Messenger RNA (mRNA)

Isolation of mRNA from the total RNA obtained from Tri-Reagent (*Section 5.2.5.i.1*) involves the following steps. Instead of re-suspending the pellet in nuclease free water at the end of the Tri-Reagent protocol, the pellet was re-suspended in 10 μ l of Elution buffer. To this 1ml of the Micro-FastTrack™ 2.0 Binding buffer was added and heated to 65°C for 5 minutes and then placed immediately on ice for exactly 1 minute. Once complete the normal procedure shown in *Appendix A Section A.9.1* was followed, however the protocol starts at transfer of the mixture to the oligo(dT) cellulose vial.

ii.1) Red Cell lysis Buffer (Promega UK ltd, Southampton, UK) and SV Total RNA Isolation System (Promega UK ltd, Southampton, UK) – tRNA.

Once the samples were thawed the (5ml) blood was centrifuged at 600 xg for 5 minutes at RT and the supernatant removed. To the supernatant an equal volume of red blood cell lysis solution was added (5ml) (Promega UK ltd, Southampton, UK) and mixed in thoroughly, this was repeated. The sample was then centrifuged for 5 minutes at 600 xg and all but 1/10 of the supernatant was discarded (500 μ l left).

The sample was then lysed by adding 700 μ l of SV RNA lysis buffer and again thoroughly mixed. To this 1400 μ l SV dilution buffer was added and mixed in by inverting a number of times. The sample was then held at 70°C for 3 minutes, after which the sample was centrifuged at 10,000 xg for 10 minutes at RT. This lysate was then transferred to a sterile tube for purification.

ii.2) SV Total RNA Isolation System

The solution collected either from the *Section 5.2.5.i.1* or *Section 5.2.5.ii.1* was then purified using the following procedure. Firstly 800 μ l of 95% ethanol was added to the sample and this was gently mixed by pipetting, once thoroughly mixed this was transferred

to a spin column assembly and centrifuged at 10,000 xg for 1 minute (continue until all solution transferred). Once all the solution was transferred, 600µl of SV RNA wash solution was added to the spin basket and again centrifuging was performed at 10,000 xg for 1 minute at RT. The flow was then discarded and a master mix containing 40µl Yellow core buffer, 5µl 0.09M NaCl₂ and 5µl DNase 1 enzyme made up.

This master mix was added to the spin column (ensuring the membrane is covered) and incubated for 15 minutes at RT. Once complete 200µl SV DNase Stop Solution was added and the sample was centrifuged at 10,000 xg for 1 minute. Then 600µl SV RNA wash solution was added and again centrifuged at 10,000 xg for 1 minute with the flow discarded. This was repeated with 250µl SV RNA wash solution after which the spin column was transferred to a new 1.5ml-elution tube. Then 100µl of nuclease free water was added, and the flow through was kept as this contained the purified RNA.

iii) RBC Lysis Solution (Gentra Systems) and RNA Isolation Kit Purescript® (Gentra Systems, Minneapolis USA) - tRNA

This isolation kit is divided into a number of stages these are

iii.1 Cell Lysis

iii.2 Protein-DNA Precipitation

iii.3 RNA Precipitation

iii.4 RNA Hydration

iii.1) Cell Lysis

Once the blood sample (5ml) was thawed it was transferred to a 15ml centrifuge tube and three times the amount of RBC Lysis (15ml) solution was added (Gentra Systems). This mixture was then inverted and left to stand at RT for 10 minutes, after which it was inverted again. Once complete the mixture was then centrifuged for 20 seconds at 15,000 xg and the supernatant removed, apart from approximately 200µl of residual liquid. The tube was then vortexed to re-suspend the pellet and the same amount of Cell lysis solution as blood (5ml) was used to re-suspend the pellet, this was done by pipetting up and down three times.

iii.2) Protein-DNA Precipitation

The Protein-DNA precipitation Solution (1.6ml) at a volume 1/3 less than the total blood volume was added, to remove protein-DNA. This mixture was inverted gently and then placed on ice for 10 minutes after which the sample was centrifuged at 15,000 xg for 3 minutes.

iii.3) RNA Precipitation

After protein-DNA precipitation, RNA precipitation was performed. The supernatant was removed and 100% Isopropanol was added at an equal volume to the blood volume. This solution was then mixed gently fifty times and centrifuged at 15,000 xg for 5 minutes, after which a small white pellet should be visible. The supernatant was then poured away and 70% ethanol at an equal volume to the blood sample was applied. The tube was then inverted several times to wash the pellet and again centrifuged at 15,000 xg for 2 minute. The ethanol was then drained off and the pellet was allowed to air dry for 15 minutes.

iii.4) RNA Hydration

The sample was then hydrated by adding 100µl RNA hydration solution and was left on ice to re-hydrate for 30 minutes. The sample was then vortexed vigorously for 5 seconds and carefully transferred to a fresh tube, which was then stored at -80°C until required.

5.2.6 Statistical Analysis.

An analysis of variance (ANOVA) was then performed on the urine pellet immunocytochemistry results, the PSA serum reading, and the RT-PCR results from the urine samples. To identify if a significant different in MCM2, 5 and 7 expression occurs between the different prostatic conditions, and to identify if PSA serum testing can significantly identify difference between neoplasia and non-neoplastic conditions. A Bonferroni test was also performed for MCM2, MCM5, MCM7 and PSA to assess the homogeneity of the different prostate conditions. Therefore a *P-value* of <0.05 indicates that the results are considered to be significant. Although sequencing was not performed

on the PCR products produced by these experiments, Simone Degan, (2004) did perform sequencing on his samples when designing the MCM2, MCM5 and MCM7 primers.

5.3 Results

5.3.1 Current Diagnostic Test: PSA for the Detection of Prostate Cancer.

The least invasive technique currently used to detect prostatic adenocarcinoma is PSA, and this is measured via serum testing (total PSA measured). It is currently postulated that if a patient has a serum PSA level higher than that stated in the age standardised range in *Table 1.2*, then they are believed to have prostatic adenocarcinoma. To evaluate the efficiency of PSA, all patients that consented to take part in the trial had their serum PSA levels recorded (*Raw data Appendix F Table f.1*). As most of the clinical patients fell into age range five (Please ref. to *Appendix F Table f.2* for PSA range), which represents patients over the age of sixty, the PSA range 0–4.5ng/ml was used as the ‘normal’, to tabulate the data.

Table 5.1 and Figure 5.1 Summaries the number of BPH and cancerous consented patients that fall into one of the three PSA ranges. Range used :-0.0–4.5 ‘normal’, 4.6–9.9 ‘inconclusive’ and 10> ‘cancerous’.

Table 5.1. The serum PSA levels which BPH (Benign) and Cancerous (Cancer) consented patients fall into (Range used :-0.0 – 4.5 ‘normal’, 4.6 – 9.9 ‘inconclusive’ and 10> ‘cancerous’). The PSA levels are in ng/ml and sample number is n=70

PSA Level	Benign	Cancer
1) 0.0 - 4.5	25	11
2) 4.6 - 9.9	9	5
3) 10>	6	14

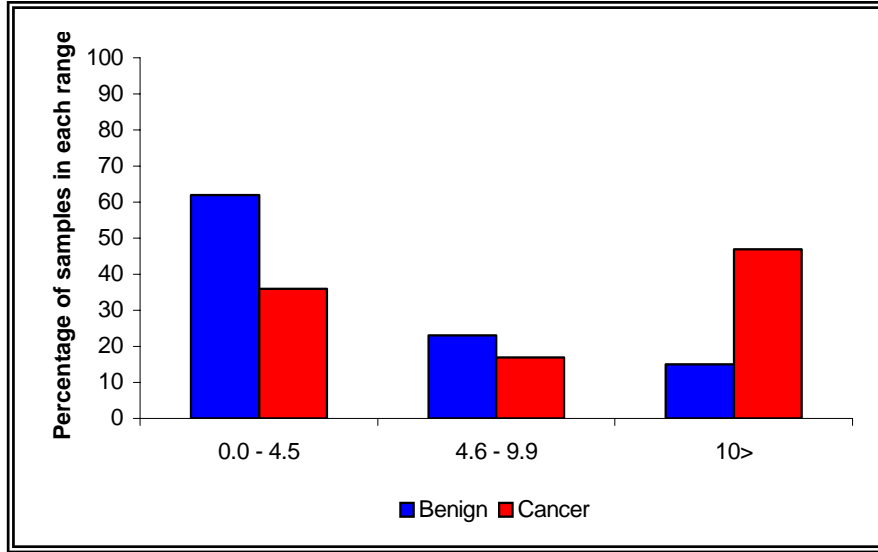


Figure 5.1. The percentage of BPH (Benign) and cancerous (cancer) patients consented for the trial, which fall into one of the three serum PSA levels. The sample number is n=40 for BPH patients and n=30 for cancerous patients. PSA readings are in ng/ml.

These results show that slightly more BPH patients (65%) fall in the ‘normal’ range of 0.0-4.5ng/ml compared to adenocarcinoma patients (37%), and that slightly more adenocarcinoma patients (47%) fall into the PSA range of >10ng/ml than BPH patients (15%). There is however a problem with the mid-range (4.6-9.9ng/ml), as slightly more BPH patients (23%) fall in this range than adenocarcinoma patients (17%). This consequently means that 35% (>4.5ng/ml) of BPH patients go through unnecessary biopsy, and that 37% (<4.5ng/ml) of adenocarcinoma patients could be potentially missed, if PSA testing alone is implemented. From these results it is clear that PSA serum testing only has 63% sensitivity and 60% specificity, in identify prostate cancer in this study (using the raw data *Appendix F Table f.1*).

A statistical analysis was then performed on the raw data (*Appendix F Table f.1*), to assess if a significant difference could be recorded between different prostatic conditions, by their serum PSA readings (ng/ml).

Figure 5.2, Table 5.2 and 5.3 Show the results for the statistical analysis on the patients' serum PSA readings (ng/ml), for different prostatic conditions.

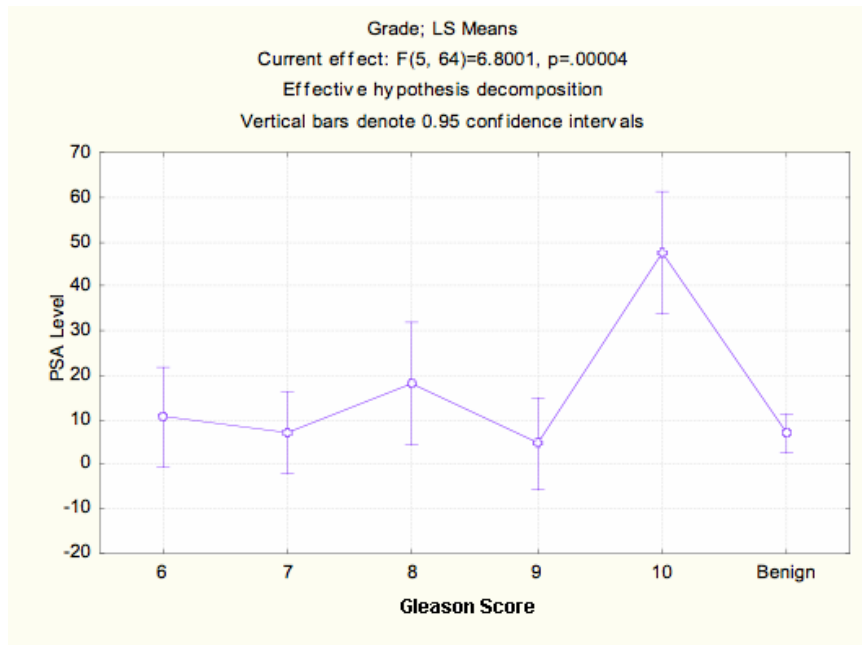


Figure 5.2. A Line graph of the mean serum PSA level (ng/ml) obtained for each Gleason Score (Score) and BPH (Benign), with an error bar showing their 95% confidence Limit. Sample number N=70.

Table 5.2. A One-way Anova for serum PSA readings (ng/ml) against prostatic score (BPH- Gleason Score 10). Raw data shown in Appendix F.1 and *P-value* <0.05 = significant difference is seen between the Scores and the Serum PSA reading. Univariate Tests of Significance for PSA Level.

<i>PSA</i>	N	Degrees of Freedom	Analysis of variance (ANOVA)	
			F	P
Intercept	1	1	50.000	<0.0001
Score	6	5	6.800	<0.0001
Total	70	64		

Table 5.3. Shows the Bonferroni test on the serum results, all the scores that are homogenous for serum PSA are shown by **** and as you can see there are 2 homogenous groups (1 and 2).

Bonferroni Test		
Score**	Homogenous Groups*	
	PSA	
	1	2
Benign	****	
6	****	
7	****	
8	****	****
9	****	
10		****

* = Indicates Homogenous groups, variable PSA
 ** Score: - Benign (Benign Prostatic Hyperplasia):
 6 (Gleason Score 6): 7 (Gleason Score 7) :
 8 (Gleason Score 8): 9 (Gleason Score 9):
 10 (Gleason Score 10)

The statistical analysis reveals that there is a significant difference ($p\text{-value} = 0.000038$) between the amount of PSA secreted into the serum by different prostatic conditions. With the Bonferroni Test (Table 5.3) showing, that Gleason score 10 is significantly different to BPH, and to Gleason score 9, 7 and 6, but not significantly different to Gleason score 8.

These results indicate that serum PSA testing is not efficient at distinguishing most neoplastic conditions from non-neoplastic conditions. As Gleason scores 6-9 are not significantly different in serum PSA readings from BPH, with only Gleason score 10 producing significantly more PSA.

5.3.2 New Diagnostic Techniques: Clinical Sample- Evaluation of Urine Pellets Immunocytochemistry

Urine samples collected for the trial were separated into two groups; half were processed for RT-PCR, while the other half were cytospun onto slides. ICC (*Section 5.2.4*) was then performed on the twenty seven cytospun slides for antibodies to MCM2, 5, 7 and PSA, to assess if the immunoreactivity differs between neoplastic and non-neoplastic conditions. *Table 5.4 and Figure 5.3*, shows the ICC results obtained for the cytospun urine pellets for antibodies to MCM2, 5, 7 and PSA, using the staining criterion shown in *Table 3.1*, while *Figure 5.4-5.12* demonstrates some of the staining seen visually. Raw data showing ICC

Table 5.4. The mean values obtained for Scores 6 - 10, BPH and healthy volunteers (*Raw data Appendix E Table e.4*), when urine pellets were cytospun and ICC was performed for antibodies to MCM2, 5, 7 and PSA. The scoring system is shown in *Table 3.1*. Sample Numbers: Scores 6-10 n=4 (2 patients for each score 2X slides), BPH n= 20 (10 BPH patients x 2 slides), Healthy Males n= 8 (4 healthy male volunteers x2 slides), and Healthy Females n= 6 (3 healthy female volunteers x 2 slides).

results, shown in *Appendix F Table f.4* and standard error of the mean values, shown in *Appendix F Table f.5*.

