# Quantitative Reverse Transcriptase Polymerase Chain Reaction in the Molecular Staging of Prostate Cancer

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**List of Abbreviations:**

| | |
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| BM | Bone marrow |
| BPH | Benign prostatic hyperplasia |
| CaP | Carcinoma of the prostate |
| CEA | Carcinoembryonic antigen |
| CK | Cytokeratin |
| CMC | Circulating melanoma cell |
| CT | Computed tomography |
| Ct | Critical threshold |
| CTCs | Circulating tumour cells |
| DHT | Dihydrotestosterone |
| DNA | Deoxynucleic acid |
| dNTP | Deoxynucleotide-triphosphates |
| DRE | Digital rectal examination |
| EBRT | External beam radiotherapy |
| ECM | Extracellular matrix |
| ERSPC | European randomised screening for prostate cancer |
| EORTC | European Organisation for Research & Treatment of Cancer |
| EZH2 | Enhancer of Zeste homologue 2 |
| FCM | Flow cytometry |
| FISH | Fluorescent in situ hybridisation |
| GAPDH | Glyceraldehyde-3-phosphate |
| GPI | Glycosylphosphatidylinositol |
| HK | Human kallikrein |
| HRPC | Hormone refractory prostate cancer |
| IMRT | Intensity modulated radiotherapy |
| KLK | Kallikrein |
| LH-RH | Luteinizing hormone releasing hormone |
| LN | Lymph node |
| LoD | Limit of detection |
| MRC | Medical Research Council |
| mRNA | Messenger ribonucleic acid |
| MRI | Magnetic resonance imaging |

**List of Abbreviations (continued)**

| | |
|---|---|
| PB | Peripheral blood |
| PcG | Polycomb group |
| PECs | Prostate epithelial cells |
| PET | Positron emission tomography |
| PIN | Prostatic intraepithelial neoplasia |
| PLCO | Prostate, Lung, Colorectal & Ovary |
| PLND | Pelvic lymph node dissection |
| PSA | Prostate specific antigen |
| PSCA | Prostate stem cell antigen |
| PSAD | Prostate specific antigen density |
| PSMA | Prostate specific membrane antigen |
| qRT-PCR | Quantitative reverse transcriptase polymerase chain reaction |
| RNA | Ribonucleic acid |
| RP | Radical prostatectomy |
| RT | Reverse transcription |
| RTOC | Radiation Therapy Oncology Group |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SWOG | South West Oncology Group |
| SCA-2 | Stem cell antigen 2 |
| SEER | Surveillance Epidemiology & End Results |
| TNM | Tumour node metastasis |
| TRUS | Trans- rectal ultrasound |
| TYR | Tyrosinase |
| uPA | Urokinase-type plasminogen activator |
| UV | Ultraviolet |

# THE UNIVERSITY OF MANCHESTER

**ABSTRACT OF THESIS** submitted by David G Ross for the Degree of MD and entitled Quantitative Reverse Transcriptase Polymerase Chain Reaction in the Molecular Staging of Prostate Cancer. August 2007.

Prostate cancer (CaP) is the most common cancer in men in the UK and its incidence is increasing. The natural history of the disease is very variable, in some men progressing rapidly while in others it will run a more indolent course. In early disease localised to the prostate, radical treatment options offer potential cure however these come with considerable potential morbidity and a significant proportion of patients will relapse despite such interventions. This suggests the presence of microscopic disease beyond the prostate, not clinically detectable using current staging modalities.

The presence of circulating prostate epithelial cells (PECs) in the peripheral blood (PB) or bone marrow (BM) of men with CaP may represent micrometastatic disease and thus be indicative of systemic cancer spread and potentially a poor outcome from localised therapies or conservative surveillance protocols. Reverse transcriptase polymerase chain reaction (RT-PCR) can detect the presence of such cells through amplification of the prostate specific messenger RNA that they express. Studies to date have however produced conflicting results.

In this study, molecular staging using RT-PCR was approached by designing and optimising a quantitative methodology, targeting five prostate markers, PSA, PSMA, PSCA, HK2 and $DD3^{PCA3}$, to detect PECs in both PB and BM. Assay *in vitro* sensitivities were 1 PEC/1ml of PB PSA, 10 PECs PSMA, 1000 PECs PSCA, 1000 PECs HK2 and 100 DD3 RNA molecules /1ml PB for $DD3^{PCA3}$.

Samples from 98 men with clearly characterised CaP and disease free controls were evaluated. Only $DD3^{PCA3}$ was entirely prostate specific. Marker expression was seen in control samples for the other markers although satisfactory specificity was achieved, except PSCA, by the application of marker expression thresholds. Clinically, PSA and HK2 appeared the most promising targets. PSMA utility was limited by its specificity, particularly in BM, while $DD3^{PCA3}$ was detected in only eight patients. General trends of increasing marker expression were seen with increasing clinical disease stage. Greatest mRNA yields were seen in men with advanced disease and patterns of high marker expression were seen to be associated with the hormone escaped state, both metastatic and metastasis free. None of the assays could differentiate between localised and locally advanced cancers or identify poor prognosis groups in early disease. The combination of data from multiple markers and paired PB and BM sampling improved overall target yield, yet their more extensive evaluation was limited by poor clinical sensitivity and issues of specificity.

Quantitative RT-PCR is a potentially useful tool for the molecular detection of circulating tumour cells in CaP. In this study positive RT-PCR status was associated with clinically advanced and hormone escaped disease. Assay sensitivity and specificity limited its utility in localised cancers, although only long term clinical follow up will clarify the significance of PECs in these patients. The use of multiple markers, quantitative techniques and two site sampling are interesting additions to standard methodologies although large studies with long follow up, using robust methodologies will be required to define their utility.

**Declaration**

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

David G Ross
MB ChB MRCS(Eng)

**Copyright Statement**

Dedicated to GG and ESR

For understanding, loving and believing in me

"And then to sleep with a prayer for the beloved in your heart
and a song of praise upon your lips."

Kahlil Gibran

The Prophet, On Love

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# Chapter 1: Introduction

## 1.1 Prostate Cancer

### 1.1.1 Prostate Structure and Function

The prostate is a glandular pelvic organ situated between the pelvic floor inferiorly and the base of the bladder superiorly (figure 1.1). It is pierced posteriorly by the two ejaculatory ducts which open into the prostatic urethra as it traverses the gland. The prostate is enclosed by a capsule composed of collagen, elastin and smooth muscle.

The function of the prostate is as an exocrine gland expelling 1.5 to 2 ml of prostatic secretions into the seminal plasma during ejaculation, the role of which is in liquefaction of semen[1].

### 1.1.2 Incidence

Prostate cancer (CaP) is the most common cancer in men in the UK with 24,700 new cases being diagnosed and accounting for 9,800 deaths annually [2]. It typically affects men beyond middle age and although mortality from CaP remains relatively constant its incidence is increasing. This continuing increase in prevalence reflects changes in western population demographics, greater disease awareness, improved diagnostic techniques and the increased use of prostate specific antigen (PSA) as a screening tool. Some of these factors have certainly resulted in more early stage disease being detected which is of particular importance as these patients can undergo potentially curative treatment.

### 1.1.3 Aetiology

The risk of prostate cancer, particularly early onset disease, is strongly affected by family history and hereditary susceptibility is considered the strongest risk factor. Studies suggest that 5% to 10% of all prostate cancer and as much as 30% to 40% of early onset disease is a result of familial disease. A number of putative chromosome loci have been mapped (HPC1, PcaP, HPCX, CAPB, HPC2, HPC20). However no significant genes have been cloned [3].

Some dietary components have been consistently shown in epidemiological studies to be associated with CaP. High dietary intake of calcium, fatty acids and red meat are associated with an increased risk of CaP while phyto-oestrogens, lycopene, selenium and vitamin E may have some degree of preventative action [4].

### 1.1.4 Demographics

There are differences in the incidence of CaP between both ethnic populations and countries, with the highest incidence being among African-American men in the USA and the lowest levels in Asia especially China. Migration studies suggest that these are real ethnic differences and not simply environmental or health care dependent. For example when Japanese men move from Japan to the USA, the incidence of prostate cancer increases but only to about 50% of the rate in white Americans and to 25% of that for African-Americans [5].

The incidence of CaP rises markedly with increasing age in men beyond late middle age. The Cancer Research UK statistics from 2003 (figure 1.2), demonstrate that around 85% of all cases of CaP were diagnosed in men over the age of 65 [2]. With UK population longevity increasing CaP will prove to be a progressively significant health care burden.

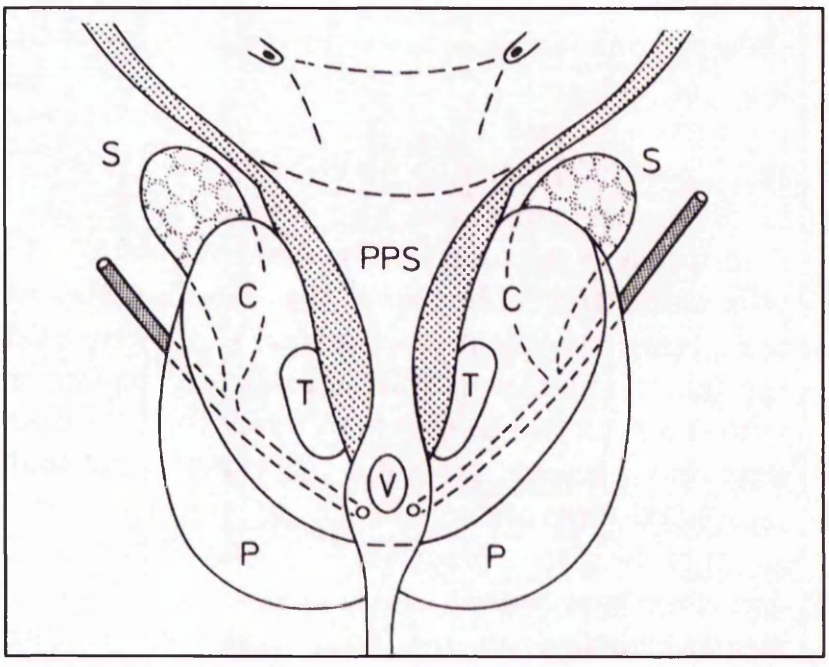



**Figure 1.1:** Zonal Anatomy and Relations of the Human Prostate. (P) peripheral zone, (C) central zone, (T) transitional zone, (S) seminal vesicles, (PPS) pre-prostatic sphincter, (V) verumontanum.

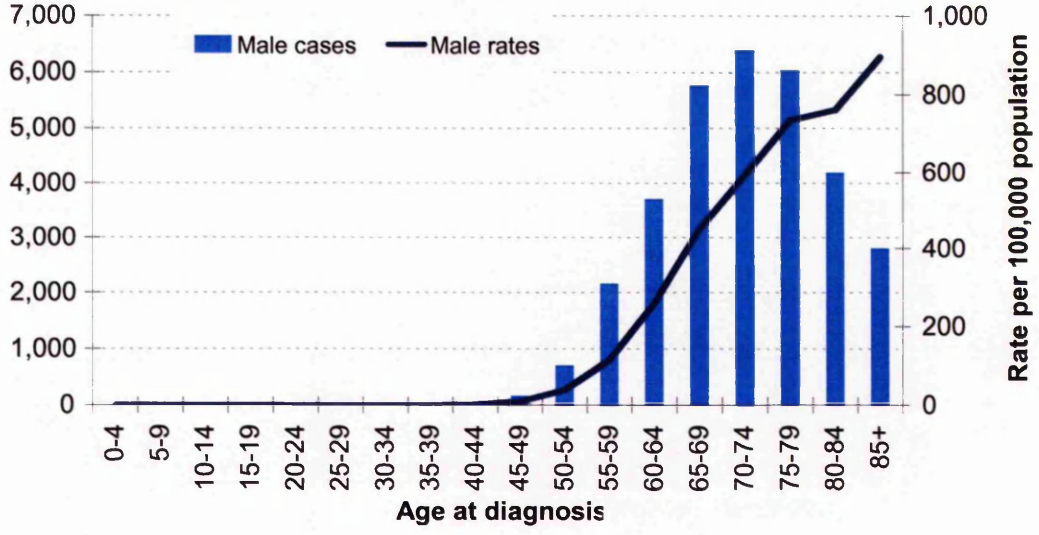



**Figure 1.2:** Number of new cases and age specific incidence rates for CaP in the UK in 2003 [2].

### 1.1.5 Stage at Diagnosis

In the pre- PSA era George [6] (1988) reported that 58% of patients presented with metastatic CaP. With the introduction of serum PSA measurements the proportion of patients presenting with metastases has fallen. In their 1997 study Johansson *et al* [7] observed that 25% of men presented with metastases while 47% had localised disease and 28% locally advanced disease.

### 1.1.6 Natural History

The natural history of CaP is notoriously variable and difficult to predict. Some men have purely incidental disease that runs a very passive course, while others have far more aggressive CaP resulting in metastases and death.

Necropsy studies have highlighted the high incidence of asymptomatic incidental prostate cancers in elderly males dying from other causes [8,9]. This has long been recognised: Franks [10], in 1954, proposed the theory of latent carcinoma of the prostate, i.e. disease with low malignant potential that remains sub-clinical for the lifetime of the host. Others have however stressed the malignant potential of this incidentally diagnosed disease [11,12].

A number of groups have looked at the natural history of CaP in patient groups managed by watchful waiting. George [6] studied the natural history of patients free from metastatic disease (M0), observing a disease specific survival of 80% and 75% at 5 and 7 years respectively, compared with 13% at 5 years for those presenting with metastases. Although only 11% developed metastases, 84% had palpable local progression. A high rate of clinical progression was also reported by McLaren *et al* [12], in untreated patients with clinically localised disease. After 2 years 40% of patients with T1 disease and 51% of patients with T2 disease had clinically detectable progression. Following a further year, 60% of those with T2 disease had progressed. Similarly Adolfsson *et al* reported a 67% five year local progression rate in expectantly managed M0 cancers [13]. The risks of developing metastases and CaP specific death were again low in this series.

Adolfsson *et al* also studied 11 500 men diagnosed between 1965 and 1993 with clinically localised CaP within the Finnish Cancer Registry [14]. They concluded that around half of men with localised cancers will die from other causes and 30% have disease that will run an indolent course. Of those surviving beyond 10 years 5% will eventually succumb to CaP.

Johansson *et al* [7] reported on the fifteen year outcome in all patients presenting with CaP. The disease specific mortality for the whole group was 31%. However this was heavily weighted by those with metastases at presentation, 78% of whom died from CaP. Patients with metastasis negative locally advanced disease were hormone manipulated at presentation and thus their disease natural history was not elucidated. For men with clinically localised disease managed by watchful waiting, 33% progressed, 13% developed metastases and 11% died from their disease. Compared with patients over 80 years, men younger than 61 years at diagnosis were far more likely to develop metastases (36% Vs 5%), and die from their disease (44% Vs 25%). This highlights the great importance of age and co-morbidity on clinical course.

Aus *et al* [15] suggested a more aggressive disease natural history in their study of the cause of death in 514 men with M0 CaP. Over 50% of patients died of their disease in the first 10 years and 63% of those who survived beyond 10 years would eventually succumb to CaP. The methodology of this study however selected a cohort containing a high proportion of high grade cancers and when the data was adjusted accordingly, only those men with poorly differentiated disease had a low disease specific survival.

Thus disease stage and grade has a significant bearing on disease natural history. Chodak *et al* [16] performed a pooled analysis of 828 patients with clinically localised disease from six non-randomised studies. Men with poorly differentiated disease had a ten year disease specific survival of 34% compared to 87% for those with well or moderately differentiated CaP. Similarly metastasis free survival for men with low grade disease was 81% versus only 26% for high grade cancers. These findings were confirmed by Lu-Yao and Yao[17] who utilised the Surveillance Epidemiology and End Results (SEER) database to evaluate 19,898 men

managed conservatively over a ten year period. They showed a ten fold increase in the risk of dying of grade 3 CaP compared with grade 1 disease, with ten year disease specific survival rates of 93%, 77% and 45% for grades 1, 2 and 3 respectively.

These studies observing the natural history of CaP represent disease diagnosed prior to the advent of widespread PSA testing. There is no published long term survival data for the PSA-era. Thus the utility and accuracy of these historic series in contemporary practice remains to be determined. PSA use has introduced a significant lead time estimated at between 3 and 14 years depending upon disease grade [18,19]. Cancers are therefore being diagnosed at a far earlier stage and often treated with aggressive radical therapy despite the knowledge that a significant proportion would not significantly impact on the patient if managed conservatively. The importance in clearly determining the natural history of PSA detected CaP cannot be overemphasised. Achieving this may however be hampered by modern approaches to CaP management where, expectant monitoring has tended to be reserved for those with a limited life expectancy.

## 1.2 Presentation and Diagnosis of Prostate Cancer

### 1.2.1 Presentation

Traditionally the majority of patients diagnosed with CaP would present with lower urinary tract voiding symptoms due to bladder outflow obstruction. George [6] (1988) reported that 90% of CaP patients were referred for treatment of prostatic symptoms or relief of acute or chronic urinary retention. In contemporary practice a much greater number of men are diagnosed following a serum PSA estimation either as part of the assessment of mild voiding symptoms in primary care or as a screening procedure.

Formal PSA screening is not established clinical practice in the UK and the evidence for its introduction remains inconclusive. Bartsch *et al* observed that the fall in CaP mortality was significantly higher in the Tyrol region, where PSA screening was freely available, compared to the rest of Austria where it was not [20].

Conversely Lu-Yao *et al* could show no significant difference in CaP mortality between high and low PSA screened populations [21]. Both of these reports lack the potency of well designed trials and it is hoped that the three ongoing prospective randomised studies ERSPC (European Randomised Screening for Prostate Cancer), PLCO (Prostate, Lung, Colorectal and Ovary) and the UK ProTecT trials will give a definitive answer to the screening issue.

### 1.2.2 Diagnosis

The diagnosis of prostate cancer requires acquisition of a history, examination and special investigations. Patient history may detect lower urinary tract symptoms or symptoms due to more advanced disease, while family history may identify patients at increased risk of CaP. The crux of the physical examination is digital rectal examination (DRE) which allows assessment of the prostate and ano-rectum.

Initial investigations include an estimation of serum levels of PSA. Its use increases CaP detection by 81% compared to DRE alone [22]. PSA is however organ rather than disease specific, yet despite its lack of cancer specificity its use has radically improved the diagnosis of early stage CaP and enables men with clinically undetectable disease to be identified.

Most patients with either a raised PSA or abnormal DRE findings proceed to trans-rectal ultrasound (TRUS) guided biopsy of the prostate. Biopsy tissue is assessed histologically to confirm or exclude the diagnosis of CaP. A negative biopsy cannot absolutely exclude the diagnosis of CaP and patients with raised PSAs, particularly those at an increased risk, should be followed up appropriately with serial PSA estimations and repeat biopsy as indicated. In men with a serum PSA between 4 and 10 ng/ml, Djavan *et al* detected cancers in 10% of repeat biopsies [23].

### 1.2.3 Pathology

#### 1.2.3.1 Adenocarcinoma

The histological cell type of most prostate cancers is adenocarcinoma. The majority of these develop in the peripheral zone of the gland[24] and over 85% of tumours are multifocal [25]. Tumours are graded using the Gleason scoring system, which describes five levels of disorder of cellular architecture as shown in table 1.1 and figure 1.3. Prostate cancers are invariably heterogenous and the Gleason score is stated as the two predominant areas of cellular architecture to give a sum score (minimum 2 and maximum 10) [26].

#### 1.2.3.2 Prostatic Intraepithelial Neoplasia

Prostatic Intraepithalial neoplasia (PIN) consists of architecturally benign prostatic ducts and acini lined by cytologically atypical cells [27]. High grade PIN displays moderate to severe dysplasia and is regarded as the most likely precursor lesion of many intermediate to high grade prostatic adenocarcinomas [28]. When carcinoma is present there is an increase in the size and number of PIN foci, and with increasing amounts of high grade PIN there are a greater number of multifocal carcinomas [27]. The incidence of carcinoma on repeat biopsy after an initial diagnosis of isolated high grade PIN is between 22% [29] and 100% [30].

| Gleason Grade | Histological Features |
|---|---|
| Grade 1 | Small uniform glands with minimal nuclear changes |
| Grade 2 | Medium sized acini separated by stromal tissue but more closely arranged |
| Grade 3 | Marked variation in glandular size and organisation and general infiltration of the stroma |
| Grade 4 | Marked cellular atypia and extensive infiltration |
| Grade 5 | Sheets of undifferentiated cancer cells |

**Table 1.1:** Features of Gleason Grades [26].

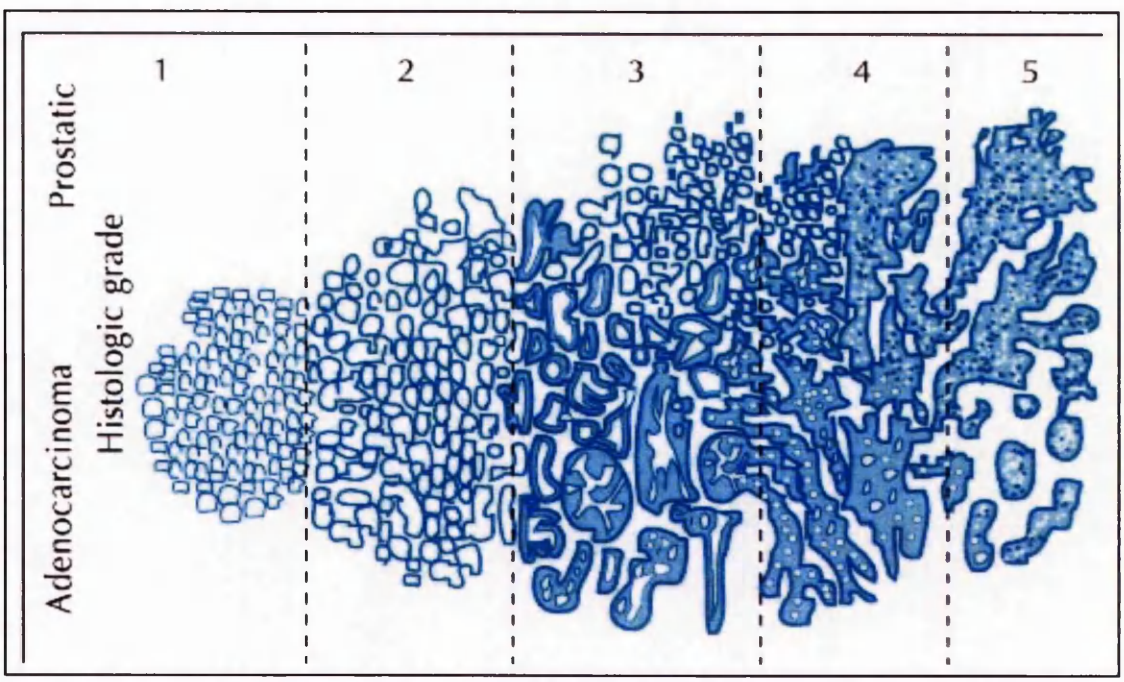



**Figure 1.3:** The Histological Architecture Grades Used in the Gleason Scoring System for CaP.

## 1.3 Prostate Markers

### 1.3.1 Prostate Specific Antigen (PSA)

PSA is an androgen regulated 33 KDa serine protease and a member of the family of human tissue kallikreins located on chromosome 19 [31]. It was first isolated and purified by Wang *et al* in 1979 [32]. PSA is primarily produced by prostate ductal and acinar epithelium and its function is in liquefaction of the seminal coagulum by its action in the fragmentation of seminogelin [33].

PSA is mainly prostate specific although it is expressed at very low levels in breast, thyroid, salivary gland and lung tissues [34]. However, PSA is organ not disease specific, being expressed by normal prostate epithelial cells, benign prostatic hyperplasia (BPH) and overexpressed by malignant epithelial cells. Therefore the presence of PSA in male blood serum is normal but defining the level at which PSA becomes indicative of cancer is extremely complex. Elevations in serum PSA are seen with adenocarcinoma of the prostate as well as in benign conditions including BPH, prostatitis or following urinary retention. Ejaculation [35], prostatic massage [36], prostate biopsy [37] and urethral instrumentation have also been shown to elevate PSA levels. Historically serum levels of less than 4.0 ng/ml were considered as normal. However, contemporary practice has moved towards the use of age specific PSA ranges as detailed in section 1.3.1.2.

#### 1.3.1.1 Clinical Utility of PSA

Despite its lack of prostate cancer specificity, PSA is still currently the best tumour marker available in clinical practice, with increasing serum levels correlating with an increasing risk of prostate cancer. Of men with a serum PSA between 4 and 10 ng/ml, 22% will be found to have prostate cancer on biopsy, with this figure rising to 67% for those with a PSA above 10 ng/ml [38]. Thus over 75% of men with a PSA between 4 and 10 ng/ml will have negative biopsies and conversely around 22% of men with a PSA <4 ng/ml will be harbouring CaP [39]. The risk of CaP at these low PSA levels falls with diminishing serum PSA being 27% between 3.1 and 4.0 ng/ml and 6.6% at 0.5 ng/ml or less [40]. This illustrates both the utility and limitations of PSA in diagnosing CaP. A number of approaches have been investigated to address these issues of specificity and to attempt to make serum

PSA a more discriminating tumour marker for detecting clinically significant cancers.

#### 1.3.1.2 Age Specific PSA

The inter-relationships between serum PSA, patient age and prostate volume were investigated by Oesterling *et al* [41]. PSA increased with both age and prostate volume, while volume also correlated directly with patient age. This resulted in the establishment of age-specific PSA reference ranges; for men aged 40 to 49 years, <2.5 ng/mL; for 50 to 59 years, <3.5 ng/mL; 60 to 69 years, <4.5 ng/mL; and 70 to 79 years, <6.5 ng/mL. Subsequently the appropriateness of age specific PSA has been questioned since it significantly increases the number of negative biopsies performed in the lower age range while failing to diagnose a significant proportion of cancers in men in their 60s and 70s [42]. Whether these cancers were clinically significant or not remains to be determined.

#### 1.3.1.3 Free PSA

The majority of serum PSA is complexed to proteins, predominantly alpha1-antichymotrypsin [43], and it has been demonstrated that as the percentage of free PSA decreases the probability of CaP increases. In a retrospective study, Catalona *et al* [44] claimed to reduce the number of negative biopsies by 31% while still diagnosing over 90% of cancers by using a percentage free PSA level of 23% as cut off for biopsy. More recently, percentage free PSA has been shown to predict CaP development in patients with a serum PSA between 4 and 10 ng/ml [45], and to improve CaP detection in high risk populations with serum PSA levels within normal limits [46].

#### 1.3.1.4 PSA Velocity

Despite variability in serial serum PSA measurements in men with no evidence of CaP, a PSA velocity, the rate of change of serum PSA, at a level greater than 0.75 ng/ml per year is strongly suggestive of a diagnosis of CaP [47]. Lynn *et al* [48] have more recently demonstrated the utility of short term PSA velocity in predicting prostate biopsy histology, while Fang *et al* [49] have identified a significant association between PSA velocity and the risk of CaP in men with serum PSA <4ng/ml.

PSA velocity may also have a utility in predicting treatment outcome following surgery and radiotherapy for CaP. D'Amico *et al* showed that a pre-treatment PSA velocity of >2ng/ml/yr was associated with a higher risk of CaP specific and overall mortality in men undergoing RRP [50], and for patients with both high and low risk CaP being treated with EBRT [51]. In hormone escaped metastatic cancer being treated with cytotoxic, cytostatic or combination therapy, an increasing PSA velocity has been shown to be significantly associated with shorter survival [52].

#### 1.3.1.5 PSA Density

As stated previously serum PSA increases with increasing prostate volume. PSA density (PSAD) is the ratio of serum PSA to the volume of the prostate. The principle of PSAD is that in a patient with a high PSA associated with a small gland the PSA production cannot be attributed to BPH, thus this is more likely to indicate CaP. In a prospective study of 700 patients Catalona *et al* [42] using PSAD cutoff of 0.078 could detect 95% of cancers with a specificity of 19%. An inverse relationship between free PSA and PSAD was also noted and indeed the two measures performed almost identically, the advantage of free PSA being in the avoidance of TRUS measurements and the inter-observer variability associated with this.

#### 1.3.1.6 Role of PSA in Staging of CaP

This is detailed in section 1.5.1.2

#### 1.3.1.7 Role of PSA in Patient Monitoring and Surveillance

Serial serum PSA measurements are the mainstay of monitoring patients following potentially curative treatments, during active surveillance and watchful waiting, and in monitoring response to hormone manipulation. Following radical prostatectomy PSA levels are expected to become undetectable since all PSA producing prostate tissue has been excised. Indeed a detectable serum PSA is indicative of a poor outcome. Radiotherapy treatment leaves a prostate remnant *in situ* and thus PSA remains detectable at low levels. As is discussed in section 1.6.1 the PSA nadir level is the baseline for subsequent PSA measurements. In patients managed by watchful waiting, hormone therapy is withheld until the serum PSA reaches a

threshold level. This value is not clearly defined in the literature. However, in clinical practice it is between 20 and 30 ng/ml. The role of PSA in active surveillance protocols is detailed in section 1.6.1.9.

### 1.3.2 Other Prostate Markers

#### 1.3.2.1 The Human Glandular Kallikrein Family

The 15 members of the human kallikrein gene family are a sub-group of the serine protease family, whose genes are localised to chromosome 19q13.4 and their genes and proteins have a number of common structural features [53]. Human kallikreins 2, 3, 4 and 15 are differentially expressed in CaP.

##### 1.3.2.1.1 Human Kallikrein 3 (PSA)

See section 1.3.1

##### 1.3.2.1.2 Human Kallikrein 2 (HK2)

HK2 is a serine protease with trypsin-like enzymatic activity, which is secreted into seminal fluid. It has an 80% sequence homology with PSA (Human Kallikrein 3). Its expression has been found to be increased in prostatic intraepithelial neoplasia (PIN) and to a greater degree in prostate cancer [54]. Furthermore, HK2 expression was shown to be associated directly with the Gleason grade of the primary tumour. Foci of prostate cancer metastatic to the lymph nodes have been found to demonstrate the highest level of expression [55].

##### 1.3.2.1.3 Human Kallikrein 4 (HK4)

Using RT-PCR, the mRNA coding for this 22 KDa protease has been detected predominantly in benign and malignant prostate tissue although low levels can be seen in testicular, mammary, adrenal, uterine and thyroid tissues [56,57]. At the mRNA level HK4 expression is higher in benign than malignant prostate [58]. Its expression by the LNCaP cell line is androgen sensitive and it is not seen in the androgen independent cell lines PC-3 and DU-145 [56].

##### 1.3.2.1.4 Human Kallikrein 15 (HK15)

The kallikrein 15 (KLK) gene has a 41% similarity with KLK3 and encodes for the 28 KDa protease HK15. This is expressed in thyroid, prostate, salivary and

adrenal glands as well as the colon, testis and kidney [59]. Its expression is hormonally regulated, being up-regulated by steroid hormones, particularly androgens, in LNCaP cells [59]. HK15 is up-regulated in over 80% of prostate tumours compared with matched benign prostate and there is a trend towards up-regulation in more advanced and aggressive tumours [60].

#### 1.3.2.2 Prostate Specific Membrane Antigen (PSMA)

PSMA is a 100 KDa glycoprotein that is located on the plasma cell membrane of normal and prostate cancer cells [61]. It is predominantly prostate specific although low, yet significant, amounts have been detected in normal salivary gland, brain and small intestine [62,63]. Wright *et al* [64] demonstrated upregulation in high grade primary tumours and in both lymph node and bone metastases. Its expression in the commonly used prostate cancer cell lines is limited to the androgen sensitive LNCaP [62]. PSMA expression is hormone sensitive, being downregulated in LNCaP cells by dihydrotestosterone, and indeed its mRNA expression in CaP is highest in hormone deprived states [62].

#### 1.3.2.3 Prostate Specific Stem Cell Antigen (PSCA)

PSCA is a glycosylphosphatidylinositol (GPI) anchored glycoprotein expressed on the cell surface. Its closest homologue, stem cell antigen 2 (SCA-2), is a marker of immature thymic lymphocytes. It is predominantly prostate specific, although a low level of expression has been seen in placenta, kidney and small intestine (around 1% of the level seen in prostate). Within normal prostate tissue, *in situ* hybridisation demonstrates that PSCA expression is heterogeneous but restricted predominantly to a subset of normal basal cells [65]. Tran *et al* [66] suggest that PSCA marks the transition between a transformation susceptible highly proliferative state and a more differentiated state and may therefore identify a unique subpopulation of cells. It is expressed in high grade PIN, suggesting that up-regulation of PSCA is an early event in carcinogenesis, and in greater than 80% of prostate cancers, including both androgen dependent and independent tumours. The androgen receptor negative, androgen independent prostate cancer cell lines PC3 and DU145 also showed PSCA expression [65].

Compared with normal prostate, PSCA is over-expressed in around 40% of tumours. The level of tissue PSCA expression correlates with grade, stage and progression to androgen independence. Comparison of paired primary tumours and bone metastases demonstrated increased expression in the bone samples and up to 87% of bone metastases express PSCA [67] [68].

#### 1.3.2.4 $DD3^{PCA3}$

$DD3^{PCA3}$ is a non-coding gene whose mRNA expression is extremely prostate specific [69]. Moreover, De Kok *et al* [70] and Hessels *et al* [71] demonstrated a median upregulation in its expression of between 34 and 66-fold from benign to malignant prostate tissues. Its expression in CaP metastases remains undetermined. CaP cell line expression is limited to LNCaP cells [69]. Importantly for its use in RT-PCR studies utilising peripheral blood samples, $DD3^{PCA3}$ mRNA could not be detected in leukocytes [70]. Hessels *et al* [71] recently described a $DD3^{PCA3}$ based molecular urine analysis technique which demonstrated a 67% sensitivity and 83% specificity in diagnosing CaP in men with a PSA >3 ng/ml.

#### 1.3.2.5 Hepsin

Hepsin is a 51 kDa transmembrane serine protease which is highly expressed in the liver and to a lesser extent in the prostate, pancreas, lung, thyroid, and pituitary [72]. It is up-regulated in prostate cancers, being expressed only at very low levels in the luminal epithelia of benign glands [73]. At the mRNA level, Stephan *et al* [74] observed hepsin overexpression in 90% of primary cancers compared with matched benign tissue. Molecular profiling of Gleason 4/5 tumours showed Hepsin to be the most up-regulated gene with an average 34 fold increase compared with BPH and its expression increases with increasing malignant tumour phenotype [75] [76].

#### 1.3.2.6 Prostasin

Prostasin is 40 kDa GPI-anchored serine protease [77] ubiquitously expressed in human prostate, liver, salivary gland, kidney, lung, pancreas, colon, bronchus and kidney although not by lymphocytes and polymorphonuclear cells. Prostate glands and seminal vesicles show over 20-fold higher prostasin levels than any other tissue [78]. While expression is maintained in LNCaP cells, it is downregulated in the

invasive androgen insensitive cell lines DU-145 and PC-3. Restoration of expression in the two latter cell lines reduces their *in vitro* invasive capabilities [79]. Takahashi *et al* [80] demonstrated that prostasin expression was inversely related to prostate tumour grade and was significantly lower in metastatic and hormone refractory cancers.

### 1.3.2.7 Urokinase-Type Plasminogen Activator (u-PA)

u-PA is a serine protease which forms part of the plasminogen activation system. The role of u-PA and various other proteases in the degradation of extracellular matrix (ECM), an essential step in tumour cell metastasis, has been widely reported and reviewed [81]. Specific to prostate cancer, u-PA is highly expressed in the invasive prostate cancer cell lines PC-3 and DU-145, but not by the non-invasive LNCaP cells [82]. Using an *in vitro* co-culture system, Hart *et al* [83] demonstrated that the inhibition of u-PA reduced prostatic cell colony growth in bone marrow stroma. Van Veldhuizen *et al* [84] showed increased expression of u-PA in 70% of primary tumours with extracapsular extension compared with 27% of localised cancers, while Kirchheimer *et al* [85] reported 1.5 times u-PA upregulation from primary tumour to the matched bone metastasis. Primary tumour u-PA expression has also been shown to correlate with the presence of bone metastases and cause specific survival [86].

### 1.3.2.8 Enhancer of Zeste Homolog 2 (EZH2)

EZH2, a Polycomb Group (PcG) protein, is the human homologue of the *Drosophila* protein Enhancer of Zeste. PcG proteins repress gene expression, silencing genes at the level of chromatin structure [87]. EZH2 is highly expressed in early embryogenesis but its expression decreases as cells differentiate [88]. In the prostate, amounts of EZH2 mRNA and protein are increased from normal prostate through primary tumours to metastatic prostate cancers [89]. Varambally *et al* [89] demonstrated that in patients with clinically localised CaP, those with primary tumours which expressed higher concentrations of EZH2 showed a worse prognosis. Rhodes *et al* [90] used combined primary tumour EZH2 and E-cadherin status to predict failure following radical prostatectomy. EZH2 is expressed by the cell lines LNCaP, PC-3 and DU-145 [89].

## 1.4 Staging of CaP

### 1.4.1 Staging System

Prostate cancer is staged using the tumour, nodes and metastasis (TNM) system as detailed in table 1.2 [91]. This describes the extent of local tumour, loco-regional lymph node involvement and the presence of distant tumour metastases. The tumour (T) staging is divided into impalpable T1 disease, palpable disease that remains within the confines of the prostatic capsule, T2, and disease that is more locally advanced T3 and T4. The most important distinction is between localised disease, T1 and T2, and more advanced disease.

## 1.5 Current Staging Modalities

The aim of clinical staging is to predict the true pathological tumour stage, identifying those whose disease is truly confined within the limits of the prostate.