Gleason Score	MCM2	MCM5	MCM7	PSA
6	1.25	1.00	0.00	0.50
7	0.75	1.00	0.75	1.50
8	1.50	1.50	0.25	2.00
9	1.50	1.50	0.50	1.75
10	1.50	2.00	0.00	1.50
BPH	0.32	0.53	0.00	1.79
Healthy Male	0.50	1.00	0.00	1.38
Healthy Female	0.66	1.17	0.00	1.83

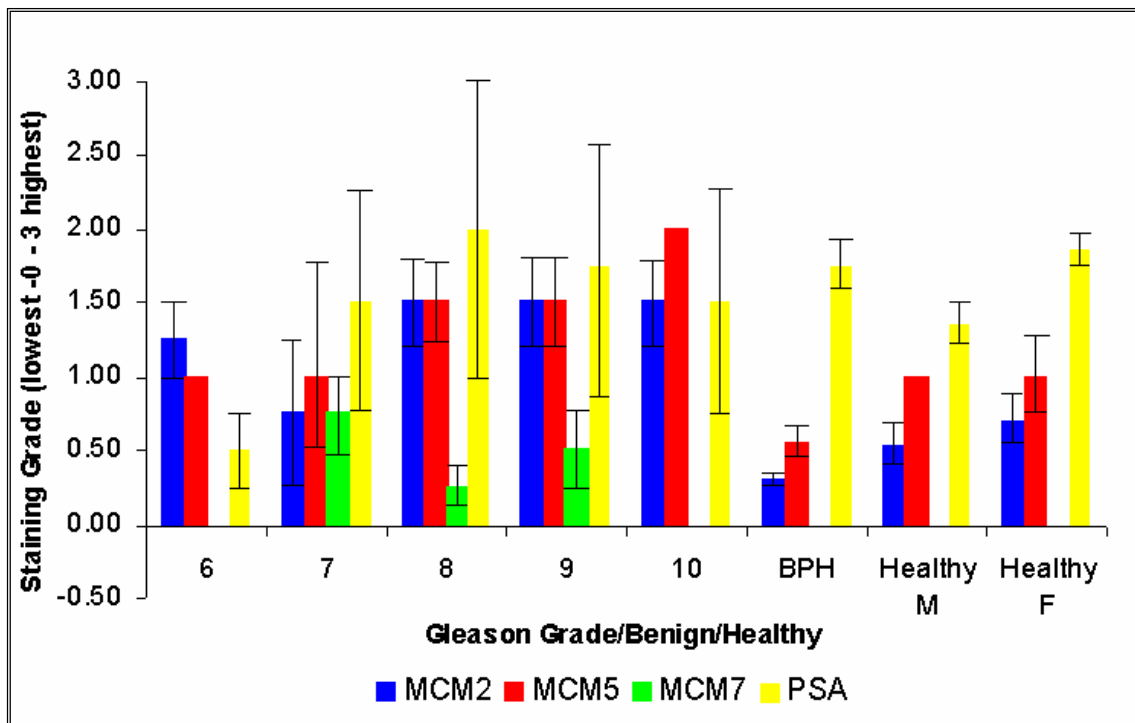


Figure 5.3. The mean staining ability given and standard error for each Gleason Scores 6-10, BPH and healthy volunteers, when urine pellets were cytospun and immunohistochemistry for antibodies to MCM2, MCM5, MCM7 and PSA were assessed. The scoring system is shown in *Table 3.1*. Gleason Scores 6-10 n=4 (2 patients for each score x 2 slides), BPH n= 20 (10 BPH patients x 2 slides), Healthy Males n= 8 (4 healthy male volunteers x2 slides), Healthy Females n= 6 (3 healthy female volunteers x 2 slides).

The results indicate that antibodies to MCM2 and 5 seem to show slightly more cellular staining for neoplastic conditions than non-neoplastic conditions and that the immunoreactivity seems to increase with Gleason score. For MCM7 however only Gleason score 7, 8 and 9 show immunoreactivity, with no immunoreactivity detected for BPH, healthy volunteers or Gleason score 6, 10. PSA however does not seem to show any increase in immunoreactivity between Gleason score, BPH and healthy volunteers.

It is important to realise that the error bars do overlap between groups, which may indicate that some Gleason grade are not different to each other or BPH, healthy volunteers. It is feasible however that the large error bars are due to low sample numbers and not due to

insignificant results. Therefore until the trial numbers are increased, it is hard to speculate if these findings are truly representative of what is expected.

A statistical analysis was then performed on the IHC raw data for the urine pellets, to assess if a significant difference occurs between neoplastic and non-neoplastic conditions for MCM2, 5, 7 and PSA. *Table 5.5* shows the Bonferroni test results for MCM2, 5, 7 and PSA.

Table 5.5. Statistical results for MCM2, 5, 7 and PSA, when a Bonferroni test was performed on the raw data for the number of cytopsun urine pellets stained. The table shows which scores are homogenous to each other and which are significantly different to each other. Homogenous groups shown by **.**

Bonferroni test									
Score**	MCM2		Homogenous Groups *				PSA		
	1	2	MCM5		MCM7				
	1	2	1	2	1	2	3	1	
Healthy	****	****	****	****	****			****	
Benign	****		****		****			****	
6	****	****	****	****	****	****		****	
7	****	****	****	****			****	****	
8		****		****	****	****	****	****	
9		****	****	****		****	****	****	
10		****		****	****	****		****	

* = Indicates Homogenous groups, variables MCM2, MCM5, MCM7 and PSA
 ** Score: - Healthy - Healthy Males/Females : Benign (Benign Prostatic Hyperplasia):
 6 (Gleason Score 6) : 7 (Gleason Score 7) : 8 (Gleason Score 8) : 9 (Gleason Score 9) :
 10 (Gleason Score 10)

The statistical analysis reveals that a significant difference is seen for MCM2 (p -value=0.000034), MCM5 (p -value=0.00023) and MCM7 (p -value=0.000002) but not for PSA (p -value=0.34). From the Bonferroni test (*Table 5.5*) a significant difference occurs between BPH to Gleason scores 8 - 10 for MCM2, between BPH to Gleason scores 8 and 10 for MCM5 and between BPH/healthy volunteers and Gleason scores 7 - 9 for MCM7.

These results therefore indicate that high-scores (Gleason Score 8-10), seems to be distinguishable using antibodies to MCM2 and 5, while intermediate/high-scores are distinguishable using antibodies to MCM7. It is also possible to postulate that intermediate-

scores may also be significantly different to BPH for MCM2 and 5 (mean staining ability *Figure 5.3*), but until sample numbers are increased it is impossible to determine. Finally PSA is not significantly different between BPH, healthy volunteers and cancerous conditions, as all the scores fall into one homogenous group.

There is also controversy with the staining results from the healthy volunteers, as they seem to show a higher immunoreactivity than expected. There are however two possible explanations for these findings and these are 1) the age of the healthy volunteers and 2) the time between processing. To therefore assess how processing time and storage affect cellular viability, please ref. to **page 137**.

Although the statistical analysis for this translation study does not show a defined difference between cancerous conditions to BPH and healthy volunteers, it does show promising results and it is important to realise that sample numbers are low and that this could affect the statistical outcome.

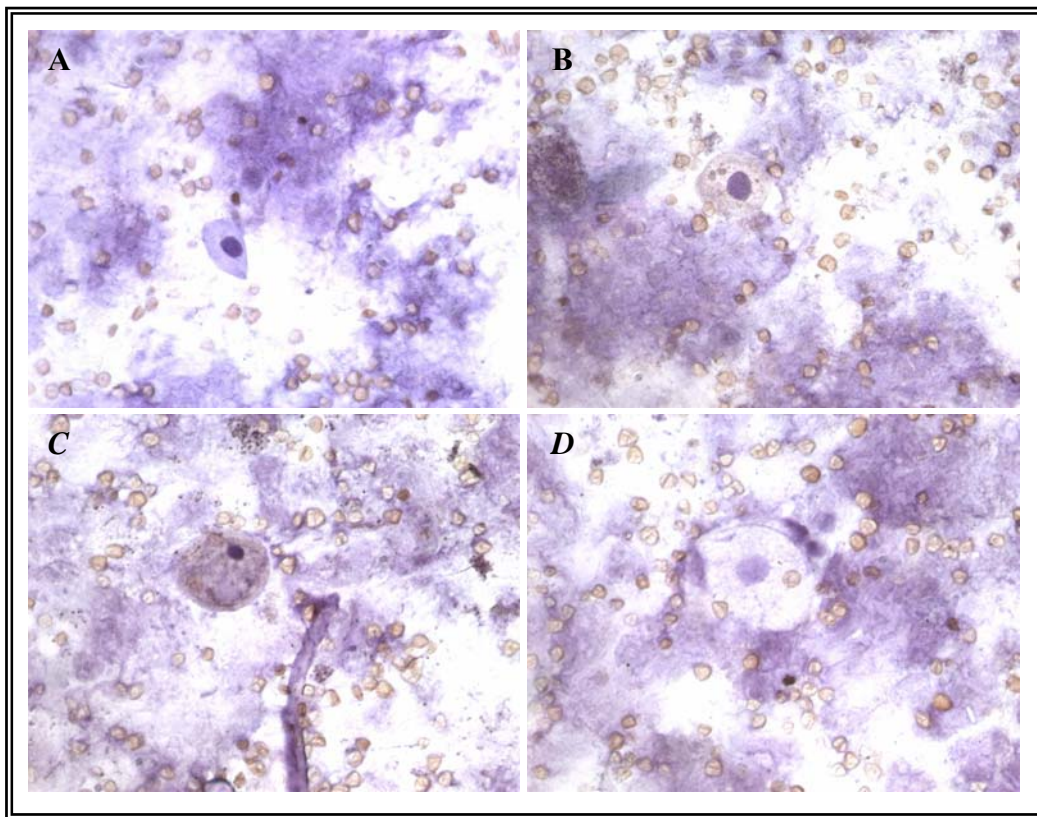


Figure 5.4. ICC results obtained for urine pellets of Gleason Score 10 cytospun onto slides and then stained using antibodies to B=MCM2, C= MCM5, D=MCM7 and A= Negative. Magnification x400. Brown precipitate indicates positive cells.

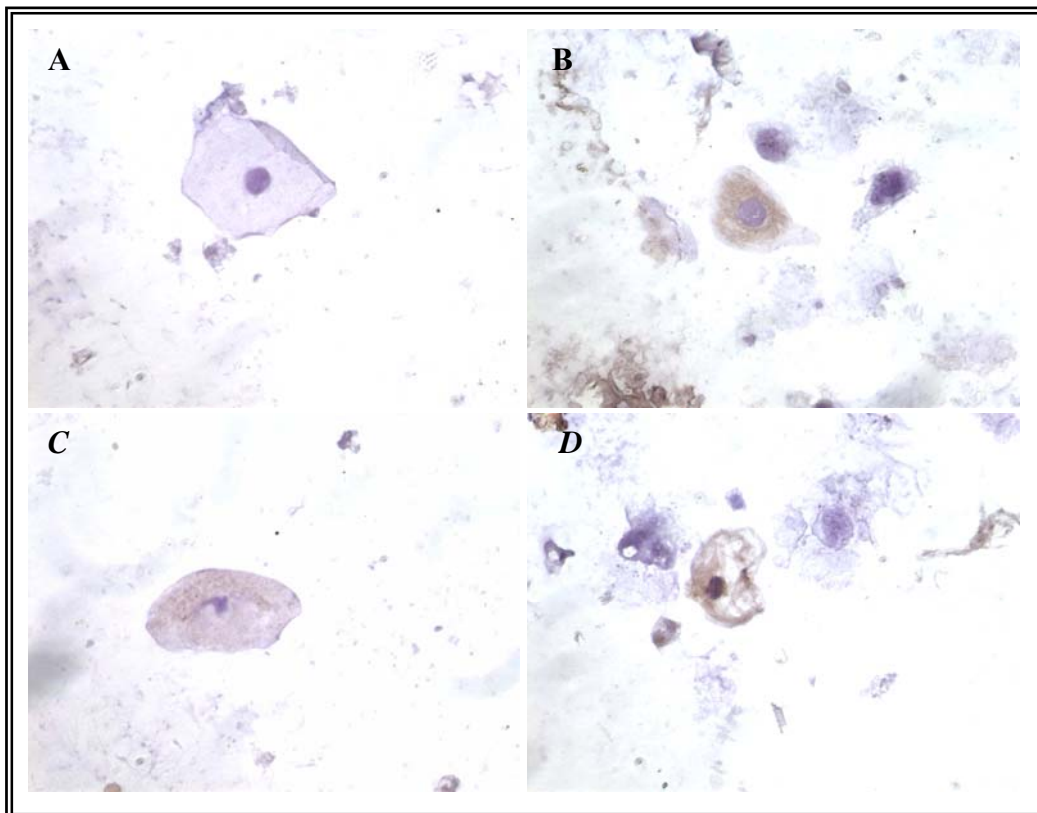


Figure 5.5. ICC results obtained for urine pellets of Gleason Score 9 cytospun onto slides and then stained using antibodies to B=MCM2, C= MCM5, D=MCM7 and A= Negative. Magnification x400. Brown precipitate indicates positive cells

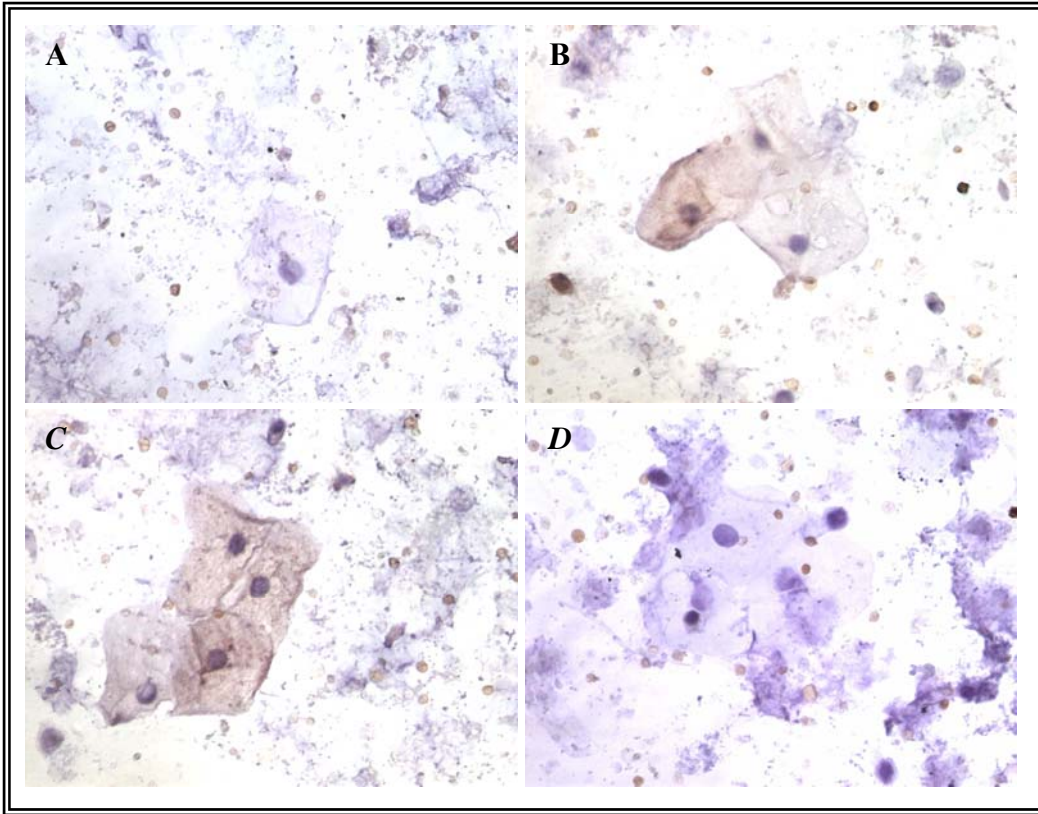


Figure 5.6. ICC results obtained for urine pellets of Gleason Score 8 cytospun onto slides and then stained using antibodies to B=MCM2, C= MCM5, D=MCM7 and A= Negative. Magnification x400. Brown precipitate indicates positive cells

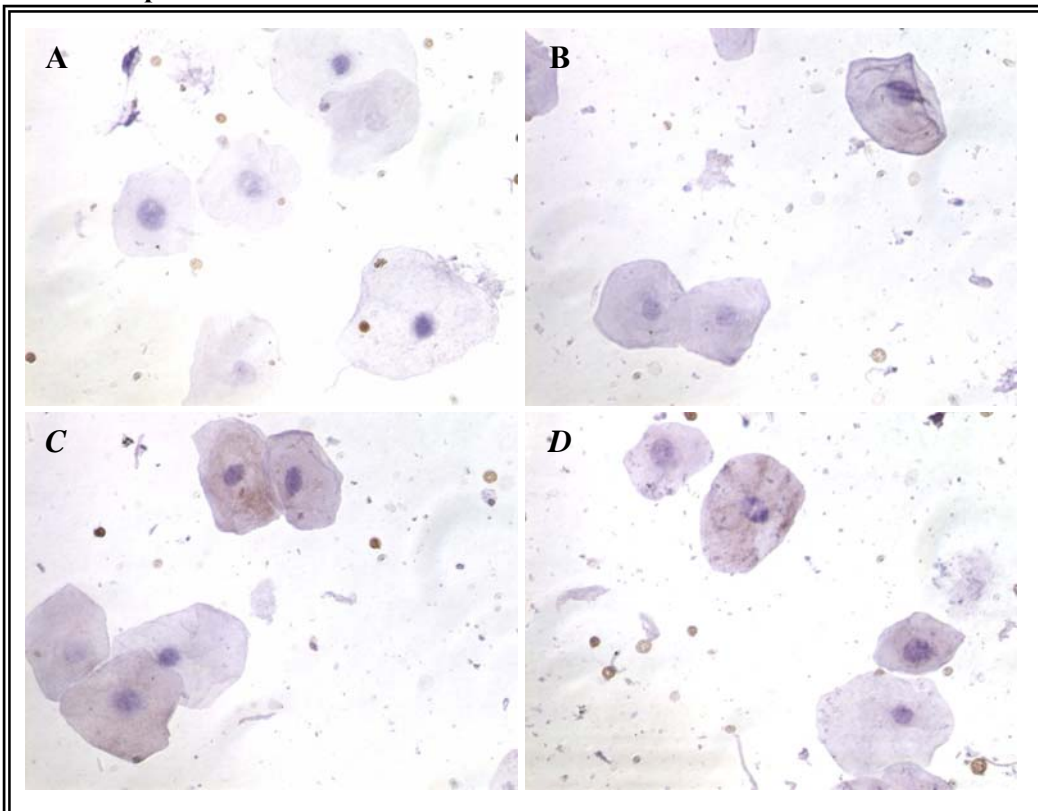


Figure 5.7. ICC results obtained for urine pellets of Gleason Score 7 cytospun onto slides and then stained using antibodies to B=MCM2, C= MCM5, D=MCM7 and A= Negative. Magnification x400. Brown precipitate indicates positive cells.

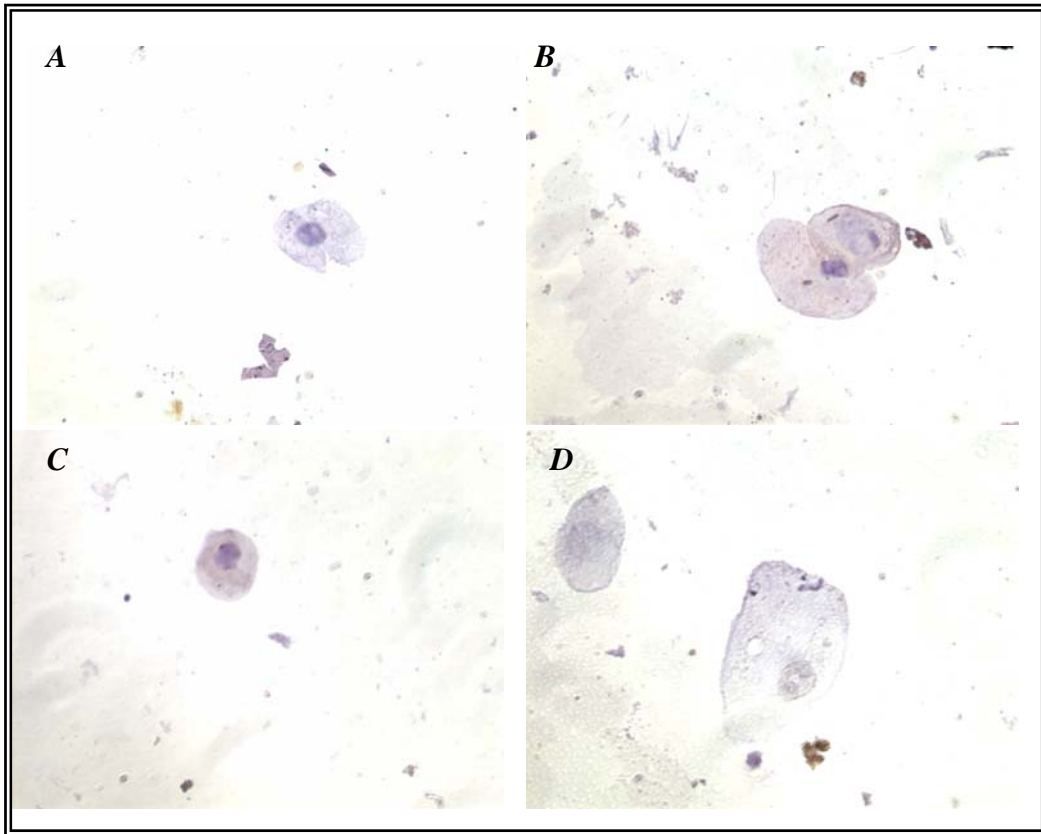


Figure 5.8. ICC results obtained for urine pellets of Gleason Score 6 cytospun onto slides and then stained using antibodies to B=MCM2, C= MCM5, D=MCM7 and A= Negative. Magnification x400. Brown precipitate indicates positive cells.

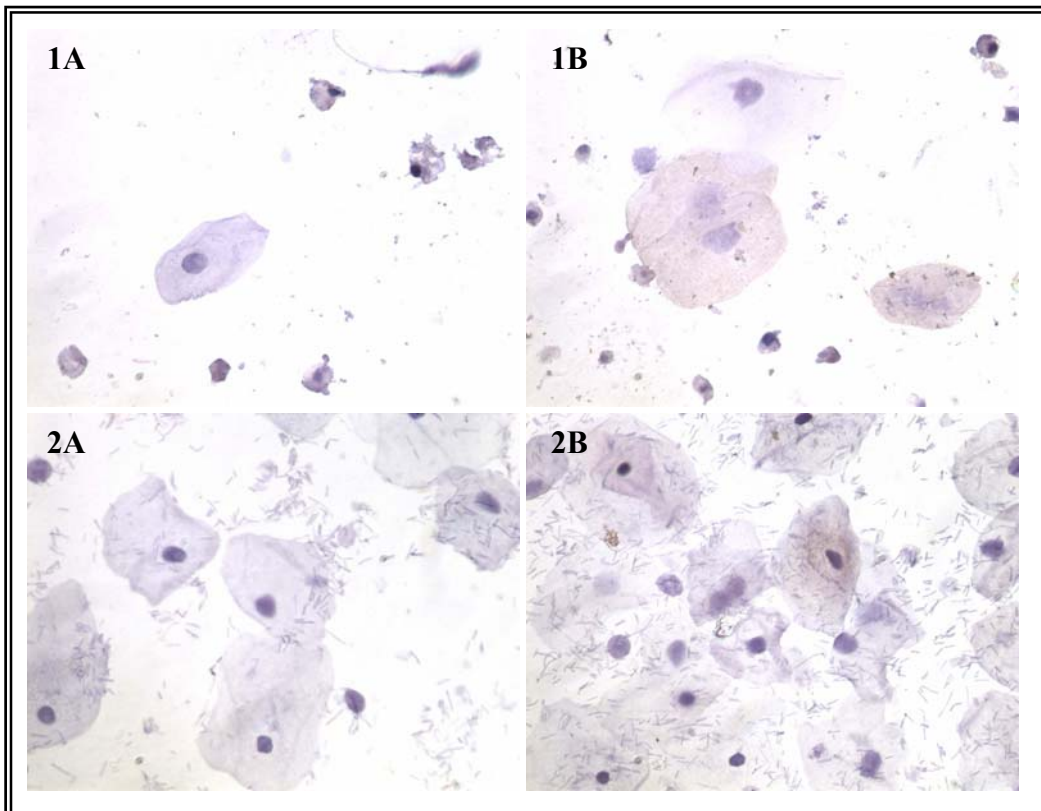


Figure 5.9. ICC results obtaining for urine pellets cytospun and stained using antibodies to Polyclonal PSA on pellets from BPH and healthy volunteers. 1 = BPH, 2 = Healthy Volunteer, A = Negative, B = Positive. Magnification x400. Brown precipitate indicates positive cells.

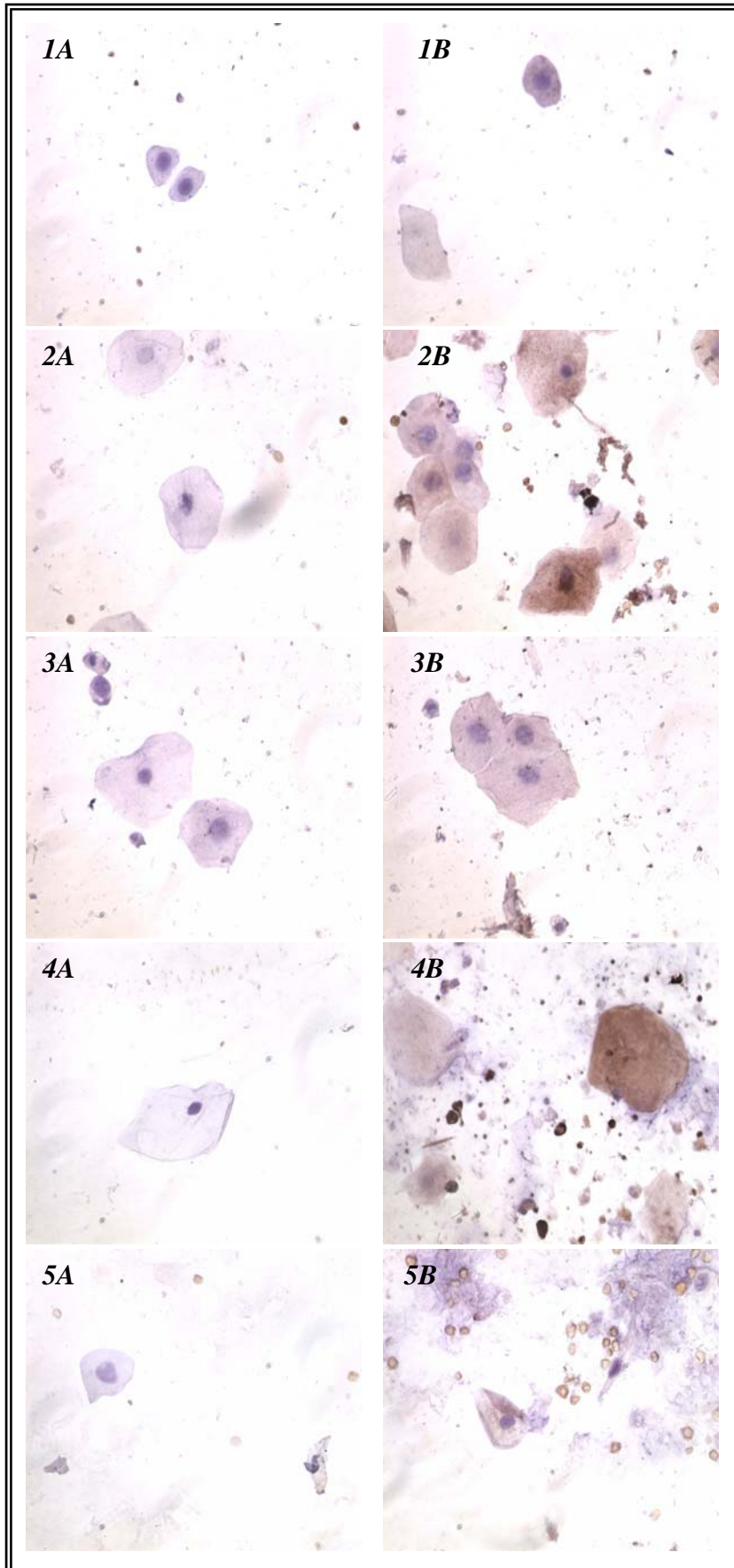


Figure 5.10. ICC results for urine pellets using Ab to Polyclonal PSA on urine pellets extracted from adenocarcinoma. 1 = Score 6, 2 = Score 7, 3 = Score 8, 4 = Score 9, 5 = Score 10, A = Negative, B = Positive. Magnification x400. Brown precipitate indicates positive cells.

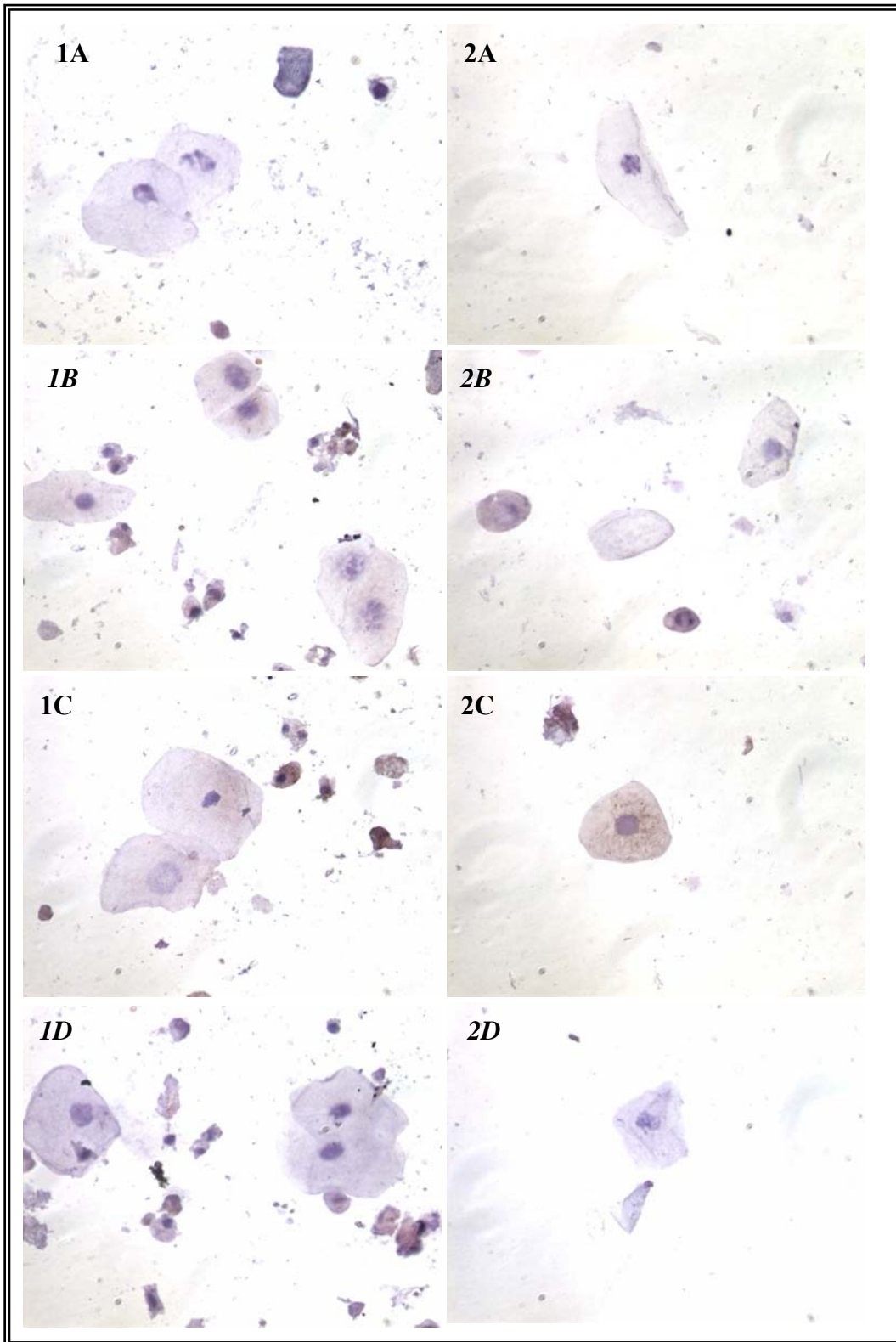


Figure 5.11. ICC results obtaining for antibodies to B=MCM2, C=MCM5 and D=MCM7 A= Negative on urine pellets extracted from 1 = BPH patient, 2 = BPH patient. Magnification x400. Brown precipitate indicates positive cells.