### 1.5.1 Staging of Local Disease

#### 1.5.1.1 Digital Rectal Examination

Although DRE is the standard technique for the clinical assessment of extent of local disease it has been shown to underestimate pathological stage in a significant proportion of patients [92]. The sensitivity of DRE in predicting organ confined disease is around 50% in all patients and lower in those with a serum PSA < 4ng/ml [93].

| TNM | Description |
|---|---|
| **T1** | **Clinically inapparent tumour not palpable or visible by imaging** |
| T1a | Tumour found by chance in <5% excised tissue |
| T1b | Tumour found by chance in >5% excised tissue |
| T1c | Tumour confirmed by needle biopsy |
| T0 | No local tumour detectable |
| **T2** | **Tumour confined within the prostate** |
| T2a | Tumour limited to half one lobe or less |
| T2b | Tumour spread to half or more of one lobe but not both |
| T2c | Tumour has spread to both lobes |
| **T3** | **Tumour extends through the prostatic capsule** |
| T3a | Extracapsular extension (unilateral or bilateral) |
| T3b | Tumour invades one or both seminal vesicles |
| **T4** | **Tumour attached to or invaded adjacent structures other than the seminal vesicles** |
| **N** | **Regional lymph nodes** |
| Nx | Loco-regional lymph nodes cannot be evaluated |
| N0 | No lymph node involvement |
| N1 | Regional lymph node metastasis |
| **M** | **Distant metastases** |
| Mx | Distant metastases cannot be evaluated |
| M0 | No distant metastases |
| M1 | Distant metastases present |
| M1a | Non-regional lymph node(s) |
| M1b | Bone(s) |
| M1c | Other site(s) |

**Table 1.2:** TNM Staging System for CaP [91].

#### 1.5.1.2 PSA

Serum PSA levels have been shown to correlate strongly with pathological stage particularly at levels below 4 ng/ml and above 20 ng/ml [94]. In radical prostatectomy series 75% of men with a preoperative serum PSA of 4 or less will have truly localised disease however this is the case for only 53% with a PSA between 4 and 10 ng/ml [92]. For levels in the intermediate range there is considerable overlap between stages thus limiting PSA's utility as an independent staging tool in this range.

#### 1.5.1.3 Trans Rectal Ultrasound Scan

Classically prostate tumours appear as hypoechoic areas in the peripheral zone of the prostate on TRUS. However, its utility in staging and diagnosis is greatly restricted as only 60% of tumours are visible. The staging accuracy of TRUS reported in the literature is between 46% and 63% [95-97].

#### 1.5.1.4 Computed Tomography (CT)

CT has no significant advantage over TRUS in local staging and in distinguishing T2 from T3 disease it has sensitivity, specificity and accuracy of 30%, 79% and 51% respectively [98].

#### 1.5.1.5 Magnetic Resonance Imaging (MRI)

Body coil MRI again is no better at staging the extent of local disease than TRUS [95,96]. The use of endo-rectal coil MRI improves resolution of imaging however its utility remains limited by poor sensitivity and a high false positive rate [99].

#### 1.5.1.6 Multivariate Analysis

In an attempt to address the limitations of individual preoperative variables in predicting pathological stage Partin *et al* [92] combined three variables, PSA, Gleason score and clinical stage on DRE using logistic regression analysis to construct nomograms. Their ability to predict pathological stage in men with clinically localised CaP was validated in a multi-centre study, the correct prediction for organ confined disease, isolated capsular penetration, seminal vesicle invasion and lymph-node metastases were 67.3%, 59.6%, 79.6% and 82.9% respectively

[94]. More recently these nomograms have been updated to reflect CaP in a contemporary population [100].

### 1.5.2 Lymph Node Metastases

Lymph node metastases are present in up to 25% of patients presenting with CaP depending on stage. The incidence of lymph node involvement in patients with clinically localised disease has been reported at between 2% and 6% [100,101]. Staging modalities available to assess loco-regional lymph node involvement are surgical, open or laparoscopic pelvic lymph node dissection (PLND), CT, MRI and positron emission tomography (PET).

PLND is the most accurate and reliable technique but also the most invasive and therefore associated with morbidity. Standard CT and MRI, although non-invasive, depend on size criteria for the detection of lymph node disease thus limiting sensitivity. The sensitivity of CT in detecting lymph node metastases is 25% to 75% [102,103]. Levran *et al* reported that of 409 men undergoing radical prostatectomy, 15 had pathological lymph node involvement despite all having negative preoperative CT scans [104]. Two dimensional MRI techniques hold little advantage over CT. However, Jager *et al* reported a three dimensional technique with a sensitivity and specificity of 75% and 98% respectively [105]. The use of superparamagnetic nanoparticles in conjunction with high resolution MRI improved sensitivity to 90.5% with specificity of 97.8% however this technology remains in the research arena [106]. PET has not been widely used in prostate cancer staging however de Jong *et al* found $^{11}$C-choline PET to be more accurate in direct comparison to conventional CT and MRI, reporting a sensitivity of 80%, a specificity of 96% and an accuracy of 93% [107].

The low positive yields and expense of all the above techniques make their use appropriate only in those at high risk of lymph node metastases [108].

### 1.5.3 Detection of Bone Metastases

Distant metastases in prostate cancer most commonly arise in bone. Radionuclide bone scintigraphy remains the most widely used technique to assess the skeleton for metastases due to its combination of sensitivity, availability and ability to evaluate the entire skeleton. However, skeletal changes detected on scintigraphy are not CaP specific and may require further confirmatory investigations. It has also been highlighted that routine bone scanning is inappropriate in many low risk asymptomatic patients, the positive yield being <5% for all men with a PSA between 4 and 20 ng/ml and <2% if associated with a Gleason score <6 [108,109]. Gleason score, PSA and clinical stage are all predictors of a positive bone scan [110]. MRI has also been shown to be sensitive and specific in the detection of bone metastases, and indeed, it may detect small metastatic deposits and those without cortical involvement earlier than bone scintigraphy [111].

## 1.6 Treatment of CaP

Appropriate management of patients with CaP is primarily dependent on accurate clinical staging as only those with truly localised (T1-T2) disease have potentially curable cancer. Treatment options are also influenced by other patient and disease factors.

### 1.6.1 Localised CaP

The treatment options for men with organ confined prostate cancer are radical prostatectomy, radical radiotherapy, active surveillance and watchful waiting with delayed hormone manipulation. Only prostatectomy and radiotherapy, encompassing external beam radiotherapy (EBRT) and interstitial radiotherapy (brachytherapy), are therapies with curative intent.

#### 1.6.1.1 Comparison of Treatments with Curative Intent

There is intense debate regarding treatment choices in localised disease and indeed studies have shown significant speciality bias in treatments used [112]. There are very few randomised trials directly comparing the treatment modalities and

these have been criticised for their design. Surgery, external beam radiotherapy and brachytherapy have been shown to be equivalent treatment options by reports comparing single treatment series and retrospective analyses but these are hampered by patient group selection bias, differing study end points and the fact that only surgical series have pathological staging data [113-115].

### 1.6.1.2 Radical Prostatectomy

Radical prostatectomy (RP) is a surgical procedure to remove the prostate gland, the contained tumour and the seminal vesicles. It can be performed via the retropubic, transperineal or laparoscopic routes. It is only a potentially curative operation if the disease is localised with no extracapsular spread or seminal vesicle involvement. Despite improvements in surgical technique [116], there is a finite mortality risk and a significant risk of complications particularly urinary incontinence (<10%) and erectile dysfunction (10-90%). Incontinence may be caused by direct myogenic sphincter damage or sphincter denervation due to damage to the somatic and autonomic branches of the pudendal and pelvic nerves at the apex of the prostate. Damage to the cavernosal nerves results in impotence. Thus RP is not commonly performed in men with a life expectancy of less than 10 years or those with significant co-morbidities.

Over the past decade in the UK there has been a twenty fold increase in the use of RP [117] and in a randomised trial a significant disease specific survival advantage of RP over watchful waiting has been demonstrated, relative risk 0.56, as well as a 14% lower incidence of metastasis formation in the surgically managed group [118,119].

### 1.6.1.3 Outcome following Radical Prostatectomy

Metastasis free survival rates at 10 and 15 years post surgery of 74%-87% and 61%-82% respectively have been reported [120-122]. However between 17% and 35% of men undergoing RP will experience biochemical recurrence (PSA >0.2ng/ml) with long term follow up [120,123,124]. Pound *et al* [121] investigating the natural history of PSA recurrence, showed that 34% of patients with biochemical recurrence would progress to develop metastases at a median time of 8 years,

with both the time until and the likelihood of this occurring being dependent on time to PSA failure, tumour Gleason score and PSA doubling time.

#### 1.6.1.4 Prediction of Outcome

A number of factors have been identified that can predict disease progression following RP; high pre-operative PSA [125,126], high percentage of biopsy core involved with tumour [127], high tumour grade [126,128], extracapsular extension [126,128-130], seminal vesical invasion [130,131], positive surgical margins [128,130,131] and lymph node metastases [125,131,132]. Further to this, nomograms utilising pre- and post-operative parameters can be used to determine biochemical recurrence free survival probabilities for men with clinically localised CaP undergoing RP [133].

It can thus be seen that outcome is very much dependent upon accurate pre-operative disease staging. This has been shown to be inaccurate as currently 20-30% of patients have their disease upstaged following pathological assessment of surgical specimens [134-136]

#### 1.6.1.5 External Beam Radiotherapy

EBRT is a non-invasive potentially curative treatment modality for localised CaP. Traditional standard dose delivery of 60-70 Gy is associated with higher rates of biochemical failure than dose ≥72 Gy [137]. Initial implementation of dose escalation was predictably limited by dose toxicity, since conventional EBRT used only square or rectangular 'non-anatomic' fields, thus large volumes of non-target tissues received high doses of radiation [138]. Conformal radiotherapy directly images the prostate using CT or MRI scanning and subsequent to this the individual radiation beams are shaped or conformed to produce a three dimensional patient specific volume for treatment. This optimises irradiation treatment dose to the target cancer while minimising the dosage to normal sensitive tissues and allowing dose escalation [139]. Intensity modulated radiotherapy (IMRT) is an advanced form of conformal radiotherapy which allows increased doses of over 80Gy to the target.

Patients undergoing EBRT for CaP are at risk of gastrointestinal, genitourinary and sexual side effects, given the close proximity of the prostate to the rectum, bladder, neurovascular bundles and penile erectile tissue. In a cohort of 377 patients from the European Organisation for Research and Treatment of Cancer (EORTC) trial 22863, who received a dose of 70 Gy, 23% experienced Radiation Therapy Oncology Group (RTOG) grade ≥2 urinary or intestinal complications. There were only 4 (1%) treatment related deaths and 5% of patients experienced grade 3 or 4 late toxicity [140]. With regards erectile function, Beard *et al* reported that although the use of conformal irradiation reduced sexual side effects compared to conventional EBRT, 20% of patients in this group still reported no erections and 60% inadequate erections [141].

Radiation dose is a significant determinant of treatment outcome however prognostic indicators such as serum PSA, biopsy Gleason score and clinical stage should be considered in treatment planning. Pollack *et al* found that dose escalation from </=67Gy to >67-77Gy improved 4-year recurrence free survival for all risk groups with localised CaP, however only those with a baseline serum PSA >10ng/ml benefited from doses >77Gy [142]. In addition to dose escalation, neoadjuvant, concomitant and adjuvant hormone manipulation has also been shown to improve recurrence free and overall survival in high risk patients [143,144].

### 1.6.1.6 Outcome Following EBRT

5-year disease free survival rates for patients with clinically localised disease have been reported of between 65% and 89% [114,145-148]. These figures are dependent on the outcome predictor characteristics of the patients included in each cohort. Zietman *et al* reported overall 10-year disease free survival of 40% for localised disease treated with conventional radiotherapy [149]. Using high dose (81Gy) IMRT Zelefsky *et al* achieved 8-year actuarial PSA relapse-free survival rates for men in favorable, intermediate and unfavorable risk groups of 89%, 78% and 67% respectively [148].

### 1.6.1.7 Brachytherapy

Prostate brachytherapy involves the implantation of radioisotope seeds emitting short-range radiation into the prostate gland under TRUS guidance. The localised dose of radiation has resulted in reports of a lower incidence of rectal and bladder radiation effects than EBRT and improved potency rates compared to RP [150]. Khaksar *et al* reported that 62% of men, potent prior to brachytherapy treatment, remained potent at 2 years, although 60% were using phosphodiesterase inhibitors [151]. In this cohort of 300 patients, there was a 7% acute urinary retention rate, 2.7% going on to require TURP, and 5.7% developed urethral strictures. With regards late toxicity, Zelefsky *et al* reported 7% grade 2 and 1% grade 3 or 4 rectal toxicity, while for urinary toxicity the rates were 19% and 4% respectively [152].

Five-year disease free survival rates of 69% to 96% have been reported for localised disease[114,147,152,153]. Beyer *et al* [153] suggest that outcome is comparable with EBRT for Gleason <7 tumours, while EBRT has an outcome advantage for higher grade tumours.

### 1.6.1.8 Predictors of Outcome Following Radiation Treatment

As outlined previously, pre-therapy factors significantly associated with disease outcome are clinical tumour stage, Gleason score of the biopsy specimen and pre-treatment serum PSA [154,155]. The most significant predictor of long term outcome is the level of the post treatment PSA nadir. In Critz *et al's* series, patients achieving a post treatment nadir of 0.5 ng/ml or less had a 5 year disease free survival of 93% compared with only 26% for those achieving a nadir of 0.6 to1.0 ng/ml [156]. Following PSA failure, PSA doubling time has been demonstrated to predict time to prostate cancer specific death [157].

### 1.6.1.9 Active surveillance

Active surveillance is a strategy that aims to select only those men with significant cancers for curative therapy, reducing the burden of treatment related side effects without compromising survival. Clinically, this entails, close monitoring with early radical treatment in those with signs of progression. Patients managed with active surveillance must be suitable candidates for radical curative treatment, with low or intermediate risk disease.

The described patient monitoring protocols are varied. Hardie *et al* [158] used serial serum PSA measurements combined with clinical evaluation (DRE), while Choo *et al* [159] also performed repeat prostate biopsy. Similarly, there is no consensus on the definition of disease progression and the criteria for intervention. Biochemical, clinical and histological indicators of progression have all been used. These include a short PSA doubling time, local progression on DRE and pathological upgrading on repeat biopsy [158,159]. At 5 years, 79% of the Royal Marsden cohort of 80 patients remained treatment free and there had been no CaP deaths[158], while 66% of the larger Canadian cohort remained on active surveillance with a disease specific survival of 99.3% at 8 years [160]. Further large studies with long term follow up will help to define the role of active surveillance in the management of CaP; identifying suitable patient populations and standardising protocols, with clear criteria for intervention.

### 1.6.1.10 Watchful Waiting

Watchful waiting entails having no immediate treatment but subsequently undergoing delayed hormone manipulation at the time of disease progression. The appropriate use of this management regimen is controversial and reflects the uncertainty regarding disease natural history highlighted previously. There are few randomised trials comparing treatment with curative intent with watchful waiting for localised CaP and reported studies of conservatively managed patients often select older patients with low grade disease.

Bill-Axelson *et al*, in comparing RRP with expectant management for clinically localised disease, showed a 5% CaP specific survival advantage as well as more substantial reductions in local and metastatic disease progression in the surgically

treated group [119]. As was noted with natural history studies, the study patient population does not reflect contemporary populations. In this cohort only 12% of patients had T1c disease. More recently Parker *et al* used statistical modelling to apply pre-PSA clinical data to current practice [18]. They predicted a survival benefit of less than 1% for radical treatment over conservative management for men with low grade screen detected CaP, although greater advantages were seen in those with higher grade cancers.

The definitive answer regarding the role of 'watch and wait' will only be known with the reporting of contemporary series, yet it is clear that patient and disease factors must be taken into consideration including patient age, disease stage and degree of differentiation. In modern urological practice CaP is being detected in younger men at earlier stages. Aus *et al* [15] found that in men younger than 65 at the time of diagnosis of non-metastatic CaP, 75% of those managed without curative intent would subsequently die from CaP. In a study evaluating 451 men with localised CaP, Albertsen *et al* [161] showed that survival in low grade disease was not significantly different from the general population however men with higher grade tumours (Gleason scores 5 to 10) experience a progressively increasing loss of life expectancy.

### 1.6.2 Management of Locally Advanced Disease

EBRT remains the treatment of choice for patients with locally advanced non-metastatic CaP (T3/4Nx M0). The addition of neoadjuvant hormone ablation has been shown to be associated with an improvement in local control and a lower incidence of distant metastases [144]. Bolla *et al* demonstrated a relapse free survival advantage of 78% versus 40% for radiotherapy with concomitant/adjuvant hormone therapy compared to radiotherapy alone [162]. Although watchful waiting has previously been advocated in this group, a Medical Research Council (MRC) trial, comparing immediate versus deferred hormone therapy in patients with locally advanced and asymptomatic metastatic disease, reported advantages in the immediate treatment group in terms of disease progression, development of metastases, local and distant complications and survival [163].

### 1.6.3 Management of Advanced Disease

Patients with advanced disease can be considered in two groups, hormone naïve or hormone refractory, dependent upon their treatment history.

#### 1.6.3.1 Hormone Naïve CaP

Since Huggins and Hodges' discovery of the hormone responsive nature of metastatic prostate cancer, endocrine manipulation has remained the mainstay in management of advanced disease [164]. Hormone ablation is achieved through the use of luteinizing hormone releasing hormone (LH-RH) agonists, antagonists, anti-androgens, oestrogens or surgical orchidectomy. This is not a curative treatment modality, the disease usually becoming hormone refractory within 18 to 24 months [165]. LH-RH therapy offers a survival advantage over conservative management, disease specific survivals of 26 months compared to 13 months [166]. Again, deferred hormone manipulation is not appropriate in advanced disease [163].

#### 1.6.3.2 Hormone Refractory CaP (HRPC)

HRPC is an incurable disease and patients may face a considerable range of problems consequent upon progressive disease. These include lower urinary tract malfunction, ureteric obstruction, skeletal dysfunction, bone marrow insufficiency, rectal infiltration, lymphoedema and pain. Treatment is thus focused on managing specific disease manifestations as well as general palliative and supportive measures. In addition there are various secondary therapies which may impact on survival or more importantly quality of life. Indeed, any survival benefit of such treatment regimens must be carefully balanced against their potential side effects.

##### 1.6.3.2.1 Second-Line Endocrine Treatment

Following failure of primary endocrine therapy suppression of testicular androgens should be maintained as androgens can continue to promote tumour growth in this setting [167]. Fowler *et al* reported a 50% decrease in PSA levels in over half of patients with progressive disease following orchidectomy [168]. Thus patients whose

primary treatment has been LH-RH agonist monotherapy or orchidectomy, should be commenced on additional anti-androgen therapy.

Disease progression following combined androgen blockade should initially be addressed by withdrawal of the anti-androgen while maintaining testosterone suppression as up to 20% of patients will show a biochemical response [169-171].

#### 1.6.3.2.2 Third-Line Endocrine Treatment

Oestrogens [172], glucocorticoids [173,174] and adrenal androgen inhibitors such as ketoconazole [175,176] have all been used as third line agents with varying degrees of PSA and symptomatic response being reported.

#### 1.6.3.2.3 Chemotherapy

Historically the role of chemotherapy in HRPC has been limited by poor disease response rates compounded by high degrees of treatment toxicity [177]. More contemporary studies have demonstrated a definite role for chemotherapy in appropriately selected patients. Initially combined therapy with mitoxantrone and prednisolone, which is well tolerated, was shown to have a significant palliative, although not survival, advantage over steroid and supportive treatment alone [174,178]. The first studies to demonstrate a survival advantage in HRPC used docetaxel combined with prednisolone [179] or estramustine [180]. Both randomised controlled trials compared outcomes using taxane chemotherapy with established combined mitoxantrone and prednisolone treatment. In the TAX 327 study, although only around a third of patients responded to treatment, the docetaxel plus prednisolone cohort benefited from a median survival advantage of 2.4 months as well as significant improvements in symptom scores, PSA response and quality of life measures [179]. The combination of docetaxel and estramustine used in the SWOG 99-16 trial was more toxic however a survival advantage of 2 months was also shown [180]. Docetaxel chemotherapy is now the chemotherapeutic agent of choice in HRPC however the optimal timing of its use is yet to be determined.

## 1.7 Metastases

### 1.7.1 The Metastatic Process

Metastasis is a multistep, selective process regulated by a number of different mechanisms (Fig 1.4) [181]. It involves a series of sequential steps and only a subset of cells will survive each of these. In the primary tumour, growth must be progressive with extensive angiogenesis for the tumour to become greater than 2mm in diameter. This must be followed by local invasion and intravasation of tumour cells. Cells escaping into the circulation are carried by blood flow to secondary sites where they arrest by size restriction in the capillary beds of the new organ. Exiting the circulation relies on adherence and extravasation processes. The subsequent further development of these tumour cells depends on cell responses to the microenvironment.

This process is highly inefficient with most tumour cells entering the circulation being rapidly eliminated, and less than 0.1% of cells surviving to go on to form metastases [182]. Similarly, it has been estimated that the number of intravenously injected tumour cells necessary to establish distant metastases is $1x10^5$ [183,184]. This is due to the heterogenous nature of primary tumours in which different tumour cell clones from the same primary have different metastatic potential [185].

The formation of metastases is not entirely circulation dependent. Ewing's [186] entirely blood flow dependent model of metastasis highlights the importance of the circulation in tumour cell dissemination yet cannot fully explain the pattern of all tumour metastases. Paget's [187] 'seed and soil' model demonstrated that successful metastasis depends on both the nature of the tumour cell and the environment in which it ends up.

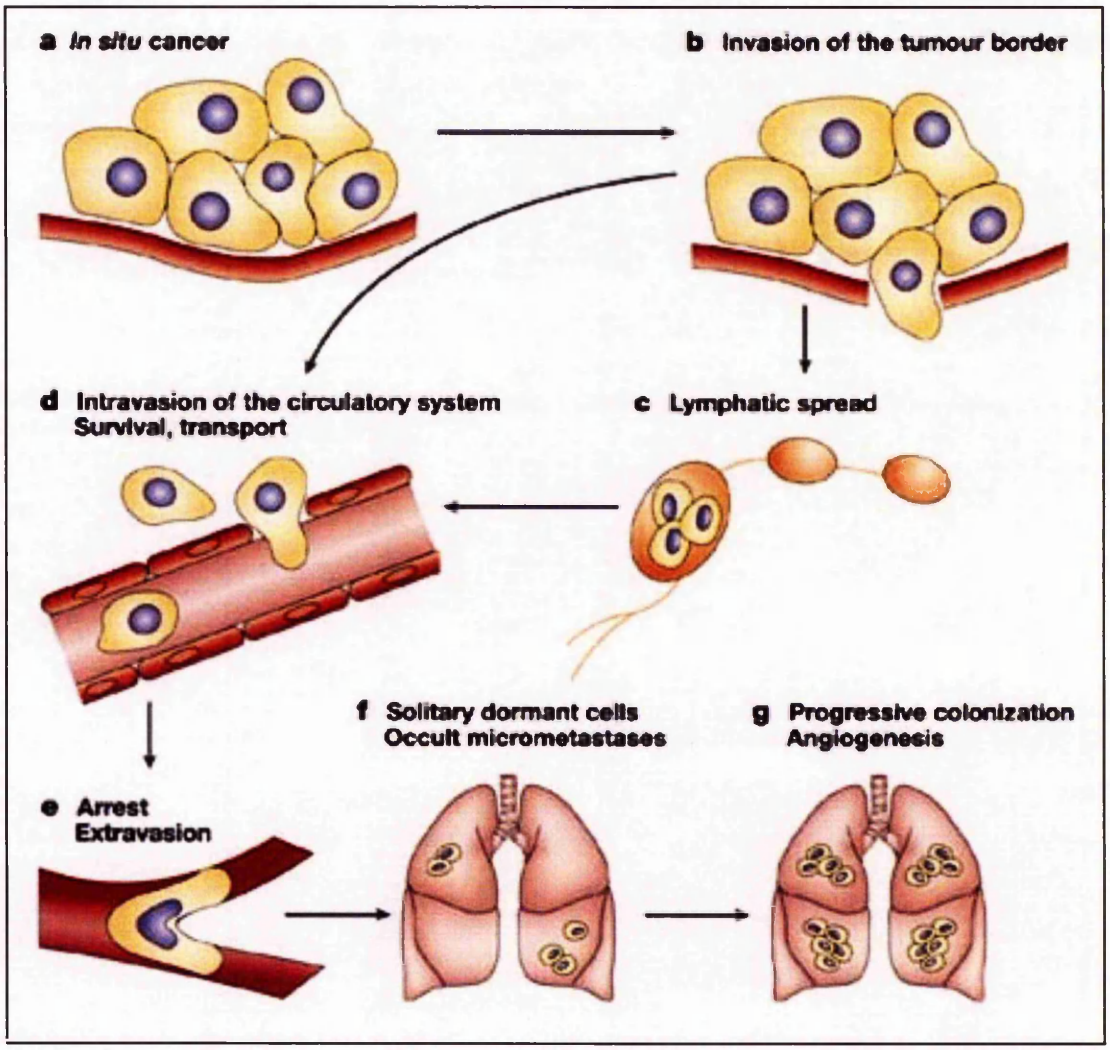



**Figure 1.4:** Schematic diagram of the metastatic process [181].

### 1.7.2 Micrometastatic Disease

It is clear that an early event in the formation of metastases is the dissemination of tumour cells into the circulation. Current standard staging modalities aim to detect established metastases present at secondary sites, a far later stage in the metastatic process, and are limited by their requirement for a significant critical tumour volume. The detection of disseminated circulating tumour cells (CTCs) or 'micrometastases' may be a far earlier sign of metastatic disease and thus have a significant impact on patient outcome.

## 1.8 Detection of Disseminated Tumour cells

Various approaches have been employed in detecting the presence of micrometastases in peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) in a variety of solid tumours. These include immunocytochemistry (section 1.10.1), flow cytometry (section1.10.2) and reverse transcription polymerase chain reaction (RT-PCR). These techniques may be used in conjunction with cell enrichment and extraction techniques such as immunomagnetic enrichment or density gradient centrifugation.

## 1.9 The Reverse Transcription Polymerase Chain Reaction (RT-PCR)

### 1.9.1 Amplification

The polymerase chain reaction (PCR) is a molecular technique that enables the amplification of tiny amounts of specific sequences of nucleic acids. It was first described by Saiki in 1985 [188], in the development of a prenatal diagnostic test for sickle cell anaemia. PCR amplification involves two nucleotide primers that flank the DNA segment to be amplified and repeat cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences and extension of the annealed primers and sequence of interest by a DNA polymerase enzyme. This in essence doubles the amount of the target DNA in each cycle and thus there is exponential accumulation of this fragment. The later addition of a thermostable DNA polymerase isolated from *Thermus aquaticus* simplified the

procedure while also improving both its sensitivity and specificity allowing the amplification of a single target molecule in $10^5$ or $10^6$ cells [189].

The addition of a reverse transcription (RT) step extended the utility of the technique, enabling messenger (mRNA) to be the nucleotide target of interest [190,191]. This was first used in the diagnosis of chronic myeloid and acute lymphocytic leukaemias by the detection of leukaemia specific mRNAs. Figure 1.5 illustrates the principle of the RT-PCR technique in detecting prostate epithelial cells. Total RNA is extracted from the cell including the mRNA message coding for the sequence of interest, ensuring the absence of any genomic DNA. Reverse transcription can be performed to either produce a cDNA bank of copies of all the RNA in the sample using random hexamers or oligo-dt primers, or only cDNA copies of the target gene using gene-specific primers.

For the PCR stage gene specific primers are used. These are often designed to span exon-exon boundaries to prevent the amplification of any genomic DNA contamination. To the same end, a DNase enzyme treatment step may be included in the RNA extraction protocol. A complete absence of genomic DNA is desirable as it may produce false positive results due to amplification of DNA target genes or pseudogenes and its amplification may compete with the efficient amplification of target cDNA.

The advantage of the RT-PCR technique is that it detects only the genes that are being actively transcribed by the cells in the sample, not simply the presence of a genomic sequence.

### 1.9.2 Product Visualisation and Identification

Typically PCR products are separated by agarose gel electrophoresis and visualised using the fluorescent dye ethidium bromide viewed under ultraviolet (UV) illumination. Ethidium bromide intercalates between the stacked bases of DNA and its fixed position and close proximity to the bases causes bound dye to display an increased fluorescent yield compared to dye in free solution. The product size is deduced by comparison to a standard DNA marker run on the same agarose gel.

To ensure that the PCR reaction is amplifying the actual sequence of interest, the product's identity can be confirmed by a number of methods including restriction analysis, DNA sequencing and Southern blotting.

### 1.9.3 Quantitative PCR

Traditional PCR is a purely qualitative end point technique, with product detection and identification occurring at the end of all the amplification cycles. The end point is variable from sample to sample and gels cannot resolve these variabilities in yield. Theoretically there is a quantitative relationship between the amount of starting target and the amount of PCR product at any given cycle number. Various techniques have been used to add a quantitative dimension to PCR. Sokoloff *et al* [192] developed a $P^{32}$ labelled assay, while O'Hara *et al* [193] used quantitative ethidium bromide densitometry but neither has shown clinical value. Alternative methods co-amplify endogenous standards in semiquantitative assays or exogenous standards which are amplified by the same primers as the target molecule with the same amplification efficiency [194]. The results from the latter have demonstrated reliable quantitation of prostate markers.

### 1.9.4 Real Time PCR

Real time PCR allows quantitation through the measurement of product accumulation on a cycle-by-cycle basis. This is achieved by the detection of fluorescence, measured in the exponential phase of the reaction. This is the optimal point for data analysis since at later stages there is greater reaction variability as reagents are consumed. The fluorescence is produced either by SYBR Green or product specific nucleotide probes.

SYBR Green is an intercalating dye that fluoresces following complete polymerisation of the product, binding to double stranded DNA in a non-specific manner. Double dye nucleotide probes consist of a single stranded probe sequence that is complimentary to one of the strands of the amplicon. A fluorophore is attached at one end and a quencher at the other, each of which

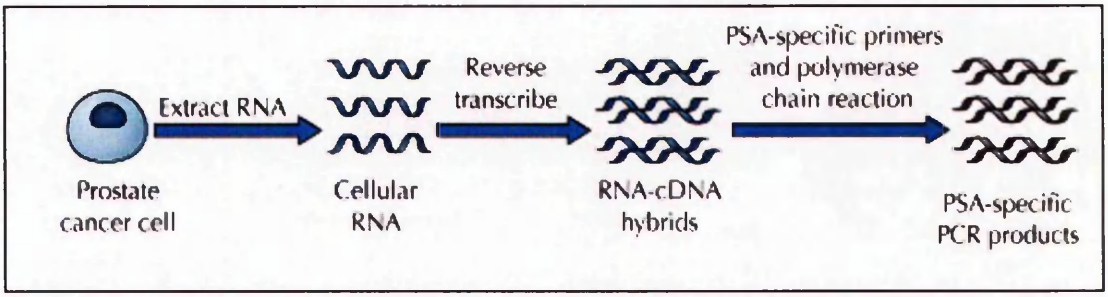



**Figure 1.5:** Schematic representation of RT-PCR detection of mRNA coding for PSA.

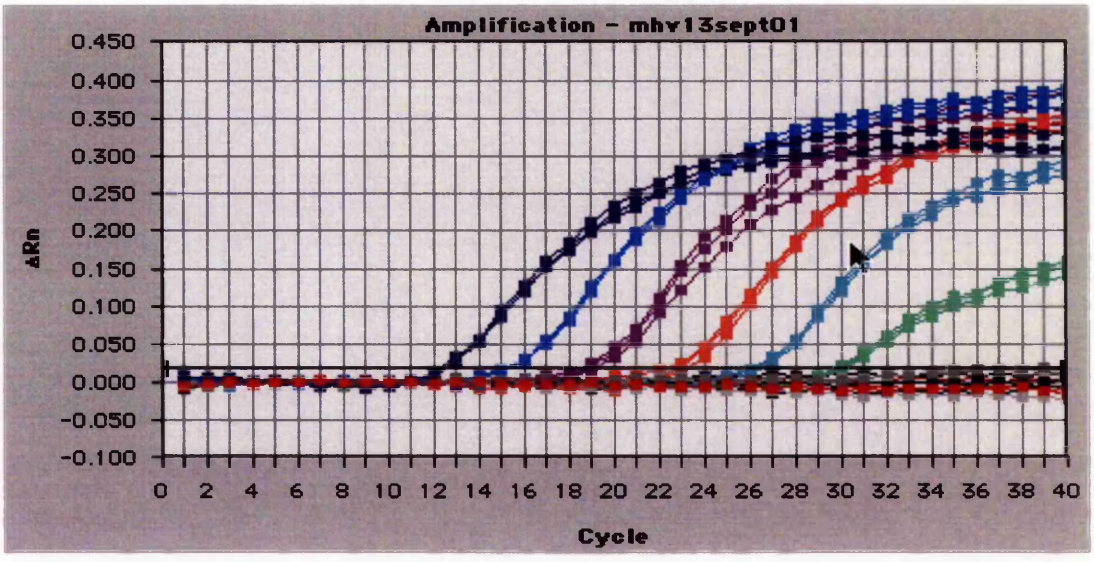



**Figure 1.6:** Real time plot for serial dilutions of input DNA

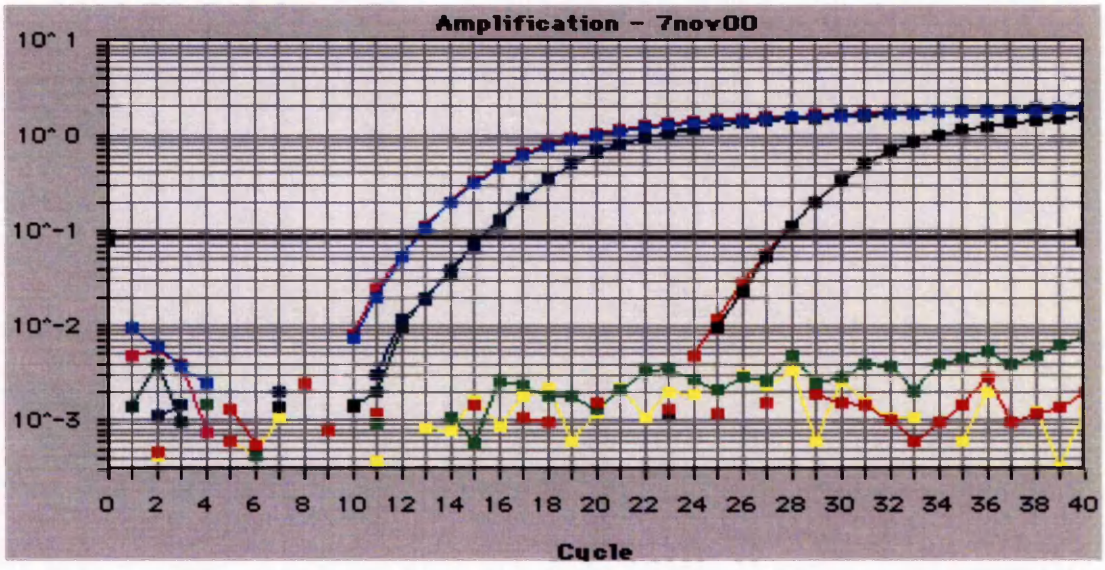



**Figure 1.7:** Typical real time RT-PCR plot - fluorescence plotted on a logarithmic scale

fluoresces at a different wavelength. When joined by the nucleotide backbone only the quencher fluoresces. During the annealing step of the PCR reaction the probe binds to the target amplicon. As the Taq polymerase enzyme extends from the primer it displaces the fluorophore and releases it into solution resulting in irreversible increase in fluorophore fluorescence and decrease in that of the quencher. The incorporation of probes improves assay specificity, avoids non-product signal due to primer dimer and alleviates the need to confirm product identity by product dissociation.

The fluorescent signal detected is recorded during every cycle and represents the amount of product amplified to that point in the amplification reaction. The key to the technique is the Critical threshold (Ct), the first point or cycle at which the signal is first recorded as statistically significant above background [195]. The greater the amount of input target, the earlier the reaction reaches the Ct.

Figure 1.6 shows the real time results for a log dilutional series of input DNA. Each point represents the measured fluorescence for that reaction at the end of each cycle and hence the accumulation of product. The black horizontal line is the threshold value. With increasing input target DNA the curve moves to the left, crossing the Ct at an earlier cycle number.

With fluorescence plotted on a log scale as in figure 1.7, the threshold value is set 10 standard deviations above background, and the effect of differing concentrations of input target on Ct can clearly be seen. The more dilute reactions reaching the critical value at a later cycle. Control reactions such as those represented by the green or yellow plots remain well below the threshold line.

Quantification is achieved in either a relative or absolute manner. Relative quantification relies on the amplification of an endogenous control gene in addition to the gene of interest. The control's expression should be greater than that of the marker of interest and remain constant in proportion to total RNA. Suitable controls are ß-actin, which is expressed in most cell types coding for a cytoskeleton protein, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) a ubiquitously

expressed gene. The use of such 'housekeeping gene' amplification also ensures the integrity and efficiency of the reverse transcription reaction.

Absolute quantification requires an internal standard whose sequence is such that it is amplified by the target specific primers and with the same efficiency as the amplicon of interest. Known numbers of copies are amplified to produce a standard curve to allow quantification in 'unknown' samples.