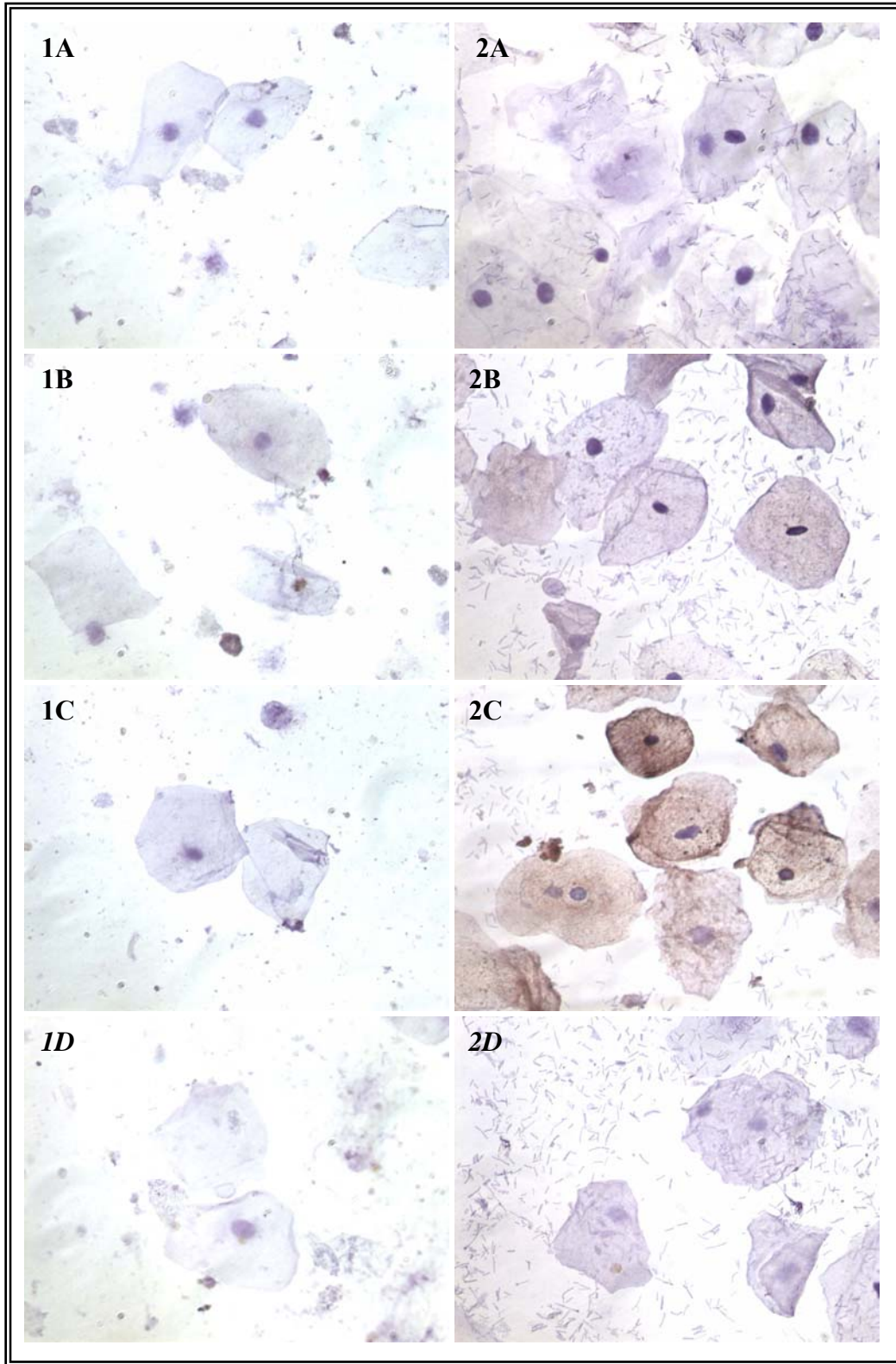


Figure 5.12. ICC results obtaining for antibodies to B=MCM2, C=MCM5 and D=MCM7 A= Negative on urine pellets extracted from 1 = Healthy volunteer patient, 2 = Healthy volunteer patient. Magnification x400. Brown precipitate indicates positive cells.

RT-PCR

RNA was then extracted from the remaining thirty five urine pellets, which were collected from cancerous, BPH and healthy volunteers (*Appendix A Section A.10.2*), the quantity of RNA was then calculated and RT-PCR was performed on a set amount, to assess the expression of MCM2, 5, 7 and β -Actin (*Ref Appendix F Table f.6 for RNA quantities*). Before RT-PCR was performed, optimisation for the urine pellets was carried out, to ensure that a cycle number is chosen, where exponential amplification of the expressional region is still occurring (*Appendix F Figure f.1-2*), the cycle number decided upon is 32 cycles.

The urine pellet concentrations were then standardised and RT-PCR was performed. No expression of β -Actin, MCM2 or 7 could be achieved for the urine pellets, but MCM5 expression was obtained and the relative brightness (rbu) results are shown in *Appendix F Table f.7* and *Figure 5.13*. *Figure 5.14* Shows the ethidium bromide gel for MCM5 and *Appendix F figure f.3* shows the RT-PCR gel pictures for the negative controls.

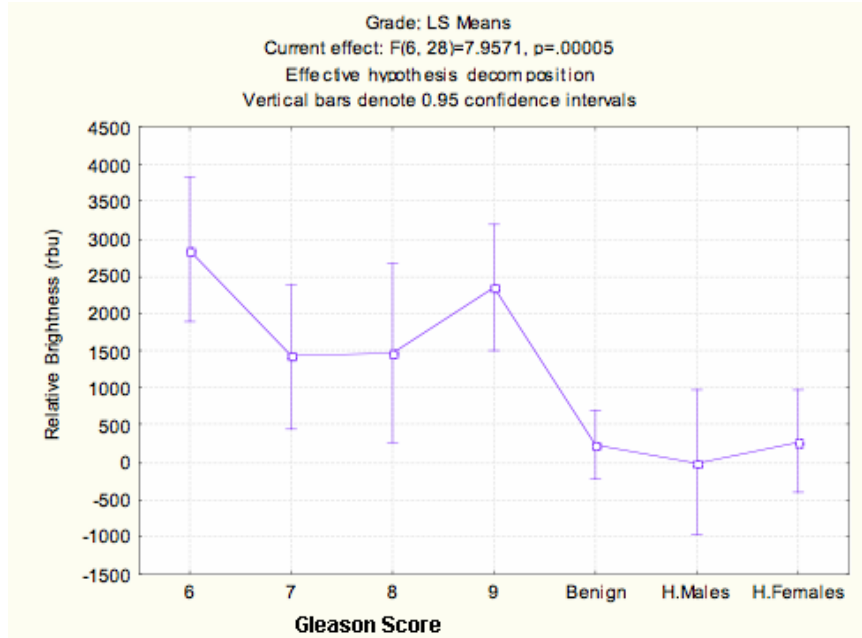


Figure 5.13. A line graph of the mean Relative brightness (rbu) obtained when RT-PCR is performed on MCM5 for 100 μ g/ μ l of RNA from cancerous, BPH and healthy urine pellets. The *p-value* = 0.00005 and the error bars show the 95% Confidence limit for each category. Samples 9 = Gleason Score 9 n=4, 8 = Gleason Score 8 n=2, 7 = Gleason Score 7 n=3, 6 = Gleason Score 6 n=6, Benign n=14, H Females= Healthy Females n=6, H Males= Healthy Males n=3.

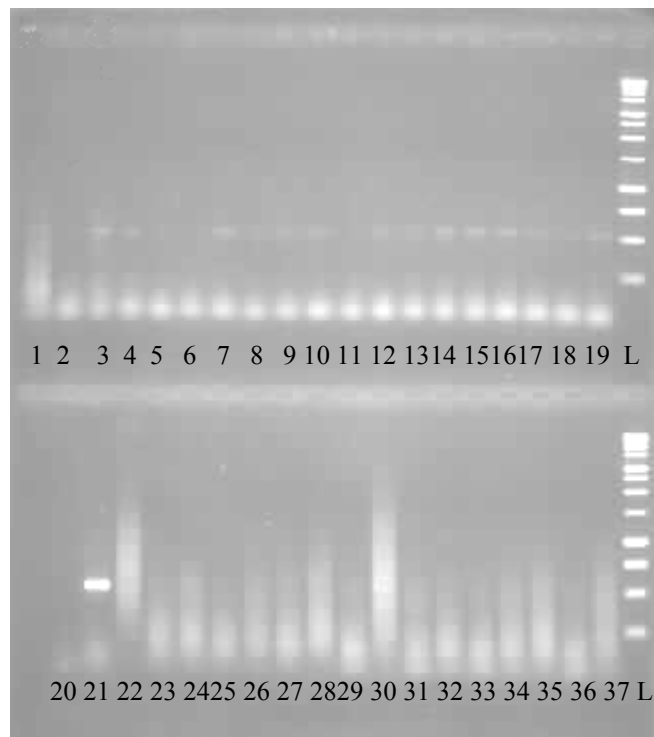


Figure 5.14. Ethidium bromide stained gel showing MCM5 RT-PCR (500bp bands visible) for RNA extracted from 35 urine pellets. L = Ladder 1 = p-129, 2 = H-013, 3 = H-015, 4 = p-004a, 5 = p-043, 6 = p-048, 7 = p-123, 8 = p-033, 9 = p-004, 10 = p-009a, 11 = p-023, 12 = p-013, 13 = p-051, 14 = p-011a, 15 = p005a, 16 = p007a, 17 = p-049, 18 = p-002a, 19 = p-005w, 20 = Negative, 21 = MCM5 extracted from 200µg/µl PNT1A 22 = H10, 23 = H008, 24 = p-034, 25 = H-014, 26 = H-009, 27 = p-008a, 28 = H-019, 29 = p-101, 30 = H-006, 31 = p-073, 32 = H-011, 33 = p-112, 34 = p-037, 35 = p-001a, 36 = p-012a 37 = p-031.

The mean rbu readings show, that an increase in MCM5 expression occurs in adenocarcinoma, there is however overlap of error bars, so it is feasible that certain Gleason scores may not be different from other scores or non-neoplastic conditions. A statistical analysis is then performed on the data and it shows (*Appendix F Table f.8-9*) that a significant difference ($p\text{-value}=0.000046$) in MCM5 expression does occur between neoplastic and non-neoplastic conditions. With the Bonferroni test (*Table f.9*) showing that Gleason scores 6 and 9 are significantly different to BPH, healthy males and healthy females, but are not significantly different to Gleason score 7 and 8. Also Gleason scores 7 and 8 are not significantly different to BPH, healthy males and healthy females. So although the rbu show increased expression of MCM5 for neoplastic conditions, the statistical analysis only proves that Gleason score 6 and 9 are significantly different to BPH and healthy volunteers.

These preliminary results for MCM5 therefore look promising, but until further analysis is performed, it is impossible to accurately determine if a significant difference is seen between adenocarcinoma and BPH.

Storage and Processing Times of Urine Pellets

There is a possibility, that storage time and temperature before processing may have affected the quality and quantity of the RNA and the urine pellet activity. Therefore the cellular number was assessed for a urine sample that is left at different storage times and temperatures *Appendix F Table f.10 and Figure 5.15.*

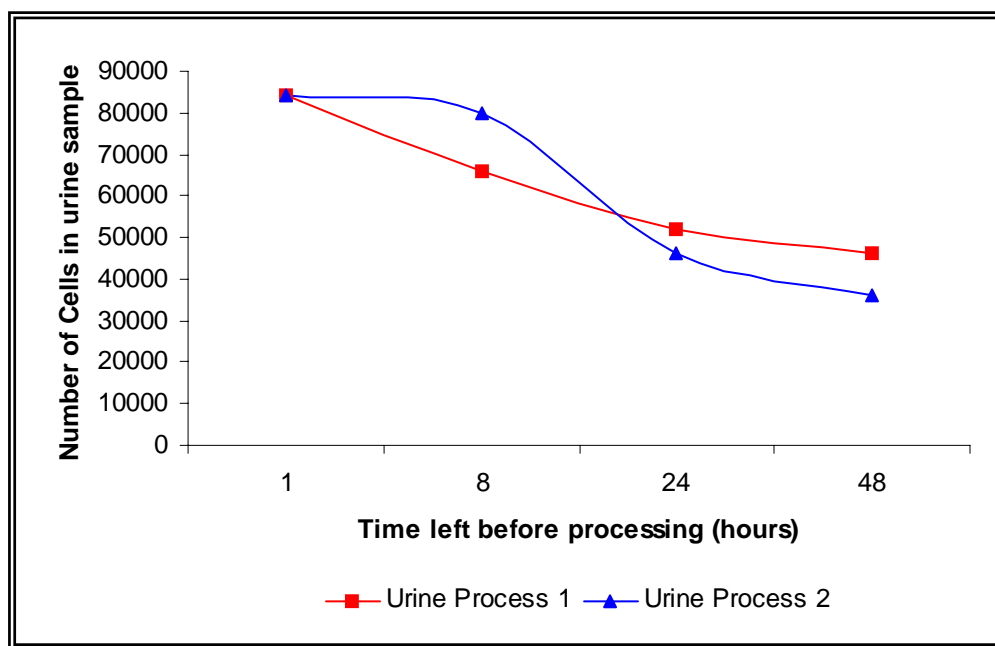


Figure 5.15. A line graph of the number of cells detected in 15mls of urine sample after two different processing techniques and four different processing times. Urine Process 1:- samples left at 4°C and then processed with 1, 8, 24 and 48 hours, Urine Process 2:- samples left at RT for 1hr/8hrs after which it was stored at 4°C until processing at 24 and 48 hours

These results show, that although temperature does not affect the cellular number, the time period between processing does, with a 50% drop recorded if the sample is not processed

until 48 hours after being voided. These results therefore enhance the importance of processing the urine samples as soon as possible.

5.3.3 New Diagnostic Techniques: Clinical Sample- Evaluation of Blood samples Optimal Technique for RNA Extraction from Frozen Blood

As the blood samples for this project were stored incorrectly for optimal isolation of RNA, a number of techniques were performed to try and find a method which would provide optimal yield. The procedures tried are shown in *Section 5.2.5*, and the results are shown in *Table 5.6*.

Table 5.6. Techniques used to try and optimise RNA yield and purity from frozen blood. Each technique was performed on two blood samples.

Technique Tested	RNA Yield and Purity	RNA Yield and Purity
1) Tri-Reagent not DNase Treatment (<i>Section 6.2.3.A.1 and 2.5.3</i>)	292µg/µl A260/280= 1.251	124µg/µl A260/280= 1.483
2) Tri-Reagent, Rneasy® Mini Column and DNase Treatment (<i>Section 6.2.3.A.1, 6.2.3.A.2 and 2.5.3</i>)	122µg/µl A260/280= 1.120	146µg/µl A260/280= 1.418
3) Tri-Reagent and SV total RNA Isolation system (<i>Section 6.2.3.C.1 and 6.2.3.C.2</i>)	20µg/µl, A260/A280= 1.398	20µg/µl A260/280= 1.061
4) Tri-Reagent and Micro-Fast Track™2.0 Kit (<i>Section 6.2.3A.1 and 6.2.3.A.3</i>)	196µg/µl A260/A280= 0.992	196µg/µl A260/280= 1.256
5) Red Cell lysis Buffer and SV Total RNA Isolation System (<i>Section 6.2.3.B</i>)	40µg/µl A260/280= 0.952	42µg/µl A260/280= 1.247
6) RBC Lysis Solution and RNA Isolation Kit Purescript® (<i>Section 6.2.3.C</i>)	56µg/µl A260/A280= 1.373	50µg/µl (sample DNase treated) A260/280= 1.106

After extraction, RT-PCR was performed using β -Actin primers to assess which technique gave optimal results. *Figure 5.16 and 5.17* Shows the ethidium bromide gels of the RT-PCR reactions for β -Actin for the different techniques. While *Table 5.7* shows the relative brightness readings (rbu) obtained, when RT-PCR is performed on β -Actin for the different techniques.

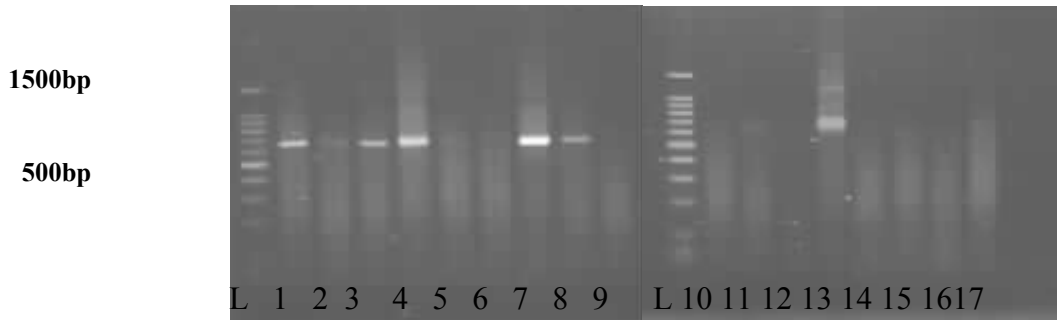


Figure 5.16. Ethidium bromide stained gel showing the RT-PCR results for β -Actin (680bp bands visible) on 100 μ g/ μ l of RNA extracted from frozen blood samples by different techniques. L= Ladder, 1= Tri-Reagent and SV total RNA Isolation system, 2= RBC Lysis Solution and RNA Isolation Kit Purescript®, 3= Tri-Reagent, RNeasy® Mini Column and DNase Treatment, 4=Tri-Reagent not DNase Treatment, 5= Tri-Reagent and Micro-Fast Track™2.0 Kit, 6= Red Cell lysis Buffer and SV Total RNA Isolation System, 7= 100 μ g/ μ l of RNA from PNT1A cell line Positive control, 8= 100 μ g/ μ l of RNA from PE/PJ41 cell line Positive Control, 9= Negative Control, 10= 1-RT, 11= 2-RT, 12= 3-RT, 13= 4-RT, 14= 5-RT, 16= 6-RT, 17= 7-RT.

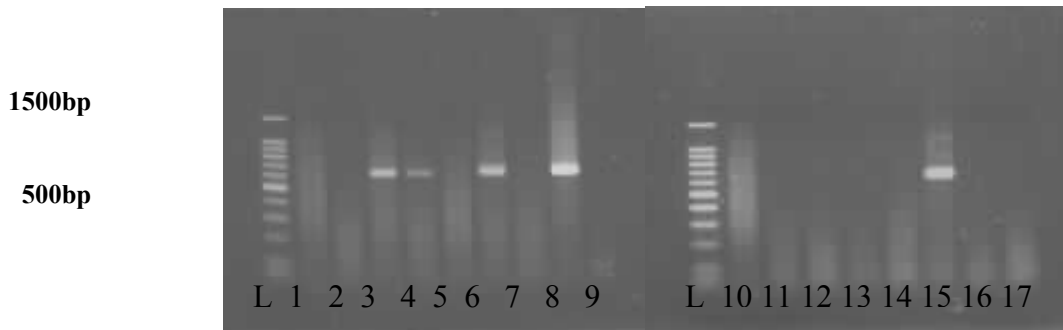


Figure 5.17. Ethidium bromide stained gel showing the RT-PCR results for β -actin (680bp bands visible) on 100 μ g/ μ l of RNA extracted from frozen blood samples by different techniques. L= Ladder, 1= Tri-Reagent and SV total RNA Isolation system, 2= RBC Lysis Solution and RNA Isolation Kit Purescript®, 3= Tri-Reagent, RNeasy® Mini Column and DNase Treatment, 4=Tri-Reagent and Micro-Fast Track™2.0 Kit, 5= Red Cell lysis Buffer and SV Total RNA Isolation System, 6= Tri-reagent not DNase treat, 7= Tri-Reagent and DNase treated, 8= 100 μ g/ μ l of RNA from PNT1A cell line Positive control, 9= Negative Control, 10= 1-RT, 11= 2-RT, 12= 3-RT, 13= 4-RT, 14= 5-RT, 16= 6-RT, 17= 7-RT.

Table 5.7. Relative brightness reading (rbu) for RT-PCR performed on β -actin on 100 μ g/ μ l of RNA extracted from frozen blood samples by different techniques (table 6.10).

Extraction Method	Raw Volume RT-PCR	Negative RT (+/-)	Raw Volume RT-PCR	Negative RT (+/-)
1) Tri-Reagent	(Not DNase treated) 17186.93	(Not DNase treated) 16773.54	(Not DNase treated) 14176.38 (DNase treated) -	(Not DNase treated) 8968.27 (DNase treated) -
2) Tri-Reagent, Rneasy® Mini Column and DNase Treatment	10901.78	-	11618.94	-
3) Tri-Reagent and SV total RNA Isolation system	14412.35	-	-	-
4) Tri-Reagent and Micro-Fast Track™2.0 Kit	5784.90	-	4475.94	-
5) Red Cell lysis Buffer and SV Total RNA Isolation System	-	-	-	-
6) RBC Lysis Solution and RNA Isolation Kit Purescript®	(Not DNase treated) 4162.32	1170.86	(DNase treated) -	-

The results show that RT-PCR does not work for technique 5, but works for one blood sample for technique 3, has DNA contamination for technique 1 and 6 and works on both blood samples for techniques 2 and 4. The technique chosen is therefore between 2 and 4.

Technique 4 is not chosen as although it produced the highest RNA yield, it took 4 hours to complete and only 10 μ l of RNA is produced. The technique decided upon is therefore technique 2, as it produced a relatively high RNA yield, took roughly 2.5hours and gave 30 μ l of sample.

RNA was then extracted using technique 2 (Table 5.6) for the fifty four blood samples, which were collected from cancerous, BPH and healthy volunteers, these were then

quantified and RT-PCR was performed to assess the expression of MCM2, 5, 7 and β -actin (Ref. *Appendix F Table f.11 for RNA yields*).

RT-PCR on Clinical Blood Samples

Before RT-PCR was performed on the blood samples they were first optimised so that a cycle number is chosen, where exponential amplification occurred (Ref. *Appendix F Table f.12 and Figures f.4-5*). The cycle numbers chosen is 35 cycles for MCM2, 5 and 7 and 32 cycles for β -Actin.

The rbu for the blood samples are shown in *Appendix F Table f.13* and No expression occurred for MCM2 or 7 by RT-PCR. Some of the blood samples for MCM5 and β -Actin do however produce results and the ethidium bromide gels show, the RT-PCR results for MCM5 and β -Actin (*Figures 5.18-5.21*). While the negative controls - Ethidium bromide gel showing PCR on RNA samples for β -Actin (No RT on RNA samples to insure No DNA contamination), are shown in *Appendix F Figure f.6-f.7*. The mean amount of MCM5 amplified by each Gleason score, BPH and healthy volunteers is shown in *Figure 5.22 and Table 5.8*.

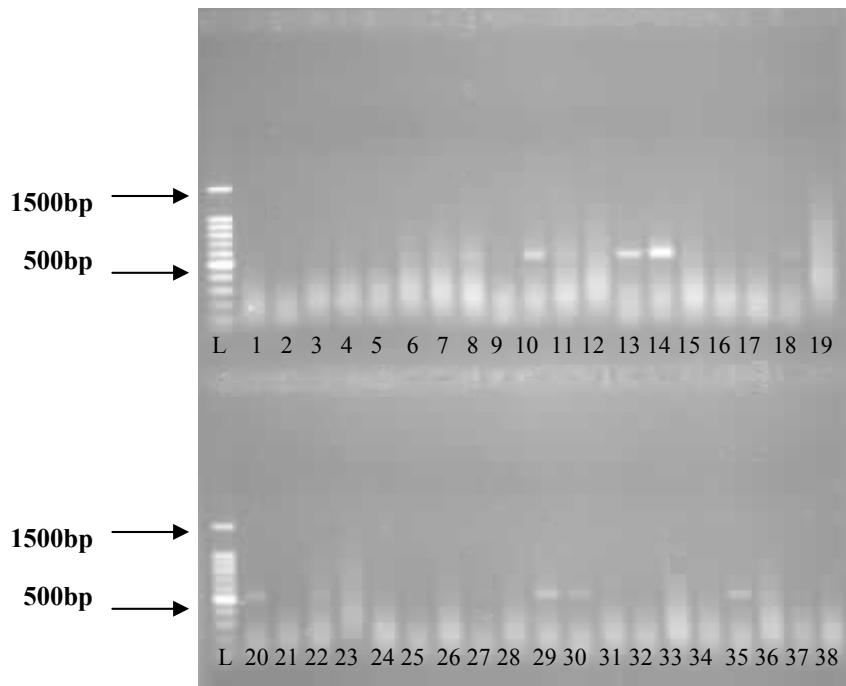


Figure 5.18. Ethidium bromide gel showing the RT-PCR results for MCM5 (500bp bands visible) tested on 50-150 μ g/ μ l of RNA extracted from blood samples. L = Ladder, 1 = P-016, 2 = P-003W, 3 = P-002, 4 = P-002W, 5 = P-032, 6 = P-073, 7 = P-123, 8 = P-005W, 9 = P-031, 10 = P-012, 11 = P-013, 12 = P-004, 13 = P-115, 14 = P-033, 15 = P-114, 16 = P-026, 17 = P-014, 18 = P-037, 19 = H-019, 20 = H-004, 21 = H-005, 22 = P-021, 23 = P-093, 24 = P-112, 25 = H-008, 26 = P-051, 27 = P-049, 28 = P-129, 29 = P-052, 30 = H-013, 31 = P-025, 32 = P-074, 33 = P-047, 34 = H-001, 35 = H-015, 36 = P-103, 37 = P-039, 38 = H-009.

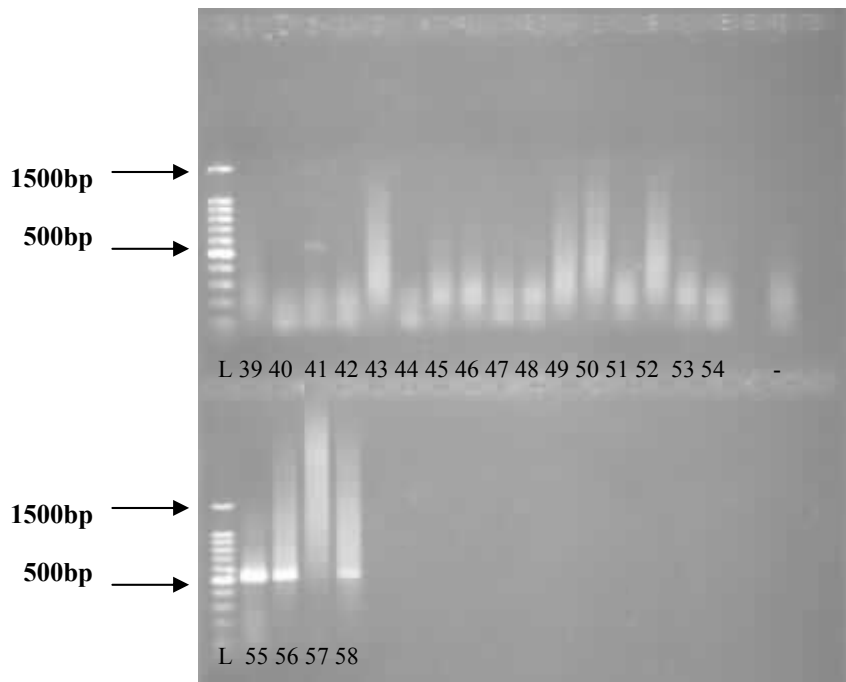


Figure 5.19. Ethidium bromide gel showing the RT-PCR results for MCM5 (500bp bands visible) tested on 50-150 μ g/ μ l of RNA extracted from blood samples. L = Ladder, 39 = H-007, 40 = P-008, 41 = H-010, 42 = H-014, 43 = P-001, 44 = P-023, 45 = P-061, 46 = P-028, 47 = H-021, 48 = P-048, 49 = H-003, 50 = P-022, 51 = P-024, 52 = H-006, 53 = H-012, 54 = P-050, - = Negative Control, 55 = Positive Control 150 μ g/ μ l of RNA from PNT1A cells, 56 = Positive Control 150 μ g/ μ l of RNA from PC-3 cells, 57 = Positive Control 150 μ g/ μ l of RNA from CaCO2 cells, 58 = Positive Control 150 μ g/ μ of RNA from OE21 cells.

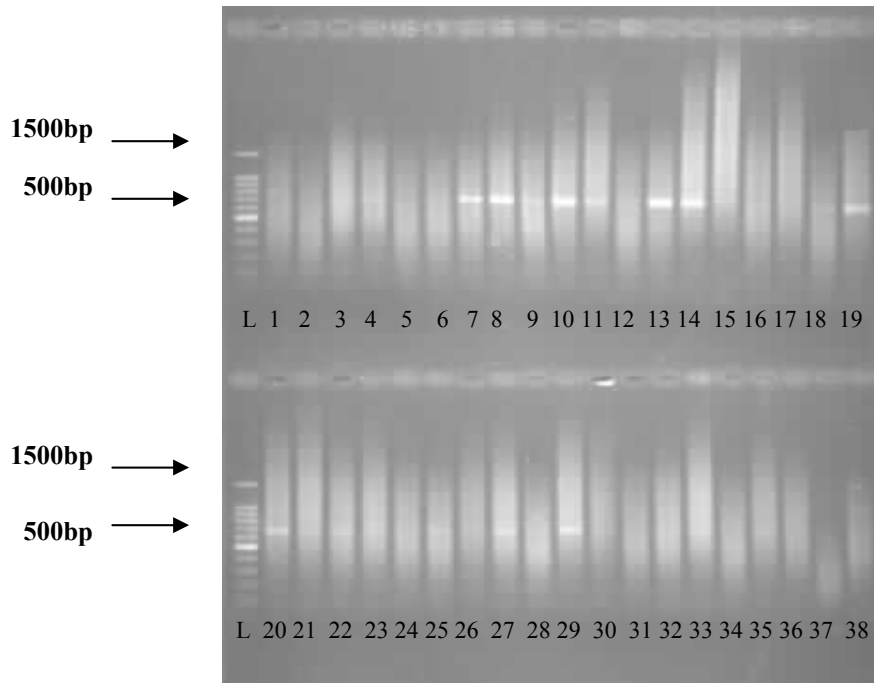


Figure 5.20. Ethidium bromide gel showing the RT-PCR results for β -Actin (680bp bands visible) tested on 50-150 μ g/ μ l of RNA extracted from blood. L = Ladder, 1 = P-016, 2 = P-003W, 3 = P-002, 4 = P-002W, 5 = P-032, 6 = P-073, 7 = P-123, 8 = P-005W, 9 = P-031, 10 = P-012, 11 = P-013, 12 = P-004, 13 = P-115, 14 = P-033, 15 = P-114, 16 = P-026, 17 = P-014, 18 = P-037, 19 = H-019, 20 = H-004, 21 = H-005, 22 = P-021, 23 = P-093, 24 = P-112, 25 = H-008, 26 = P-051, 27 = P-049, 28 = P-129, 29 = P-052, 30 = H-013, 31 = P-025, 32 = P-074, 33 = P-047, 34 = H-001, 35 = H-015, 36 = P-103, 37 = P-039, 38 = H-009.

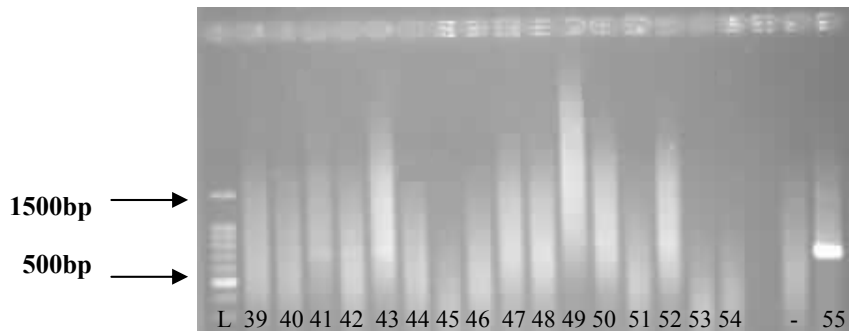


Figure 5.21. Ethidium bromide gel showing the RT-PCR results for β -Actin (680bp bands visible) tested on 50-150 μ g/ μ l of RNA extracted from blood. L = Ladder, 39 = H-007, 40 = P-008, 41 = H-010, 42 = H-014, 43 = P-001, 44 = P-023, 45 = P-061, 46 = P-028, 47 = H-021, 48 = P-048, 49 = H-003, 50 = P-022, 51 = P-024, 52 = H-006, 53 = H-012, 54 = P-050, - = Negative Control, 55 = Positive control 150 μ g/ μ l of RNA from PNT1A cells.

Table 5.8 The table shows the number of samples with No MCM5 expression, the number of samples with MCM5 expression and the percentage that gave a relative brightness reading (rbu) for each score. The mean relative brightness obtained for each score including all the samples = relative brightness reading 1 and the mean relative brightness obtained for scores only including the samples that gave a positive result = relative brightness reading 2 (Raw data Appendix F Table f.13).