#### 1.9.4.1 Product Confirmation Using SYBR Green

SYBR Green binding is non-specific, intercalating within any double stranded DNA including primer dimer. The products detected by this method can be observed by performing a melt curve in which the reaction is slowly heated from 40°C to 80°C while continuously monitoring fluorescence. The temperature at which the product 'melts' ($T_m$) is marked by a fall in fluorescence due to SYBR Green dissociation. The $T_m$ of a product is dependent on its length and sequence. Thus different products will melt, or dissociate from SYBR Green, at different temperatures which are displayed as distinct peaks when negative fluorescence is plotted against temperature.

#### 1.9.4.2 Assay Optimisation

RT-PCR assay optimisation entails balancing analytical sensitivity, the ability to detect minute amounts of target in a vast non-specific background, with specificity, avoiding the amplification of 'illegitimately' transcribed mRNA.

Thermocycling conditions can impact on assay performance. Primer annealing is temperature dependent, higher temperatures conferring greater specificity, a high degree of complementarity being required to allow binding in a high energy reaction thus minimising mispriming events. Similarly, hot start reactions prevent the polymerase enzyme binding until the reaction temperature is high enough to prevent mispriming, while two step PCR utilises one high temperature for both annealing and extension thus avoiding the lower temperatures which encourage non-target primer binding. The impact of mispriming on the efficiency of the

desired reaction is greatest during the early amplification cycles as the resultant nucleotide will be amplified throughout the remaining reaction.

The time allowed for product extension should be determined by considering the expected product size and the velocity of action of the Taq polymerase enzyme, which adds around 1000 bases per minute. This prevents the construction of longer false products such as that from the amplification of genomic DNA when using non-exon boundary spanning primers. Increasing the number of amplification cycles will improve the sensitivity of the assay. However, the tiny amounts of illegitimately transcribed RNA produced by background cells or misprimed product may become detectable.

In real time PCR assays nucleotide primers require optimisation. This is achieved by amplifying identical amounts of product using varying primer concentration ratios and identifying the reaction with the lowest Ct value. Probe optimisation is achieved similarly using serial probe dilutions in otherwise identical PCR reactions to determine the probe concentration with the lowest Ct.

The relative concentrations of all other assay components including target RNA, deoxynucleotide-triphosphates (dNTPs), magnesium ions ($Mg^{2+}$), buffers and enzyme can all impact on assay performance and may therefore require optimisation.

### 1.9.5 Use of RT-PCR in the Detection and Staging of Solid Tumours

For RT-PCR techniques to detect a circulating or occult tumour cell, the cell must have specific changes in its mRNA expression that distinguish it from the surrounding environment. Differential mRNA expression is a more common finding and has been investigated for both diagnostic and staging capabilities in melanoma, breast, colorectal, lung as well as CaP. The selection of an appropriate assay target is essential. The features of the optimal cancer cell marker are tissue specificity, cancer specificity and a relative expression relating to the cell's metastatic potential. However, even if the target message is expressed by other cell types within the body, it may be a suitable target as long as it is not normally expressed within the environment being tested.

Mori *et al* studied the detection of carcinoembryonic antigen (CEA) mRNA in LNs and PB of patients with breast and various gastro intestinal cancers. All PB control samples were negative, while 76% of patients who experienced disease recurrence after resections with curative intent were assay positive and the frequency of assay positivity increased with advancing disease stage [196].

In breast cancer, a number of investigators have used CK19, CK20, CEA, mucin-1 and mucin-2 to detect circulating tumour cells but in many their clinical significance has not been clarified and the use of these markers have been hampered by a lack of specificity [197]. Stathopoulou *et al* demonstrated a correlation between the presence of cytokeratin 19 (CK19) positive cells detected preoperatively by RT-PCR in PB samples and poor clinical outcome for patients with stage I and II breast cancer. Despite the presence of occult metastatic cells in the BM being correlated with outcome in a number of studies using immunohistochemical methods [198,199] this medium has not been studied with RT-PCR.

Colorectal cancer studies have utilised RT-PCR in detecting tumour cells in PB, BM and LNs. Markers used include CK20, CEA, mucin-1 and mucin-2. A number of small studies have suggested an association between CK20 positive cells in PB [200] and in paired PB and BM samples [201] and advancing tumour stage as well as outcome. When using paired samples the BM was more often positive than the PB, 31% Vs 17%. In a cohort of 94 patients, Hardingham *et al* demonstrated a correlation between preoperative PB mRNA positivity and reduced disease free and overall survival using a panel of four markers [202].

The clinical significance of circulating melanoma cells (CMCs) has been studied by numerous groups using RT-PCR assays. These have been based on the markers tyrosinase (TYR), p97 and MelanA/MART1 either individually or in multimarker techniques resulting in detection rates between 6% [203] and 93% [204]. In a study of 200 melanoma patients Palmieri *et al* used this panel of markers to detect CMCs in PB [205]. A positive assay result correlated with disease stage, the most common prognosticator in malignant melanoma, but was not found to be an independent prognostic indicator. Others in smaller studies have demonstrated a correlation with outcome [204].

### 1.9.6 RT-PCR in Molecular Staging of Prostate Cancer

Since its first description by Moreno *et al* [206], a considerable number of investigators have studied RT-PCR in the detection of circulating cells in patients with CaP.

Assay *in vitro* sensitivity or limit of detection is usually determined by its ability to detect known numbers of cells from prostate cancer cell lines diluted in a background of nucleated cells, usually leukocytes. An alternative, used mainly in the context of quantitative assays is to use dilutions of synthesised exogenous mRNA targets. The majority of assays achieve a sensitivity of 1 target cell in a background of $10^6$ nucleated cells [207] although a few groups report capabilities of 1 in $10^8$ [208]. In terms of clinical samples this confers limits of detection of 10 cells in a 5 ml PB sample. A typical BM sample may contain a background of up to $10^8$ cells thus requiring greater assay sensitivity.

The clinical sensitivity is determined by the assay's ability to detect circulating cells in samples from men with confirmed metastatic disease, the rationale for this being that all of these men would be expected to have disseminated cells. Reported detection rates however range between 34% [209] and 100% [210] for PB samples and between 56% [211] and 100% [207] for BM.

Assay clinical specificity relies on negative results from samples derived from females or healthy males as has been demonstrated in the vast majority of studies reported. There are exceptions, reporting significant numbers of positive controls [212 213].

The majority of assays have used PB samples although a significant number have used BM, pelvic LNs obtained at the time of surgery or paired PB and BM. Using pre-operative PB samples from a series of men undergoing radical prostatectomy Katz *et al* demonstrated a significant correlation between PSA assay positivity and the presence of capsular penetration and post-operative biochemical recurrence [214,215]. More recently, the prognostic value of RT-PCR in patients managed surgically has again been demonstrated, with assay positivity correlating with PSA

recurrence and a shorter PSA doubling time [216]. In patients managed by watchful waiting McIntyre *et al* demonstrated a significant relationship between PB RT-PCR and PSA progression [217]. Survival in men with hormone escaped CaP can also be predicted by RT-PCR, a positive assay correlating with poor outcome [218].

Using paired samples Corey *et al* detected both PSA and HK2 mRNA more often in BM 71.4% and 41.3% respectively than in PB samples 19% and 12.7% [208]. BM RT-PCR studies have in general shown better correlation with clinical parameters than PB assays. Wood *et al* assessed pre-operative BM samples form men undergoing radical prostatectomy with a PSA RT-PCR assay. Significantly more patients with pathologically confirmed extra-prostatic disease had a positive assay than those with truly localised disease [207]. A subsequent larger study by the same group demonstrated a correlation between PSA positive cell detection in BM and both post-operative serum PSA and pathological stage [219].

PSA has been the most extensively utilised mRNA target although PSMA, HK2 and PSCA have been used in descending frequency. Of the more novel markers, Shariat et al showed a significant relationship between positive preoperative RT-PCR for HK2 in patients undergoing radical prostatectomy and lymph node involvement and pathological Gleason sum. For patients whose disease progressed, a positive assay was associated with aggressive progressive features; PSA doubling time of less than 10 months, failure to respond to salvage DxT, and/or a positive metastatic work up, all of which are associated with an increased risk of developing overt metastases [220]. The only group to have utilised PSCA, Hara *et al,* demonstrated significant differences between organ confined and extraprostatic disease. It was used alongside PSA and PSMA and the findings of their work suggest that PSCA is the most promising RT-PCR marker of the three [221].

The incorporation of multiple markers into a protocol is uncommon however when used increases in positive yields have been reported [208].

Quantitative RT-PCR remains a novel addition to assay techniques. Ylikoski *et al*, using time resolved fluorometry in a quantitative RT-PCR assay for HK2 and PSA,

could differentiate between benign and malignant disease but not between different stages of CaP [222]. Real time RT-PCR was used by Straub *et al* to quantitate PSA mRNA copies in men before and after radical prostatectomy [223]. They demonstrated a significant difference in both expression rate and numbers of copies detected between men with organ confined and those with more locally advanced CaP.

There are however a large number of RT-PCR studies in which, despite good *in vitro* performance, no correlation with clinical parameters or disease course could be demonstrated.

### 1.10 Non-RT-PCR Circulating Tumour Cell Detection

#### 1.10.1 Immunocytochemistry

Immunochemical staining of isolated circulating tumour cells has been investigated by a number of groups. Ts'o *et al* reported recovering around 85% of LNCaP cells spiked into PB samples using density gradient centrifugation and immunofluorescent cell staining with monoclonal PSA antibody [224]. Clinically, 88% of men with metastatic CaP were circulating cell positive. Additionally, the isolation technique used, facilitated subsequent chromosomal analysis using fluorescent in situ hybridisation techniques (FISH). Again using PB, Brandt *et al*, demonstrated the detection of PECs in 8 out of 10 men with CaP and 1 of 4 men with BPH, through a combination of density gradient centrifugation, immunomagnetic enrichment and PSA immunocytochemistry [225].

Mueller *et al* identified PECs in BM samples taken from men prior to RRP through CK8/18 and PSA immunocytochemistry [226]. CK8/18 positive cells were detected in 45% of patients, with a trend towards higher detection rates in pT4 compared to pT2/3 cancers. Although cytogenetic analysis suggested tumour origin for these circulating cells only 12% were PSA immunocytochemistry positive.

Lilleby *et al* targeted cytokeratin positive staining cells in immunomagnetically enriched PB and BM samples of men with non-metastatic CaP prior to and

following radiotherapy [227]. 12% of PB and 20% of BM samples were positive pre-treatment, however only post-radiotherapy $CK^+$ cell status correlated with disease progression free survival.

Cytokeratin staining is used in these techniques to confirm the epithelial nature of the detected cells. Cytokeratins are components of the cytoskeleton of epithelial cells. There are at least 20 different types which are differentially expressed in specific epithelial cell types or in specific pathways of epithelial differentiation. Within the prostate, luminal cells are typically CK8 and CK18 positive while basal cells are CK5 and CK14 positive. The majority of prostate cancers are composed of CK8/18 positive, PSA secreting luminal cells, however, some cells are non-PSA secreting with a basal phenotype, CK5/14 positive [228].

### 1.10.2 Flow Cytometry (FCM)

Compared with the other techniques FCM has been less widely evaluated in its ability to detect CTCs in CaP however there has been greater experience in the breast cancer field. Hamdy *et al* used monoclonal antibody staining and FCM to detect PSA positive cells in PB of patients with newly diagnosed CaP [229]. Detection of such cells correlated with the presence of clinically detectable metastases and was a better predictor of this than serum PSA. More recently Moreno *et al* combined immunomagnetic cell enrichment with FCM to identify $CK^+/CD45^-$ CTCs in men with metastatic CaP [230]. In 26 men with hormone escaped disease the presence of 5 or more CTCs was the strongest independent predictor of survival. Similar predictive value for FCM detected $CK^+$ CTCs has been demonstrated in metastatic breast cancer using a semi-automated methodology [231].

Despite the abilities of these various techniques in detecting CTCs and the associations with clinical course demonstrated by some groups, conflicting results have prevented any such technique becoming established in routine clinical practice. The complex nature of metastasis formation may be the key to this since the simple presence of cells in the circulation does not necessarily result in metastasis formation at distant sites. Whole cell extraction has the advantage of

allowing detailed evaluation of the detected cells for viability and phenotypic characteristics predictive of behaviour. RT-PCR however has been shown in comparative studies to be the most sensitive technique [232].

### 1.11 Clinical Dilemmas in Prostate Cancer

1. Current staging modalities tend to under stage disease. Thus a significant number of men inappropriately undergo radical forms of treatment and are exposed to the risks associated with these, when the true pathological extent of their CaP precludes cure.

2. The natural history and clinical course in each individual cannot currently be accurately predicted. This results in significant numbers of patients being both over and under treated and thus exposed to unnecessary potential mortality and morbidity.

### 1.12 Aims of Research

I. To develop a quantitative real time TAQman RT-PCR assay for reliable detection and quantification of circulating prostate epithelial cell (PEC) mRNA in the blood and bone marrow of patients with CaP using a panel of five prostate specific markers.

II. To correlate quantitative PEC marker profiles in the blood and bone marrow with stage of disease.

III. To correlate PEC mRNA marker profile with current predictors of disease outcome.

IV. To validate commercially developed micro fluidic real time RT-PCR methodology compared to laboratory designed assays.

V. To evaluate the utility of the novel prostate markers HK4, HK15, EZH2, uPA, prostasin and hepsin in detecting circulating PECs using commercially designed micro fluidic RT-PCR assays.

## 1.13 Rationale for Aims

The clinical implications of the presence of circulating PECs in men with CaP have not been definitively determined. Although numerous studies have been reported, the conclusions are conflicting. Methodologies lack standardisation and described techniques remain unvalidated. RT-PCR offers the most sensitive CTC detection technique although it precludes specific study of individual intact tumour cells. Despite novel prostate molecular markers being identified, few have been evaluated in RT-PCR assays. Advances in RT-PCR technology present theoretical solutions to the inherent problems of traditional techniques, yet few have been incorporated into assays designed to detect circulating cells.

Real time RT-PCR methodology provides a number of potential advantages over standard RT-PCR techniques. Most importantly it provides a quantitative value for the target of interest and avoids downstream product processing.

Quantitative RT-PCR adds an additional dimension to molecular cell detection which has not been extensively investigated to date. A quantitative value may allow the determination of limits of marker detection that reflect significant numbers or 'types' of circulating cells which will influence outcome and disease natural history. Additionally it may provide a solution to assay specificity issues resulting from illegitimate transcription, as low level expression can be excluded by applying expression thresholds to quantitative data.

Automated product identification makes the process quicker, less labour intensive and by excluding a separate product identification step, reduces a potential source of error. Therefore, real time RT-PCR is a more practical proposition for large studies and as a viable clinical tool in a commercial laboratory.

The incorporation of a panel of prostate markers maximises cell detection, addressing the heterogeneous nature of tumour cell populations. It may also highlight which cell marker or combination of markers is associated with a certain outcome. The combination of multiple markers and quantitation may allow a

patient specific marker profile to be recorded and subsequently assessed for correlations with tumour stage, current predictors of outcome and clinical course.

Paired PB and BM sampling will potentially maximise cell yield given the propensity of CaP to metastasise to bone. Other workers have demonstrated that when using paired samples the BM sample is more often positive for tumour cells.

Micro fluidic RT-PCR provides all the advantages of real time RT-PCR while using a methodology that is fast, has reduced potential for contamination and pipetting/sampling error and can simultaneously evaluate multiple samples for multiple markers. In principle it is well suited to molecular staging, both in the evaluation of novel markers and in large scale clinical studies. It is a novel technology that has not been evaluated at all for circulating cell detection.

Although novel CaP markers have been identified, few have been used in CaP molecular staging, a factor which may account for its limited clinical success. Identifying those which are useful in detecting circulating cells will provide future directions for studies of this technique.

## Chapter 2: Materials and Methods

### 2.1 Development of *In Vitro* Prostate Cancer Model

#### 2.1.1 Cell Lines

The *in vitro* development of the RT-PCR assay required the use of prostate cells grown in culture as a prostate cancer cell model. The cell line characteristics are detailed in table 2.1.

| Cell Line | Cell Type | Origin | Hormone Sensitive | Source | Reference |
|---|---|---|---|---|---|
| LNCaP-FGC | Epithelial | Lymph Node Metastasis | Yes | ATCC | Thalmann 1994 [233] |
| PC-3 | Epithelial | Bone Metastasis | No | ECACC | Kaighn 1979 [234] |
| DU-145 | Epithelial | Brain Metastasis | No | ECACC | Stone 1978 [235] |
| PNT2-C2 | Epithelial | SV40 Transfected Normal Prostate | Yes | ECACC | Berthon 1995 |

**Table 2.1:** Prostate cell line characteristics

### 2.1.2 Cell Culture

All cell lines used in assay development were cultured in the same manner. LNCaP, PNT2 and DU-145 were cultured in RPMI 1640 (BioWhittaker UK) supplemented with 10% Fetal Calf Serum (BioWhittaker UK), 100mM Sodium Pyruvate (Invitrogen NZ), 10mM Hepes Buffer (Invitrogen NZ) and 2% L-glutamine. PC-3 cells were grown in Ham's F12 (PAA Laboratories, Austria), 7% FCS and 2% L-glutamine. The cell lines were cultured and maintained in T25 tissue culture flasks (Falcon, Becton Dickinson Labware, NJ, USA) at 37°C in an environment of 5% $CO_2$ enriched air. Cells were passaged when they reached confluency using strict aseptic technique in a laminar flow hood.

For cell harvesting, following removal of the culture medium and rinsing with phosphate buffered saline (PBS), 1ml of 0.25% trypsin was added to the culture flask and then briefly incubated at 37°C. For continued culture, cells were then resuspended in 5mls of growth medium and reseeded into fresh flasks at a 1:10 dilution. Cells required for RNA extraction were resuspended in PBS following the incubation stage and transferred to a 15ml Falcon tube for centrifugation at 800G for 5 minutes.

To investigate the effects of testosterone on LNCaP cell marker expression, separate flasks of LNCaP cells were cultured in standard conditions and media with additional 1nM dihydrotestosterone.

### 2.1.3 RNA Extraction From Cells in Culture

RNA extraction was performed in a laminar air flow hood, using RNase/DNase free Eppendorf tubes and Gilson micro pipettes with filter tips.

Following harvesting, the cell pellet was resuspended in 0.5ml of TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) and incubated at room temperature for 5 minutes. 0.2ml of chloroform were added per 1ml of lysed cells, mixed and centrifuged at 13000rpm for 15 minutes. This upper aqueous phase was aspirated, mixed with

an equal volume of isopropanol, incubated for 10 minutes at room temperature prior to centrifugation at 13000 rpm for 15 minutes. The supernatant was removed, the RNA pellet resuspended in an equal volume of 75% ethanol and again centrifuged at 13000 rpm for 15 minutes. The supernatant was discarded and the RNA pellet dried.

To ensure no DNA contamination, the pellet underwent a DNase treatment by resuspension in 43µl RN/DNase free water and the addition of 5µl of DNase buffer (1M sodium acetate, 50mM $Mg_2SO_4$, 3mM $MnCl_2$, pH 5.0) and 2µl of DNase (Roche, Mannheim, Germany). The sample was incubated at 37°C for 30 minutes and the enzyme subsequently denatured by heating to 99°C for 10 minutes.

### 2.1.4 Quantitatation of RNA

The RNA concentration of samples was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Readings were taken at wavelengths of 260nm and 280nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample using the following equation:

$$\text{Concentration } (\mu g/ml) = A_{260}/0.025$$

The ratio of the readings at 260 nm and 280 nm provided an estimation of the purity of the nucleic acid. Pure RNA samples have a 260/280 ratio of 1.9 to 2.0, protein contamination resulted in significantly lower ratio values.

### 2.1.5 RNA Storage

The RNA in solution was precipitated by three volumes of ethanol and a tenth of a volume of 3M sodium acetate pH 5.2 prior to storage under ethanol at minus 80°C.

### 2.1.6 Cell Line Marker Expression

The relative expression by the four cell lines of the prostate markers PSA, PSMA, PSCA, HK-2 and $DD3^{PCA3}$ was assessed using a real time quantitative assay as detailed in 2.3.9.

## 2.2 Prostate Cancer Model for Establishment of *In Vitro* Assay Sensitivity

### 2.2.1 Peripheral Blood Sample Collection and Prostate Cell Spiking

The *in vitro* sensitivity of the assay was determined by its ability to detect the presence of serially diluted LNCaP target cells in female blood. Blood samples were collected using PAXgene™ blood RNA tubes (PreAnalytiX GmbH, Hombrechtikon, CH) which utilise a closed vacuum system to collect 2.5ml of peripheral blood. The tube contains an additive that stabilises cellular RNA for storage up to 7 days and prepares the sample for RNA purification.

LNCaP cells were harvested from culture as described in section 2.1.2 and re-suspended in 2ml of culture medium. Cells were counted using a haemocytometer viewed by light microscopy (x100), and thus the cell concentration of the suspension calculated. Serial dilutions of LNCaP cells from $10^5$ cells to 1 cell per 100µl were made up in PBS. These were used to spike the female peripheral blood samples with 10 fold serial dilutions of LNCaP in a range from 1 to $10^5$ cells per 1 ml of blood. These were incubated at room temperature for 2 hours and then refrigerated at 4°C until RNA extraction as in method 2.2.2.

### 2.2.2 RNA Extraction From Cell Spike Samples

RNA extraction was performed using the PAXgene™ blood RNA kit following the protocol shown in figure 2.1. A nucleic acid pellet was formed by centrifugation at 3000g for 10 minutes. The supernatant was removed and the pellet rinsed with 5ml of RNase/DNase free water which was removed following a further 10 minute centrifugation at 3000g. The pellet was transferred to a screw cap microcentrifuge

tube and resuspended in 360µl of lysis buffer to which was added 40µl of proteinase K. The sample was incubated at 55°C while being agitated at 1400rpm for 10 minutes in an Eppendorf Comfort thermomixer to allow adequate protein digestion.

Following centrifugation at 12000g for 3 minutes the supernatant was passed through a Qiashredder (Qiagen Ltd,Crawley, UK), to remove high molecular weight cellular components including DNA, and was further centrifuged for 2 minutes at 12000g. 350µl of ethanol were added to the resulting supernatant and mixed by vortexing. The lysate was then applied to the PAXgene™ RNA spin column and underwent centrifugation for 1 minute at 9000g to allow selective RNA binding. 700µl of wash buffer was applied to the column and incubated at room temperature for 5 minutes, followed by centrifugation for 1 minute at 9000g. 500µl of a second wash buffer was then applied to the column and again centrifuged. The column was then dried by centrifugation at 12000g for 3 minutes.

The bound RNA was then eluted into buffer using two identical steps in which 40µl of elution buffer was applied to the column and then centrifuged for 1 minute at 9000g. All 80µl of flow through were then incubated at 65°C for 5 minutes followed by DNase treatment as in method 2.1.3.

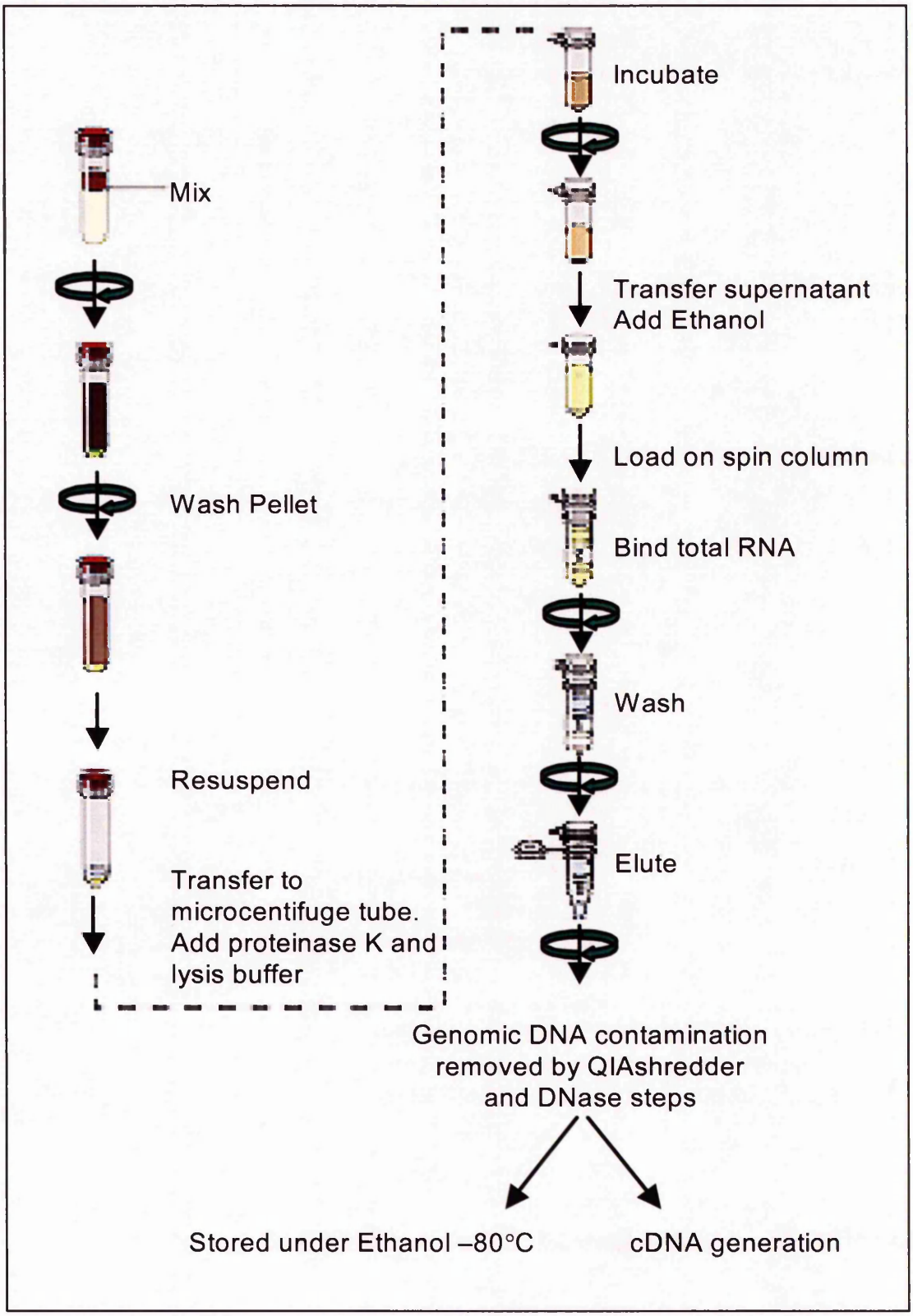



**Figure 2.1**: RNA Extraction Protocol - using PAXgene™ blood RNA kit [236].

## 2.3 Development of RT-PCR assay

### 2.3.1 Reverse Transcription

Isolated RNA was reverse transcribed to produce its complementary cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, New Jersey, USA) in the quantities shown in Table 2.2 per 400ng of RNA.

The reaction components were set up in a clean (RNA/DNA free) room prior to the addition of RNA template. Reverse Transcription was performed in RNase/DNase free 0.2ml thin walled PCR tubes using a Geneamp™ PCR system 9700 thermocycler (Applied Biosystems, New Jersey, USA).

Reaction conditions were 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C.

| Component | Amount (µl) |
|---|---|
| RNase Free Water | Variable |
| 10x TaqMan RT Buffer | 2.0 |
| 25mM Magnesium Chloride | 4.4 |
| dNTP Mixture | 4.0 |
| Random Hexamers | 1.0 |
| RNase Inhibitor | 0.4 |
| Multiscribe Reverse Transcriptase (50U/µl) | 0.5 |
| RNA | 400ng |

**Table 2.2:** Reverse transcription components

### 2.3.2 Primer Design

Forward and reverse nucleotide primers were designed for each of the five prostate markers of interest using published sequencing data [237] and Primer Express software (Applied Biosystems, New Jersey, USA). This software incorporates a function to design nucleotides specifically for use in TAQman™ real time PCR experiments.

TAQman Primer Design Requirements:

1. Primer melting temperature ($T_m$) 58-60°C
2. Primer length 9 - 40 bases
3. <2°C difference in $T_m$ between primers
4. Maximum of 2 Gs or Cs in the last 5 bases at the 3' end

In addition to the above requirements every effort was made to design primers that crossed exon-exon boundaries on the target to avoid amplification of any sample contamination with genomic DNA.

| Target | Orientation | Primer Sequence | Position |
|---|---|---|---|
| B-actin | Forward | 5'-AAGCCACCCCACTTCTCTCTAA-3' | 1491-1500 |
| | Reverse | 5'-AATGCTATCACCTCCCCTGTGT-3' | 1549-1570 |
| GAPDH | Forward | 5'- GAAGGTGAAGGTCGGAGTC-3' | 87-106 |
| | Reverse | 5'- GAAGATGGTGATGGGATTTC -3' | 217-238 |
| PSA | Forward | 5'-CAGCATTGAACCAGAGGAGTTCT -3' | 515-537 |
| | Reverse | 5'-TTGCGCACACACGTCATTG-3' | 581-599 |
| PSMA | Forward | 5'-AGTGACGAGAATTTACAATGTGATAGGT -3' | 1311-1341 |
| | Reverse | 5'-TGAGGGTCAATACCACCAAACA -3' | 1406-1427 |
| PSCA | Forward | 5'–TACTCCTGCAAAGCCCAGGT-3' | 87-106 |
| | Reverse | 5'–AGTCATCCACGCAGTTCAAGC -3' | 217-238 |

**Table 2.3:** β-Actin, GAPDH, PSA, PSMA and PSCA PCR oligonucleotide primer sequences & positions

Primers used by other groups were also utilised for HK2 [194] and DD3$^{PCA3}$ [71] as shown in table 2.4.

| Target | Orientation | Oligonucleotide Sequence | Position |
|---|---|---|---|
| HK2 | Forward | 5'-GAACCAGAGGAGTTCTTGCG -3' | 522-541 |
| | Reverse | 5'-CCCCAGAATCACCCCCACAA –3' | 665-684 |
| DD3$^{PCA3}$ | Forward | 5'-TGGGAAGGACCTGATGATACA –3' | 97-117 |
| | Reverse | 5'-CCCAGGGATCTCTGTGCTT–3' | 459-478 |

**Table 2.4:** HK2 and DD3$^{PCA3}$ PCR oligonucleotide primer sequences and positions.

### 2.3.3 Nucleotide Probe Design

Product specific nucleotide probes were designed for the PSA, PSMA, PSCA and HK2 assays using Primer Express software (Applied Biosystems, New Jersey, USA) as previously described. The Probe Sequences used are detailed in table 2.5.

TAQman Probe Design Requirements:

1. $T_m$ 10°C higher than the primer $T_m$
2. Probe length 9 – 40 bases
3. No G on the 5' end
4. < 4 contiguous Gs
5. Must not have more Gs than Cs

| Target | Probe Sequence |
|---|---|
| PSA | 5'- CCC AAA GAA ACT TCA GTG TGT GGA CCT CC -3' |
| PSMA | 5'– TGA ATC CCG GTG ACC TCC CAG AAT G –3' |
| PSCA | 5'- CGC GCG GTC CAG CAC TGC T –3' |
| HK2 | 5'- TGT GTG CTG GGC TCT GGA CAG GTGGTA A -3' |

**Table 2.5:** PCR Nucleotide Probes for PSA, PSMA, PSCA and HK2.

### 2.3.4 Qualitative PCR Reactions

Traditional qualitative PCR methods were utilised initially in confirming primer function. RNA isolated from LNCaP cells was reverse transcribed to cDNA for primer target. All PCRs were performed in triplicate and were run alongside control reactions containing no target and minus RT reactions containing LNCaP RNA prior to reverse transcription, thus identifying any component contamination or genomic DNA contamination in the RNA following the extraction process. The 'housekeeping' genes B-actin and GAPDH were co-amplified with each PCR run.

100ng of target cDNA was amplified in a 50μl reaction containing the following: 25 pmole forward primer, 25 pmole reverse primer, 1μl of dNTP mix (each at 10mM), 75 unit Taq polymerase (Promega, Madison, WI, USA) and 5μl 10x PCR buffer containing 1.5mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH9.0) and 0.1% Triton X-100 (Promega, Madison, WI, USA). Thermocycling was performed on Geneamp PCR System 9700 thermocycler (Applied Biosystems, New Jersey, USA) as follows: 95°C for 10 minutes followed by 35 cycles of 95°C for 15 seconds, 60°C for 1 minute, followed by 72°C for 10 minutes.

### 2.3.5 PCR Product Visualisation

The DNA products were separated by agarose gel electrophoresis and visualised using the fluorescent dye ethidium bromide viewed under ultraviolet (UV) illumination.

Agarose powder 2% by weight was dissolved in TAE electrophoresis buffer (Tris-acetate 0.04M, EDTA 0.001M) to which was added ethidium bromide 0.5μg/ml. This was set in a mould with a comb *in situ* to form sample wells. Once set, the gel was mounted in an electophoresis tank and covered with TAE buffer containing ethidium bromide 0.05μg/ml. Ten microlitres of PCR product mixed with 1μl of loading buffer (5% glycerol, 0.1mM EDTA, 0.025% bromophenol blue and 0.025% cyanol zylene) were loaded into the wells. The first well in each plate contained 4μl

of DNA molecular weight Marker VIII (Roche Diagnostics GmbH, Germany) in loading buffer. Electrophoresis proceeded at 80V for 30 minutes.

The resultant gel was viewed under UV light and the size of the PCR products compared to the standard of the DNA marker.

### 2.3.6 PCR Product Purification

PCR product purification was required to enable the confirmation of product identity through sequencing. The QIAquick PCR Purification Kit protocol (QIAGEN, Crawley, UK) was utilised which is designed to purify double stranded DNA fragments, ranging in size from 100bp to 10kb, from primers, polymerases and salts. The protocol incorporates a spin-column containing a silica gel membrane with selective binding properties.

125µl of binding buffer PB were added to 25µl of PCR product which was then applied to the spin column and centrifuged at 12000g for 60 seconds to bind DNA. 750µl of PE wash buffer were then applied to the column which was again centrifuged at 1200g for 60 seconds and for a further 60 seconds following discard of flow through. The DNA was then eluted from the column into a clean 1.5ml microcentrifuge tube through the addition to the membrane of 50µl of elution buffer EB (10mM Tris HCl, pH 8.5) and centrifugation at 12000g for 1 minute.

### 2.3.7 Quantification of Purified PCR DNA Product

The DNA concentration of samples was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) as in section 2.1.4.

### 2.3.8 PCR Product Sequencing

All PCR product sequencing was performed by the staff of the molecular core facility using the following protocol. Three nanograms of purified PCR product

template and 15pmol of forward or reverse primer were made up to a total reaction volume of 12µl with DN/RNase free water in a 500µl microcentrifuge tube.

### 2.3.9 TAQman™ Real Time Quantitative PCR

To allow quantitation of target RNA, the PCR primers were incorporated into a real time PCR assay. For each reaction, 100ng of cDNA was amplified in a 25µl PCR reaction with an optimised ratio of forward and reverse primers and 12.5µl of either SYBR Green Master Mix™ (Applied Biosystems, New Jersey, USA) or TAQman™ PCR Master Mix (Applied Biosystems, New Jersey, USA) with a marker specific nucleotide probe. All reactions were performed in triplicate with minus RT and target free negative controls as in section 2.3.4. Housekeeping gene amplification was performed for each sample source. The reactions were carried out in separate wells of 96 well optical plates (Applied Biosystems, New Jersey, USA). The ABI PRISM 7700® Sequence Detection System and sequence detector software (both Applied Biosystems, New Jersey, USA) were used for thermocycling, product detection and data analysis.

Reaction conditions for PCR were; an initial stage of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, followed by a single hold at 72°C for 10 minutes.

### 2.3.10 Real Time PCR Product Identification

In reactions using oligonucleotide probes, no downstream processing was required. When SYBR Green™ was used the products underwent a dissociation reaction. The products remained in the same 96 well plate as used for the PCR amplification and thermocycling was again performed using the ABI PRISM 7700® Sequence Detection System. Reaction conditions were 95°C for 15 seconds, then rapid cooling to 60°C and gradual heating over 20 minutes up to 95°C. Dissociation data was analysed using dissociation curve software (Applied Biosystems, New Jersey, USA) and negative fluorescence was plotted against temperature.

### 2.3.11 Primer Optimisation

Twenty-five microlitre PCRs containing 100ng of cDNA derived from $10^6$ LNCaP cells, 12.5µl SYBR Green Master Mix™ and variable volumes of RN/DNase free water were made up using the 9 different primer ratios detailed in table 2.6. Each reaction was run in duplicate along with standard controls. Thermocycling and product dissociation were performed as in sections 2.3.9 and 2.3.10.

| | **REVERSE PRIMER (nM)** | | |
|---|---|---|---|
| **FORWARD PRIMER (nM)** | **100** | **300** | **900** |
| **100** | 100/100 | 100/300 | 100/900 |
| **300** | 300/100 | 300/300 | 300/900 |
| **900** | 900/100 | 300/300 | 900/900 |

**Table 2.6:** Primer concentration ratios used in primer optimisation

### 2.3.12 Probe Optimisation

The specific probes for each of the markers PSA, PSMA, PSCA and HK2 were tested at the following concentrations; 10, 50, 100 and 200nM. Each 25µl reaction was run in triplicate and contained along with the probe, 12.5µl TAQman™ PCR Master Mix, forward and reverse marker specific optimised primers and 100ng of

cDNA derived from $10^6$ LNCaP cells. The details of the PCR reaction are identical to those described in section 2.3.9.

### 2.3.13 Use of Plasmid Construct in Development of $DD3^{PCA3}$ Assay

#### 2.3.13.1 PCR Detection of pMB45 IS-$DD3^{PCA3}$ DNA Plasmid

The $DD3^{PCA3}$ plasmid cDNA construct pMB45 IS-$DD3^{PCA3}$ as described by Hessels *et al* [71] (figure 2.2) was spiked into PCRs containing 100ng of cDNA derived from normal female peripheral blood in 10 fold serial dilutions from one copy to $10^6$ copies. These were made up to a total reaction volume of 25μl with $DD3^{PCA3}$ forward and reverse primers, SYBR Green Master Mix™ and DN/RNase free water. Reactions were run using the PCR and dissociation protocols detailed in methods 2.3.9 and 2.3.10.

#### 2.3.13.2 Synthesis of $DD3^{PCA3}$ RNA from pMB45 IS-$DD3^{PCA}$ Plasmid

##### 2.3.13.2.1 Plasmid Linearization

The $DD3^{PCA3}$ plasmid construct was linearized using the restriction enzyme Kpn I (Promega, WI, USA). A digest reaction containing 1μg of plasmid DNA, 0.2μl acetylated bovine serum albumin (BSA) at a final reaction concentration of 0.1mg/ml, 2μl of 10x restriction buffer (100mM Tris –HCl (pH 7.5), 500mM KCl, 70mM $MgCl_2$, 10mM dithiothreitol (DTT) at 37°C) and 5 units of Kpn I restriction enzyme, was made up to a final volume of 20μl with DN/RNase free water, and incubated at 37°C for 2 hours. The Kpn I enzyme was then heat deactivated at 65°C for 15 minutes. Plasmid digestion was confirmed by gel electrophoresis, using a 1% agarose gel and comparing product size to a concurrently run 1Kb DNA ladder (New England Biolabs, USA).

#### 2.3.13.2.2 $DD3^{PCA3}$ RNA Synthesis

T3 RNA polymerase was used to synthesise $DD3^{PCA3}$ RNA from the linear $DD3^{PCA3}$ plasmid. The reaction, carried out in a microcentrifuge tube, contained 20µl of T3 transcription 5x buffer (40mMTris (pH 7.9 at 25°C), 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl), 10µl 100mM DTT, 100 units of recombinant RNasin ribonuclease inhibitor, 20µl rNTP mix (2.5mM rATP, 2.5mM rGTP, 2.5mM rUTP, 2.5 mM rCTP in nuclease free water), 5µg of linearized plasmid DNA and 40 units of phage T3 RNA polymerase made up to a total volume of 100µl with nuclease free water, was incubated for 2 hours at 37°C. This was followed by a DNase treatment as described in section 2.1.3. The resultant RNA was quantified using nanodrop spectrophotometer as in section 2.1.4.

### 2.3.13.3 $DD3^{PCA3}$ RNA of Plasmid Origin in Establishing Assay *In Vitro* Sensitivity

Serial dilutions of $DD3^{PCA3}$ RNA copies from $10^7$ to 10 were spiked into 2.5 ml female peripheral blood samples collected in PAXgene™ blood RNA tubes. Total RNA was extracted as described in methods 2.2.2 and reverse transcription performed as in method 2.3.1. 100ng of the synthesised cDNA was amplified in an optimised real time PCR reaction (methods 2.3.9 and 2.3.10) with samples from each serial dilution spike being amplified in triplicate.

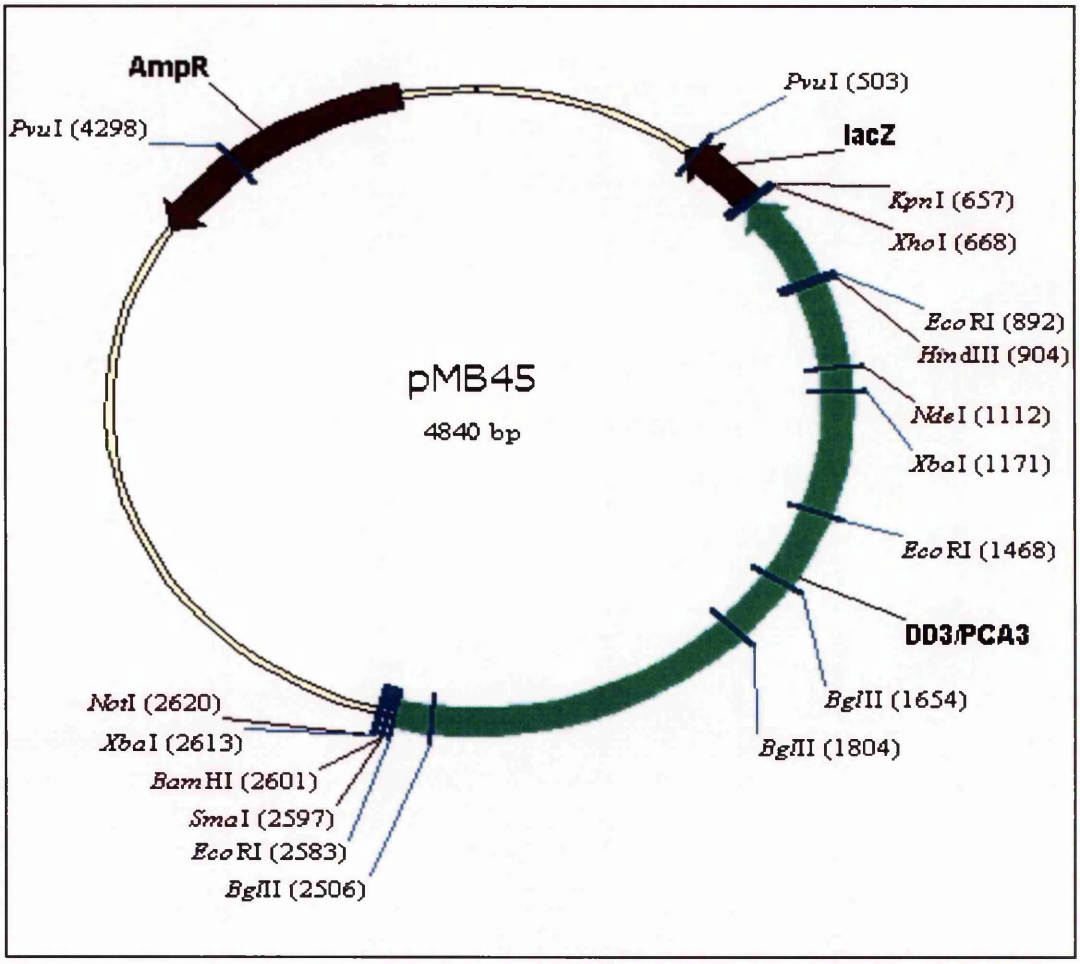



**Figure 2.2:** pMB45 IS-DD3$^{PCA3}$ plasmid construct [71].

## 2.4 Patient Samples

### 2.4.1 Patient Sample Collection

Suitable study patients with CaP and normal controls were identified through close links with the departments of urology and oncology at the Christie, South Manchester University and Hope Hospitals. All patients were enrolled with informed consent using an ethically approved protocol.

Female and normal male control patients had to be free from any form of haematological or malignant disease. CaP study patients all had fully characterised and staged disease following current best practice guidelines.

The following information regarding patients enrolled in the study was recorded from their case notes:

- Patient age
- Serum PSA level at time of sampling
- Clinical T stage at time of sampling based on DRE
- Biopsy Gleason score
- Most recent bone scan result
- Treatment received and hormone status at time of sampling
- In patients undergoing RRP – pathological stage and margin status
- Disease status at follow up

Peripheral blood samples (2.5mls) were collected using PAXgene™ blood RNA tubes (PreAnalytiX GmbH, Hombrechtikon, CH) as in section 2.2.2. Bone marrow aspirates were collected from the posterior iliac crest. This was achieved under local or general anaesthetic, using a marrow gauge needle and syringe. 2.5mls of the BM aspirate was immediately transferred to a PAXgene™ blood RNA tube upon collection.

### 2.4.2 Application of Real Time RT-PCR Assay to Patient Samples

Both PB and BM samples were handled and processed as detailed for cell spike samples (method 2.2.2), being stored at minus 80°C in the RNA and, following reverse transcription (method 2.3.1), cDNA forms.

## 2.5 Micro Fluidic RT-PCR

### 2.5.1 Micro fluidic card assay design and development

The 384 well micro fluidic cards were commercially produced by Applied Biosystems®. Endogenous control genes and target genes of interest were selected from a library of industry optimised primer and probe TaqMan RT-PCR assays, Assays-on-Demand™ (Applied Biosystems). All primer design was exon-exon boundary spanning. Primer and probe sequences were not made available for validation by Applied Biosystems. The housekeeping genes GAPDH and β-actin, and the target genes PSA, PSMA, PSCA, HK2, HK4, HK15, EZH2, hepsin, prostasin and urokinase type plasminogen activator were selected prior to card manufacture. During the manufacturing process each individual assay was loaded into an individual reaction well and dried. The micro fluidic card design is shown in figure 2.3. Each card had 8 filling ports and reservoirs, each with 48 associated RT-PCR reaction wells pre-loaded with marker specific assay. 8 individual samples could therefore be evaluated simultaneously for the expression of up to 48 gene markers. As the micro fluidic card technique had not previously been evaluated for a circulating cell detection protocol 4 replicate assays were used for each of the 10 markers of interest and the 2 housekeeping genes.

### 2.5.2 Evaluation of micro fluidic card RT-PCR

The *in vitro* performance of the micro fluidic card system was evaluated by direct comparison with the 96 well format described previously. The samples evaluated were RNA extracted from LNCaP cells in culture, female PB and serial dilutions ($10^6$ to 1) of LNCaP cells spiked into female PB. Total RNA extraction,

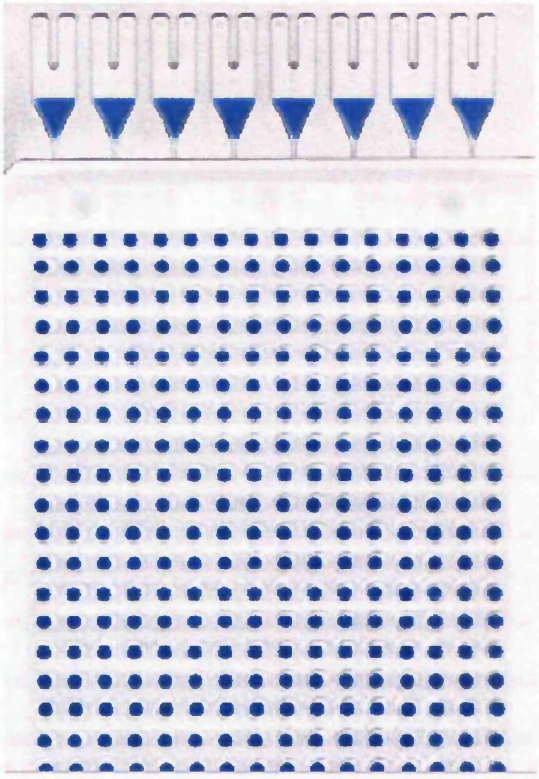
quantification and reverse transcription was performed on the above samples as described in methods 2.1.3 – 4, 2.2.1 - 2 and 2.3.1. 100ng aliquots of the resultant cDNA were then evaluated for quantitative expression of GAPDH, β-actin, PSA, PSMA, PSCA and HK2 using the TaqMan real time PCR protocol described in section 2.3.9.

100ng of the LNCaP derived cDNA was also applied to a micro fluidic protocol as follows. 100ng of cDNA in 50μl of RNase/DNase free water were added to 50μl of TaqMan® universal PCR mastermix (Applied Biosystems) and mixed by vortexing. The 100μl reaction mix was then loaded into one of the fill reservoirs on the micro fluidic card using a filter tipped micro pipette. Once all the reservoirs were filled the plate was centrifuged at 1200 rpm for 2 spins of 1 minute using a Sorvall® Legend T centrifuge with custom micro fluidic card buckets and card holders. The plates were then sealed using a micro fluidic card sealer (Applied Biosystems) and loaded onto ABI Prism 7900 HT sequence detection system for thermocycling and product detection. Reaction conditions for PCR were; an initial stage of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Product detection and data analysis was all performed using the ABI Prism 7900HT and SDS software (both Applied Biosystems).

The micro fluidic format sensitivity and reproducibility with smaller amounts of target was also evaluated using 10 fold dilutions of LNCaP cDNA from 100 ng to 0.1ng. *In vitro* sensitivity was established by assaying 100ng of cDNA from the standard LNCaP serial dilutions samples used for the 96 well format. Using cDNA derived from female PB the specificity of the technique and of each individual assay for both the novel and established markers used on the card format were established. Minus RT controls were run in separate reservoirs to ensure no genomic DNA contamination, while target free water controls ensured no contamination of the DNase/RNase free water or the universal mastermix.

The clinical performance of the micro fluidic technique was established by analysing 100ng aliquots of cDNA derived from PB and BM samples from men within the main study population.

A)



B)

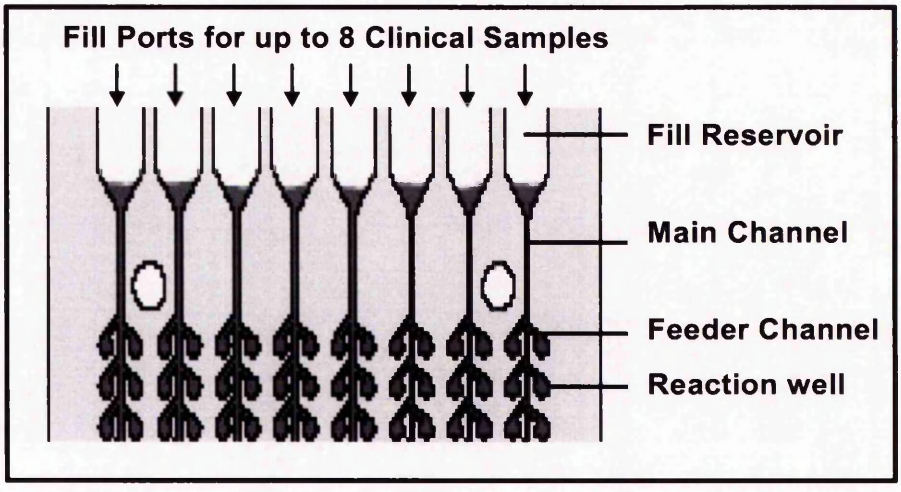



**Figure 2.3:** Micro fluidic card used for micro fluidic multi-marker RT-PCR experiments. A) photograph showing micro fluidic card. B) schematic diagram showing detail of micro fluidic card.

## Chapter 3: Results of Assay Development:

### 3.1 Establishment of cell line model

To assess the sensitivity and specificity of the primers it was necessary to set up a cell line model. The gene expression in the four prostate cell lines LNCaP, PC3, DU-145 and PNT2-C2 for the prostate markers PSA and PSMA and the housekeeping genes β-actin and GAPDH were determined. Total RNA was extracted from $10^6$ cells by Trizol extraction and cDNA produced utilising random hexamer primers in a reverse transcription reaction. A real time PCR protocol was then used to quantify cell line gene expression (figure 3.1).

GAPDH and β-actin were amplified in all the cell line samples, with product being detected at 16-19 and 21-23 cycles respectively, thus confirming the integrity of the RNA and the reverse transcription reaction (figures 3.1 & 3.2). Product identity was confirmed by their dissociation temperature ($T_m$) on the product dissociation curves, 82.6°C for GAPDH and 80.9°C for β-actin (figure 3.3). The PCR product size of these housekeeping genes was confirmed by gel electrophoresis on a 2% agarose gel, 226bp for GAPDH and 80bp for β-actin (figure 3.4).

Only the LNCaP cell line expressed PSA and PSMA with the detected fluorescence from their reactions crossing the critical threshold value at an average of 27 and 29 cycles respectively (figure 3.2). As expected from the primer design, the $T_m$ for the PSA and PSMA products were 79.7°C and 80.9°C respectively (figures 3.5 & 3.6) and the PCR product sizes, confirmed by gel electrophoresis, correlated with the expected values of 85bp for PSA and 114bp for PSMA (figure 3.7). PSCA, HK2 and $DD3^{PCA3}$ were not quantified in cell line selection work.

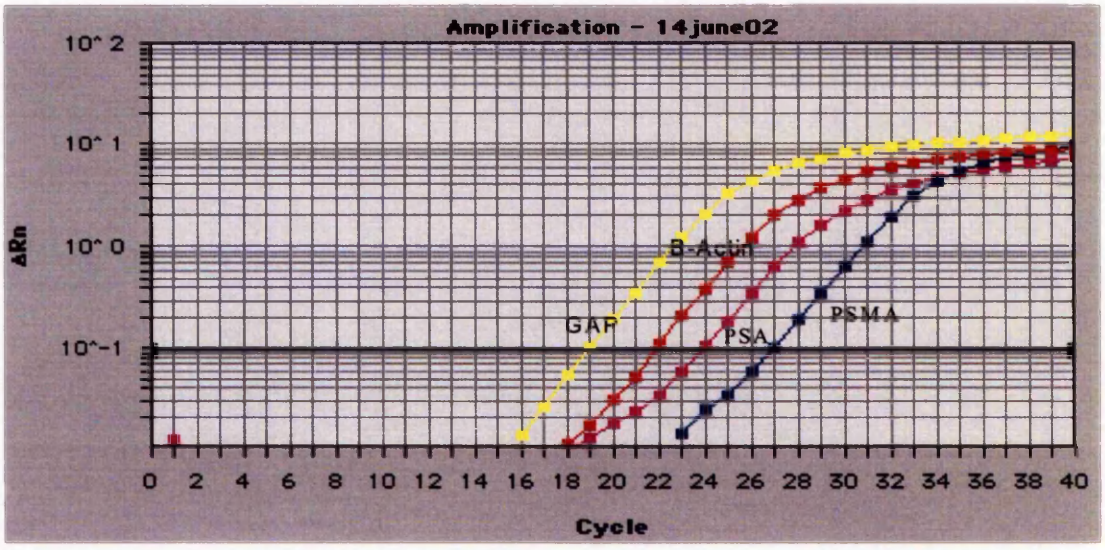



**Figure 3.1:** Typical real time PCR plots showing GAPDH, β-actin, PSA and PSMA expression in RNA isolated from $10^6$ LNCaP Cells. The Ct has been set at $10^{-1}$ as determined by negative control background fluorescence.

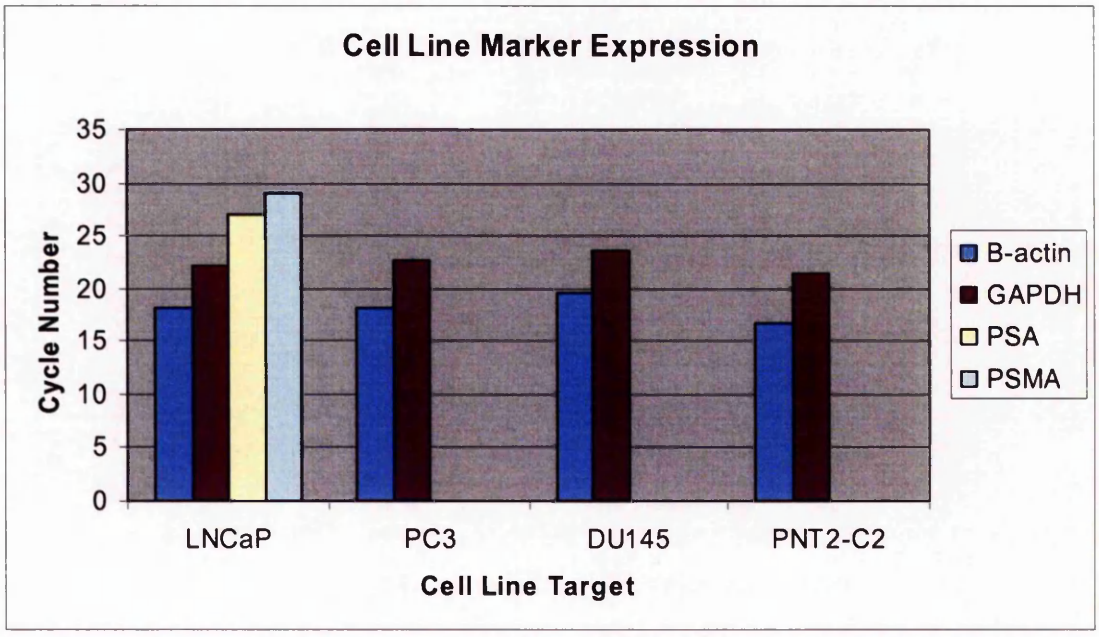



**Figure 3.2:** Bar chart showing the average Ct cycle number for the real time PCR detection of β-actin, GAPDH, PSA and PSMA mRNA as expressed in total RNA from LNCaP, PC3, DU145 and PNT2-C2 prostate cell lines.

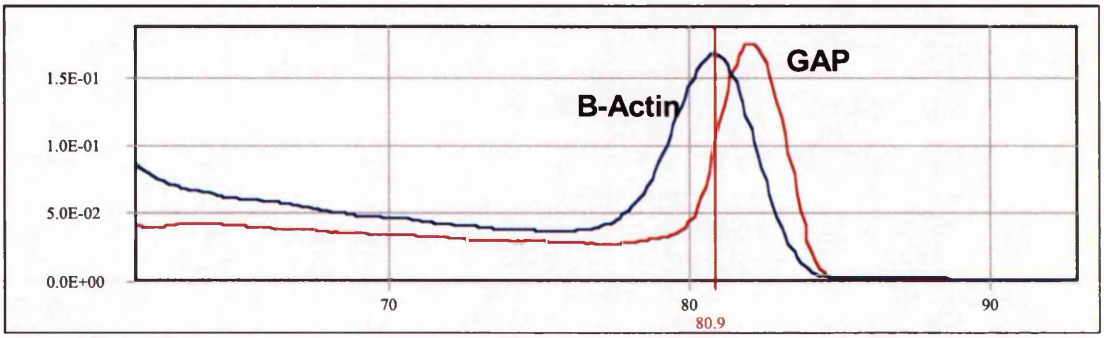



**Figure 3.3:** Dissociation curves for the 'housekeeping genes' β-actin and GAPDH. Negative fluorescence is plotted on the y-axis against temperature in degrees centigrade on the x-axis. The peak at 80.9°C is the $T_m$ for the β-actin product and the peak at 82.6°C is the GAPDH $T_m$.

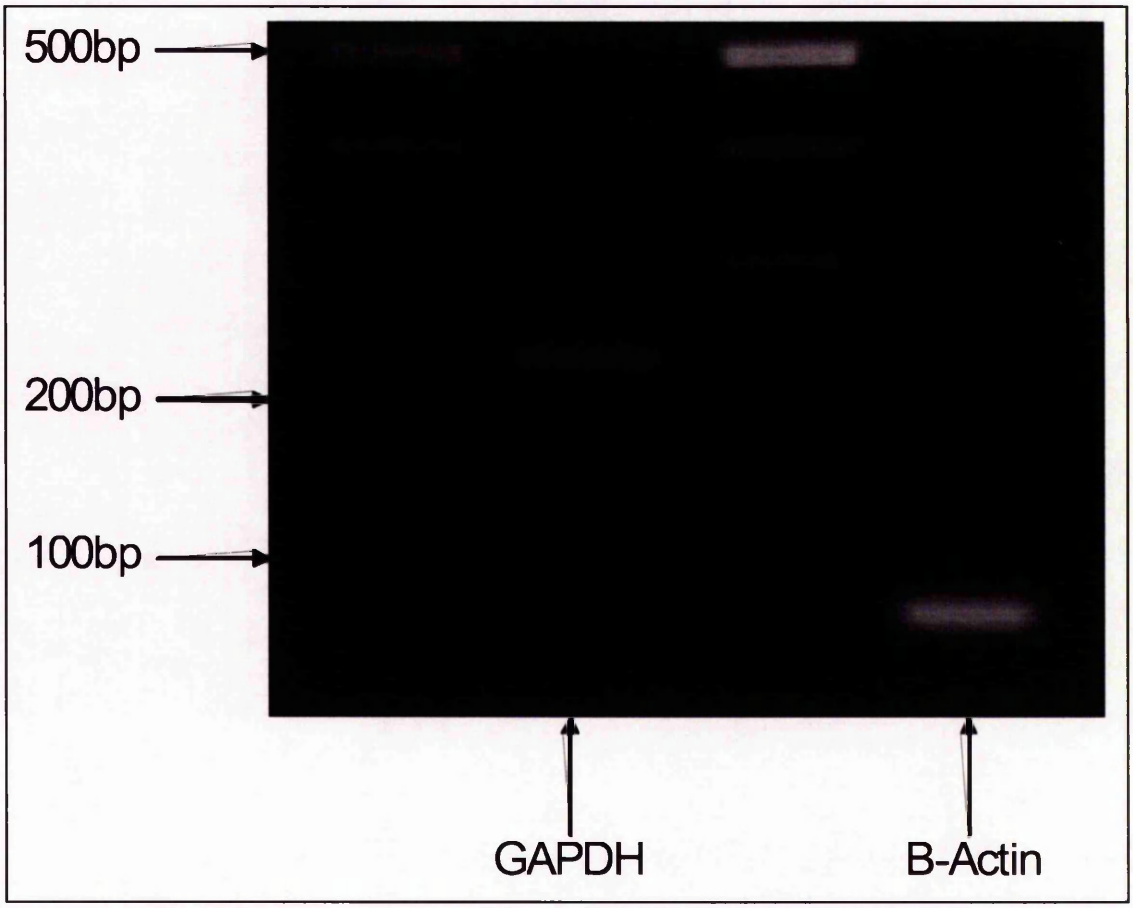



**Figure 3.4:** PCR products for the housekeeping genes GAPDH and B-Actin derived from total RNA isolated from $10^6$ LNCaP Cells viewed following electrophoresis on a 2% agarose gel with molecular weight marker in adjacent lanes.

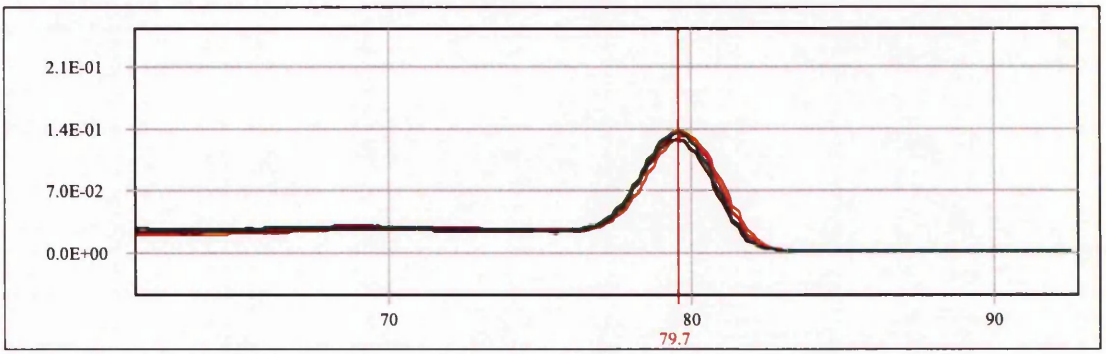



**Figure 3.5:** Dissociation curves for PSA product in multiple replicate RT-PCR reactions derived from total RNA isolated from $10^6$ LNCaP cells.

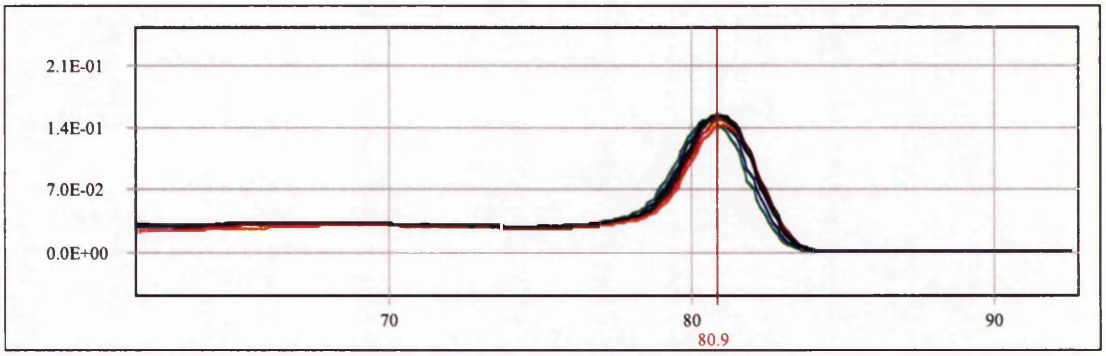



**Fig 3.6:** Dissociation curve for PSMA product in multiple replicate RT-PCR reactions derived from total RNA isolated from $10^6$ LNCaP cells.

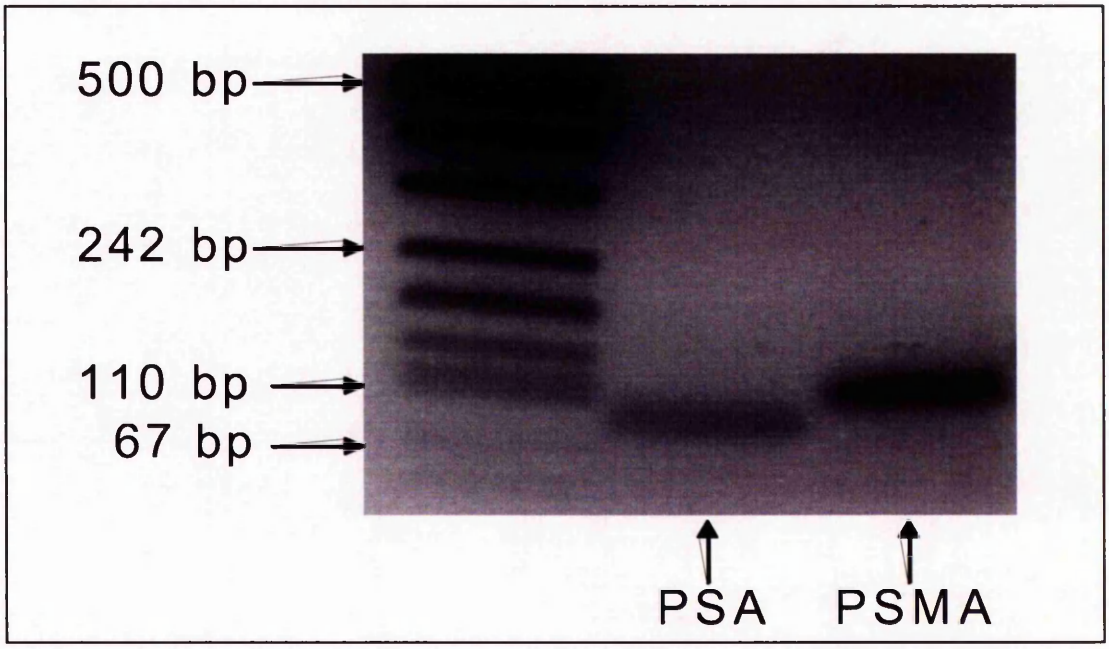



**Figure 3.7:** PCR products for the genes PSA and PSMA derived from total RNA isolated from $10^6$ LNCaP cells viewed following electrophoresis on a 2% agarose gel with molecular weight marker in lane 1.

### 3.2 Primers for Five Marker Panel

The primers designed for PSCA, HK-2 and $DD3^{PCA3}$ as detailed in section 2.3.2 were run, in addition to PSA and PSMA primers, in a real time PCR with100ng of target cDNA isolated from $10^6$ LNCaP cells. Successful co-amplification of GAPDH and β-actin housekeeping genes confirmed the RNA integrity and the efficiency of the RT reaction. Product was amplified for all genes of interest (figure 3.8). The Ct values for the different markers were as follows; PSA 19.5 cycles, PSMA 21 cycles, HK-2 22 cycles, PSCA 29.6 cycles and $DD3^{PCA3}$ 31.4 cycles. Water controls and minus RT reactions were negative for all markers thus excluding the possibility of false positive results due to genomic DNA contamination.

The identity of each product was determined by both product dissociation and agarose gel electrophoresis. HK-2, $DD3^{PCA3}$ and PSCA demonstrated $T_m$ values of 83.4°C, 79.8°C, and 88.2°C respectively (figures 3.9 – 3.11). The $T_m$ value for PSCA was 3°C higher than the predicted value of 85°C. The product sizes demonstrated on gel electrophoresis were in keeping with the expected product sizes of 85bp PSA, 114bp PSMA, 151bp PSCA, 163bp HK2 (figure 3.12) and 381bp $DD3^{PCA3}$ (not shown).

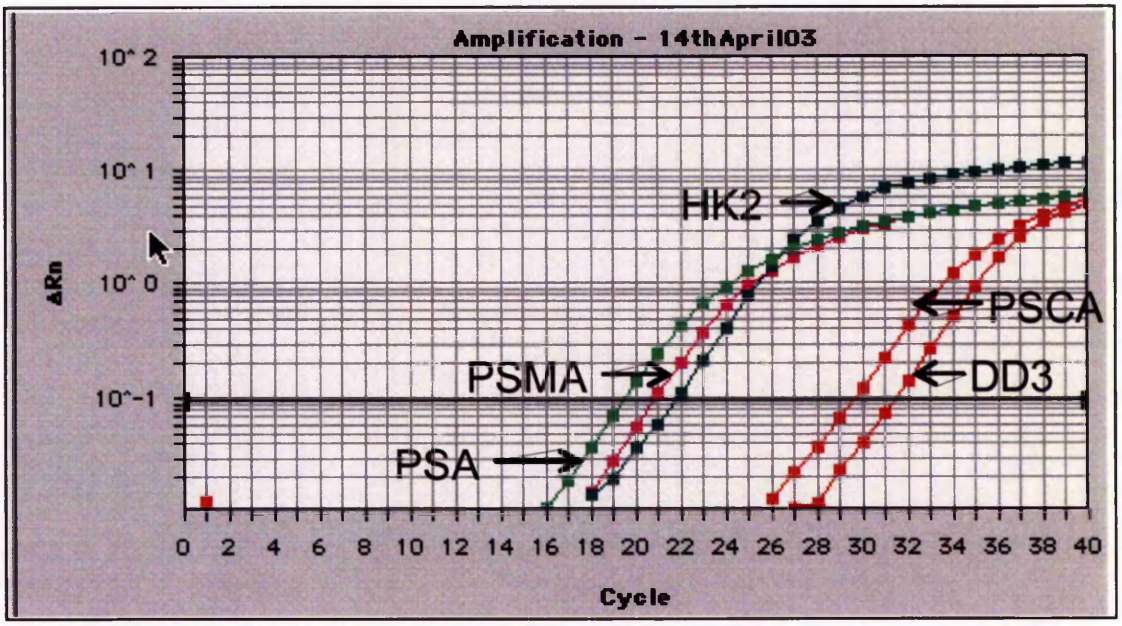



**Figure 3.8:** Real time PCR plot showing LNCaP cell line gene expression of the prostate markers PSA, PSMA, HK2, PSCA and DD3. Ct set at $10^{-1}$ to exclude background control fluorescence. Housekeeping gene amplification traces have been removed for clarity.

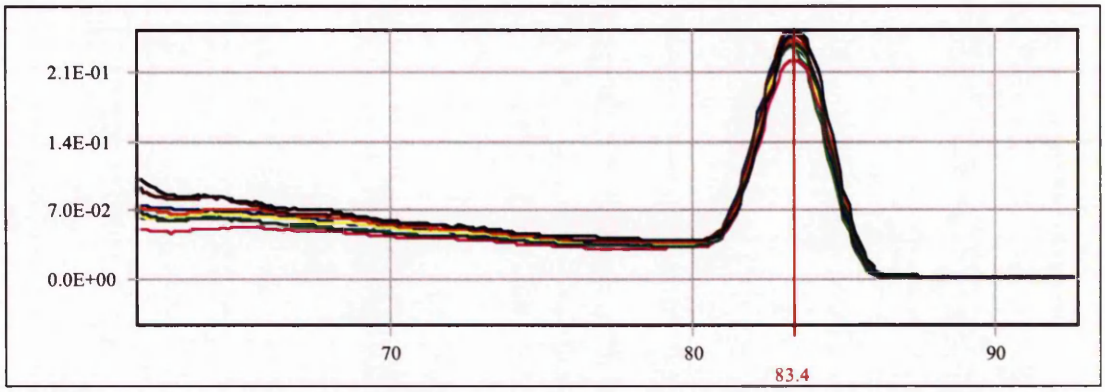



**Figure 3.9:** Dissociation curve for HK2 RT-PCR assay product generated by multiple replicate reactions from total RNA from $10^6$ LNCaP cells showing a Tm of 83.4°C. The Y-axis displays negative fluorescence plotted against temperature in degrees Centigrade.