Score	No. of samples with NO relative brightness Reading	No. samples with relative brightness Reading	Percentage of samples from each score with reading	Mean Relative brightness Reading 1	Mean Relative brightness Reading 2
6	0	3	100%	3519.27	3519.27
7	2	1	33%	2649.78	7949.35
8	1	0	0%	0.00	0.00
9	2	2	50%	1133.38	2266.76
10	3	0	0%	0.00	0.00
Benign	22	3	12%	459.79	3831.56
Healthy M	5	1	16%	393.75	2362.52
Healthy F	6	3	33%	936.84	2810.53

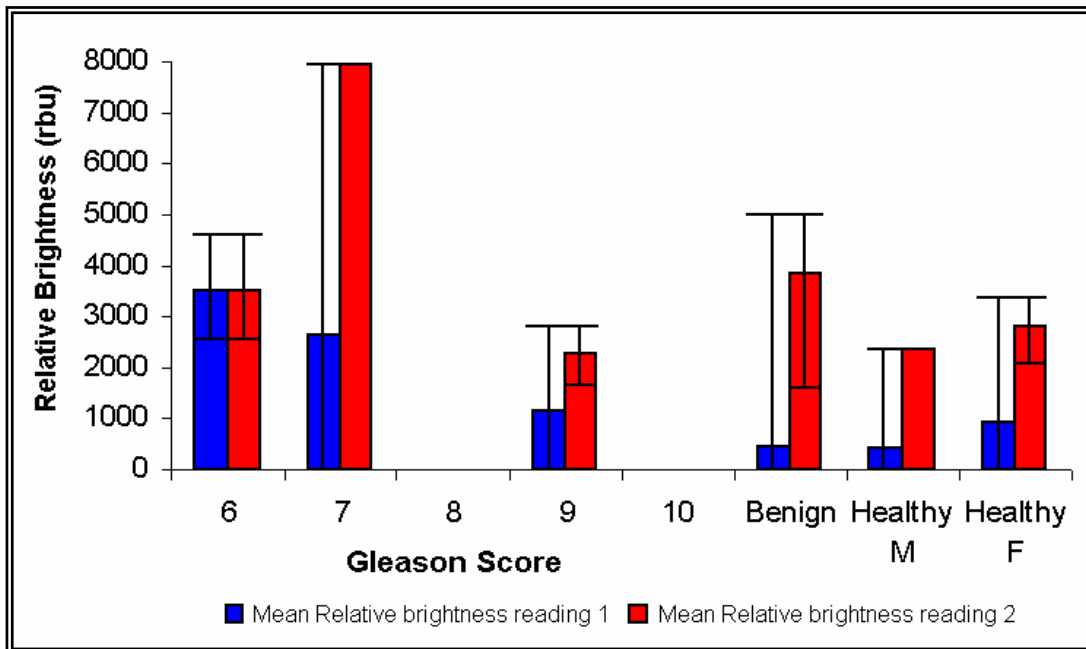


Figure 5.22. The mean relative brightness readings (rbu) obtained using RT-PCR on MCM5 from the blood samples. The Mean relative brightness Reading 1 = Is the mean relative brightness for each score which includes all the blood samples. While the Mean relative brightness Reading 2 = Is the mean relative brightness obtained for each score using only the blood samples that have produced a result by RT-PCR on MCM5.

The sample numbers are as followed for the Mean relative brightness Reading 1: - Gleason Score 6 n=3, Gleason Score 7 n=3, Gleason Score 8 n=1, Gleason Score 9 n=4, Gleason Score 10 n=3, Benign n=25, Healthy M (M=Males) n=6, and Healthy F (F=Females) n=9.

The sample numbers are as followed for the Mean relative brightness Reading 2: - Gleason Score 6 n=3, Gleason Score 7 n=1, Gleason Score 8 n=0, Gleason Score 9 n=2, Gleason Score 10 n=0, Benign n=3, Healthy M (M=Males) n=1, and Healthy F (F=Females) n=3.

The error bars show the mean range for the samples.

The tabulated RT-PCR results show (*Table 5*), that MCM5 and β -Actin are amplified by some of the blood samples. For instance six out of the thirteen cancerous blood samples amplified MCM5, three out of the twenty five BPH blood samples amplified MCM5, and four out of the fifteen healthy volunteers expressed MCM5. Also six out of the thirteen cancerous blood samples expressed β -Actin, seven out of the twenty five BPH cases expressed β -Actin, and five out of the fifteen healthy volunteers expressed β -Actin.

If *Figure 5.22 and Table 5.8*, mean relative brightness reading 1 is taken into account, then there is a possible difference in MCM5 expression between blood samples extracted from cancerous, BPH and healthy volunteers. These results show that neoplastic blood samples amplified more MCM5 to produce a higher mean rbu, than BPH and healthy volunteers, with the highest mean rbu being detected at Gleason score 6.

If however mean relative brightness reading 2 is taken into account, which does not include blood samples that do not amplify MCM5. Then the mean rbu does not show a great difference in the level of expression of MCM5, between neoplastic and non-neoplastic conditions, but again like mean relative brightness reading 1, the highest mean rbu reading is detected by a neoplastic score (Gleason Score 7).

As β -Actin is a housekeeping gene and is expressed by all cells, it is possible to postulate that only blood samples, which amplify β -actin, should be analysed for MCM5 expression, as these samples are the ones with living cells in circulation. If this rule is taken into account, and all samples that do not express β -actin are discarded, then the results in *Table 5.9 and Figure 5.23* are obtained for MCM5 expression.

Table 5.9. Percentage of blood samples shown to express MCM5 and their mean relative brightness reading for each score when RT-PCR was performed for MCM5. Only samples shown to express β -Actin counted and relative brightness assessed (Raw data *Appendix F Table f.13*).

Score	No. of samples with No relative brightness Reading	No. of samples with a relative brightness Reading	Percent expressing relative brightness reading	Mean relative brightness Reading (Total relative brightness/total No. of samples)
6	0	3	100%	3519.27
7	0	1	100%	7949.35
9	1	1	50%	1425.82
Benign	4	3	43%	1642.17
Healthy M	2	1	33%	787.51
Healthy F	1	1	50%	1684.21

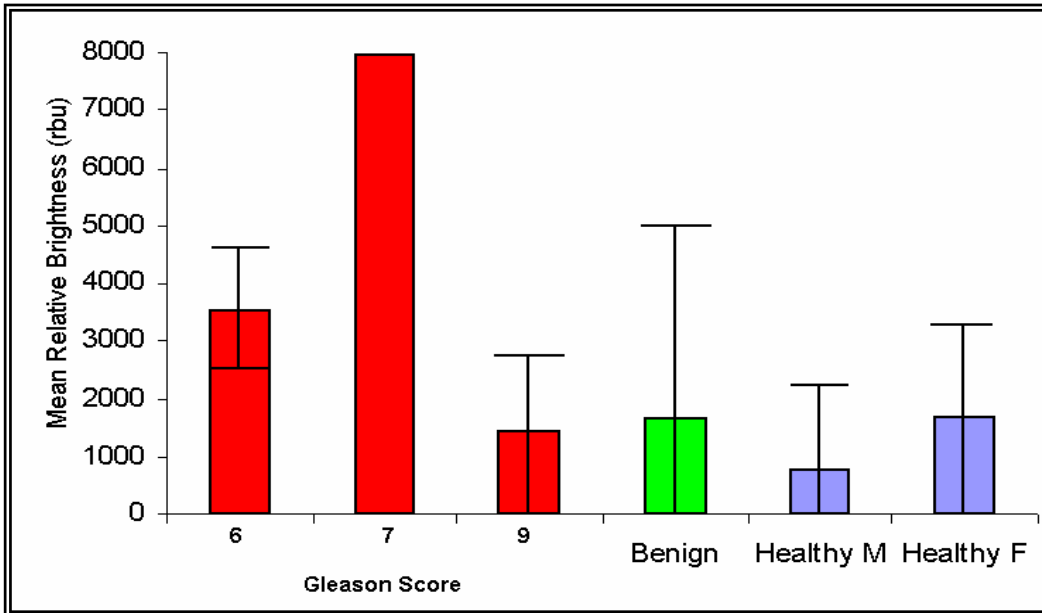


Figure 5.23. Mean relative brightness reading obtained for MCM5 from blood samples, which are shown to express β -Actin. The error bar shows the mean range and the sample numbers are as followed for Gleason Score 6 n= 3, Gleason Score 7 n= 1, Gleason Score 9 n= 2, benign n= 7, healthy M (M = male) n= 3, healthy F (F=female) n= 3.

The results show (*Figure 5.23*) that only Gleason score 6 and 7 produce a mean rbu reading for MCM5, which is higher than that obtained for BPH or healthy volunteers. There is however overlap of error bars, so it is possible that these are not truly different from one another. These results do not show a conclusive difference in mean rbu between neoplastic and non-neoplastic conditions. These preliminary results do however look promising, but

until analysis with higher sample numbers are performed, it is impossible to speculate the advantage of RT-PCR for the diagnosis of prostate adenocarcinoma.

5.4 Discussion

The results of this study show (*Figure 5.1-5.2 Table 5.1-5.3*) that serum PSA testing is unable to significantly distinguish low/intermediate grades of adenocarcinoma from BPH, with no significant difference recorded between most of the serum PSA levels, and the technique only having 63% sensitivity and 60% specificity. Fitzpatrick, *et al* (2002) stated that traditionally serum PSA levels of less than 4ng/ml are considered to be normal, while readings between 4-10ng/ml are borderline and those above 10ng/ml are strongly suggestive of prostate cancer. Benson, *et al* (1992) also suggested that if a patient's serum PSA level falls into the borderline range, they should be suspected of having organ confined disease. The results of this thesis do not agree with Benson, *et al* (1992), as they show that 23% of BPH patients fall into the borderline range compared to 17% of malignant patients. This means that a higher percentage of patients in the mid-range (4-10ng/ml) are false positive and go through unwarranted stress and anxiety. These results do however correlate with Catalona, *et al* (1991) who stated that only one quarter of men within the mid-range would actually have prostate cancers, and with Adam, *et al* (2001), who stated that the specificity of detecting a difference between BPH to prostate cancer in the grey zone (4-10ng/ml) is only 25-30%.

A significant difference was recorded for Gleason score 10, as this is shown to be significantly different to BPH, Gleason score 6, 7 and 9, with a higher level of PSA recorded. These results also show, that more BPH patients than malignant patients fall into a range of less than 4.5ng/ml (65%) and more malignant patients than BPH patients fall into a range of more than 10ng/ml (47%). There are issues with the low and high ranges, as although most BPH patients fall into the lower range and most malignant patients fall into the higher range. This study still shows that 37% of malignant patients fall in a range less than 4.5ng/ml, and 14% of BPH patients fall in a range of more than 10ng/ml, meaning that false negative and false positive results occur. These results agree with Catalona, *et al*

(1997) who found that 20% of men with serum PSA levels less than 4ng/ml, actually had prostate cancer, and with Kranse, *et al* (1999) who stated that although PSA appears to be the strongest marker of prostate cancer, by using the threshold of 4.0ng/ml, up to a third of cancers are missed.

This study shows that PSA can not distinguish approximately 40% of malignant conditions from non-malignant conditions, so its sensitivity and specificity are questionable. These results do correlate with a number of current findings, as it has been suggested that although PSA has significantly increased prostate cancer detection at an early stage, it remains highly controversial (Fitzpatrick, *et al* 2002), with PSA alone missing approximately 25% of cancers (Catalona, *et al* 1994) and all experts agree that PSA has limited specificity, as BPH can also cause an increase in PSA levels (Carter, *et al* 2004).

To try and improve diagnosis of prostate cancer, urine pellets were analysed by a translational study using MCM2, 5, 7 and PSA. The results show that an increase in staining ability occurs for MCM2, 5 and 7 in urine pellets from malignant patients (*Table 5.4-5.5 Figure 5.3-5.12*), with a significant difference recorded between BPH and Gleason scores 8-10 for MCM2, between BPH and Gleason Scores 8 and 10 for MCM5 and between BPH/healthy volunteers and Gleason scores 7, 8 and 9 for MCM7. PSA does not show any significant differences between cells from BPH, healthy volunteers or cancerous patients and even healthy female volunteers were shown to have detectable PSA staining in their urine pellet, further emphasising the fact that PSA is not prostate specific.

Although increased MCM2, 5 and 7 cellular staining in urine pellets seems to occur for prostate cancer patients, it is important to note the overlap of error bars (SE) between malignant and non-malignant patients, so it is possible that the differences seen may not be 'true'. This overlap however could be due to a number of reasons, for example it is possible that the overlap is due to the small sample size, as only twenty seven patients were assessed, also it is possible that the time between processing the samples may affect the results. *Figure 5.15* shows that samples processed straight away have a higher cellular

number, than samples left for a few hours (50% reduction if cells not processed with 48hours), therefore until a translational study is repeated, with increased sample numbers and optimal processing performed, it is hard to speculate on whether the data is significant whether the data

There are also unexpected results in the staining ability of the cells obtained in urine sediment from the healthy volunteers, for MCM2 and 5. As slightly higher cellular staining occurs than expected, causing no significant difference to be seen between the healthy volunteers and the Gleason scores (*Table 5.5*) (*Figure 5.3*). There are a number of possible reasons for these findings, and it can be postulated that either the age of the patients, or the time between processing the urine samples, affects the results. For instance when considering the age of the patients, 92% of cancerous patients are over the age of 60, while none of the healthy volunteers are, with 64% females and 50% males falling into the age range of 18-29 (*Appendix F Table f.2-3*). Therefore as the healthy volunteers are younger, it is expected that normal cellular proliferation is higher for them and therefore the staining ability is greater. Also if the time between processing is taken into account, there is a possibility that this affected the staining ability, as the healthy volunteers urine samples were processed a lot quicker (within 1 hour of voiding), than the clinical samples (could be left of up to 48 hours before processing).

Although only slightly significant differences are seen for the translational study, the preliminary findings for MCM2, 5 and 7 do seem promising and correlate with current studies. For instance, Davis, *et al* (2002) found that MCM2 increases in expression in stool samples retrieved from patients with colorectal cancer and Stoeber, *et al* (1999) (2002), found that an immunoassay for urine cells against antibodies to MCM5, showed an increase in staining for urothelial cancer cells, compared to normal bladder cells.

The results from these studies suggest that MCM2, 5 and 7 may have the potential to assist with prostate cancer diagnosis by analysis of urine sediments, and special attention should be focused on MCM7. Although it only produced detectable staining at the intermediate

score (7-9), with no staining detected for BPH or the healthy volunteers, this may be useful; positive MCM7 staining could indicate cancer. Although this may provide valuable information, a TRUS biopsy will still be required to confirm these findings unless a clearer association between the MCMs and prostate cancer is distinguished.

The cells found in urine sediment include urothelial cells and not just prostatic cells. Therefore, although prostate cells are shed into urine sediment, it is important to realise that the majority of cells will be non-prostatic and as the MCMs stain any cells performing proliferation, they may detect other cancer types. This means false positive results for BPH patients may occur, and positive results may indicate some other type of urogenital disease. For instance Stoeber *et al* (2002) found that elevated levels of MCM5 in urine sediment are highly predictive of bladder cancer. Therefore to clearly confirm if prostate cancer is detected by an increase in MCM2, 5 and 7 expression, a flexible cystoscopy could be used to exclude bladder urothelial abnormalities. However, it has been suggested by Botchkina *et al* (2005) that because of weaker cell-to-cell and cell-to-extracellular matrix contact, more cancerous cells than normal will be exfoliated into biological fluids such as urine, and as about 3-4cm of the urethra lies in the prostate, this means that more cells are expected to be shed into the urine of prostate cancer patients, compared to BPH and healthy volunteers.

Although the majority of cells in urine sediment will not be of prostatic origin, a number of studies have shown that prostatic cancer can be detected in urine sediment. For instance, Goessl *et al* (2000) stated that urothelial, renal and prostate malignancies can be detected via urine, while Stoeber *et al* (2002) found in a study of bladder cancer that testing for MCM5 also appears to detect prostate cancer cells.

A transcriptional study was then performed on the urine pellets and the results show that when RT-PCR was performed for MCM2, 5, 7 and β -Actin on urine sediment, only MCM5 produces positive results. This could be for a number of reasons: for instance, because no expression is occurring for β -Actin and this is a housekeeping gene which should ALWAYS be expressed, there is a possibility that the expression seen by MCM5 is a false

positive result and that realistically the RNA yield was too low and of poor quality to detect β -Actin, MCM2, MCM5 and MCM7. Another possible reason is that β -Actin, MCM2 and MCM7 are expressed by the cells but at a much lower level than MCM5 and this is too low to be detected by PCR. This would seem unlikely given considering β -actin's popularity as a positive control for RT-PCR. Finally, it may be that not enough replicating prostatic cells are shed into the urine sediment, so that not enough β -Actin, MCM2 or MCM7 are expressed to enable detection by PCR.

MCM5 expression was detected in urine sediment using RT-PCR and the results show an increase in the rfu in malignant conditions, compared to non-malignant conditions (*Figure 5.13*), with the technique giving positive results in 11/12 cancerous patients but only 3 of the 23 BPH patients. When an analysis of variance is performed a significant difference is then seen between Gleason score 6 and 9 to BPH and the healthy volunteers. These results indicate that MCM5 has a potential for assisting the diagnosis of prostate cancer, but sample size needs to be increased to conclusively prove this. With further study, RT-PCR testing for MCM5 in urine sediment may therefore provide another diagnostic method for prostate cancer detection.

Use of PCR-based techniques such as RT-PCR have already been shown to be a plausible method for the diagnosis of prostate cancer. For instance, increased methylation of GSTP1 has been detected in urine sediment from patients with prostate cancer (Goessl, *et al* 2001), and increased telomerase activity has also been detected in urine samples obtained from prostate cancer patients (Meid, *et al* 2001). These studies therefore show that urine analysis using PCR could be implemented for prostate cancer diagnosis, so MCM5 could show potential as a biomarker with such a technique.

Again, it is important to consider the limitations of the techniques investigated by this part of the study. As noted previously, limitations include the lack of specificity of MCMs to prostate cancer cells, with the potential for false positive results from other types of urogenital disease. However, results from the transcriptional analysis are more promising

than the translational study on the urine sediment in terms of sensitivity as detecting RNA in bodily fluid is more sensitive than protein analysis. Goessl *et al* (2000) found that in blood, 200 prostate cancer cells could be detected among 2.2×10^7 non-malignant cells, producing clear results (clear bands on an electrophoresis gel), and that as few as 20 prostate cancer cells could be detected among 2.2×10^7 non-malignant cells, producing faint bands. Therefore, since Goessl *et al* (2000) found that DNA could be detected in bodily fluid such as blood, it is also possible to speculate that these low detection levels may also be detected in urine. Again, it maybe beneficial for a flexible cytосcopy to be performed to exclude the possibility of bladder involvement on a positive result.

Finally, a transcriptional study was performed on RNA extracted from blood (tRNA extracted from blood and mRNA amplified by RT-PCR) (Table 5.8-5.9 Figure 5.22-5.23). These samples are shown to express MCM5 and β -Actin, but again like the urine pellet transcriptional study, expression of MCM2 and 7 are not detected. Although the results from this part of the study seem to confer with the results from the transcriptional study on the urine sediment, the data seems less conclusive, as only a few samples actually produced a signal (this maybe due to the suboptimal storage and extraction method used), therefore although an increase in MCM5 rbu does seem to occur with malignant conditions, it is hard to draw any conclusive conclusions (Figure 5.22, 5.23).

The blood results for MCM5 show positive results for 6/12 cancerous patients while only 7/33 BPH patients/healthy volunteers, and when only blood samples that expressed β -Actin are taken into account, 5/6 cancerous patients are positive and 5/12 BPH patients. It is therefore possible that due to the storage of the blood samples and consequently the extraction method used, the RNA yield/quality was greatly reduced and therefore affected the rbu obtained. These blood results are therefore inconclusive, and to conclusively prove if a correlation in MCM5 expression occurs in blood, this experiment needs to be performed again.

Although the data from the three techniques used in this chapter suggests a role for MCM2, 5 and 7 for prostate cancer diagnosis, a number of alterations for implementation would be required to translate these techniques into useful diagnostic tests. Firstly, for the urine sediment analysis, it is important that the samples are processed immediately after voiding to ensure optimal cellular yields, and yield may be increased by implementing prostatic massage before voiding. Prostatic massage is a term used to describe stimulation of the prostate and may increase the dislodgement of prostate cells from the gland and consequently the number of cells shed into the urine sample. This technique may potentially help increase the results obtained by both the translational and transcriptional studies on the urine sediment, but while prostate massage may increase the number of prostate cells voided it will not exclude other urogenital cells.

Secondly, when considering the RNA extracted from blood, alterations to procedure may help ensure optimal yields and quality. Collection of samples in EDTA tubes and processing within twenty four hours instead of storage at -20°C , should increase both the quality and quantity of RNA obtained.

Finally, for all three techniques it is important to reiterate that other cancerous conditions can be detected using analysis of MCMs as they are not prostate specific markers and all cells undertaking cellular proliferation will be detected. It is therefore recommended that markers which are prostate specific are used alongside the MCMs to confirm the presence of prostatic cells which as of yet there does seem to be a suitable marker.

The results in this chapter suggest that PSA is an inefficient diagnostic marker which generates lots of false positive and false negative results. The fact that PSA is not prostate specific has been further emphasised since female urine pellets were found to stain with polyclonal PSA antibodies. ICC using antibodies to MCM2, 5 and 7 on urine sediment may however have a potential for the diagnosis of prostate cancer as well as other urogenital diseases, as may the detection of MCM5 expression by RT-PCR in urine sediment. This data provides a good starting platform for the development of MCM2, 5

and 7 analyses in assessment of prostate cancer, but further work is required to clarify their potential.

Chapter 6 – Final Discussion

The aim of this thesis was to investigate MCM2, 5 and 7 expression for the potential diagnosis of prostate cancer and to assess their usefulness as tumour specific markers. This project also analysed the current diagnostic marker for prostate cancer, PSA, as there are conflicting views on its usefulness. Therefore, PSA was assessed to see how specific it was to the prostate gland, and how reliable it was at detecting prostate cancer.

For this thesis, three main objectives were set out in the introduction. These were to analyse MCM/PSA expression in cultured cells and to optimise techniques for RNA and protein analysis; to analyse MCM/PSA expression in human prostatic tissue; and to assess whether Gleason score alters MCM/PSA expression in clinical samples. All these objectives were met within this thesis, with chapter two and three explaining the expressional differences of MCM/PSA and optimising the analysis techniques; chapter four assessing the prostatic tissue and characterising the usefulness of the MCM/PSA for different prostatic disease; and finally, chapter 5 assessing the current diagnostic technique for prostate cancer (PSA ELISA), as well as assessing MCMs for the identification of prostate cancer in clinical samples.

All the aims and objectives outlined in chapter one were fulfilled, with the scientific importance and relevance of the findings being discussed in each associated chapter. It is therefore the role of the final discussion to draw together all the results, to establish the potential diagnostic application of the MCMs, and to discuss the clinical importance of the current diagnostic marker PSA. Once all the work is linked together, the end point of this project is to implicate whether the study of MCMs can be a useful diagnostic tool for prostate cancer and to speculate on how reliable serum PSA testing is.

The search for tumour markers that can diagnose cancer is a very important area, and as more is known about cancer development and consequential factors, potential molecules that can be used for diagnosis are being identified. Tumour markers therefore remain an important area of research, especially until cause and effect of disease has been

identified. Therefore knowledge into the complete picture of a disease provides information into the susceptibility, diagnosis, prognosis, predictive response and therapeutic outcome of disease (Williamson, *et al* 2001).

Currently, most techniques implemented for the diagnosis of disease use some type of protein based assays, such as PSA serum testing. Proteomic efforts are therefore an important part of functional genomics and are critical in understanding and synthesising the information produced by the human genome project (Ahram, Best *et al* 2002). However, as more information on the human genome is understood, alternative diagnostic tests based on techniques such as PCR or microarrays can be exploited for diagnostic purposes, for instance a PCR-ELISA using CD44 was used to detect bladder and colorectal cancer (Morgan *et al*, 2002).

The format of diagnosis is also changing, as movement is occurring away from conventional single marker assays, to analysis of multiple markers. This aims to provide a more comprehensive picture of what is happening and provide more information into the optimal diagnostic and prognostic tools. For instance, advances in mass spectrometry techniques, especially those that permit 'rapid fingerprinting' of multiple markers, will enhance identification and characterisation of potential cancer biomarkers (Adam, *et al* 2001).

The ultimate aim for a diagnostic marker/s is to identify disease status in a less invasive manner, with as near to 100% specificity and sensitivity as possible. Also an ideal marker would not only identify the disease from 'normal' cells, but would show some type of expressional correlation with disease status, i.e. possibly increasing in detection as disease status intensifies.

The work on PSA in this thesis was undertaken to provide further evidence as to whether PSA is exclusive to the prostate, being synthesised and secreted only by prostatic cells, and to assess whether serum PSA testing is reliable. Chapter two investigated protein expression of PSA in a number of cell lines and this chapter indicates that PSA expression is not exclusive to the prostate, as initial screening of

thirteen human cells lines by ICC shows that PSA is expressed by eight non-prostatic cell lines. Also, when western blotting was performed on six of the cell lines, even the cell line that showed no expression of PSA during ICC (the oesophageal cell line, OE21) expressed complexes of PSA, and all six cell lines not only express PSA, but secrete PSA (shown by dot blot).

The data described above needs to be considered in context, since cells grown in culture can be subject to alterations in their behaviour due to the immortalisation process and loss of their normal cellular environment. This potentially means that PSA expressed and secreted by these cells may not be truly representative of the behaviour of the cells from which they were derived *in vivo*. For instance, cell lines can have changes in their growth due to reduced contact inhibition, changes in surface receptors such as glycoproteins, and changes in their intra-cellular matrix such as disruption of their cytoskeleton. Two of the cell lines analysed were virally transformed (SV40) to enable them to grow indefinitely *in vitro* (PNT1A and HET1A cells), and this process can cause changes in the growth phenotype of cells. In addition, culture environments lack several systemic components involved in homeostasis regulation, principally those from nervous and endocrine system (Freshney, 1987), so cellular metabolism may be more constant *in vitro* than *in vivo* and not truly representative of the tissue from which the cells are derived (Freshney, 1987).

Although it would be unwise to draw firm conclusions from cell culture work alone, it does provide a starting platform for analysis of specific cell types and physiological functions. For instance, cell culturing can be advantageous as it can provide an easy system to assess virus transmission, drug control, intracellular activities, intracellular flux, ecology and cell-cell interactions (Frshney, 1987). Chapter two shows that a number of prostatic/non prostatic cells express PSA and that there is only a slight increase in expression of PSA when the PC-3 cells (cancer derived) are compared to the PNT1A cells (derived from normal tissue). These expressional changes between the two prostatic cell lines and between the other cell lines were not what was expected, as the tissue (IHC) study by Van der Crujisen-Koeter *et al*, (2001) found PSA to be produced mainly by the prostatic epithelium, with only low concentrations in

endometrium, breast tissue, adrenal, renal and female serum. The IHC data in this thesis does not agree with this and suggests that non prostatic cells produce/express PSA at a similar level to prostatic cells and therefore emphasise the problem with using this protein as a prostate specific marker.

If LNCaP cells were included in this study, a greater difference in PSA expression may have been detected, this is because the LNCaP cells are androgen dependent prostatic adenocarcinoma cells and are considered less deranged than the PC-3 cells, which are androgen independent prostatic adenocarcinoma cells. For instance Sun *et al*, (2001) states that androgen dependent prostate cancer cells can stimulate PSA production, and it has been shown that LNCaP cells express PSA at a higher intensity than androgen independent cells (DU45 – Prostate carcinoma ATCC- HTB81) (Song et al 2003).

Chapter four looked at the expressional variation of PSA in malignant conditions compared to BPH. The IHC results show that PSA was not elevated in prostate cancer, and a significant difference was only recorded between Gleason score 10 and BPH, where a drop in staining ability at Gleason score 10 occurred. These results imply that most prostatic conditions overlap in their PSA staining ability and show no conclusive difference. The work of chapter five (Section 5.3.1) was then performed to analyse the current PSA serum test and the results reveal that PSA testing had only 63% sensitivity and 60% specificity in accurately identifying prostate cancer in this set of patients and that only Gleason score 10 was shown to be significantly different to BPH, with a significant increase in serum measurement detected. A study was also performed using PSA antibodies on urine sediment (Section 5.3.2) and no significant difference could be detected between Gleason score, BPH or healthy volunteers and even female urine sediment cells seemed to express PSA.

The work in this thesis on PSA emphasises the fact that it is not prostate specific, as PSA is synthesized/secreted by non-prostatic cells, expressed by female derived cell lines (OE33, HT29, ZR 75 1) and expressed by urine sediment cells from female volunteers. In addition, serum PSA testing is approximately 40% inaccurate at correctly identifying prostate cancer patients. These findings further emphasise the unreliability

of PSA as a prostate specific tumour marker and agree with other studies such as; Malatesta *et al* (2000) who found that human placenta was shown to synthesise and secrete PSA, while Silver, *et al* (1997) that found that men born with bladder exstrophy had detectable serum PSA levels, even though these men did not have prostate cancer, They also agree with Stamey *et al*, (2004), who found that elevated serum PSA levels can be correlated to prostatic weight and non-cancerous conditions, such as BPH and with Carter and Isaacs, (2004) who found that high-grade cancer can actually produce less PSA than low-grade cancer.

Despite a number of attempts to try and improve serum PSA detection i.e. via PSA velocity, PSA density, and PSA forms, the sensitivity and specificity for the technique is still insufficient for it to be considered as an ideal tool for diagnosis of prostate cancer (Pannek, *et al* 1997). This study and others have therefore ‘highlighted’ the current problems with PSA, therefore there is a need for a marker(s) that can correctly distinguish benign growths from malignant growths and can also provide information on the potential aggressiveness of the tumour (Botchkina *et al* 2005).

The main objective of this thesis was to investigate whether MCM2, 5 and 7 could be used as potential new biomarkers for prostate cancer, so firstly a cell culture study was performed to assess MCM2, 5 and 7 expressions in prostatic cells, to obtain a better understanding of their role, and to investigate their potential as diagnostic markers. Following the *in vitro* study, prostatic tissue and clinical samples were analysed, to investigate if any expressional alteration between malignant and non-malignant conditions could be detected.

The *in vitro* work in chapter three shows that a difference in MCM2, 5 and 7 expression can be detected between PNT1A cells and PC-3 cells. However, instead of the expected increase in MCM2, 5 and 7 expression occurring in the PC-3 cells, such as that visualised for prostate adenocarcinoma tissue (Chapter 4 – IHC results), there seems to be reduced expression. This reduction in MCM2, 5 and 7 was unexpected and these findings suggest a loss of MCM function in the PC-3 cells. This is plausible since the PC-3 cells are highly aggressive neoplastic cells (androgen independent) and therefore

their normal proliferation maybe highly deranged. PC-3 cells are highly aggressive prostate cancer cells, while DU-145 cells are moderately aggressive prostate cancer cells and LNCaP cells are poorly aggressive prostate cancer cells (Nair *et al*, 2004). Therefore, if DU-145 or LNCaP cells had been included in this analysis, they may represented an intermediate level of neoplasia and shown an increase in MCM2, 5 and 7 expression, which would be more comparable to the tissue results obtained (Chapter four).

Again, it is important to remember that cellular transformation of the PNT1A cells by SV40 has been used to immortalise the cells, which may effect MCM2, 5 and 7 expression and not be representative of these cells *in vivo*. In addition, the removal of these cells from their normal homoeostatic environment may have affected MCM expression. This point emphasises the potential problems with using *in vitro* cell cultures and although this technique provides a quick and easy way of assessing cells of a specific origin, the results obtained can be variable due to the culturing conditions. It is therefore important to consider that although the PNT1A cells seem to express more MCM2, 5 and 7 than the PC-3 cells, this elevation may not be truly representative of these cells *in vivo* as most cells *in vivo* are in constant regulation to maintain a balance between cellular death and cellular growth. In cell culture however, this balance is altered so that all the cells continue to replicate indefinitely, meaning that the PNT1A cells are constantly performing proliferation and continually producing MCMs. However, although the culturing conditions may affect the expression of MCM2, 5 and 7, cell culturing is still advantageous as it can provide information on expressional difference between these cell types, which would be difficult to determine by *in vivo* means. Therefore, although it can be speculated that although the results seen may be in part due to immortalisation, important questions about MCM are raised and possible explanations should be investigated.

As part of their normal function, MCMs are loaded onto the ORC, but instead of one or two being loaded, 10-40 MCMs are loaded (Edwards *et al* 2002), far more than required. This suggests the MCMs may perform other roles within the cellular cycle, and not just replicate DNA. An alternative role for the MCMs might be to provide

genomic integrity, as a decrease in the levels of MCMs, has been demonstrated to lead to DNA damage (Bailis & Forsburg 2004). Therefore, the reduction in MCM2, 5 and 7 expression in the PC-3 cells, may be due to loss of DNA stability. This loss of DNA stability may be expected in cancerous cells, as these cells have lost co-ordination with their normal cellular functions and replicate without stimuli or listening to signals from the 'outside world'.