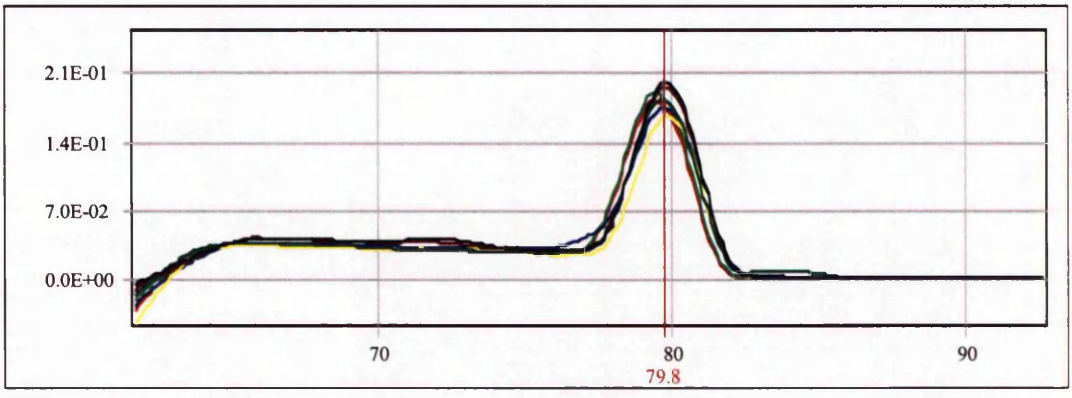



**Figure 3.10:** Dissociation curve for DD3 RT-PCR assay product generated by Taqman RT-PCR in multiple replicate reactions from total RNA from $10^6$ LNCaP cells showing a Tm of 79.8°C

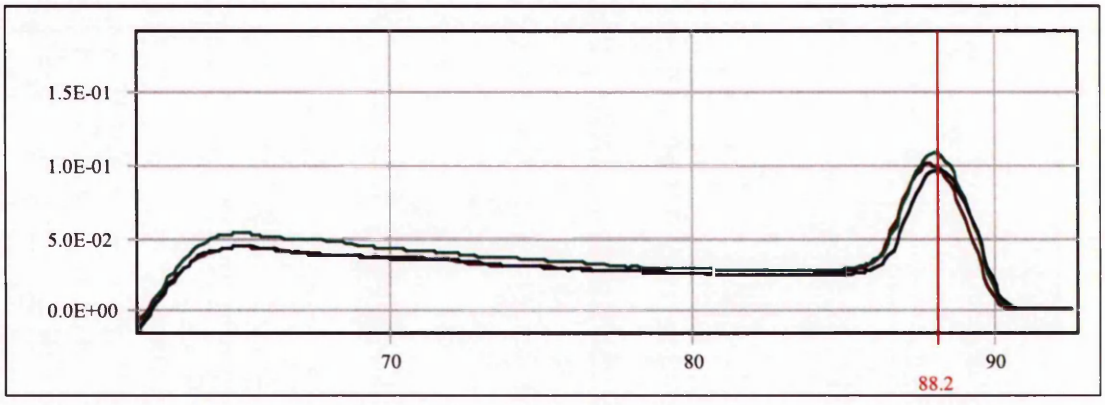



**Figure 3.11:** Dissociation curve for PSCA RT-PCR product generated by Taqman RT-PCR in multiple replicate reactions from total RNA from $10^6$ LNCaP cells showing a Tm of 88.2°C.

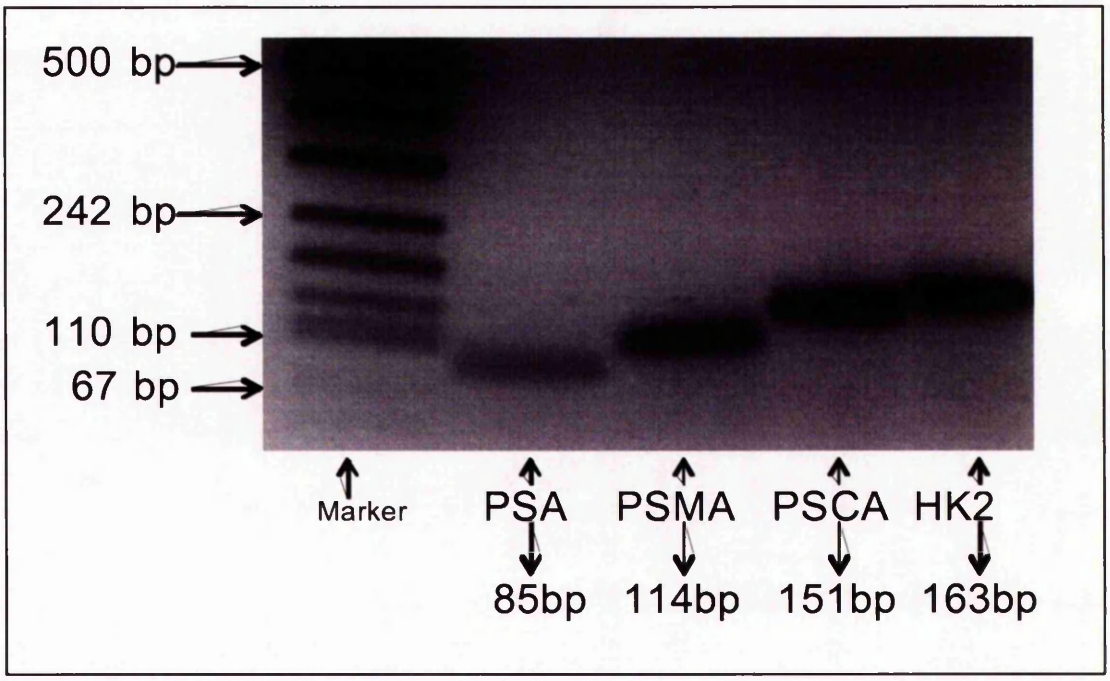



**Figure 3.12:** Real time PCR products for PSA, PSMA, PSCA and HK2 derived from RNA isolated from $10^6$ LNCaP cells viewed following electrophoresis on a 2% agarose gel. A molecular weight marker has been run concurrently in the left lane with known band sizes as shown. The expected PCR product sizes are shown below each gene.

### 3.3 PCR Product Sequences

To confirm the identity of the PCR products for the five prostate markers of interest, sequencing of the products was performed using forward and reverse primers. The sequencing data was then evaluated for sequence homology using FASTA software. This confirmed that the PCR products for all five assays were homologous with the expected sequences (figures 3.13 to 3.21).

**PSA PCR product predicted sequence:**

515-cagcat tgaaccagag gagttcttga ccccaaagaa acttcagtgt gtggacctcc

atgttatttc caatgacgtg tgtgcgcaa -599

**Sequencing of PSA PCR product with reverse primer:**

```
EM_HUM:AF335478; AF335478 Homo sapiens prostate-specif  (1945 nt)
rev-comp initn: 385 init1: 385 opt: 402  Z-score: 473.2  bits: 96.7
E(): 6e-17
banded Smith-Waterman score: 402;  97.619% identity (97.619% similar)
in 84 nt overlap (84-1:1754-1837)

                  100        90        80        70        60
PSA-              NNANAGNCCGCGCAAATGAGCGTTTAACCAGAGGAGTTCTTGACCCCA
                                     ::: :: ::::::::::::::::::::::
EM_HUM GGGACCACCTGCTACGCCTCAGGCTGGGGCAGCATTGAACCAGAGGAGTTCTTGACCCCA
          1730      1740      1750      1760      1770      1780

          50        40        30        20        10
PSA-   AAGAAACTTCAGTGTGTGGACCTCCATGTTATTTCCAATGACGTGTGTGCGCAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_HUM AAGAAACTTCAGTGTGTGGACCTCCATGTTATTTCCAATGACGTGTGTGCGCAAGTTCAC
          1790      1800      1810      1820      1830      1840

EM_HUM CCTCAGAAGGTGACCAAGTTCATGCTGTGTGCTGGACGCTGGACAGGGGGCAAAAGCACC
          1850      1860      1870      1880      1890      1900
```

**Figure 3.13:** PSA PCR assay product sequencing using the reverse primer. The expected sequence for the PSA PCR product is shown - size 85bp. The forward and reverse primer sequences are shown in red font. The sequencing data using the PSA reverse primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the PSA gene sequence, confirming product identity.

**PSMA PCR product predicted sequence:**

```
                                                    1311- tgaagtgaca

agaatttaca atgtgatagg tactctcaga ggagcagtgg aaccagacag atatgtcatt

ctgggaggtc accgggactc atgggtgttt ggtggtattg accctca -1427
```

**Sequencing of PSMA PCR product with forward primer:**

```
EM_HUM:M99487; M99487 Human prostate-specific membrane  (2653 nt)
 initn: 305 init1: 305 opt: 311  Z-score: 375.0  bits: 78.9 E():
1.3e-11
banded Smith-Waterman score: 311;  95.522% identity (95.522% similar)
in 67 nt overlap (25-91:1363-1429)

                     10        20        30        40        50
PSMA          TTCANTGTGATTCTNGTCCTTATCCCGNACAGATATGTCATTCTGGGAGGTCAC
                                      ::  ::::::::::::::::::::::::::
EM_HUM GTGATAGGTACTCTCAGAGGAGCAGTGGAACCAGACAGATATGTCATTCTGGGAGGTCAC
           1340      1350      1360      1370      1380      1390

           60        70        80        90
PSMA    CGGGACTCATGGGTGTTTGGTGGTATTGACCCTCAAAA
        :::::::::::::::::::::::::::::::::::: :
EM_HUM CGGGACTCATGGGTGTTTGGTGGTATTGACCCTCAGAGTGGAGCAGCTGTTGTTCATGAA
           1400      1410      1420      1430      1440      1450

EM_HUM ATTGTGAGGAGCTTTGGAACACTGAAAAAGGAAGGGTGGAGACCTAGAAGAACAATTTTG
           1460      1470      1480      1490      1500      1510
```

**Figure 3.14:** PSMA PCR assay product sequencing using the forward primer. The expected sequence for the PSMA PCR product is shown, spanning the boundary between exons 9 and 10 - size 114bp. The forward and reverse primer sequences are shown in red font and exon-exon boundary in blue font. The sequencing data using the PSMA forward primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the PSMA gene sequence, confirming product identity.

**PSMA PCR product predicted sequence:**

1311- tgaagtgaca

agaatttaca atgtgatagg tactctcaga ggagcagtgg aaccagacag atatgtcatt

ctgggaggtc accgggactc atgggtgttt ggtggtattg accctca -1427

**Sequencing of PSMA PCR product with reverse primer:**

```
EM_HUM:AF007544; AF007544 Homo sapiens prostate-specif  (93525 nt)
rev-comp initn: 123 init1:  95 opt: 157  Z-score: 160.2  bits: 44.7
E(): 0.34
banded Smith-Waterman score: 157;  85.965% identity (85.965% similar)
in 57 nt overlap (77-21:36229-36279)

            100         90         80         70         60         50
PSMA-  AGGCCCCTGCCNGNAAAAGNNAGNTTNTTTAGTGACCGAGAATTTACCAATGTGATAGGT
                                        ::::: :::::::::: ::::::::::::
EM_HUM AGTCAAGATGCACATCCACTCTACCAATGAAGTGA-CGAGAATTTA-CAATGTGATAGGT
    36200      36210      36220      36230       36240       36250

             40         30         20         10
PSMA-  ACTCTCCAGGAGGAGCCACGTGGAACCCACGACCAGAGATGTCAGTT
       ::::::   :::::::   ::::::::
EM_HUM ACTCTC--AGAGGAGC--AGTGGAACCAGGTAAAGGAATCGTTTGCTTAGCAAATATTGA
      36260       36270       36280      36290      36300      36310
```

**Figure 3.15:** PSMA PCR assay product sequencing using the reverse primer. The expected sequence for the PSMA PCR product is shown, spanning the boundary between exons 9 and 10 - size 114bp. The forward and reverse primer sequences are shown in red font and exon-exon boundary in blue font. The sequencing data using the PSMA reverse primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the PSMA gene sequence, confirming product identity.

**PSCA PCR product predicted sequence:**

```
                        87- tact cctgcaaagc ccaggtgagc aacgaggact

gcctgcaggt ggagaactgc acccagctgg gggagcagtg ctggaccgcg cgcatccgcg

cagttggcct cctgaccgtc atcagcaaag gctgcagctt gaactgcgtg gatgact -238
```

**Sequencing of PSCA PCR product with forward primer:**

```
EM_HUM:BC065183; BC065183 Homo sapiens prostate stem c  (1025 nt)
 initn: 705 init1: 705 opt: 708  Z-score: 702.6  bits: 138.9 E(): 1.9e-29
banded Smith-Waterman score: 708;  97.959% identity (97.959% similar) in
147 nt overlap (5-151:82-228)

                                          10        20        30
PSCA                                TTANTGTTAAGCCCAGGTGAGCAACGAGGACTGC
                                       ::  ::::::::::::::::::::::::::
EM_HUM CAGCCAGGCACTGCCCTGCTGTGCTACTCCTGCAAAGCCCAGGTGAGCAACGAGGACTGC
            60        70        80        90       100       110

          40        50        60        70        80        90
PSCA   CTGCAGGTGGAGAACTGCACCCAGCTGGGGGAGCAGTGCTGGACCGCGCGCATCCGCGCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_HUM CTGCAGGTGGAGAACTGCACCCAGCTGGGGGAGCAGTGCTGGACCGCGCGCATCCGCGCA
           120       130       140       150       160       170

         100       110       120       130       140       150
PSCA   GTTGGCCTCCTGACCGTCATCAGCAAAGGCTGCAGCTTGAACTGCGTGGATGACTAAACN
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::: :
EM_HUM GTTGGCCTCCTGACCGTCATCAGCAAAGGCTGCAGCTTGAACTGCGTGGATGACTCACAG
           180       190       200       210       220       230


PSCA   N

EM_HUM GACTACTACGTGGGCAAGAAGAACATCACGTGCTGTGACACCGACTTGTGCAACGCCAGC
           240       250       260       270       280       290
```

**Figure 3.16:** PSCA PCR assay product sequencing using the forward primer. The expected sequence for the PSCA PCR product is shown - size 151bp. The forward and reverse primer sequences are shown in red font. The sequencing data using the PSCA forward primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the PSCA gene sequence, confirming product identity.

**PSCA PCR product predicted sequence:**

```
                        87- tact cctgcaaagc ccaggtgagc aacgaggact

gcctgcaggt ggagaactgc acccagctgg gggagcagtg ctggaccgcg cgcatccgcg

cagttggcct cctgaccgtc atcagcaaag gctgcagctt gaactgcgtg gatgact -238
```

**Sequencing of PSCA PCR product with reverse primer:**

```
EM_HUM:BC065183; BC065183 Homo sapiens prostate stem c  (1025 nt)
rev-comp initn: 535 init1: 535 opt: 535  Z-score: 536.5  bits: 107.6
E(): 3.4e-20
banded Smith-Waterman score: 535;  100.000% identity (100.000%
similar) in 107 nt overlap (108-2:76-182)

                                    110       100        90        80
PSCA-                                NTTTACTCCTGCAAAGCCCAGGTGAGCAACGAG
                                     :::::::::::::::::::::::::::::::
EM_HUM GCCCTGCAGCCAGGCACTGCCCTGCTGTGCTACTCCTGCAAAGCCCAGGTGAGCAACGAG
         50        60        70        80        90       100

              70        60        50        40        30        20
PSCA-  GACTGCCTGCAGGTGGAGAACTGCACCCAGCTGGGGGAGCAGTGCTGGACCGCGCGCATC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_HUM GACTGCCTGCAGGTGGAGAACTGCACCCAGCTGGGGGAGCAGTGCTGGACCGCGCGCATC
         110       120       130       140       150       160

              10
PSCA-  CGCGCAGTTGGCCTCCTA
       ::::::::::::::::::
EM_HUM CGCGCAGTTGGCCTCCTGACCGTCATCAGCAAAGGCTGCAGCTTGAACTGCGTGGATGAC
         170       180       190       200       210       220
```

**Figure 3.17:** PSCA PCR assay product sequencing using the reverse primer. The expected sequence for the PSCA PCR product is shown - size 151bp. The forward and reverse primer sequences are shown in red font. The sequencing data using the PSCA reverse primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the PSCA gene sequence, confirming product identity.

**HK2 PCR product predicted sequence:**

522- gaaccagag gagttcttgc

gccccaggag tcttcagtgt gtgagcctcc atctcctgtc caatgacatg tgtgctagag

cttactctga gaaggtgaca gagttcatgt tgtgtgctgg gctctggaca ggtggtaaag

acacttgtgg gggtgattct gggg -684

**Sequencing of HK-2 PCR product with forward primer:**

```
EM_HUM:AF188746; AF188746 Homo sapiens prostrate kalli  (2830 nt)
 initn: 630 init1: 630 opt: 630  Z-score: 685.5  bits: 137.1 E():
6.2e-29 banded Smith-Waterman score: 630;  100.000% identity (100.000%
similar) in 126 nt overlap (11-136:534-659)

                                    10        20        30        40
HK2                              CCTTTGCTTAGTGTGAGCCTCCATCTCCTGTCCAATGACA
                                    :::::::::::::::::::::::::::::::
EM_HUM AGGAGTTCTTGCGCCCCAGGAGTCTTCAGTGTGTGAGCCTCCATCTCCTGTCCAATGACA
           510       520       530       540       550       560

               50        60        70        80        90       100
HK2     TGTGTGCTAGAGCTTACTCTGAGAAGGTGACAGAGTTCATGTTGTGTGCTGGGCTCTGGA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_HUM TGTGTGCTAGAGCTTACTCTGAGAAGGTGACAGAGTTCATGTTGTGTGCTGGGCTCTGGA
           570       580       590       600       610       620

              110       120       130       140       150
HK2     CAGGTGGTAAAGACACTTGTGGGGGTGATTCTGGGGANCNNNNNCCCCCC
        ::::::::::::::::::::::::::::::::::
EM_HUM CAGGTGGTAAAGACACTTGTGGGGGTGATTCTGGGGGTCCACTTGTCTGTAATGGTGTGC
           630       640       650       660       670       680

EM_HUM TTCAAGGTATCACATCATGGGGCCCTGAGCCATGTGCCCTGCCTGAAAAGCCTGCTGTGT
           690       700       710       720       730       740
```

**Figure 3.18:** HK-2 PCR assay product sequencing using the forward primer. The expected sequence for the HK2 PCR product is shown - size 163bp. The forward and reverse primer sequences are shown in red font. The sequencing data using the HK2 forward primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the HK2 gene sequence, confirming product identity.

**HK2 PCR product predicted sequence:**

```
                                          522- gaaccagag gagttcttgc

gccccaggag tcttcagtgt gtgagcctcc atctcctgtc caatgacatg tgtgctagag

cttactctga gaaggtgaca gagttcatgt tgtgtgctgg gctctggaca ggtggtaaag

acacttgtgg gggtgattct gggg -684
```

**Sequencing HK-2 PCR product with reverse primer:**

```
EM_HUM:AF188747; AF188747 Homo sapiens prostrate kalli  (2867 nt)
rev-comp initn: 344 init1: 344 opt: 604  Z-score: 669.8  bits: 134.1
E(): 4.5e-28 banded Smith-Waterman score: 604;  96.992% identity
(96.992% similar) in 133 nt overlap (133-1:495-625)


                                      130       120       110
HK-2-                               NNNTTTGAACCAGAGGAGTTCTTGCGCCCCAGGA
                                    : ::::::::::::::::::::::::::::
EM_HUM CCACCTGCTACGCCTCAGGCTGGGGCAGCATCGAACCAGAGGAGTTCTTGCGCCCCAGGA
          470       480       490       500       510       520

        100        90        80        70        60        50
HK-2-  GTCTTCAGTGTGTGAGCCTCCATCTTCCTGTCCAATGACATGTGTGCTAGAGCTTACTCT
       :::::::::::::::::::::::: :::::::::::::::::::::::::::::::::
EM_HUM GTCTTCAGTGTGTGAGCCTCCATC-TCCTGTCCAATGACATGTGTGCTAGAGCTTACTCT
          530       540       550       560       570       580

         40        30        20        10
HK-2-  GAGAAGGTGACAGAGTTCANGTTGTGTGCTGGGCTNCTGGACA
       :::::::::::::::::: :::::::::::::: :::::::
EM_HUM GAGAAGGTGACAGAGTTCATGTTGTGTGCTGGGCT-CTGGACAGGTGGTAAAGACACTTG
          590       600       610       620       630       640

EM_HUM TGGGGTGAGTCATCCCTACTCCCAACATCTGGAGGGGAAAGGGTGATTCTGGGGGTCCAC
          650       660       670       680       690       700
```

**Figure 3.19:** HK-2 PCR assay product sequencing using the reverse primer. The expected sequence for the HK2 PCR product is shown - size 163bp. The forward and reverse primer sequences are shown in red font. The sequencing data using the HK2 reverse primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the HK2 gene sequence, confirming product identity.

**DD3$^{PCA3}$ PCR product predicted sequence:**

```
                                        97- tggg aaggacctga tgatacagag
gaattacaac acatatactt agtgtttcaa tgaacaccaa gataaataag tgaagagcta
gtccgctgtg agtctcctca gtgacacagg gctggatcac catcgacggc actttctgag
tactcagtgc agcaaagaaa gactacagac atctcaatgg caggggtgag aaataagaaa
ggctgctgac tttaccatct gaggccacac atctgctgaa atggagataa ttaacatcac
tagaaacagc aagatgacaa tataatgtct aagtagtgac atgtttttgc acatttccag
ccccttaaa tatccacaca cacaggaagc acaaaaggaa gcacagagat ccctggga -478
```

**Sequencing of DD3$^{PCA3}$ PCR product with forward primer:**

```
EM_PAT:BD082485; BD082485 PCA3, PCA3 genes, and method  (3582 nt)
 initn: 813 init1: 760 opt: 859  Z-score: 850.0  bits: 168.2 E():
3.3e-38. banded Smith-Waterman score: 859;  98.895% identity (98.895%
similar) in 181 nt overlap (11-190:299-478)

                                    10        20        30
DD3                           GGGGGAATAGAAGGCTGCTGACTTT-CCATCTGAGGCCAC
                                      :::::::::::::: ::::::::::::::
EM_PAT ACATCTCAATGGCAGGGGTGAGAAATAAGAAAGGCTGCTGACTTTACCATCTGAGGCCAC
      270       280       290       300       310       320

      40        50        60        70        80        90
DD3    ACATCTGCTGAAATGGAGATAATTAACATCACTAGAAACAGCAAGATGACAATATAATGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PAT ACATCTGCTGAAATGGAGATAATTAACATCACTAGAAACAGCAAGATGACAATATAATGT
      330       340       350       360       370       380

     100       110       120       130       140       150
DD3    CTAAGTAGTGACATGTTTTTGCACATTTCCAGCCCCTTTAAATATCCACACACACAGGAA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PAT CTAAGTAGTGACATGTTTTTGCACATTTCCAGCCCCTTTAAATATCCACACACACAGGAA
      390       400       410       420       430       440

     160       170       180       190
DD3    GCACAAAAGGAAGCACAGGAGATCCCTGGGANN
       ::::::::::::::::: ::::::::::::
EM_PAT GCACAAAAGGAAGCACA-GAGATCCCTGGGAGAAATGCCCGGCCGCCATCTTGGGTCATC
      450       460       470       480       490       500

EM_PAT GATGAGCCTCGCCCTGTGCCTGGTCCCGCTTGTGAGGGAAGGACATTAGAAAATGAATTG
       510       520       530       540       550       560
```

**Figure 3.20:** DD3$^{PCA3}$ PCR assay product sequencing using the forward primer. The expected sequence for the DD3$^{PCA3}$ PCR product is shown - size 381bp. The forward and reverse primer sequences are shown in red font. The sequencing data using the DD3$^{PCA3}$ forward primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the DD3$^{PCA3}$ gene sequence, confirming product identity.

**DD3$^{PCA3}$ PCR product predicted sequence:**

```
                                        97- tggg aaggacctga tgatacagag
gaattacaac acatatactt agtgtttcaa tgaacaccaa gataaataag tgaagagcta
gtccgctgtg agtctcctca gtgacacagg gctggatcac catcgacggc actttctgag
tactcagtgc agcaaagaaa gactacagac atctcaatgg caggggtgag aaataagaaa
ggctgctgac tttaccatct gaggccacac atctgctgaa atggagataa ttaacatcac
tagaaacagc aagatgacaa tataatgtct aagtagtgac atgtttttgc acatttccag
ccccttaaa tatccacaca cacaggaagc acaaaaggaa gcacagagat ccctggga -478
```

**Sequencing of DD3$^{PCA3}$ PCR product with reverse primer:**

```
EM_PAT:BD082483; BD082483 PCA3, PCA3 genes, and method  (1872 nt)
rev-comp initn: 382 init1: 382 opt: 747  Z-score: 823.3  bits: 162.4
E(): 1.9e-36. banded Smith-Waterman score: 747;  88.649% identity
(88.649% similar) in 185 nt overlap (193-10:75-258)

                                        190       180       170
DD3-                                 TTTGGNNGGGCCCGNTGAGACACAGGTGAGA
                                     : ::  :: :: : ::: ::: ::::::::
EM_PAT AGCCGAGGGAGACCAGGAAGATCTGCATGGTGGGAAGGACCTGATGATACAGAGGTGAGA
           50        60        70        80        90       100

        160       150       140       130       120       110
DD3-   NATAAGAAAGGCTGCTGACTTTACCATCTGNGGCCACACATCTGCTGNAATGGAGATAAT
       :::::::::::::::::::::::::::::: ::::::::::::::: ::::::::::::
EM_PAT AATAAGAAAGGCTGCTGACTTTACCATCTGAGGCCACACATCTGCTGAAATGGAGATAAT
         110       120       130       140       150       160

        100        90        80        70        60        50
DD3-   TANCATCACTAGGAAACAGCAAGATGACAATATAATGTCTAAGTAGTGACATGTTTTTGC
       :: :::::::: :::::::::::::::::::::::::::::::::::::::::::::::::
EM_PAT TAACATCACTA-GAAACAGCAAGATGACAATATAATGTCTAAGTAGTGACATGTTTTTGC
         170       180       190       200       210       220

         40        30        20        10
DD3-   ACANTCNCNGCCCC-NTAANTNTCCACACACACAGCTCCCAGTG
       ::: :  : ::::: ::: : ::::::::::::
EM_PAT ACATTTCCAGCCCCTTTAAATATCCACACACACAGGAAGCACAAAAGGAAGCACAGAGAT
           230       240       250       260       270       280

EM_PAT CCCTGGGAGAAATGCCCGGCCGCCATCTTGGGTCATCGATGAGCCTCGCCCTGTGCCTGG
           290       300       310       320       330       340
```

**Figure 3.21:** DD3$^{PCA3}$ PCR assay product sequencing using the reverse primer. The expected sequence for the DD3$^{PCA3}$ PCR product is shown - size 381bp. The forward and reverse primer sequences are shown in red font. The sequencing data using the DD3$^{PCA3}$ reverse primer is shown below. This has been evaluated using FASTA nucleotide homology software and is aligned with the DD3$^{PCA3}$ gene sequence, confirming product identity.

### 3.4 Primer Optimisation

Table 3.1 illustrates the impact of altering the ratios of forward and reverse primer concentrations on sensitivity of PSA, PSMA, PSCA and HK2 assays in detecting target in 100ng of LNCaP derived RNA. The left hand column shows the primer concentration ratio used in each reaction with the consequent numeric Ct value for each marker being shown in subsequent columns. Each reaction was performed in triplicate on two separate occasions and the Ct values shown are the mean values. The concentration with the lowest Ct value is the optimal ratio which correlates to nanomolar concentration ratios of forward to reverse primers of 300:900, 900:900, 300:300 and 900:900 for PSA, PSMA, PSCA and HK2 respectively.

| Primer Concentration | CYCLE NUMBER | | | |
|---|---|---|---|---|
| Ratio Forward:Reverse (nM) | PSA | PSMA | PSCA | HK2 |
| 100:100 | 21.3 | 34.6 | 27.1 | 28.1 |
| 300:100 | 29.3 | 33.1 | 26.5 | 27.2 |
| 900:100 | 21.9 | 34.2 | 29.1 | 26.6 |
| 100:300 | 20.4 | 32.4 | 26.4 | 28.4 |
| 300:300 | 18.8 | 31.9 | 25.8 | 27.4 |
| 900:300 | 19.1 | 30.6 | 25.9 | 27.0 |
| 100:900 | 19.2 | 32.1 | 26.1 | 28.6 |
| 300:900 | 18.6 | 32.2 | 25.5 | 27.6 |
| 900:900 | 21.2 | 30.2 | 28.0 | 26.3 |

**Table 3.1:** Primer optimisation – the effect of varying primer concentration ratios on assay sensitivity utilising 100ng of LNCaP derived total RNA. The shaded cell identifies the optimal ratios.

### 3.5 Optimisation of LNCaP Cell Line

LNCaP cell line marker expression has been shown by other groups to be altered by cell culture conditions and by prolonged time in culture. To assess the effect of cell culture and dihydrotestosterone (DHT) on marker gene expression in LNCaP cells, RNA was isolated from low passage cells (P14), high passage cells (P93) and high passage cells cultured in the presence of 1nM DHT (P93+DHT). All five genes, PSA, PSMA, PSCA, HK2 and $DD3^{PCA3}$, were expressed in LNCaP cells under all cell culture conditions examined (figure 3.22).

Expression of the house keeping genes β-actin and GAPDH was determined to allow normalisation between cell culture conditions. Once standardised to ß-actin and GAPDH expression there was little variation in the levels of gene mRNA expression (figure 3.23). Slight variation in PSCA expression was seen in the different cell samples being highest in P93 LNCaP cells with a Ct value of 28.2 while the Ct values for P93 cells cultured with 1nM DHT and P14 cells were 29.3 and 30.0 respectively.

### 3.6 Nucleotide Probes

#### 3.6.1 Probe Optimisation

The optimal probe concentrations for the PSA, PSMA, PSCA and HK2 assays were identified using sequentially increasing concentrations of probe from 10nM to 200nM in RT-PCR reactions with 100ng of target cDNA from $10^6$ LNCaP cells. For all markers there was a stepwise decrease in Ct as probe concentration increased. The greatest improvements in performance were observed as probe concentration was increased from 10nM to 50nM although further small improvements were seen with further concentration increases. The impact of probe concentration was most pronounced for PSCA with a fall in Ct of over 6 cycles as probe concentration increased from 10nm to 200nM (figures 3.24 to 3.26). 200nM probe concentrations gave optimal performance for all assays.

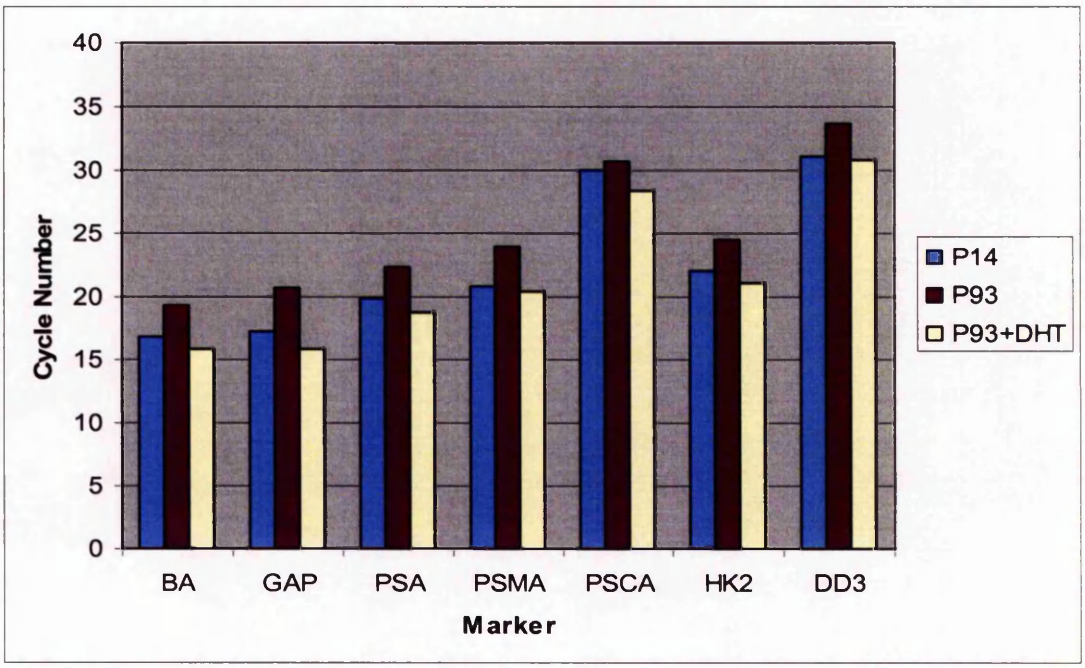



**Figure 3.22:** Prostate marker gene expression of early and late passage LNCaP cells cultured in standard conditions and late passage cells stimulated with 1nM DHT for 120 hours prior to RNA isolation. Cycle number is inversely proportional to marker mRNA expression.

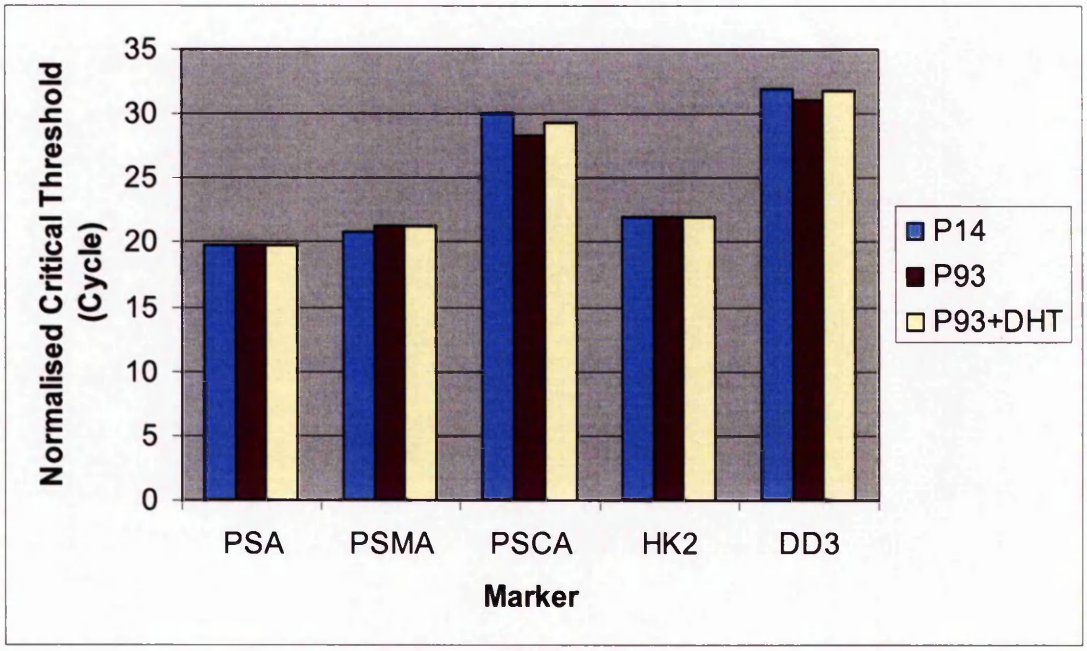



**Figure 3.23:** Prostate marker gene expression for P14, P93 and P93+DHT 1nM LNCaP cells normalised to ß-actin expression. Cycle number is inversely proportional to marker mRNA expression. Cell passage number and DHT stimulation have little overall impact on marker mRNA expression.

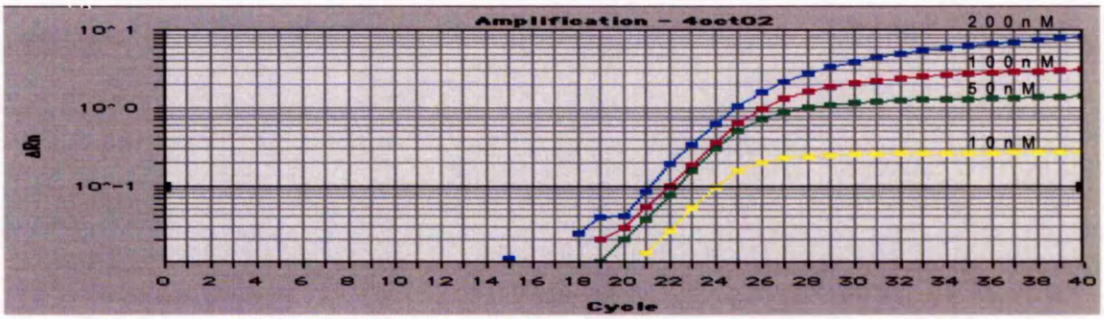



**Figure 3.24:** Taqman RT-PCR reaction using serial probe concentrations for PSA expression in 100ng LNCaP RNA with optimal primer concentration ratios of 300nM : 900nM, forward to reverse. The plots are from single reactions at each concentration while overall assay performance at each probe concentration was calculated from the mean Ct values for replicate reactions.

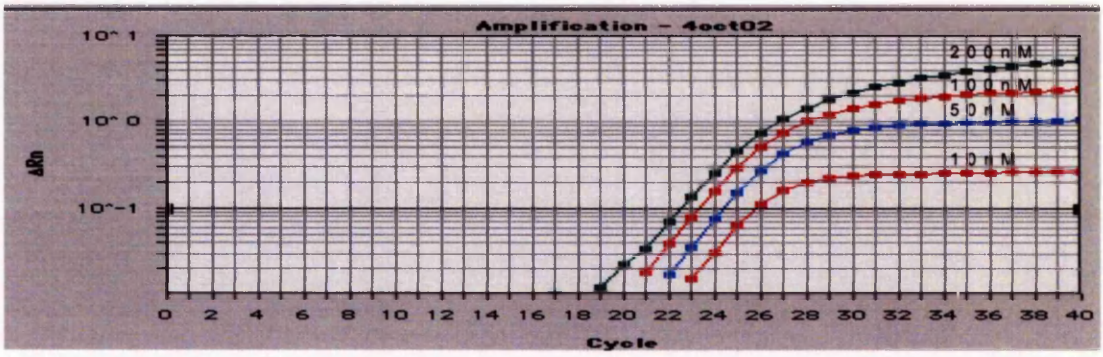



**Figure 3.25:** Taqman RT-PCR reaction using serial probe concentrations for PSMA expression in 100ng LNCaP RNA. The plots are from single reactions at each concentration while overall assay performance at each probe concentration was calculated from the mean Ct values for replicate reactions.