In chapter four, MCM2, 5 and 7 expression was assessed in prostatic tissue to assess whether these molecules could be potential tumour markers. These results show that a correlation was detected between epithelial nuclei staining pattern for MCM2, 5 and 7, and disease status. For instance;

- BPH – MCM2, 5 and 7 show low staining ability (0-4% staining),
- Low Gleason Score (Score 4) – MCM2 and 5 show an increase in staining ability (4-25%), while MCM7 shows no difference in staining ability,
- Intermediate Gleason Score (Score 5-7) – MCM5 and 7 show a continued increase in staining ability, while for MCM2 staining (Score 6-7) starts to increase,
- High Gleason Score (Score 8-10) – MCM2, 5 and 7 all show an increase in staining ability compared to BPH.

This staining pattern therefore shows that MCM5 and 7 can distinguish most Gleason scores from BPH, while MCM2 can only distinguish mid/high scores from BPH.

These results suggest that MCM5 and 7 may be useful diagnostic markers, as they seem to increase in immunoreactivity as the proliferative state of the tissue increases, while MCM2 shows approximately the same staining intensity between Gleason scores, making it hard to distinguish differences between mid-scores, MCM7 directly increases in expression as adenocarcinoma increases, while MCM2 does not appear to be a great diagnostic marker, as only high-grade neoplasia shows increased immunoreactivity compared to BPH.

These results therefore agree with a number of other studies. For instance, MCM proteins are more frequently expressed in cells from malignant tissue, than in cells from

normal tissue, suggesting MCMs may be good indicators of cancer (Yoshida and Inoue 2003). MCM5 and 7 has been found to increase in expression in prostate cancer compared to normal tissue (Freeman *et al*, 1999, Padmanabhan *et al*, 2004), while MCM2 has been shown to be a poor diagnostic marker but suggested by other studies as a independent predictor of disease free survival (Meng, *et al* 2001). Therefore, while MCM5 and 7 appear to be potential diagnostic markers, the reduced staining of MCM2 may be of importance, as this has been suggested by other studies to indicate disease free survival and therefore may have a prognostic potential. Prostate cancer is a variable disease and can be highly aggressive in some, while slow growing and non invasive in others, therefore if an increase in MCM2 staining (IHC) is detected, it is believed to indicate poor prognosis. MCM2 may therefore be a useful prognostic marker able to distinguish aggressive forms from slow growing forms by the amount of MCM2 staining present and its implementation may aid in deciding the optimal treatment pathway to select.

The staining pattern of the MCMs may potentially be beneficial for use in histopathology, as currently it can be hard to distinguish malignancy by H&E staining alone. As a result, intermediate scores of prostate cancer can be misdiagnosed so suboptimal treatment pathways may be implemented. Therefore, as MCM5 and 7 were shown to be useful diagnostic markers as most Gleason scores show a higher immunoreactivity compared to BPH, these could be used to distinguish between benign and cancerous conditions, and MCM7 may provide additional information in order to distinguish the correct Gleason scores. Not only this, but as MCM2 has been suggested by other studies to be over expressed in cancers with poor prognosis, it would also be a beneficial to include this marker to indicate cancer aggressiveness and help implement optimal treatment.

IHC using the MCMs has clearly demonstrated a difference between cell cycle activity in both BPH (mainly localised to the basal cells) and adenocarcinoma (epithelium), and that there is a clear increase in the frequency of MCM nuclei staining with Gleason score. The MCMs therefore seems to correlate with a number of other prostate cancer biomarkers, such as the antibody cocktail AMACR(P504S)/34[beta]E12 and p63 (*Ref.*

Section 4.1). There is however, a major difference between this antibody cocktail and MCM2, 5 and 7 and this is because when using the AMACR(P504S)/34[beta]E12 and p63 antibody cocktail there are positive/negative markers for neoplasia as well as positive/negative markers of BPH, therefore this cocktail containing AMACR(P504S)/34[beta]E12 and p63 maybe slightly more advantageous than using the MCMs collectively. Leav *et al*, (2003) found that by using P504S, p63 or 34[beta]E12 it was possible to define areas of transition from hyperplasia to carcinoma, Molinie *et al*, (2004) found that a combination of P504S and p63 lead to a reduction in the risk of false negatives, reducing the percentage of ambiguous lesions that would normally required additional biopsies and Jiang *et al*, (2005) found that a cocktail containing 3 antibodies (AMACR(P504S)/34[beta]E12 and p63) is a simple and easy assay that could be used for routine diagnosis of prostate cancer.

IHC is not without limitations as false negative and false positive results can occur, and the staining scheme used is highly dependent on the examiners interpretation of the slides. It is also important to extend the analysis of MCM2, as although in this thesis it does not appear to show any diagnostic relevance, it has been stated by numerous other studies to show prognostic potential (*Ref. Section 4.4*). Therefore, if MCM2 is shown to be significantly linked to prostate cancer severity and an increase in staining undoubtedly indicated poor prognosis, this marker may be highly beneficial for implementation during histological analysis as it may indicate the aggressiveness of the disease and therefore the optimal treatment pathway.

Due to the potential usefulness of the tissue result, a study was then performed on urine and blood obtained from the prostatic patients, to identify if a less invasive method for detection of MCMs could be developed. The three methods used were urine pellet protein analysis, urine pellet RNA transcript analysis and blood sample RNA transcript analysis. From these approaches only the first two produced useful results. For the third technique it is suspected that no useful results were obtained as the blood samples were stored incorrectly, so RNA yields were sub-optimal and inconclusive results obtained. Both the translational and transcriptional studies on the urine sediment produced results, with the techniques showing that increased expression of MCMs occurs with disease

progression. These studies may therefore provide a starting platform, from which further analyses can be performed.

From the results shown in chapter five, the mRNA based technique (RT-PCR) reveals increased MCM5 detection for urine sediment taken from prostate cancer patients (*Figure 5.13*). This technique was shown to identify 11/12 cancerous patients and only misdiagnosed 3/23 BPH patients, with the highest rbu reading being detected at Gleason score 6. However, when a statistical analysis was performed on the rbu data for MCM5, only Gleason scores 6 and 9 were actually found to be significantly different to BPH.

There is however, a possibility that as only thirty-five urine pellets are assessed, of which twelve were malignant and twenty three non-malignant the sample size may not have been sufficient. To overcome this, a larger cohort of patients requires assessment to conclusively prove the effectiveness of this as a potential diagnostic method. Not only this, but it would be advantageous if the urine sediment are collected and processed as soon as possible, to reduce loss of cellular number. If this study was repeated and produced similar findings, then MCM5 expression in urine sediment by RT-PCR may provide a potential testing strategy for prostate cancer detection.

In the RT-PCR analysis, the differences were identified semi-quantitatively (rbu recorded) in urine sediment, therefore to identify if a real difference was present between malignant and non-malignant tissues it may be beneficial to perform a real-time PCR. If these results then indicate an amplification difference, between malignant and non-malignant states then it may be possible to develop PCR-based technology to prostate cancer diagnosis.

There are a number of drawbacks to using urine sediment for prostate cancer detection, since the urine sediment will not only contain prostate cells, but will also contain other urogenital cells. These other urogenital cells may interfere with the results, especially if another type of cancer is present. However Murphy *et al* (2001), showed that the

number of prostate cancer cells found in urine sediment is higher in men with prostate cancer.

An mRNA based assay may also be problematic, as exfoliating cells need to be separated from the suspension, RNA needs to be extracted from the cells, and reverse transcription and the polymerase chain reaction need to be performed. This technique can be very time consuming and labour intensive and RNA is prone to both degradation and contamination, which can cause either false positive or negative results. The use of this technique in district hospitals may therefore be difficult, as they may not have adequate facilities, or the budget to perform it. Although PCR analysis can have its drawbacks and is potentially more prone to contamination, and therefore errors, than protein-based techniques, this type of technique is already used in some district hospitals such as Northampton General Hospital where PCR is used to detect cystic fibrosis” (Personal Communication: John O’Donnell, 2005).

Although it may be possible to implement some type of PCR-based technique for diagnosis of prostate cancer, it would be more advantageous if a protein-based technique/assay could be developed, as this would be easier to implement in a hospital-based environment. The protein-based technique (ICC, chapter 5) for this project shows that increased expression of MCM2, 5 and 7 occurs in urine sediment from prostate cancer patients (*Figure 5.3*) compared to BPH patients. When a statistical analysis was then performed, a significant difference was recorded between Gleason scores 8-10 to BPH for MCM2, between Gleason scores 8 and 10 to BPH for MCM5 and between Gleason score 7 and 9 to BPH and healthy volunteers for MCM7 (*Table 5.5*). These results indicate that although the staining ability of urine cells increases with prostatic neoplasia, a significant difference was only seen between high-grades for MCM2, 5 and intermediate/high grade for MCM7. These low statistical differences could be due to small sample size, as large error bars were present showing overlap of scores and only twenty-seven urine pellets were analysed.

To provide further evidence for the usefulness of urine pellet analysis by ICC , a larger cohort with optimal urine sample processing needs to be studied. If subsequent findings

demonstrated a significant difference between malignant and non-malignant conditions, this study would then provide a starting platform for a number of different protein based techniques. Again it is important to notice that urine sediment is not just prostate specific and that other urogenital conditions may be detected by translational studies, using the MCMs. In addition, although the protein-based assay does produce results for MCM5, the results are not as distinctive as those produced using RT-PCR, which is probably because PCR-based methods are more sensitive than histological and cytological studies (Botchkina *et al*, 2005).

As the ICC results showed an increase in staining of MCM2, 5 and 7 in urine samples from prostate cancer, it would also be expected that if a protein extraction was performed followed by MCM analysis of some description, MCM2, 5 and 7 protein expression would also be higher in cancerous patients than BPH patients. This would allow an ELISA or similar technology that detects MCM2 and/or 5 and/or 7 to be developed and implemented for the diagnosis of prostate cancer. This type of immunoassay has already been developed for MCM5 in urine sediments, and the results indicate that an increase in MCM5 expression occurs in urothelial cancer (Stoeber, *et al* 1999). Therefore, if a protein based assay using all three MCMs can be developed and is shown to detect differences in MCM2, 5 and 7 between prostate cancer and BPH, this technique could potentially lead to a new diagnostic procedure for prostate cancer. The development of a sensitive lateral flow assay could be one possibility which is very easy to use in a clinical setting. Any such assay would however require some method to exclude results from urogenital cells, or a prostate specific marker could be used to ensure prostatic cells are present. The technique must then be able to indicate the difference not only between BPH and adenocarcinoma, but also between Gleason scores.

A protein-based assay would be very advantageous for the diagnosis of prostate cancer in a hospital environment. Not only are these techniques quick and easy to use by dipping into the patient's sample, but they are less prone to degradation than RNA and DNA-based assays, and also have a low risk of contamination compared to RNA/DNA based techniques.

There are however a number of limitations to using MCMs and the major limitation is the fact that MCMs are not prostate specific and identify any type of urogenital proliferation. Therefore, when considering the implementation of the MCMs for the diagnosis of prostate cancer, it is extremely important that this is taken into account and it would therefore be impossible to implement the MCMs as a prostate cancer specific diagnostic technique on its own. Examination of a biopsy would still be essential. Therefore this technique using the MCMs may play a role alongside PSA to help clinicians to decide if a biopsy is required, or be implemented as a technique that diagnoses all types of urogenital cancer. In this respect, MCMs seem less advantageous than other prostate markers, such as uPM3™ which can distinguish BPH from prostate cancer and is also prostate specific so will not pick up other urogenital cancers. UPM3™ is the first urine based genetic test that is highly specific for detecting prostate cancer (Bostwick *et al*, 2006), the assays shows excellent clinical performance and its specificity is far superior to tPSA (Tinzl *et al*, 2004).

In summary, the work from this thesis provides further evidence of the problems with PSA with the *in vitro* results showing that PSA it is not specific or sensitive at identifying prostate cancer, being expressed and secreted by numerous other non-prostatic cell lines of which three were of female origin. The IHC results show that BPH expresses similar levels of PSA when compared to cancerous tissue and that serum PSA analysis suggests that PSA serum measurement misdiagnosis around 40% of prostate patients.

Chapter three suggests that MCM2, 5 and 7 may perform more roles in prostate cells than just replication of DNA, and that in high grade prostate cancer cells (PC-3), reduced expression of MCMs are detected compared to 'normal' prostate cells (PNT1A). The tissue results indicate that MCM5 and 7 are markers with potential use in histopathology providing diagnostic potential, while MCM2 is less conclusive as a diagnostic marker, but has been shown by other studies to have prognostic potential.

Finally the clinical analysis reveals that by performing a translational study using MCM2, 5 and 7 and by performing a transcriptional study using MCM5 from urine sediment, a less invasive diagnostic method could be developed within the limitations that MCMs are not prostate specific tumour markers and may also detect any urogenital cancers. This however could be advantageous, as it would mean more than one urogenital site can be assessed for neoplasia using the MCMs in urine sediment, as long as a subsequent differential diagnostic strategies allow the locality of neoplasia to be identified.

Final Conclusion and Suggestions for Future Work

The work presented in this thesis has adhered to the aims and objectives set out in the introduction. The results suggest that MCM2, 5 and 7 are increasingly expressed in malignant conditions compared to non-malignant conditions in prostatic tissue, and that MCM5 and 7 may have diagnostic potential, whilst MCM2 does not appear to have significant diagnostic potential and has been suggested by other studies to have prognostic potential. The study of these molecules in clinical samples may therefore aid the histopathologist in allocating Gleason score and therefore hopefully minimise ambiguous results.

The work performed in cultured cell lines shows that MCM expression is less in prostatic adenocarcinoma (PC-3) than 'normal' prostatic (PNT1A) cells, therefore the MCMs are suggested to play more roles within cells than just replication of genetic material, and their function is more complex than initially believed.

The preliminary results for the clinical samples (Blood and urine) show that a protein based technique to detect MCM2, 5 and 7 and an RNA based technique to detect MCM5 may provide further information to aid in the diagnosis of prostate cancer and other urogenital tract problems. In addition, the current diagnostic technique based on serum PSA measurement has been shown to have a number of problems as non prostatic cell lines/female urine sediment are shown to express and secrete PSA, non-malignant tissue is shown to express PSA, and serum PSA testing causes misdiagnosis of around 40% of patients.

The work in this thesis has emphasised the importance of developing new diagnostic techniques for prostate cancer, due to the problems with PSA and PSA serum measurement and highlights the potential use of MCMs for prostate cancer diagnosis.

A large amount of additional clinical and molecular work has been suggested as a result of this project and is summarised below:

Clinical Studies: MCMs for Diagnosis

MCM2 and 5 need to be investigated in a large cohort of urine samples by translational means, while MCM5 needs to be investigated in a large cohort of urine samples by transcriptional means. By increasing sample size, it should be possible to accurately determine the usefulness of detection of these markers as diagnostic techniques. It may also be beneficial to perform the transcriptional and translational analysis on patients that have prostatic massage performed before voiding urine, to increase dislodgement of prostatic cells, which may in turn increase the signals obtained.

For the clinical use, it is important that urine samples are processed within one hour of voiding; this will ensure that the maximum cellular number and viability are obtained. In addition, a study of age-matched healthy volunteers versus clinical patients would be beneficial.

As MCM expression was found to be inconclusive for blood samples, it would also be beneficial to perform this part of the study again, ensuring samples are stored and processed correctly, so that the optimal RNA yields/quality are obtained.

By continuing the work in this thesis in the manner suggested above, it may be possible to develop a protein/molecular based technique which identifies prostate cancer in either blood or urine samples.

Molecular Studies: Function of the MCMs

As prostatic cells have expressional differences between cancerous and normal cell lines, it may be beneficial to investigate whether the MCMs play more roles than just DNA replication.

This type of analysis could be undertaken by performing a number of techniques:

- 1) The cells could be synchronised by chemical blockers instead of FCS deprivation, to stop the cellular cycle at particular phases. The protein from such cells could then be extracted at these particular phases, and analysed either by immunoblot or a 2D

gel electrophoresis, to help quantify differences in amounts seen between normal and malignant conditions. These protein extractions could also be fractionated into their nuclear and cytoplasmic components, so that when the protein is assessed between the two cell lines, the locality of MCMs' expression can also be taken into account.

- 2) Cells could again be synchronised either by FCS deprivation or chemical blockers, and other cellular cycle proteins could be assessed, such as Cdt1 (loading factor), Geminin (cell cycle/MCM inhibitor), and/or ORC proteins (loading factor). Assessing these molecules may give further clues into the alterations in the cell cycle of cancerous cells compared to normal cells as well as clues into the MCMs role and requirements during the cell cycle..

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