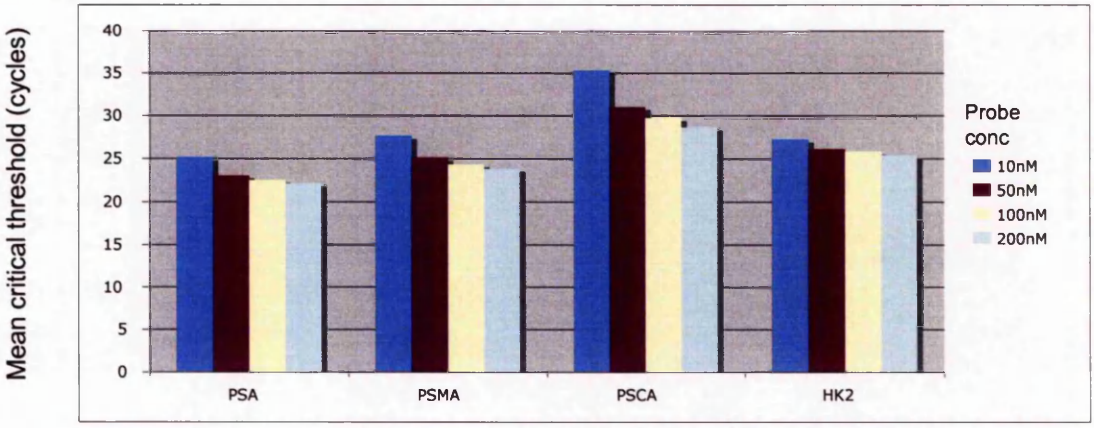



**Figure 3.26:** Probe optimisation for PSA, PSMA, PSCA and HK2 RT-PCR assays. RT-PCR reactions were run for each of the markers using optimised primer ratios with increasing probe concentrations from 10nM to 200nM. Bar chart showing the mean Ct cycle value for replicate reactions using each probe concentration for each marker.

### 3.6.2 Problems Encountered With Probes

#### 3.6.2.1. Minus RT Control Reactions

The minus RT reaction is a control to ensure that the primers are not binding to and amplifying genomic DNA contaminants in the extracted RNA. Genomic DNA contaminants were not detected using a SYBR Green based assay for any of the primer sets. However, when product detection relied on product specific probes, fluorescence was detected in these reactions reaching a high enough level to cross the critical threshold. The fluorescence plots produced by these minus RT reactions were not in an exponential phase when they crossed the critical threshold level and did not plateau. Figure 3.27 illustrates this finding for the PSA RT-PCR assay.

#### 3.6.2.2 Comparison of SYBR Green and Gene Specific Probe Sensitivity

Comparative experiments investigated the impact of non-specific SYBR Green or gene specific fluorescent probe use on the sensitivity of the assay in detecting PSA, PSMA and PSCA in 100ng of cDNA from LNCaP cells. For all three assays the incorporation of a probe increased the cycle number at which the reactions crossed the Ct compared to an otherwise identical reaction using SYBR Green (figure 3.28). Probe use decreased the sensitivity by 0.8 cycles for PSA, 1.7 cycles for PSMA and 2.2 cycles in the PSCA assay. The impact of mode of product detection on *in vitro* assay performance is shown in section 3.7.1 for PSA, 3.7.2 for PSMA and 3.7.4 for HK2.

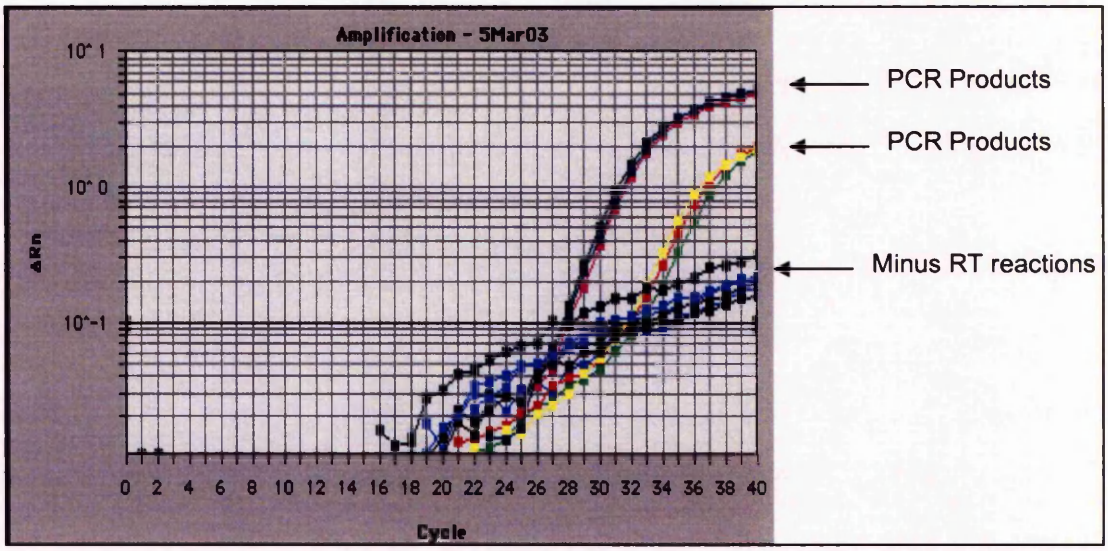



**Figure 3.27:** PSA assay using probes - cDNA and minus RT controls derived from $10^3$ and $10^2$ LNCaP cells spiked in female peripheral blood were amplified by Taqman PCR assay for PSA using product specific probe for product detection. The six PCR reactions containing cDNA target show exponential fluorescence plots, crossing the Ct during the exponential phase. The minus RT control reactions produce gradually increasing fluorescence on a cycle by cycle basis however this is not in a typical exponential amplification pattern.

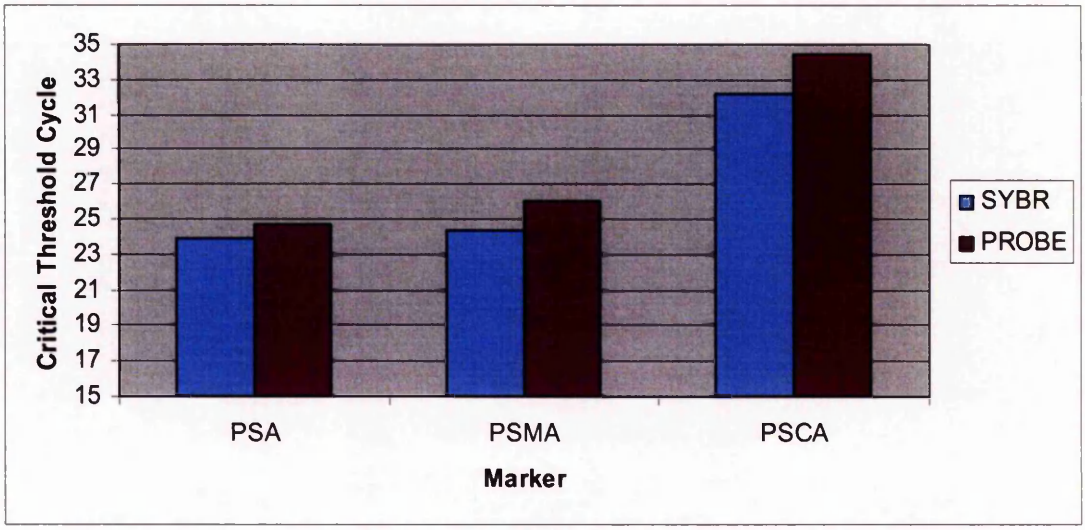



**Figure 3.28:** Gene specific probe or SYBR Green - impact of mode of product detection on PSA, PSMA and PSCA RT-PCR assay performance. Bar chart showing mean Ct cycle values for replicate assay reactions using 100ng of LNCaP RNA target and optimal primer concentrations with either nucleotide probe or SYBR green.

### 3.7 Assay Sensitivity – The Detection of LNCaP Cells in a Peripheral Blood Background

The *in vitro* sensitivity of the assay for each marker was determined by its capability in detecting serial dilutions of LNCaP cells, from 1 to $10^5$, spiked into a background of female peripheral blood. The sensitivity was assessed using SYBR Green fluorescence for all markers and additionally using probes for PSA, PSMA and HK2.

#### 3.7.1 PSA Assay

##### 3.7.1.1 PSA Assay Using SYBR Green

PSA primers reliably detected 10 LNCaP cells per ml of female blood with 1 cell being detected in one of each set of triplicate reactions (figure 3.29). The reaction containing $10^4$ LNCaP cells (yellow plot) crossed the Ct at the lowest cycle number. With decreasing numbers of spiked LNCaP cells the reactions cross the Ct at increasing cycle numbers in a stepwise manner to the right. The limit of detection for this assay was 1 LNCaP cell per ml of female blood (green plot). Female blood controls, target free controls and minus RT reactions demonstrated no detectable signal confirming specificity and absence of genomic DNA contamination. Housekeeping genes were also successfully amplified for each sample.

##### 3.7.1.2 PSA Assay Using Nucleotide Probe

When nucleotide probes were used for PSA RT-PCR product detection, the Ct values were higher for each LNCaP serial dilution compared to those obtained using SYBR Green. Figure 3.30 shows the mean Ct values recorded in replicate reactions, targeting PSA mRNA from different numbers of LNCaP cells spiked into 2.5 ml of female PB using either SYBR Green or PSA probe. Both probe and SYBR Green could detect 10 cells, however the Ct value was lower, i.e. higher sensitivity, with SYBR Green 35.5 Vs 37.2 cycles. Only SYBR Green could detect 1 cell in a female PB background. Therefore, the incorporation of nucleotide probes decreased PSA assay *in vitro* sensitivity.

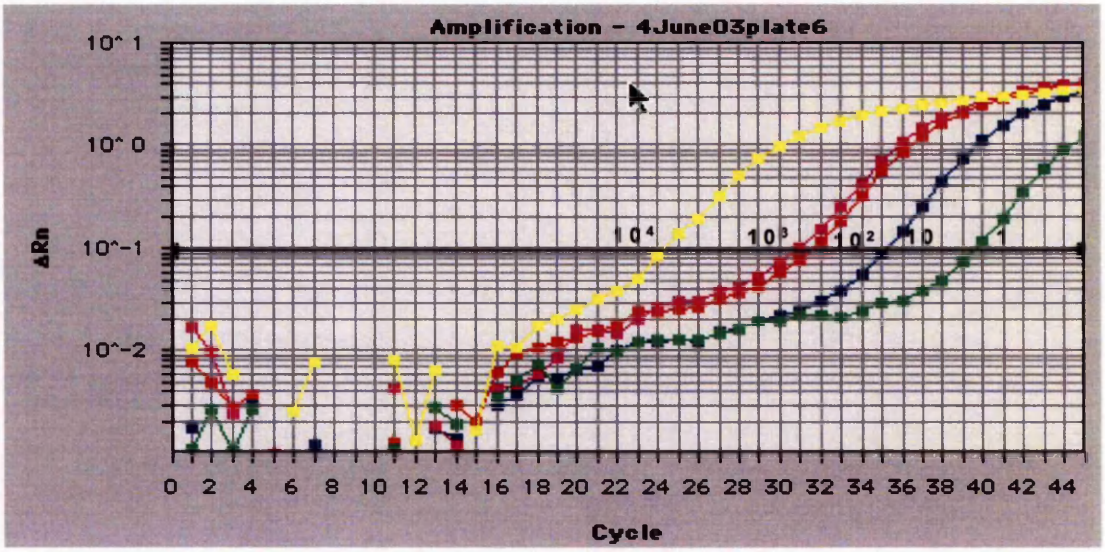



**Fig 3.29:** Sensitivity for PSA assay using SYBR Green, 100ng of total RNA from 2.5 ml female PB spiked with $10^4$, $10^3$, $10^2$, 10, 1 LNCaP was assessed using optimal primer concentration ratio of 300nM forward : 900nM reverse. Reactions containing RNA from $10^4$, $10^3$, $10^2$, 10 and 1 LNCaP were positive, the amplification plots crossing the critical threshold (black line).

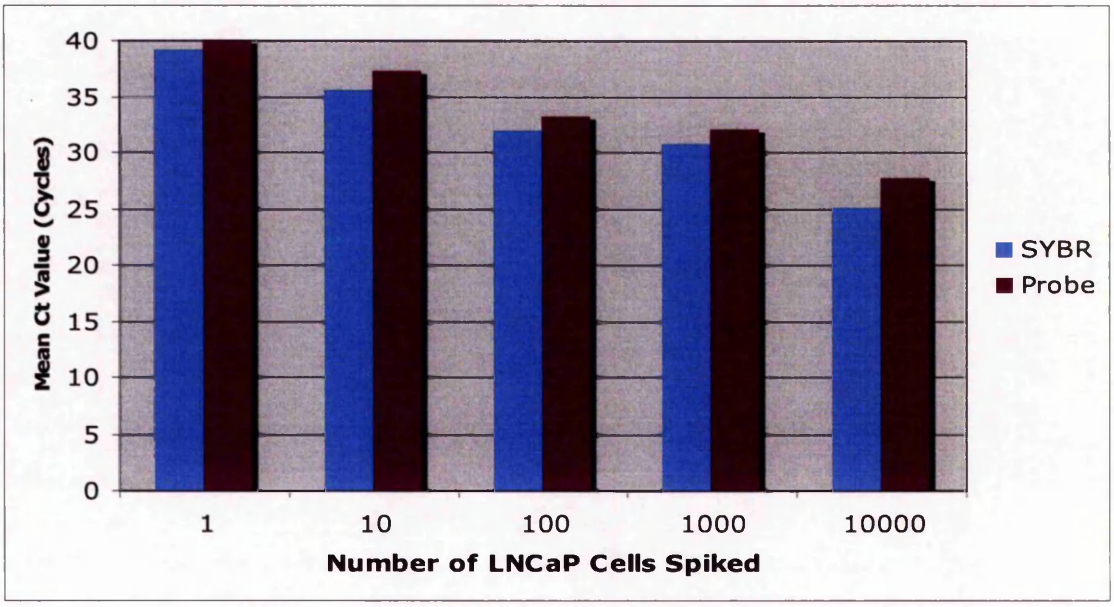



**Figure 3.30:** Comparison of PSA assay sensitivity when using either SYBR Green or nucleotide probes for product detection of PSA in 100ng of RNA isolated from serial dilutions of LNCaP cells in a background of female PB. The left column in each pair illustrates the mean Ct value for the replicate reactions using SYBR Green while the mean Ct value for reactions using probe is represented by the right column. The mean Ct values for the reactions using SYBR Green were lower than for those using probe for all cell spike concentrations, and only the SYBR Green based assay could detect PSA target in the 1 cell sample.

### 3.7.2 PSMA Assay

#### 3.7.2.1 PSMA Assay Using SYBR Green

Using PSMA primers for reactions containing cDNA derived from cell spiked female PB samples containing $10^5$ down to 10 cells (figure 3.31), 100 cells were reliably detected whilst 10 cells were detected in one of each triplicate reaction. All control and minus RT reactions were negative producing no specific product. B-actin and GAPDH targets were successfully amplified from the source cDNA.

#### 3.7.2.2 PSMA Assay Using Nucleotide Probe

As was observed for PSA, the use of nucleotide probes for PSMA RT-PCR product detection, reduced assay *in vitro* sensitivity, the Ct values being higher for each LNCaP serial dilution compared to those obtained using SYBR Green. Figure 3.32 shows the mean Ct values recorded in replicate reactions, targeting PSMA mRNA from different numbers of LNCaP cells spiked into 2.5 ml of female PB using either SYBR Green or PSMA probe. Both probe and SYBR Green could detect 100 cells, however the Ct value was lower, i.e. higher sensitivity, with SYBR green 32.4 Vs 38.2 cycles. Only SYBR Green could detect 10 cells in a female PB background.

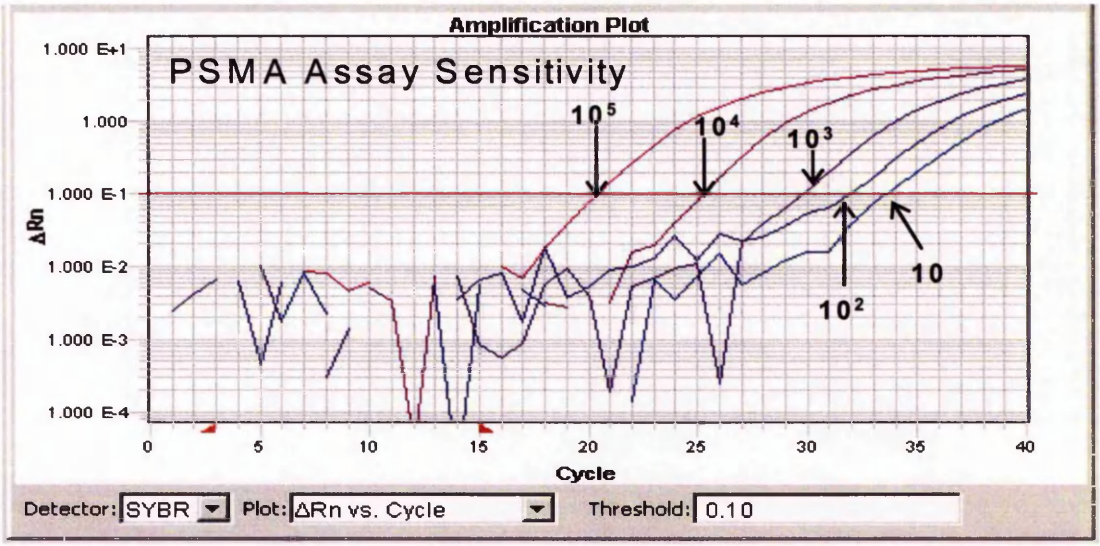



**Fig 3.31:** Sensitivity for PSMA assay using SYBR Green. 100ng of total RNA from 2.5 ml female PB spiked with $10^5$, $10^4$, $10^3$, $10^2$, 10, 1 LNCaP was assessed using optimal primer concentration ratio of 900nM forward : 900nM reverse. The reactions containing RNA from $10^5$, $10^4$, $10^3$, $10^2$ and 10 LNCaP were positive, the amplification plots crossing the critical threshold (red line).

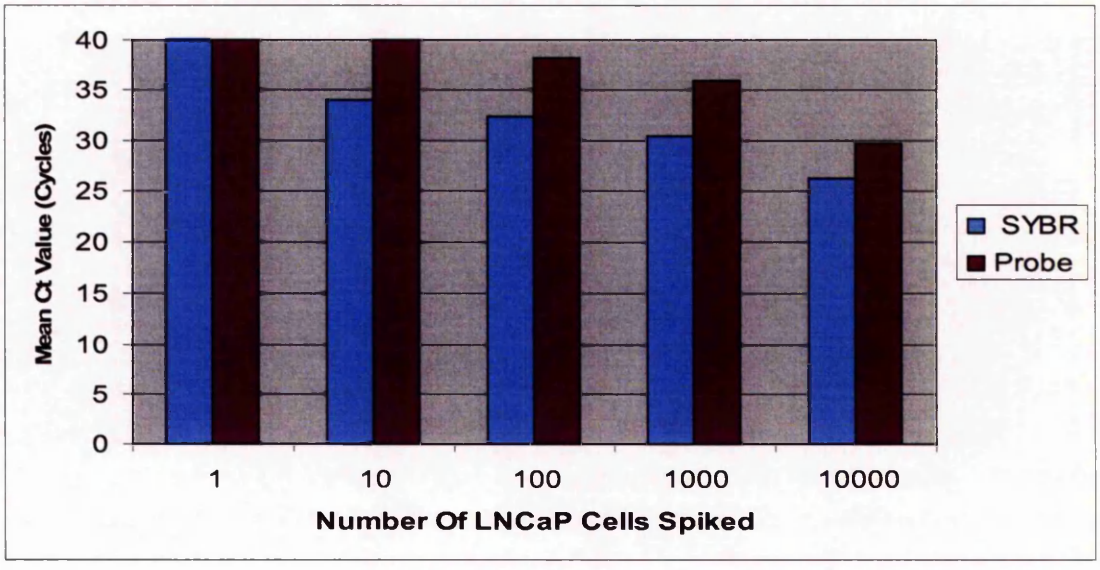



**Figure 3.32:** Comparison of PSMA assay sensitivity when using either SYBR Green or nucleotide probes for product detection of PSMA in 100ng of RNA isolated from serial dilutions of LNCaP cells in a background of female PB. The left column in each pair illustrates the mean Ct value for replicate reactions using SYBR Green while the mean Ct value for reactions using probe is represented by the right column. The mean Ct values for the reactions using SYBR Green were lower than for those using probe for all cell spike concentrations, and only the SYBR Green based assay could detect PSMA target in the 10 cell sample.

### 3.7.3 PSCA Assay

The PSCA assay displayed a limit of detection of 100 cells in 2.5ml of female blood but only one in every three reactions could detect LNCaP cells at this concentration. In figure 3.33 the amplification plots from the reactions using cDNA from the $10^5$, $10^4$ and $10^3$ cell spike samples cross the critical threshold. Background fluorescence was observed in the 10 and 1 cell reactions, the fluorescence levels remaining well below the Ct without a rapid exponential increase. Control reactions containing cDNA from normal female blood alone however gave positive assay results. Minus RT reactions were negative as were target free controls. Housekeeping genes were successfully amplified.

Repeat assays using fresh assay components and target reproduced PSCA detection in female PB. Interrogation of sequence databases looking for alternative targets with which our primers might bind and amplify failed to identify another target.

### 3.7.4 HK2 Assay

#### 3.7.4.1 HK2 Assay Using SYBR Green

When the HK2 primers were used with cDNA from cell spiked female peripheral blood samples all of the reactions containing HK2 primers crossed the Ct within a range of three cycles, 27 and 30 cycles, including water and minus RT control reactions (figure 3.34). When the HK2 PCR reaction products were dissociated the samples containing 0 to 100 cells proved to be entirely primer dimer with a Tm around 71°C. The $10^3$ and $10^4$ cell spike samples contained product with a Tm around the expected 83.5°C but also a significant amount of primer dimer at the lower Tm (figure 3.35).

#### 3.7.4.2 HK2 Assay Using Nucleotide Probe

The addition of the nucleotide probe for HK2 product detection addressed the problems of primer dimer detection in the SYBR Green protocol. Using HK2 specific primers and probe to detect serial dilutions of LNCaP cells in female PB, $10^2$ cells per 2.5 ml of blood could be reliably detected (figure 3.36).

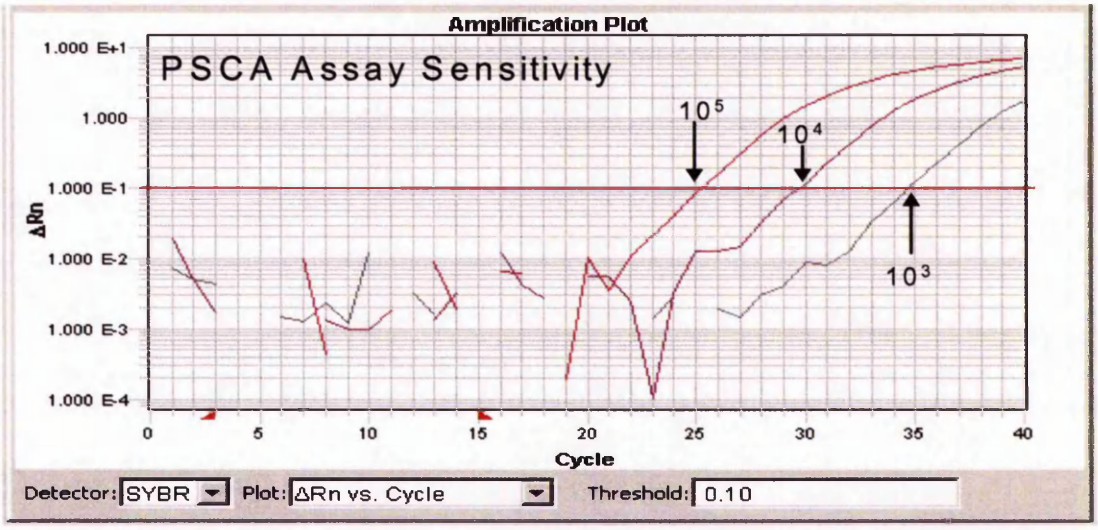



**Figure 3.33:** Sensitivity for PSCA assay using SYBR Green. 100ng of total RNA from 2.5 ml female PB spiked with $10^5$, $10^4$, $10^3$, $10^2$, 10, 1 LNCaP was assessed using optimal primer concentration ratio of 300nM forward : 300nM reverse. The reactions containing RNA from $10^5$, $10^4$ and $10^3$ LNCaP were positive, the amplification plots crossing the critical threshold (red line).

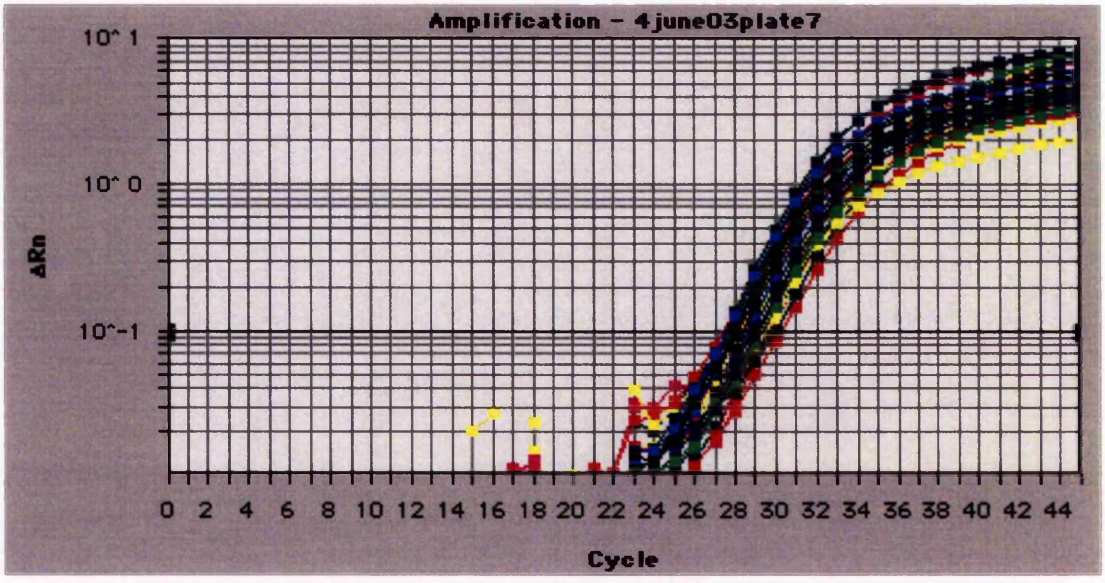



**Figure 3.34:** Hk2 assay using SYBR Green. Fluorescence from all the reactions containing HK2 primers, including water and minus RT controls, cross the the critical threshold.

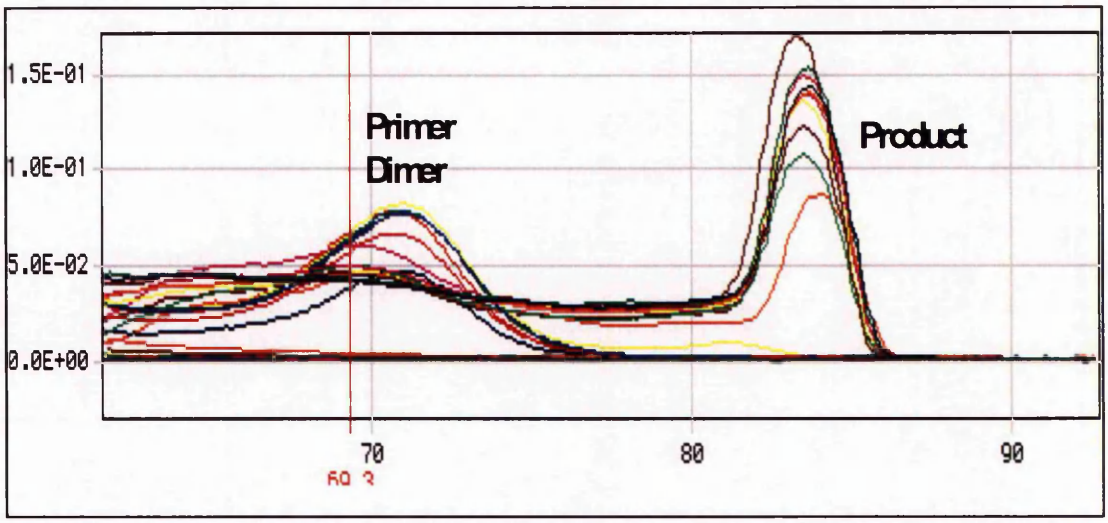



**Figure 3.35:** Dissociation curve of HK2 assay products using SYBR Green. The negative fluorescence peaks at 83.5°C are desired product (right), however for many of the reactions there is either only a primer dimer peak at 71°C or an additional peak (double peak trace) at this lower temperature.

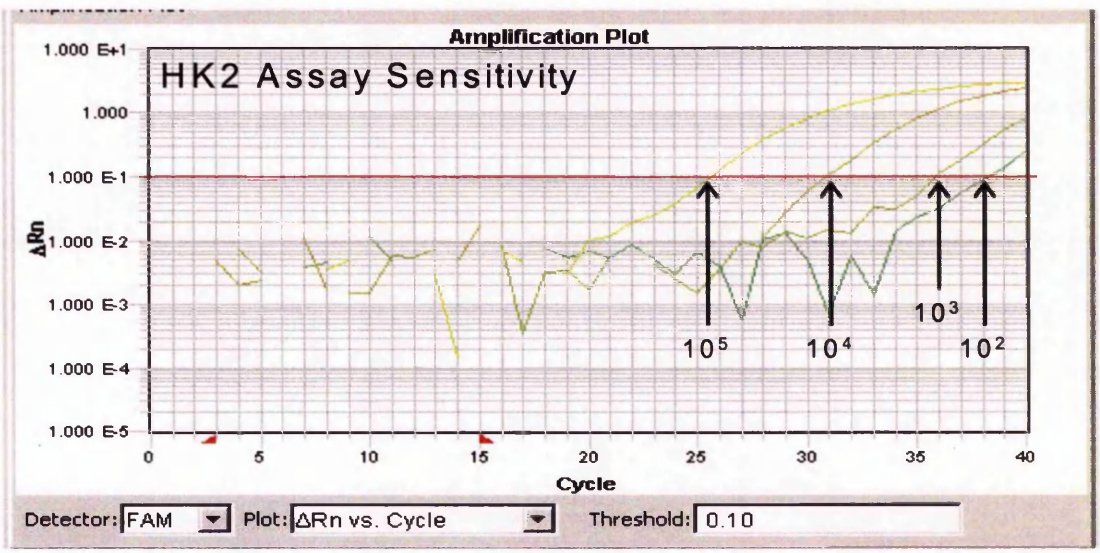



**Figure 3.36**: Real time RT-PCR plots showing sensitivity of HK2 assay using nucleotide probe. 100ng of total RNA from 2.5 ml female PB spiked with $10^5$, $10^4$,$10^3$, $10^2$, 10 and 1 LNCaP cell was assessed using optimised primer, 900nM forward and 100nM reverse, and probe concentrations. The reactions containing RNA from $10^5$, $10^4$, $10^3$ and $10^2$ LNCaP were positive, the amplification plots crossing the critical threshold (red line).

### 3.7.5 $DD3^{PCA3}$ Assay

The $DD3^{PCA3}$ assay failed to detect $10^5$ LNCaP cells in a 2.5ml female peripheral blood background. Housekeeping genes, β-actin and GAPDH were detected in all samples and the negative control reactions were clear. The primers for the $DD3^{PCA3}$ marker were able to detect $DD3^{PCA3}$ gene expression in RT-PCR reactions containing 100ng of cDNA derived from the total RNA isolated from $10^6$ LNCaP cells and 100ng of cDNA derived from total RNA extracted from prostate needle biopsy cores from men with CaP. However when up to $10^5$ LNCaP cells were diluted in a background of $10^6$-$10^8$ female PB nucleated cells then no $DD3^{PCA3}$ was detected.

## 3.8 $DD3^{PCA3}$ Based RT-PCR Assay Sensitivity Using Plasmid

The standard LNCaP cell spike model for assay validation could not be used for $DD3^{PCA3}$ target, as concentrations of up to $10^5$ cells per 2.5ml of female PB were not detected using the $DD3^{PCA3}$ assay. An alternative approach was to use a $DD3^{PCA3}$ sequence inserted in a plasmid. This provided a $DD3^{PCA3}$ DNA target, serial dilutions of which could be used to confirm assay function and integrity. Additionally, complementary $DD3^{PCA3}$ RNA could be synthesised from the DNA sequence, which in turn could be spiked, in serial dilutions, into female PB. The *in vitro* performance of the $DD3^{PCA3}$ assay could then be formally evaluated using the standard RT-PCR protocol.

### 3.8.1 Detection of Serial Dilutions of $DD3^{PCA3}$ Plasmid DNA

To confirm that the $DD3^{PCA3}$ assay could detect and amplify $DD3^{PCA3}$ DNA target, serial dilutions of $DD3^{PCA3}$ plasmid copies were spiked in a background of 100ng of cDNA derived from female PB. Figure 3.37 demonstrates the ability of the $DD3^{PCA3}$ assay to detect serially diluted $DD3^{PCA3}$ plasmid copies, from $10^6$ to 1 copy. $10^6$ plasmid copies were detected with a critical threshold of 20 cycles. As the number of plasmid copies decreased the Ct value increased, with 1 copy being detected at 35 cycles. Product identity was again confirmed by product dissociation, Tm 79.8°C, and reaction integrity by housekeeper amplification while

non-plasmid target sources were excluded by the standard negative control reactions.

### 3.8.2 Linearization of pMB45 IS-DD3$^{PCA3}$ Plasmid Using Kpn I

Now that it had been confirmed that the DD3$^{PCA3}$ assay could amplify DD3$^{PCA3}$ DNA plasmid target, the next stage was to synthesise DD3$^{PCA3}$ RNA. The T3 RNA polymerase required linear DNA to produce an RNA transcript of the DD3$^{PCA3}$ DNA message. This was achieved using the restriction enzyme Kpn I. Successful linearization of the plasmid was confirmed by gel electrophoresis, a 4800bp product being identified (figure 3.38).

### 3.8.3 Detection of Serial Dilutions of DD3$^{PCA3}$ RNA Transcribed from Plasmid

Using cDNA derived from female peripheral blood spiked with serial dilutions of DD3$^{PCA3}$ RNA copies, the DD3$^{PCA3}$ assay could detect target from the samples containing from $10^7$ DD3$^{PCA3}$ copies at 26.5 cycles, down to 100 DD3 RNA copies at 38.7 cycles (figure 3.39).

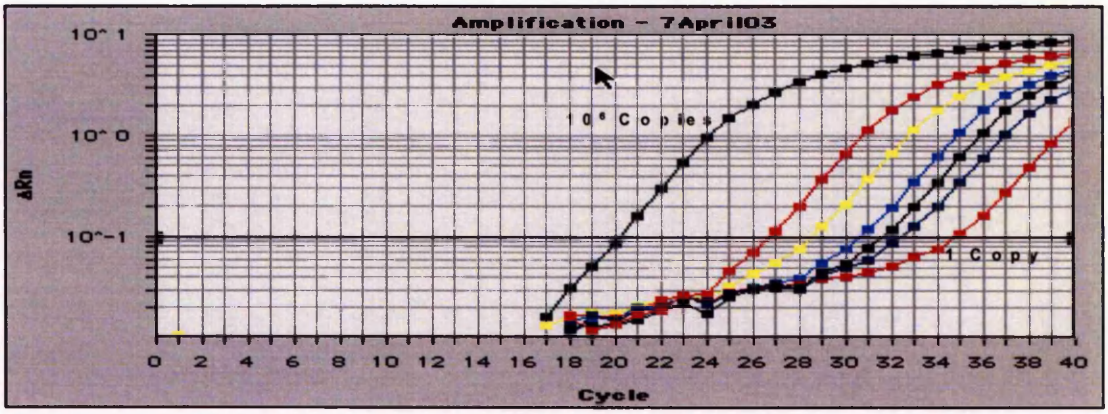



**Figure 3.37:** $DD3^{PCA3}$ PCR assay. The assay was used to detect serial dilutions ($10^6$ copies to 1 copy) of $DD3^{PCA3}$ plasmid construct in 100ng female blood derived cDNA. Successful amplification of $DD3^{PCA3}$ target was achieved for all dilutions. The Ct values for the individual reactions increased from 20 cycles for $10^6$ plasmid copies to 35 cycles for 1 plasmid copy.

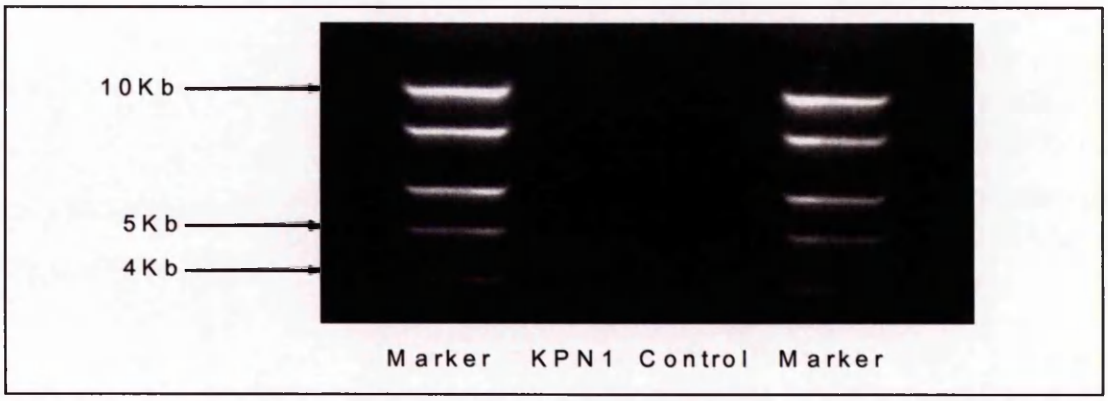



**Figure 3.38:** Confirmation of plasmid pMB45 IS-$DD3^{PCA3}$ linearisation by Kpn I restriction enzyme digest. Digest was electrophoresed on a 0.8% agarose gel.

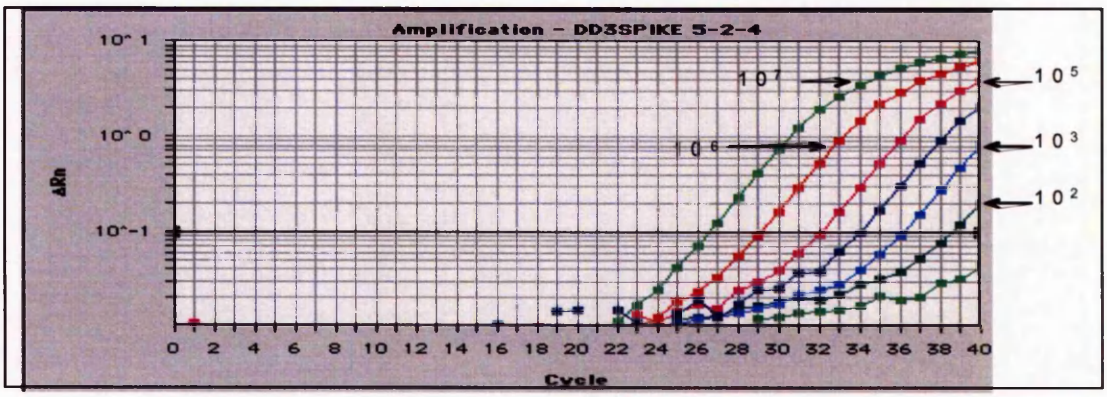



**Figure 3.39:** Real time PCR plots illustrating the ability of the $DD3^{PCA3}$ assay in detecting serial dilutions of $DD3^{PCA3}$ RNA copies spiked into 2.5ml of female PB. Dilutions from $10^7$ to $10^2$ $DD3^{PCA3}$ RNA copies per 2.5ml of PB were detected. Reaction Ct values increased from 26.5 cycles for reactions containing RNA $10^7$ copies to 38.7 cycles for $10^2$ RNA copies.

### 3.9 Overview of *in Vitro* RT-PCR Assay Sensitivities

The assay formats that would be used for the evaluation of PB and BM samples from patients with CaP and disease free controls displayed limits of detection of 1 LNCaP cell:$10^6$ nucleated PB cells for PSA, 1:$10^5$ PSMA, 1:$10^4$ PSCA and 1:$10^4$ HK2. The $DD3^{PCA3}$ assay could detect 100 target RNA molecules per ml of PB (Table 3.2).

| **Assay Target** | ***In Vitro* Sensitivity (LNCaP : PB nucleated cells)** |
|---|---|
| **PSA** | 1:$10^6$ |
| **PSMA** | 1:$10^5$ |
| **PSCA** | 1:$10^4$ |
| **HK2** | 1:$10^4$ |
| **$DD3^{PCA3}$** | 100 RNA molecules/ml of PB |

**Table 3.2:** *In Vitro* performance of the RT-PCR assays for the markers PSA, PSMA, PSCA, HK2 and $DD3^{PCA3}$. The limits of detection for the PSA, PSMA, PSCA and HK2 assays were defined by the maximum number of background PB nucleated cells in which 1 LNCaP cell could be detected by the marker specific RT-PCR assay. The PSA assay was the most sensitive, detecting 1 LNCaP cell in a background of $10^6$ PB nucleated cells. The *in vitro* sensitivity of the $DD3^{PCA3}$ assay was defined by the minimum number of $DD3^{PCA3}$ RNA molecules that it could detect per 1ml of female PB. This cannot be directly compared to the *in vitro* sensitivities for the other 4 prostate markers.

## Chapter 4: Analysis of Patient Samples

Samples and clinical information were collected from 99 men with prostate cancer. 99 PB samples and 84 BM samples were collected. 98 PB and 83 BM samples were available for RT-PCR analysis as the samples for PT88 were damaged during initial RNA extraction. All PB and BM samples were processed by total RNA extraction within 7 days of collection. Subsequently 6µg of each RNA sample was reverse transcribed with the remainder being stored under ethanol at -80°C.

### 4.1 Clinical Groupings

Clinical information for each patient was evaluated to group patients in terms of clinical disease status as follows:

1. Localised Disease
    DRE – T1 or T2
    Serum PSA </=10ng/ml
    Isotope bone scan – negative
    Treatment - untreated

2. Locally Advanced Disease
    DRE – T3 or T4
    Serum PSA - any
    Isotope bone scan – negative
    Treatment - untreated

OR

    DRE T1 or T2 & serum PSA >10ng/ml
    Isotope bone scan – negative
    Treatment - untreated

3. Hormone manipulated non-metastatic with rising PSA

DRE – T1-T4

PSA – rising

Isotope bone scan – negative

Treatment – hormone manipulation

4. Untreated Metastatic

DRE T1-T4

PSA – any

Isotope bone scan – positive

Treatment – untreated

5. Metastatic Hormone Escaped

DRE T1-T4

PSA – rising

Isotope bone scan - positive

Treatment - hormone manipulation with PSA progression

The study patient population clinical and demographic information are shown in table appendix 1. By applying the above criteria the population was classified as is shown in table 4.1, 40 patients having clinically localised disease, 33 locally advanced, 6 hormone escaped without metastases, 7 untreated metastases and 12 hormone escaped metastatic CaP. Of the patients with localised disease, 24 underwent RRP, 10 brachytherapy, 5 EBRT and 1 was managed expectantly. Four men from the locally advanced group were also treated surgically with RRP.

| Clinical Group | Number of Pts | PB Sample | Paired PB & BM |
|---|---|---|---|
| Localised | 41 | 40 | 38 |
| Locally Advanced | 33 | 33 | 25 |
| M0 Hormone Escaped | 6 | 6 | 6 |
| M1 Untreated | 12 | 12 | 9 |
| M1 Hormone Escaped | 7 | 7 | 5 |
| **Totals** | 102 | 101 | 86 |

**Table 4.1:** Study patient population groupings and samples available for RT-PCR analysis. The total study population of 99 patients were assigned to one of 6 clinically defined disease status groups. 98 of the 99 sets of samples were suitable for RT-PCR analysis, 83 included paired PB and BM samples. 41 men had clinically localised disease, 33 locally advanced CaP and 19 patients bone scan proven bone metastases.

## 4.2 RT-PCR Analysis of Clinical Samples

Eighty three paired PB and BM samples and 15 solitary PB samples from the previously described population were analysed for the 5 markers of interest using quantitative RT-PCR assays as described in section 2.4.2. Seventeen PB and 28 BM samples from CaP free controls were similarly evaluated.

### 4.2.1 Overview of Raw Data

All of the obtained RT-PCR data is detailed in appendix 2. Appendix 2.1 shows the qualitative RT-PCR results for all patient and control samples, while appendix 2.2 displays all of the Ct values for each replicate reaction for each marker for all clinical samples. For all samples analysed by RT-PCR there was successful coamplification of the housekeeper genes GAPDH and β-actin, confirming cDNA integrity. Minus RT controls were clean, excluding genomic DNA contamination, and negative water (cDNA free) controls ensured that reaction components were contaminant free.

When considering purely qualitative outcomes for all 98 patients, 75 (76%) were RT-PCR positive for at least one marker in the PB sample. Of the 83 BM samples, 76 (92%) were positive for at least one marker. When PB and BM were considered in combination for these 83 men, 80 (96%) had one marker assay positive. Of those 80, 58 were marker positive in both PB and BM, 18 were positive in BM but not PB and 4 had target detected in PB only. Table 4.2 illustrates the proportion of samples that were positive for at least one marker in each of the clinical groups. The vast majority, between 70% and 100%, of samples were marker positive and the trend was towards increasing rates of positivity when BM samples were evaluated.

Twenty eight BM aspirates (24 male, 4 female) and 17 PB samples (10 male, 7 female) from CaP free control patients were analysed. Twenty seven (96%) of the BM samples and 14 (82%) of the PB samples were RT-PCR positive for at least one marker.

| | Positive RT-PCR Reactions | | |
|---|---|---|---|
| Clinical Group | PB | BM | Paired Samples |
| Number of samples | 101 | 86 | 86 |
| Localised | 78% | 90% | 97% |
| Locally advanced | 70% | 96% | 96% |
| Hormone Escaped & Bone Scan Neg | 83% | 100% | 100% |
| Untreated Metastases | 75% | 78% | 89% |
| Hormone escaped & Metastases | 100% | 100% | 100% |
| Total Patients | 75% | 92% | 97% |
| CONTROLS | 82% | 96% | N/A |

**Table 4.2:** Patient & control PB and BM samples RT-PCR positive for any marker. The patient population has been divided by clinical criteria. High degrees of sample RT-PCR positivity is demonstrated throughout the groups for both PB and BM although the proportions are slightly higher in the BM groups.

### 4.2.2 Evaluation of Individual Markers in Peripheral Blood

Purely qualitative RT-PCR data for five markers used concurrently showed high levels of marker detection in PB and BM samples from both men with prostate cancer and disease free controls. Table 4.3 shows the percentage of PB samples in each clinical group and controls that were RT-PCR positive for each marker. The expression/detection of individual markers in the study population allowed further evaluation of the methodology. Data and analysis for each marker is described individually below.

| Clinical Group | PSA | PSMA | PSCA | $DD3^{PCA3}$ | HK2 |
|---|---|---|---|---|---|
| Localised | 25% | 43% | 73% | 5% | 13% |
| Locally advanced | 39% | 30% | 61% | 0 | 6% |
| Hormone escaped & BS negative | 50% | 83% | 50% | 0 | 17% |
| Untreated metastases | 42% | 50% | 67% | 0 | 8% |
| Hormone escaped & metastases | 100% | 57% | 29% | 29% | 86% |
| Controls | 12% | 47% | 71% | 0 | 18% |

**Table 4.3:** Percentage of PB samples RT-PCR assay positive for each of the five markers of interest in each clinical group.

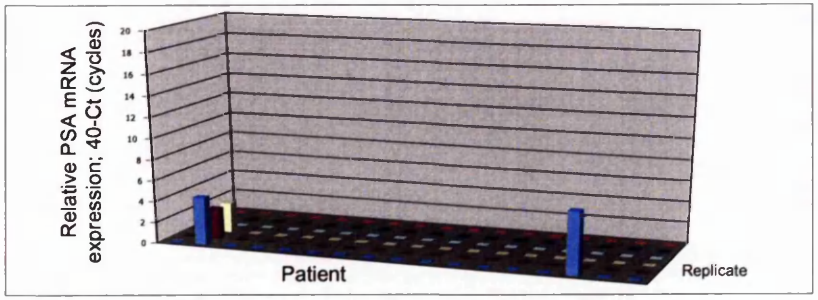
#### 4.2.2.1 Evaluation of PSA Assay in Peripheral Blood

The percentage of PB samples from each clinical and control group which were RT-PCR assay positive for PSA were evaluated prior to incorporating the quantitative data. 25% of men with localised disease, 39% with locally advanced, 50% with hormone escaped non-metastatic, 42% with untreated metastases and all patients with hormone escaped metastatic cancer were PSA RT-PCR positive (Table 4.4).

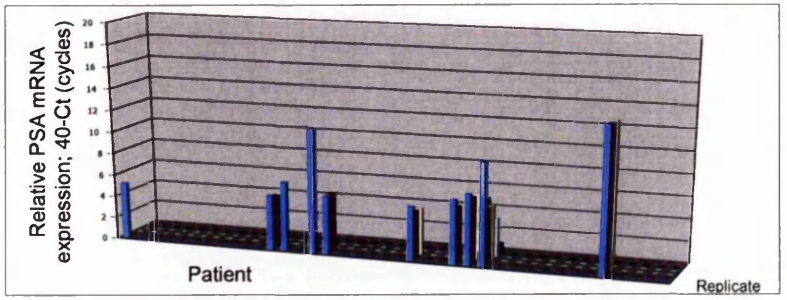
Each patient sample was evaluated for PSA expression using six replicate RT-PCR reactions, each of which gave a quantitative Ct cycle value. To allow comparison of relative levels of marker expression in each sample, each individual reaction result was expressed as (40-Ct). The higher this value, the higher the levels of marker specific mRNA in the initial sample. These quantitative values were additionally standardised to account for variations in the amounts of input cDNA by correcting to the combined mean expression levels of the 2 house keeper genes, β-actin and GAPDH. Figure 4.1 shows the corrected (40-Ct) values for each of the six replicate reactions for each individual patient or control. Clinical groups are shown in separate graphs.

| Clinical Group | % PSA RT-PCR +ve |
|---|---|
| Localised | 25 |
| Locally Advanced | 39 |
| Mo Hormone Escaped | 50 |
| M1 Untreated | 42 |
| M1 Hormone Escaped | 100 |
| Controls | 12 |

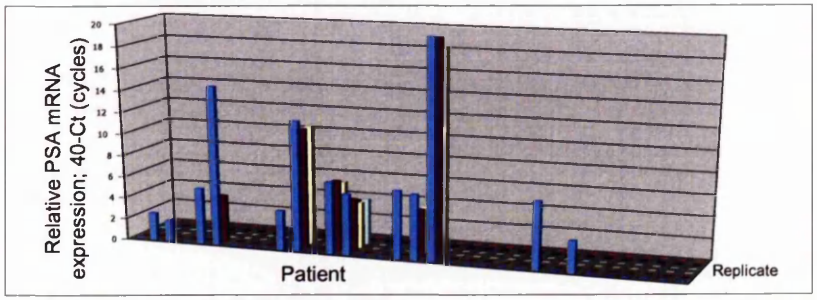
**Table 4.4:** PSA RT-PCR in PB. The percentage of individuals whose PB sample was PSA RT-PCR positive in any of 6 replicate reactions is shown for each clinical and the control group. Any reaction in which fluorescence was detected above the critical threshold was considered to be a positive assay result.



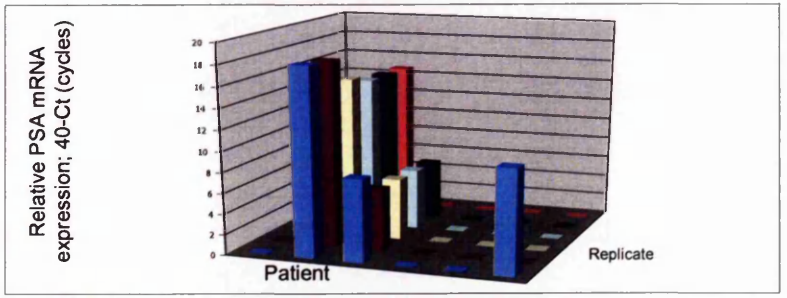

1. PB Controls n=17



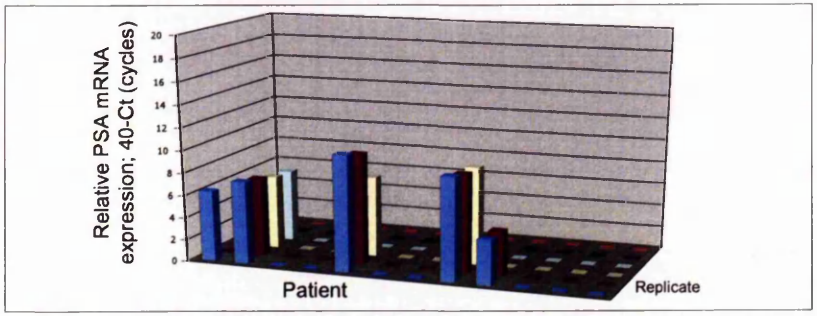

2. Clinically localised disease n=40



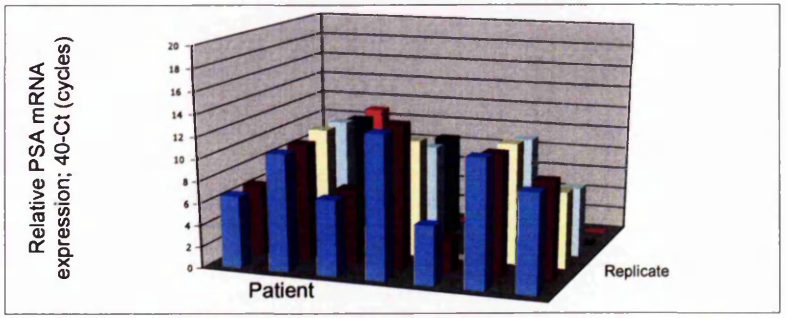

3. Locally advanced disease n=33




4. M0 hormone escaped disease n=6




5. Untreated metastatic disease n=12




6. Hormone escaped metastatic disease n=7

**Figure 4.1:** PSA RT-PCR for PB samples for clinical groups and controls. Each graph displays the data for a specific clinical or control group. Each series of 6 bars on the z-axis displays the quantitative RT-PCR values (40-Ct) for each of the 6 replicate reactions performed for each patient sample. These values have been standardised to mean expression of β-actin and GAPDH.

In the control group only 2 individuals (12%) were PSA RT-PCR positive, one having only one positive reaction while the second had 3 positive assays. The relative expression values, (40-Ct), for all the positive reactions were less than 6. The localised disease samples showed higher total numbers of patients with positive results (25%). There was a larger range of 40-Ct values, 0 to 13.3 cycles, compared to the controls and 8 of the 10 positive samples had more than 1 positive reaction. For those with locally advanced disease the overall proportion of patients with positive results was higher than the localised group, 39%. Seven of the 13 PSA positive samples were only positive in a single replicate, while 5 samples had 3 or more positive replicates. Of the 6 patients with hormone escaped non-metastatic CaP, 3 were PSA RT-PCR positive (50%). One sample was only positive for a single replicate while the other two had 5 and 6 replicates positive respectively. Samples from men with hormone escaped metastatic disease were all assay positive in at least 2 of the replicates, 3 samples having 4, 1 sample 5 and 2 samples 6 positive replicates. Expression in this group was generally more wide spread and uniform although none of the individual reactions reached the high (40-Ct) values seen in a small number of individual patients in the less clinically advanced groups.

The raw quantitative data described above provides information on the relative amounts of marker target present in each PB sample. The 6 number format expressed for each sample is however difficult to interpret and utilise. Thus further analysis was used to produce a single quantitative value for each PSA RT-PCR positive sample, which accounted for both the number of positive replicates and the relative expression, (40-Ct) value, in each of these positive assays. As is

discussed in section 6.4.1 replicate assay reactions were required to ensure representative sampling and thus evaluation of the clinical samples, which were very dilute solutions with small numbers of target molecules in a vast background of non-target RNA. Therefore the sum of all the individual positive replicate (40-Ct) values for each sample was taken and used to express overall relative PSA mRNA expression. This methodology of summing the Ct values was applied for all other marker analysis.

Figure 4.2a shows the Σ(40-Ct) values for all the PSA RT-PCR positive patients and controls. Samples which were assay negative have been excluded. Data are displayed in clinical groups, by colour coding, to show the relative marker expression within and between groups.

The quantitative RT-PCR values were then used to optimise the assay for the clinical setting by maximising clinical specificity while maintaining maximal clinical sensitivity. Using Σ(40-Ct) values, 12% of the control PB samples were PSA RT-PCR positive with Σ(40-Ct) values of 6 cycles and 10 cycles. Control positivity in RT-PCR methodology may reflect background illegitimate transcription which should also be corrected for in the patient population. In figure 4.2a it can be seen that a number of patient samples have Σ(40-Ct) values of </=6 cycles (7 patients), or </=10 cycles (13 patients). In figure 4.2b Σ(40-Ct) thresholds of 6 and 10 cycles have been set to exclude false positive assay results. The 6 cycle threshold reduced control group positives to 6% while maintaining 100% positivity in patients with hormone escaped metastatic disease. Five patients in the locally advanced group were below this threshold and 1 of the localised patients.

When a threshold of 10 cycles was used then all control PB samples were PSA RT-PCR negative and the percentage of positive samples in the localised and locally advanced groups fell from 25% to 20% and 39% to 18% respectively. However the clinical sensitivity also decreased with 2 patients with clinically proven metastases falling below this threshold (Table 4.5 and Figure 4.2 b & c). The threshold value of 6 was therefore used for further data analysis.

With the Σ(40-Ct) threshold set at 6 cycles, the relative mRNA PSA expression for the patient and control population are shown in figures 4.3a and 4.3b and table 4.6. The scatter plot figure 4.3b shows a general trend of increasing PSA mRNA expression from left, controls, to right, hormone escaped metastatic CaP and this correlation between PSA mRNA expression, Σ(40-Ct), and advancing clinical disease stage was significant (Spearman rho p=<0.0001). Mean relative PSA mRNA expression increased from 0.25 cycles in the control group to 2.6 cycles in patients with localised disease and 4.2 cycles in the locally advanced group although these differences did not reach statistical significance. Expression was highest at 32.5 cycles in men with metastatic hormone escaped cancer, while mean levels of 19.9 cycles and 5.5 cycles were seen in PB from men with non-metastatic hormone escaped CaP and untreated metastases respectively (figure 4.3c). The Kruskal-Wallis test confirmed significant differences between groups within the study population (p=<0.0001). PSA mRNA expression was significantly higher in patients with metastatic hormone escaped CaP than in controls (Mann-Whitney p=<0.0001) and all other patient groups other than those with non-metastatic hormone escaped disease. The observed differences between controls

and those patients with untreated metastases (Mann-Whitney p=0.02) and non-metastatic hormone escaped CaP (Mann-Whitney p=0.015) came close to significance. No other significant differences between clinical groups or controls were demonstrated (full data shown in appendix 3).

Within the group of 28 patients who underwent RRP, the PB PSA RT-PCR assay alone could not identify those individuals who would have positive surgical margins, a pathological Gleason score of 7 or higher or would experience early biochemical treatment failure.

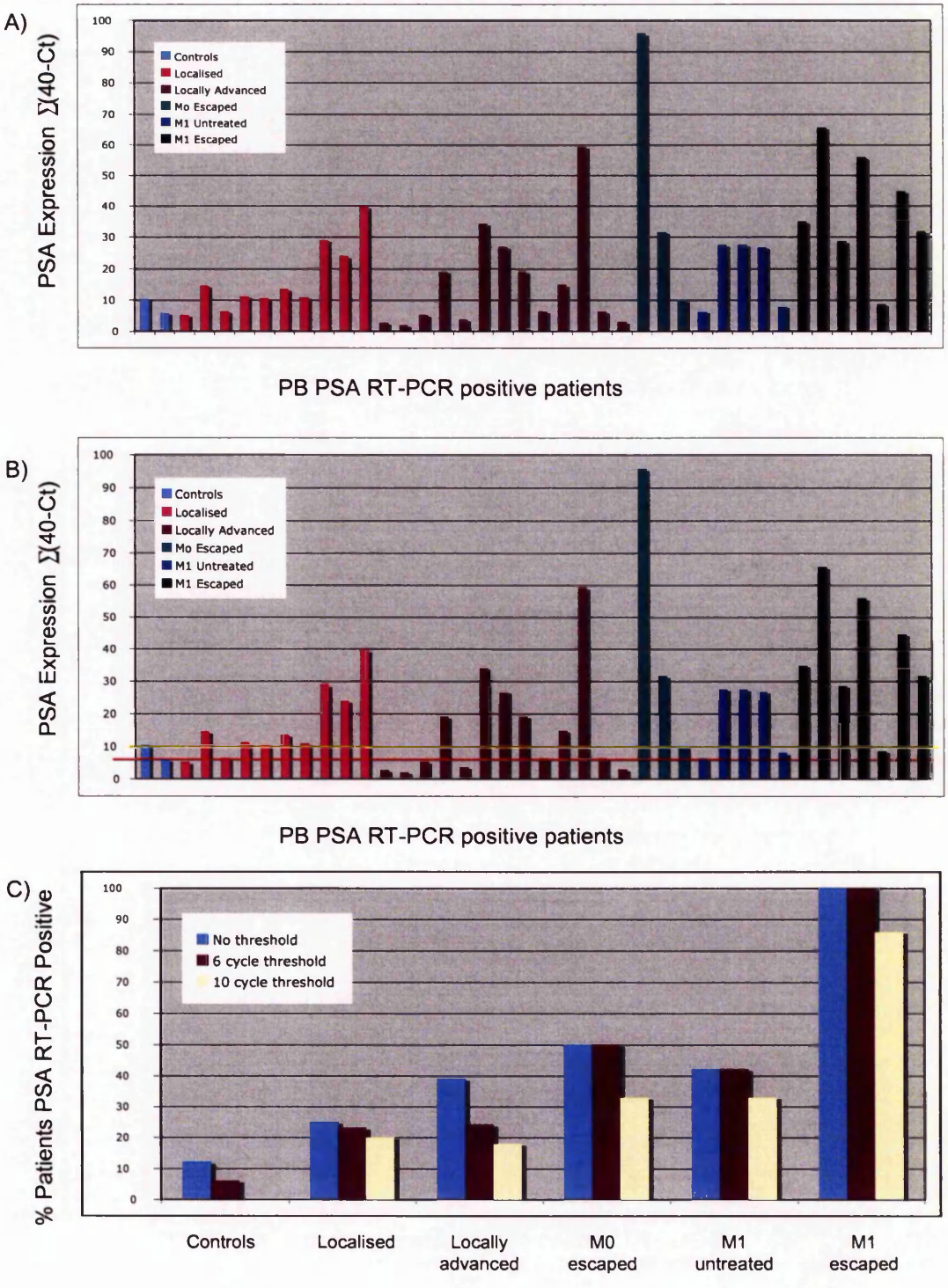



**Figure 4.2:** PSA RT-PCR evaluation of PB samples from the study population. A) Bar chart displaying the relative quantitative expression of PSA mRNA in PB samples that were PSA RT-PCR positive standardised to the expression of the housekeeping genes GAPDH and ß-actin. Marker negative samples have been excluded. Samples are displayed in clinical groups from controls on the left progressing to hormone escaped metastatic disease on the right. B) Σ(40-Ct) threshold of 6 & 10 cycles (red and yellow lines respectively have been applied to the RT-PCR results. At 6 cycles the clinical specificity is improved without any significant impact on clinical sensitivity. C) Bar chart showing the impact of the applied thresholds on the percentage of PB samples from patients in each group that were PSA RT-PCR positive.

| Clinical Group | % PSA RT-PCR +ve No Threshold | % PSA RT-PCR +ve Threshold = 6 | % PSA RT-PCR +ve Threshold = 10 |
|---|---|---|---|
| Localised | 25 | 23 | 20 |
| Locally Advanced | 39 | 24 | 18 |
| Mo Hormone Escaped | 50 | 50 | 33 |
| M1 Untreated | 42 | 42 | 33 |
| M1 Hormone Escaped | 100 | 100 | 86 |
| Controls | 12 | 6 | 0 |

**Table 4.5:** Percentage of PB samples PSA RT-PCR positive using different expression level thresholds. Thresholds of 6 and 10 cycles were applied to the sum of (40-Ct) values for each of the 6 replicate PSA RT-PCR reactions for each PB sample. Increasing threshold values improved clinical specificity by reducing positive control reactions to 6% and 12% respectively, while clinical sensitivity was reduced as fewer patients with clinically proven metastases were assay positive.

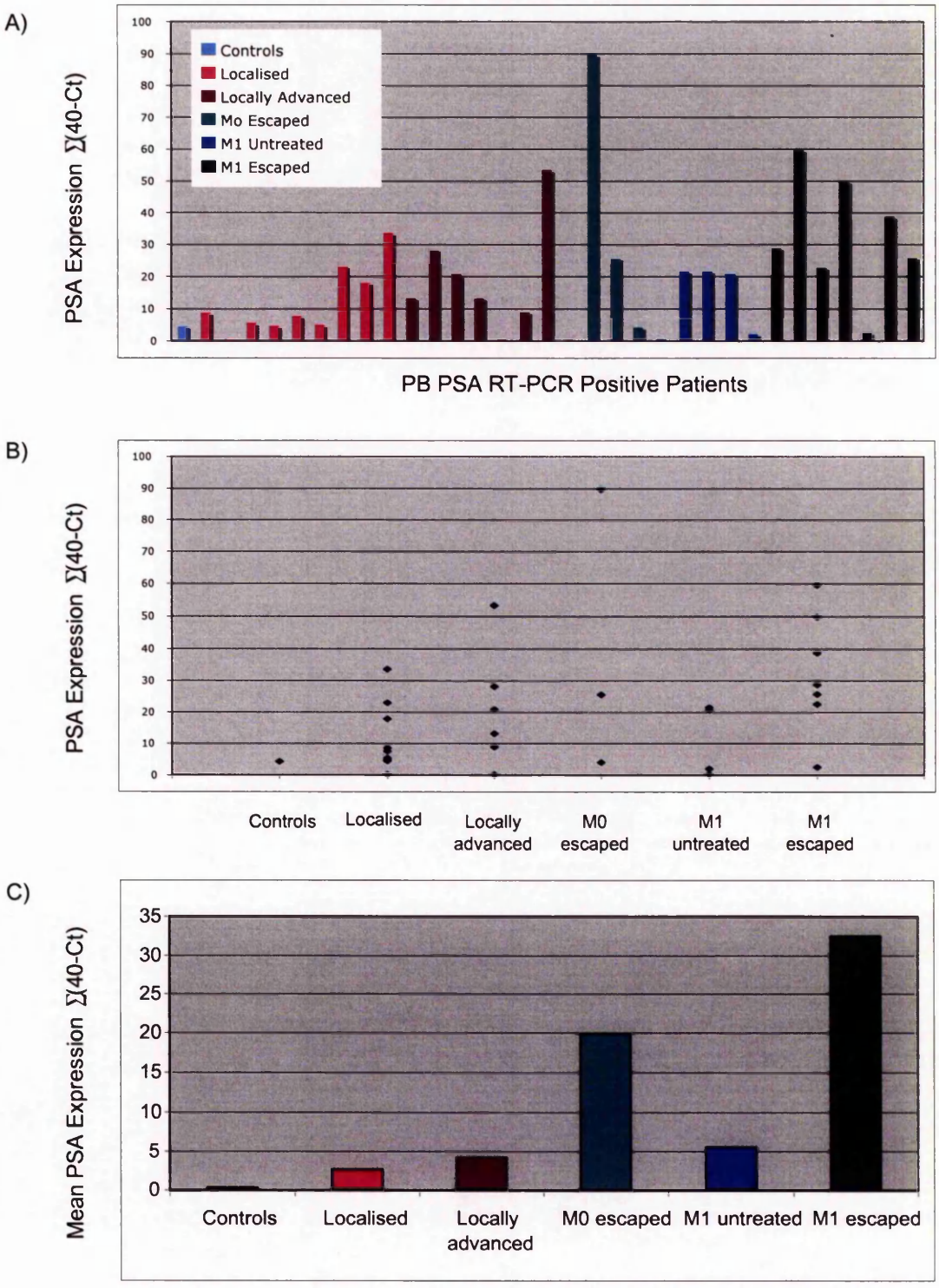



**Figure 4.3:** PB PSA RT-PCR assay with a Σ(40-Ct) threshold of 6 cycles applied. A) Bar chart showing relative PSA mRNA expression in all PB samples that were PSA assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. B) Scatter plot displaying the distribution of relative PSA mRNA expression within the study population. C) Bar chart displaying mean relative PSA mRNA expression in control and clinical groups.

| | % PSA RT-PCR +ve | Mean Σ(40-Ct) | Median Σ(40-Ct) | Range Σ(40-Ct) |
|---|---|---|---|---|
| **Localised** | 23 | 2.6 | 0 | 0 - 33.6 |
| **Locally advanced** | 24 | 4.2 | 0 | 0 – 53.3 |
| **Non-metastatic hormone escaped** | 50 | 19.9 | 2.0 | 0 – 89.6 |
| **Untreated metastases** | 42 | 5.5 | 0 | 0 – 21.5 |
| **Metastatic hormone escaped** | 100 | 32.5 | 28.7 | 2.5 – 59.4 |
| **Controls** | 6 | 0.25 | 0 | 0 – 4.3 |

**Table 4.6:** PB PSA RT-PCR assay with a threshold of 6 cycles applied. Percentages of positive samples, mean, median and range of PSA expression expressed as Σ(40-Ct) are shown for controls and each clinical group.

#### 4.2.2.2 Evaluation of HK2 RT-PCR Assay in Peripheral Blood

The HK2 assay was positive in 18% of PB controls and in 14% of the entire patient population. With the patient samples classified by clinical stage 12% of localised disease, 3% of locally advanced and 17% of non-metastatic hormone escaped cancer were HK2 RT-PCR positive. For all metastatic disease, 37% of samples were positive, and this rose to 86% in those with hormone escaped metastases. The relative HK2 mRNA expression detected in each PB sample for all patient and control samples is shown in figure 4.4a.

As with the PSA data, to take into account positive assay results in PB samples from control patients, Σ(40-Ct) thresholds were applied to the data. Cut off levels of 3.1 and 6 cycles were chosen as these excluded 67% and 100% of the positive control samples respectively. Figure 4.4b shows Σ(40-Ct) values plotted for all PB samples that were HK2 RT-PCR positive. The above thresholds have been plotted on the complete data series, the red line being set at 3.1 cycles and the yellow line at 6 cycles.

Applying the thresholds to the data improved clinical specificity as described above. A cut off of 3.1 cycles reduced the percentage of positive samples in the localised group from 12% to 10%, and the higher threshold reduced this further to 5%. The lower threshold had no impact on the locally advanced group however a cut off of 6 cycles excluded all patients. Neither threshold changed the marker positive rates in non-metastatic hormone escaped disease or untreated metastatic CaP. One patient with hormone escaped metastases was below both thresholds therefore reducing the percentage of samples positive from 86% to 71% (table 4.7 & figure 4.4c).

The 3.1 cycle threshold was used for further data analysis as it provided satisfactory specificity while maintaining maximal marker detection in patient samples. The Σ(40-Ct) values corrected to this threshold are plotted for every positive PB sample in figure 4.5a and mean and range of expression for each group are displayed in table 4.8. Mean HK2 expression for controls was 0.1

cycles. Patients with hormone escaped metastatic disease were well represented in this HK2 positive group and their mean HK2 expression was 7.8 cycles. Mean HK2 expression was higher than controls in all clinical groups with the exception of the locally advanced group (figure 4.5b). The scatter plot figure 4.6c displays individual HK2 RT-PCR result for each patient in each group. Overall there was a positive correlation between increasing clinical disease stage and increasing HK2 expression (Spearman rho p=0.02).

Statistical comparison of the clinical and control groups using the Kruskal-Wallis test confirmed a significant difference in relative HK2 expression in PB samples within the entire population (p=<0.0001). Comparison of individual groups however demonstrated that only the hormone escaped metastatic disease patients had significantly higher HK2 expression than controls and other patient groups (all data shown in appendix 3).

Of the men treated with RRP, only four showed PB HK2 mRNA expression and there was no significant association with margin positivity, higher gleason score or early biochemical failure. Interestingly, the patient with the highest HK2 PB expression had positive surgical margins, a pathological Gleason score of 8 and a rising PSA post surgery.

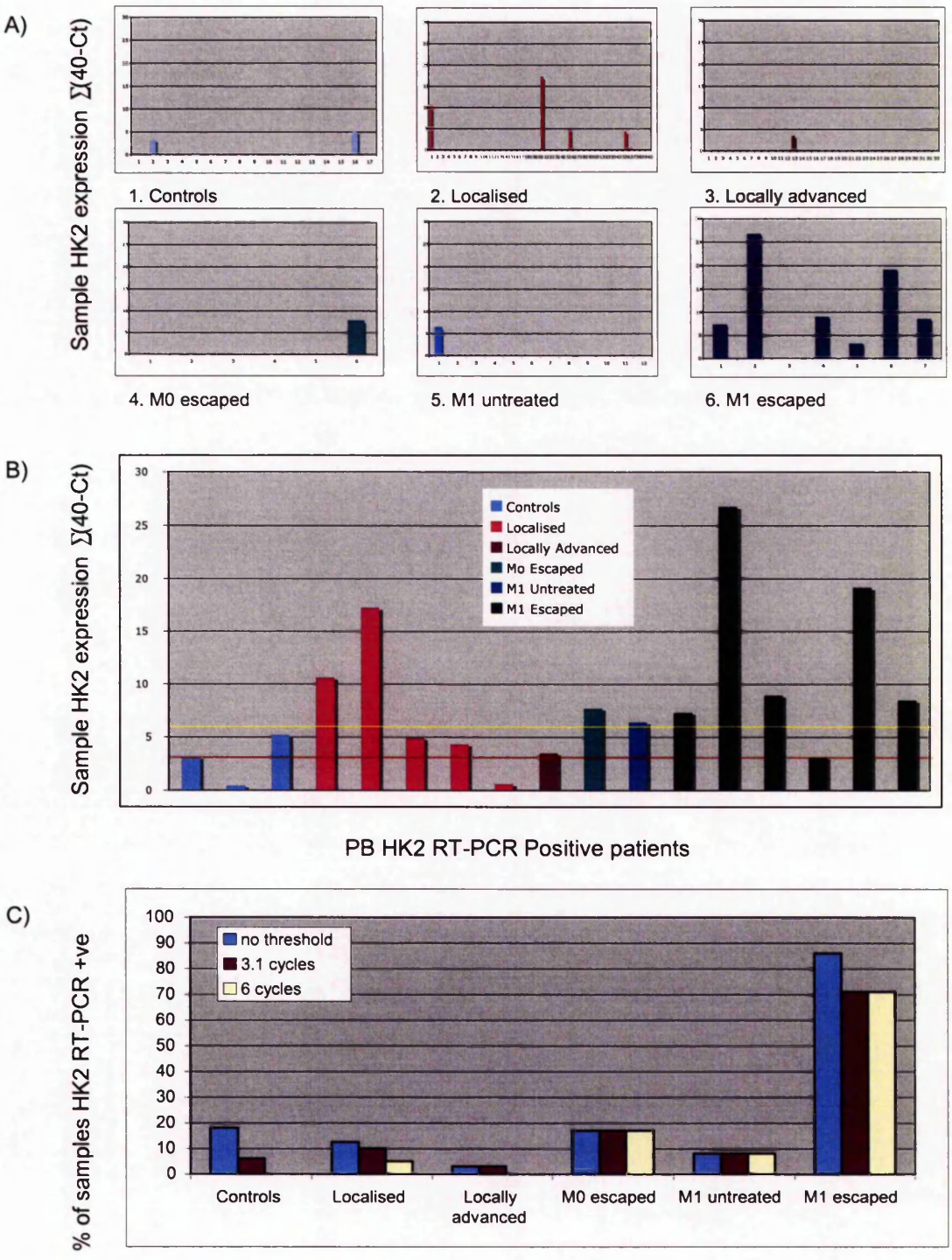



**Figure 4.4:** HK2 RT-PCR evaluation of PB samples from the study population. A) Bar charts showing relative quantitative expression of HK2 mRNA in all clinical samples standardised to the expression of the housekeeping genes GAPDH and ß-actin. Each clinical group is displayed in a separate bar chart. B) Bar chart displaying relative expression of HK2 mRNA in all assay positive samples in clinical and control groups. Σ(40-Ct) thresholds of 3.1 cycles (red line) and 6 cycles (yellow line) are shown but have not been applied to the data. These would exclude 67% and 100% of HK2 positive control samples respectively. C) Bar chart illustrating the impact of these two threshold levels on the percentage of PB samples from control and patient groups that are HK2 assay positive.

| Clinical Group | % HK2 RT-PCR +ve No Threshold | % HK2 RT-PCR +ve Threshold = 3.1 | % HK2 RT-PCR +ve Threshold = 6 |
|---|---|---|---|
| Localised | 12% | 10% | 5% |
| Locally advanced | 3% | 3% | 0 |
| Hormone escaped & bone scan negative | 17% | 17% | 17% |
| Untreated metastases | 8% | 8% | 8% |
| Metastases & hormone escaped | 86% | 71% | 71% |
| Controls | 18% | 6 | 0 |

**Table 4.7:** Percentage of patient and control PB samples HK2 RT-PCR positive. The percentage of PB samples from each group which were HK2 positive before and after applying thresholds of 3.1 cycles and 6 cycles which excluded two thirds and all positive control samples respectively.

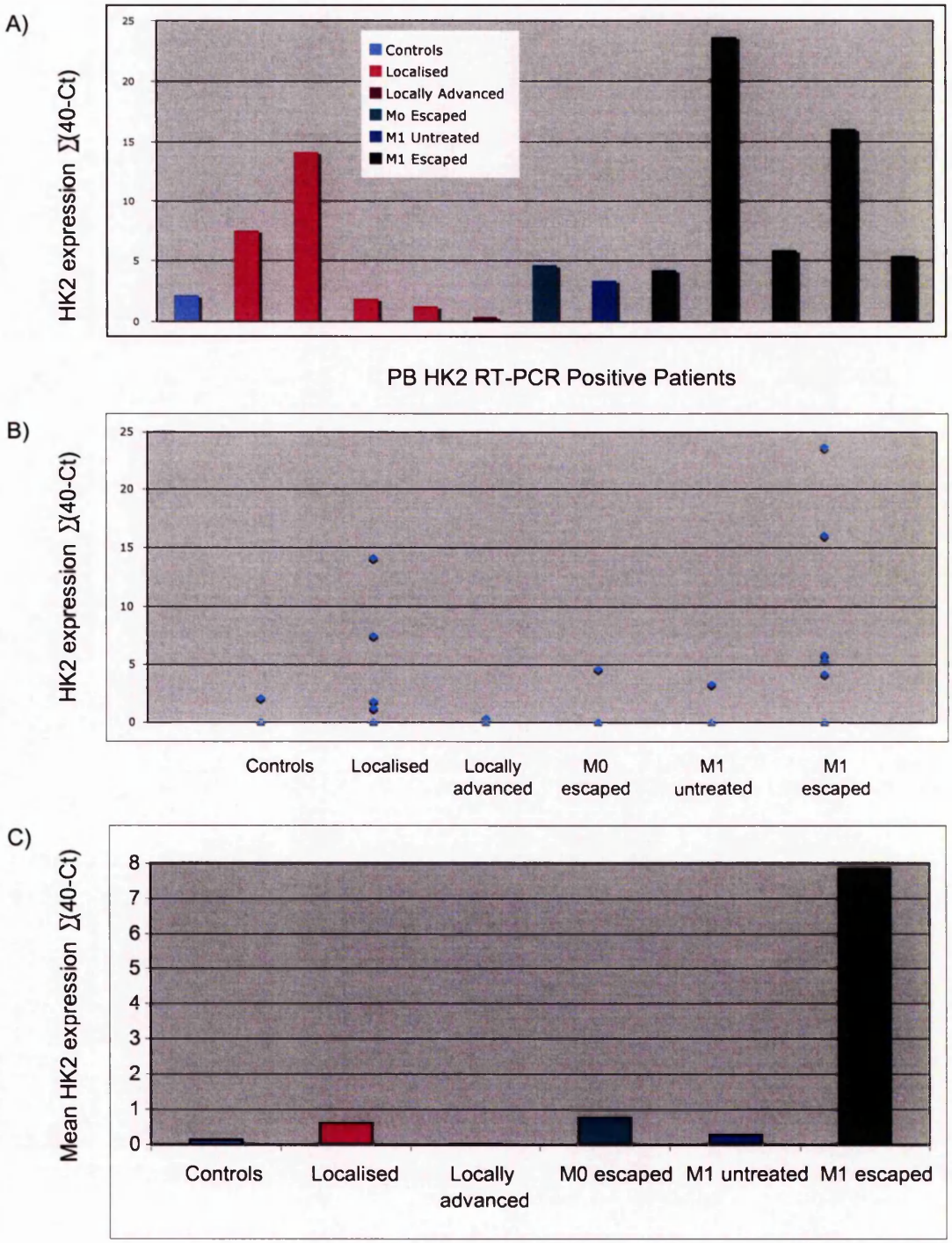



**Figure 4.5:** PB HK2 RT-PCT assay with a Σ(40-Ct) threshold of 3.1 cycles applied. A) Bar chart showing relative HK2 mRNA expression in all PB samples that were HK2 assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. B) Scatter plot displaying the distribution of relative HK2 mRNA expression within the study population. C) Bar chart displaying mean relative HK2 mRNA expression in control and clinical groups.

| | % HK2 RT-PCR +ve | Mean HK2 mRNA expression (cycles) | Range of HK2 mRNA expression (cycles) |
|---|---|---|---|
| Localised | 10 | 0.6 | 0 – 14.1 |
| Locally advanced | 3 | 0.01 | 0 – 0.4 |
| Non-metastatic hormone escaped | 17 | 0.8 | 0 – 4.6 |
| Untreated metastases | 8 | 0.3 | 0 – 3.3 |
| Metastatic hormone escaped | 71 | 7.8 | 0 – 23.6 |
| Controls | 6 | 0.1 | 0 – 2.1 |

**Table 4.8:** PB HK2 RT-PCR assay – percentage of samples HK2 positive, range and mean HK2 mRNA expression for control and patient groups with a Ct threshold of 3.1 cycles applied.

#### 4.2.2.3 Evaluation of PSMA Assay in Peripheral Blood

Peripheral blood analysis showed 42% of patient and 47% of control samples were PSMA RT-PCR +ve. The Σ(40-Ct) values for all PB samples are displayed in figure 4.6a. There were 8 out of 17 control PB samples that were PSMA positive with a range of Σ(40-Ct) values between 3.7 and 36 cycles. Five of these were less than 8 cycles, 6 less than 9 cycles and only one sample was above a level of 14 cycles (figure 4.6b). Therefore these threshold levels were applied to the data to determine the optimal threshold value for specificity and sensitivity.

The effect of the thresholds on the percentage of PSMA positive samples from patients in each clinical group is shown in figure 4.6c. Of the localised disease samples, 43% were initially PSMA RT-PCR positive. This was reduced to 20% when <8 or <9 cycles were excluded and 13% at <14 cycles. For the locally advanced CaP group the reductions were from 30% of samples being PSMA positive to 15%, 12% and 9% with the thresholds applied. Sixty seven percent of the non-metastatic hormone escaped group were PSMA positive which remained unchanged with a threshold of 8 cycles, while this was reduced to 50% when those samples with a Σ(40-Ct) value of <9 or <14 cycles were excluded. Of the 19 patients with metastatic disease were PSMA RT-PCR positive. The levels of expression in these samples were generally low with only 3 samples having Σ(40-Ct) levels above 9 cycles and only a single sample above 14 (figure 4.6b). Therefore for those with untreated metastases although 50% were PSMA RT-PCR positive initially, this was reduced to 17% with thresholds of 8 and 9 cycles, and no samples had relative expression of PSMA above 14 cycles. The percentage of PB samples that were RT-PCR positive from men with hormone escaped metastatic disease was reduced from 58% to 28% at 8 cycles and 14 % with both 9 and 14 cycle thresholds.

A Σ(40-Ct) threshold of 9 cycles was utilised for subsequent data analysis which gave 12% positivity in control samples while maintaining a significant marker positive patient population, 20% localised, 12% locally advanced, 50% non-metastatic hormone escaped, 17% untreated metastases and 14% hormone

escaped metastases. Table 4.9 gives an overview of expression in the study population. Figures 4.7a&b show the relative levels of PSMA expression, Σ(40-Ct), for all PB samples that were positive for this marker with a 9 cycle threshold applied. Although the only PSMA positive sample from a patient with hormone escaped metastatic CaP had the highest relative PSMA expression, at 58.3, the low levels of expression in the other PSMA positive samples from those with metastases and the low detection rates in these patients with advanced disease is striking. In patients with non-metastatic CaP, 8 samples show moderate to high relative PSMA expression. One of the two positive control samples had an unusually high Σ(40-Ct) value of 27 cycles, thus mean PSMA expression in the controls was 1.9 cycles, higher than that in the locally advanced and untreated metastases groups. The other patient groups had higher mean PSMA expression: localised 2.4 cycles, non-metastatic hormone escaped 11.2 cycles and metastatic hormone escaped 8.3 cycles (figure 4.7c).

Overall there was no clear pattern to PSMA expression and no significant correlation between marker expression and advancing clinical disease stage was identified. The observed differences in PSMA mRNA expression between PB samples from controls and patient groups and between patient groups were not statistically significant (full data appendix 3).

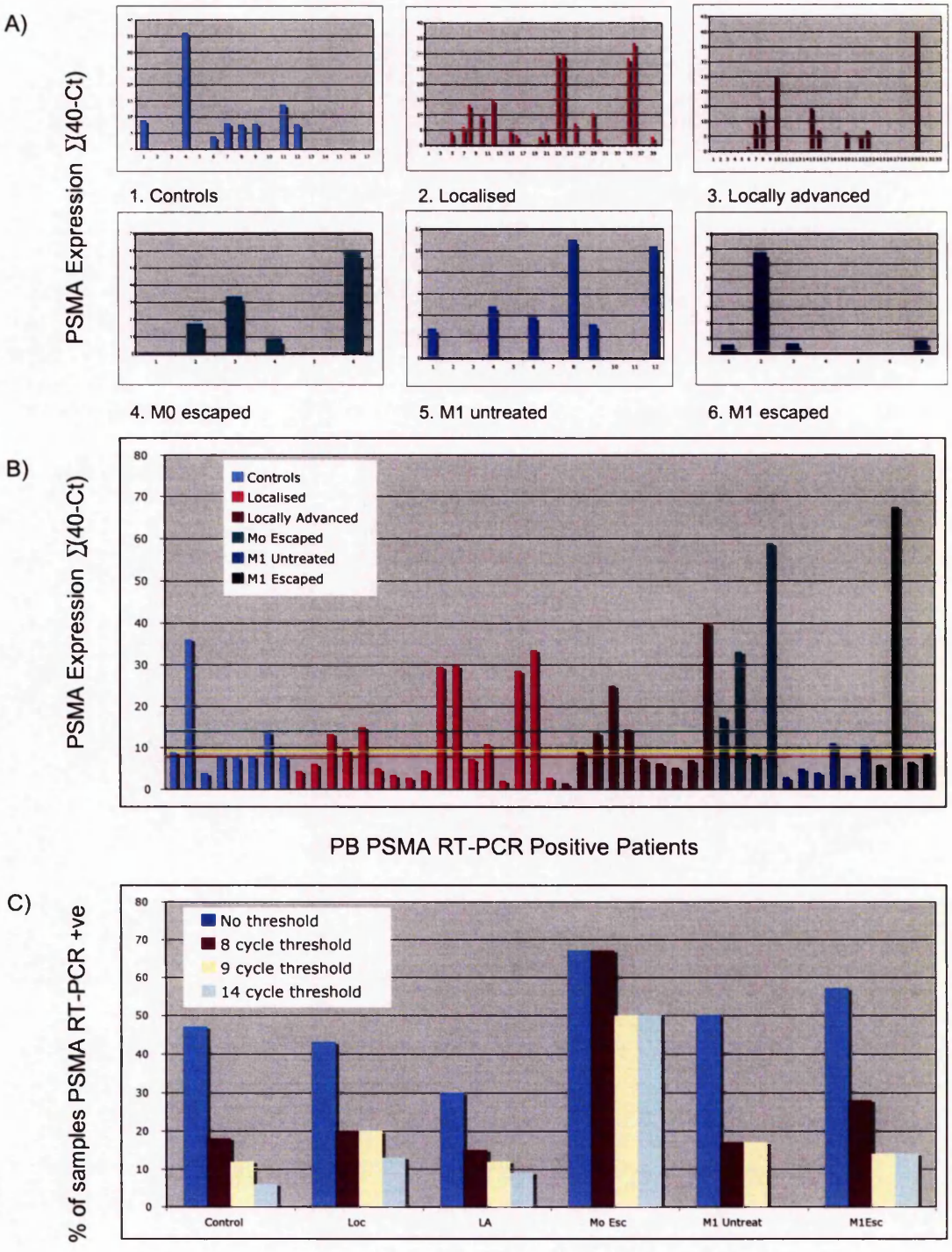



**Figure 4.6:** PSMA RT-PCR evaluation of PB samples from the study population. A) Bar charts showing relative quantitative expression of PSMA mRNA in all clinical samples standardised to the expression of the housekeeping genes GAPDH and ß-actin. Each clinical group is displayed in a separate bar chart. B) Bar chart showing Σ(40-Ct) for all PB samples that were PSMA RT-PCR positive. Threshold levels are shown at 8 (red line), 9 (yellow line) and 14 (green line) cycles. Samples are displayed in their respective clinical groups. C) Bar chart showing the impact of these Σ(40-Ct) threshold levels on the percentage of PB samples that were PSMA RT-PCR positive in the control and clinical groups. The 14 cycle threshold reduced false positives in the control group to 6% however only 12 samples from the entire CaP patient population showed PSMA mRNA expression above this level. The 9 cycle threshold allowed acceptable specificity (13% positive controls) combined with significant numbers of assay positive samples from the study cohort.

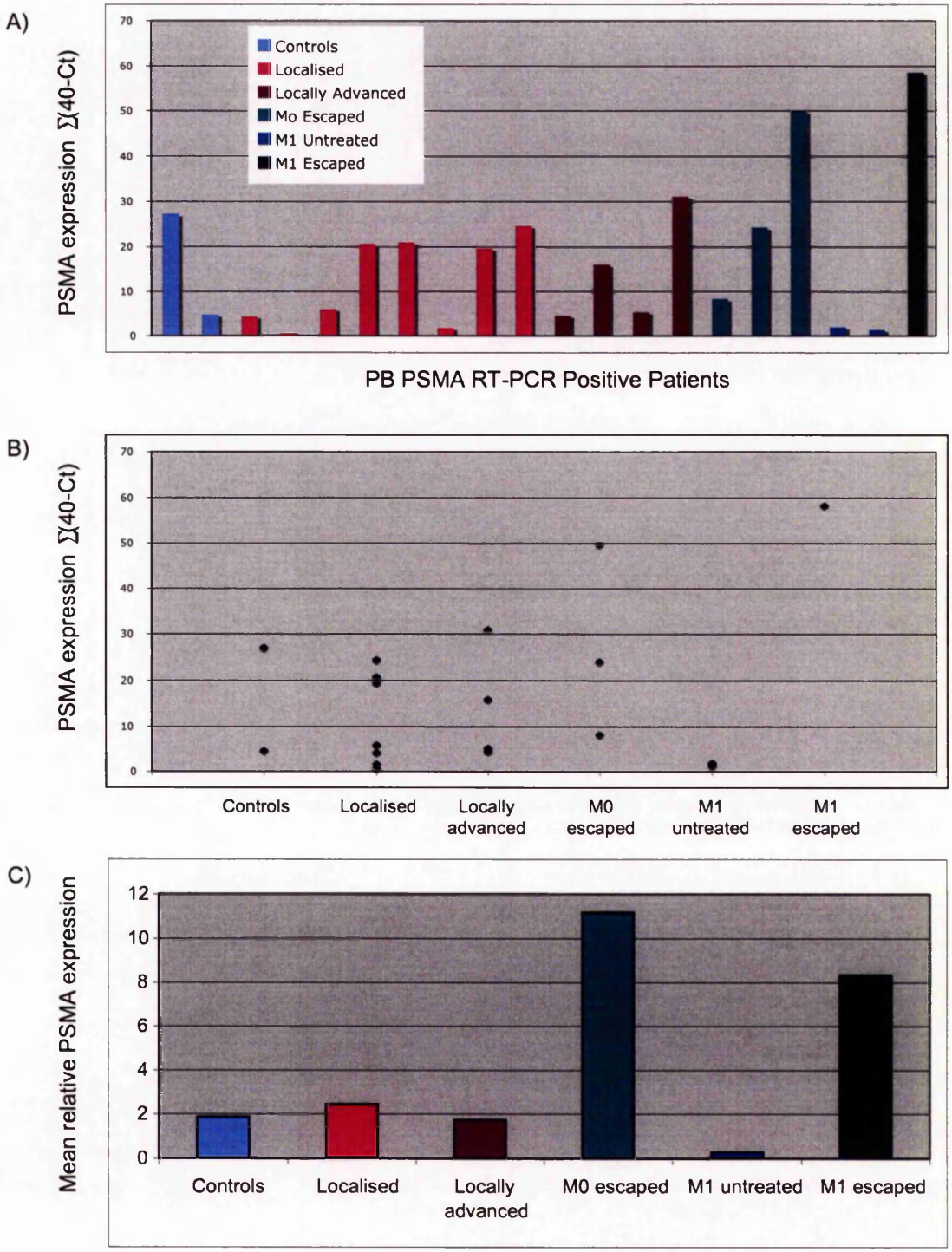



**Figure 4.7:** PB PSMA RT-PCT assay with a Σ(40-Ct) threshold of 9 cycles applied. A) Bar chart showing relative PSMA mRNA expression in all PB samples that were PSMA assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. B) Scatter plot displaying the distribution of relative PSMA mRNA expression within the study population. C) Bar chart displaying mean relative PSMA mRNA expression in control and clinical groups.

| | % PSMA RT-PCR +ve | Mean PSMA mRNA expression (cycles) | Range of PSMA mRNA expression (cycles) |
|---|---|---|---|
| Localised | 20 | 2.4 | 0 – 24.5 |
| Locally advanced | 12 | 1.7 | 0 – 30.9 |
| Non-metastatic hormone escaped | 50 | 11.2 | 0 – 49.8 |
| Untreated metastases | 17 | 0.3 | 0 – 2.0 |
| Metastatic hormone escaped | 14 | 8.3 | 0 – 58.0 |
| Controls | 13 | 1.9 | 0 – 27.0 |

**Table 4.9:** PB PSMA RT-PCR assay – showing the proportions of PSMA positive samples, mean PSMA and ranges of PSMA expression in each group with a Ct threshold of 9 cycles applied.

#### 4.2.2.4 Evaluation of PSCA RT-PCR Assay in Peripheral Blood

Peripheral blood analysis showed that 62% of patient samples were PSCA RT-PCR positive however PSCA target was also detectable in 71% of disease free controls. Seventy three percent of patients with localised disease, 61% with locally advanced disease, 50% with non-metastatic hormone escaped cancer, 67% with untreated metastases and 29% with hormone escaped metastatic CaP were PSCA RT-PCR positive. Figures 4.8a and 4.9a&b display the distribution of relative PSCA expression throughout the study population. Not only were a high proportion of control samples positive for PSCA target but the amounts of mRNA present were generally comparable to the CaP groups (figure 4.9c). When $\Sigma$(40-Ct) thresholds were applied to the data to exclude background control marker expression there remained little difference between the control and disease groups even at threshold limits as high as 25 cycles (figure 4.8b). Figure 4.8c shows the effect of expression thresholds of 15, 20 and 25 cycles on the percentages of samples PSCA RT-PCR positive in the control and patient groups. Only patients with non-metastatic hormone escaped CaP had a higher proportion of samples PSCA RT-PCR positive than the controls when using all of the thresholds, however this accounted for a very small number of patients and there was no significant difference in the relative expression levels in the positive controls and individual patients (figure 4.9c).

As PSCA expression was found to be as significant in PB samples from disease free controls as from men with CaP, PSCA was not used for further data analysis (table 4.10).

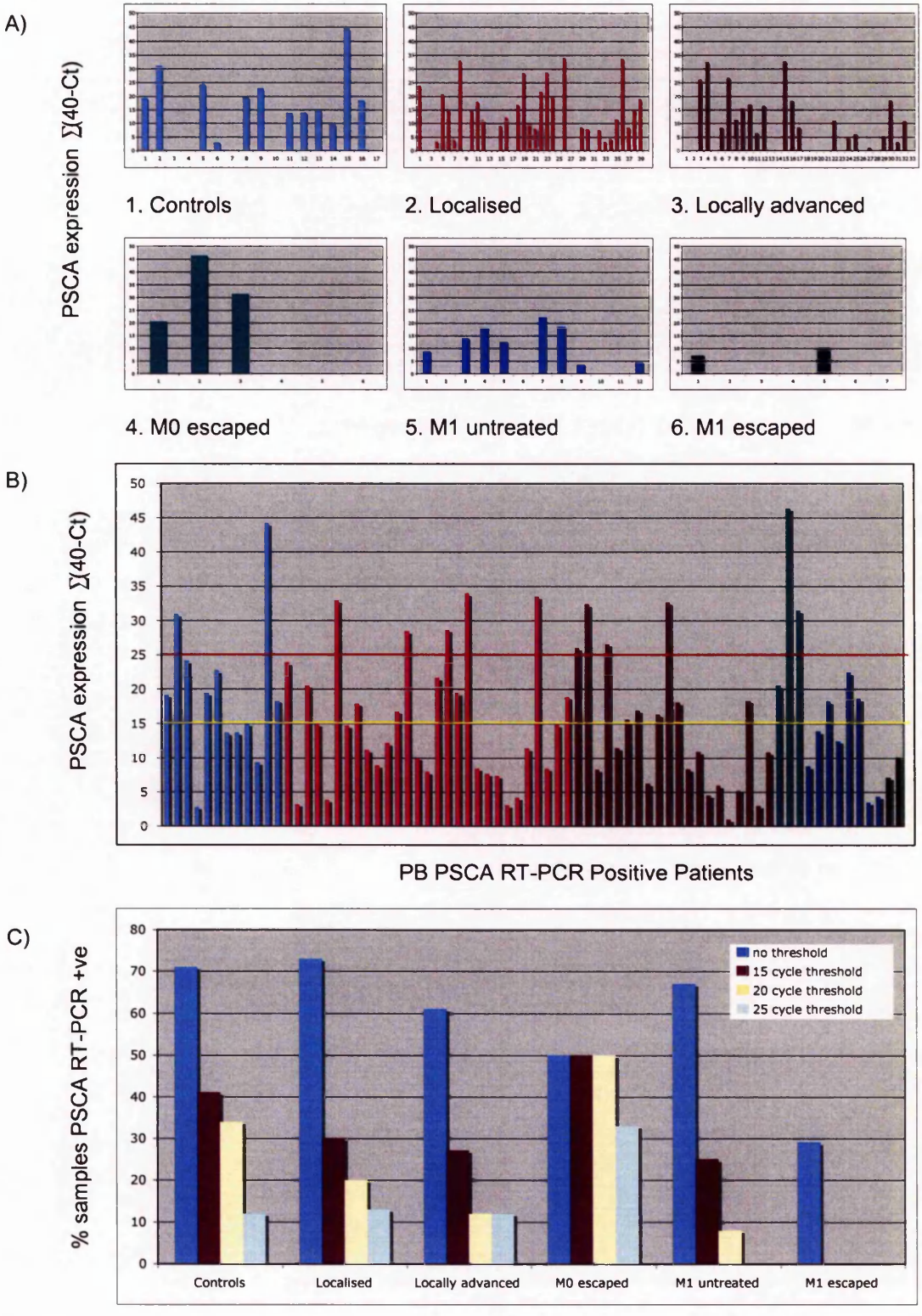



**Figure 4.8:** PSCA RT-PCR evaluation of PB samples from the study population. A) Bar charts showing relative quantitative expression of PSCA mRNA in all clinical samples standardised to the expression of the housekeeping genes GAPDH and ß-actin. Each clinical group is displayed in a separate bar chart. B) Bar chart displaying relative expression of PSCA mRNA in all assay positive samples in clinical and control groups. Σ(40-Ct) thresholds of 15 cycles (yellow line), 20 cycles (black line) and 25 cycles (red line) are shown but have not been applied to the data. C) Bar chart illustrating the impact of these three threshold levels on the percentage of PB samples from control and patient groups that are PSCA assay positive

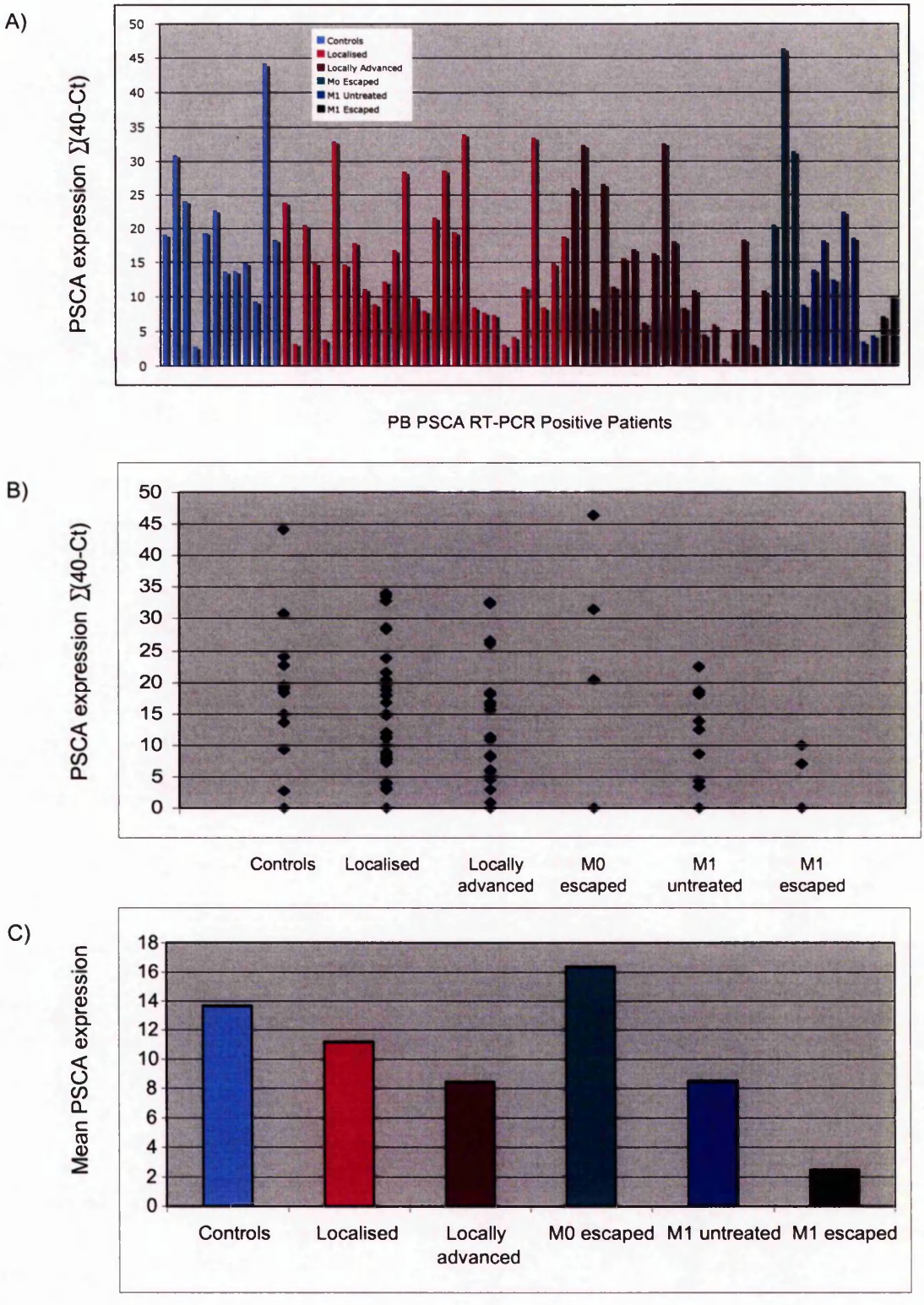



**Figure 4.9:** PB PSCA RT-PCR assay. A) Bar chart showing relative PSCA mRNA expression in all PB samples that were PSCA assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Patients are displayed in clinical groups and all negative samples have been excluded B) Scatter plot displaying distribution of relative PSCA mRNA expression within study population. C) Bar chart displaying mean relative PSCA mRNA expression in control and clinical groups.

| | % PSCA RT-PCR +ve | Mean PSCA mRNA expression (cycles) | Range of PSCA mRNA expression (cycles) |
|---|---|---|---|
| Localised | 73 | 11.1 | 0 – 32.9 |
| Locally advanced | 61 | 8.4 | 0 – 32.3 |
| Non-metastatic hormone escaped | 50 | 16.4 | 0 – 31.4 |
| Untreated metastases | 67 | 8.5 | 0 – 22.4 |
| Metastatic hormone escaped | 29 | 2.4 | 0 – 10.0 |
| Controls | 71 | 13.7 | 0 – 30.8 |

**Table 4.10:** PB PSCA RT-PCR assay – showing the proportions of PSMA positive samples, mean PSCA and ranges of PSCA expression in each group.

### 4.2.2.5 Evaluation of $DD3^{PCA3}$ RT-PCR Assay in Peripheral Blood

No $DD3^{PCA3}$ mRNA was detected in the PB from any of the control patients. This allowed a zero threshold to be applied as the clinical specificity was 100%. Four patient samples were $DD3^{PCA3}$ RT-PCR positive, 2 with localised disease and 2 with hormone escaped metastatic disease which equate to 5% and 29% positivity for the respective clinical groups. The relative quantitative $DD3^{PCA3}$ expression measures were 9.3 and 8.2 cycles for the patients with localised disease and 6.8 and 92.7 for those with hormone escaped metastases. Due to the small numbers, no clear correlation between $DD3^{PCA3}$ expression and disease status could be demonstrated. Stastistically there were significant differences between groups within the population, Kruskal-Wallis p=0.008, however paired tests directly comparing groups failed to reach significance (appendix 3).

The clinical details of the four PB $DD3^{PCA3}$ RT-PCR positive patients are shown in table 4.11. Those with clinically localised disease both had T2 Gleason 3+4=7 CaP with serum PSA levels of 7ng/ml at the time of sampling. PT18 with $DD3^{PCA3}$ expression of 9.3 was treated with RRP. Pathologically the lateral resection margins were positive and he later relapsed biochemically. The other patient was treated with EBRT and had a low and stable serum PSA at follow up. The two patients with hormone escaped metastatic disease with detectable PB $DD3^{PCA3}$ target both deteriorated rapidly with rising serum PSA levels. Both died within 4 months of sampling.

| Patient ID | Clinical Group | Age | Serum PSA | Biopsy Gleason Score | Clinical stage | $DD3^{PCA3}$ relative expression |
|---|---|---|---|---|---|---|
| PT18 | Localised | 62 | 7 | 3+4=7 | T2Mo | 9.3 |
| PT22 | Localised | 72 | 7 | 3+4=7 | T2Mo | 8.2 |
| PT21 | Metastatic hormone escaped | 72 | 656 | 4+3=7 | TxM1 | 92.7 |
| PT43 | Metastatic hormone escaped | 66 | 117 | 3+3=6 | TxM1 | 6.8 |

**Table 4.11:** Clinical data for patients with $DD3^{PCA3}$ RT-PCR positive PB samples. Only 4 men from the patient cohort were $DD3^{PCA3}$ RT-PCR positive. Two of these patients had clinically localised, T2, Gleason score 3+4=7 disease with serum PSA levels of 7 ng/ml. The remaining 2 patients had hormone escaped metastatic CaP with serum PSA levels of over 100ng/ml.

### 4.2.3 Evaluation of Individual Markers in Bone Marrow

The raw RT-PCR results for BM for each marker are summarised in table 4.12. The PSA assay showed a combination of good clinical specificity and sensitivity. For HK2 and $DD3^{PCA3}$, all BM control samples were negative. Using HK2 60% of patients with hormone escaped metastatic disease were RT-PCR positive, while the $DD3^{PCA3}$ assay detected target in only 2 of the patients with clinically identified metastases. The performance of each individual marker is detailed below.

| Clinical Group | PSA | PSMA | PSCA | $DD3^{PCA3}$ | HK2 |
|---|---|---|---|---|---|
| Localised | 32% | 82% | 76% | 3% | 11% |
| Locally advanced | 32% | 84% | 84% | 4% | 16% |
| Hormone escaped & BS negative | 67% | 100% | 83% | 0 | 33% |
| Untreated Metastases | 33% | 78% | 67% | 22% | 44% |
| Hormone escaped & metastases | 80% | 80% | 80% | 0 | 60% |
| Controls | 14% | 86% | 93% | 0 | 0 |

**Table 4.12:** Percentage of BM samples RT-PCR positive for each of the five prostate markers PSA, PSMA, PSCA, $DD3^{PCA3}$ and HK2.

### 4.2.3.1 Evaluation of PSA RT-PCR Assay in Bone Marrow

PSA RT-PCR was positive in 14% of control BM samples, 32% of localised and locally advanced CaP patients and 67% of those with non-metastatic hormone escaped cancer. Of those with proven metastases 33% of the untreated group and 80% of those who were hormone escaped were positive (figure 4.10a & table 4.13).

The quantitative RT-PCR results were again used to optimise clinical assay performance. Σ(40-Ct) thresholds of 6 and 9 cycles were applied to all positive assay results (figure 4.10b). A 6 cycle threshold reduced percentage positivity in the control group from 14% to 7% with a further reduction to 4% when the threshold was increased to 9 cycles. Both threshold values reduced the percentage of samples positive in the localised group from 32% to 13%, while in those with locally advanced disease the 6 and 9 cycle thresholds saw reductions in percentages of positive samples from 32% to 20% and 12% respectively. Σ(40-Ct) values for all samples in the other clinical groups were above both threshold levels (figure 4.10c & table 4.13). Therefore a threshold level of 9 cycles was used for final data analysis.

With the threshold of 9 cycles applied, Σ(40-Ct) values for all PSA RT-PCR positive samples are displayed in figure 4.11a. Generally the Σ(40-Ct) values are higher in the patients with metastatic disease with 70% of samples reaching over 80 cycles. In those with non-metastatic cancer two thirds of positive samples have Σ(40-Ct) values below 20 cycles while the remaining 4 patients show relative expression levels between 35 and 45 cycles.

In comparing PSA RT-PCR levels between groups the scatter plot, figure 4.11b, demonstrates both intra and inter group variability in detectable PSA expression and there is a significant positive correlation between PSA RT-PCR and increasing clinical stage, Spearman rho p=<0.0001. Mean PSA Σ(40-Ct) was 0.53 cycles for controls, localised 2.7, locally advanced 2.0, non-metastatic hormone escaped 12.4, untreated metastases 28.4 and metastatic escaped 43.9 (table 4.14 & figure

4.11c). Significant differences were demonstrated in PSA RT-PCR results between the control groups and patients with more advanced CaP, non-metastatic hormone escaped (Mann-Whitney p=0.0001), untreated metastases (Mann-Whitney p=0.01) and metastatic hormone escaped (Mann-Whitney p=<0.0001). The observed differences between other clinical groups and controls were not significant. Expression in the patients with hormone escaped or metastatic CaP was higher than in the groups with clinically localised or locally advanced disease although this only reached significance for the non-metastatic (Mann-Whitney p=0.003 & p=0.003) and metastatic (Mann-Whitney p=0.0003 & p=0.001) hormone escaped groups (data shown appendix 3).

| Clinical Group | PSA RT-PCR +ve No threshold applied | % PSA RT-PCR +ve Threshold = 6 | % PSA RT-PCR +ve Threshold = 9 |
|---|---|---|---|
| Localised | 32% | 13% | 13% |
| Locally advanced | 32% | 20% | 12% |
| Hormone escaped & bone scan negative | 67% | 67% | 67% |
| Untreated metastases | 33% | 33% | 33% |
| Metastases & hormone escaped | 80% | 80% | 80% |
| Controls | 14% | 7% | 4% |

**Table 4.13**: PSA RT-PCR evaluation of BM samples from the study population. Impact of Σ(40-Ct) threshold levels of 6 and 9 cycles on the percentage of control and patient BM samples that were PSA RT-PCR positive.

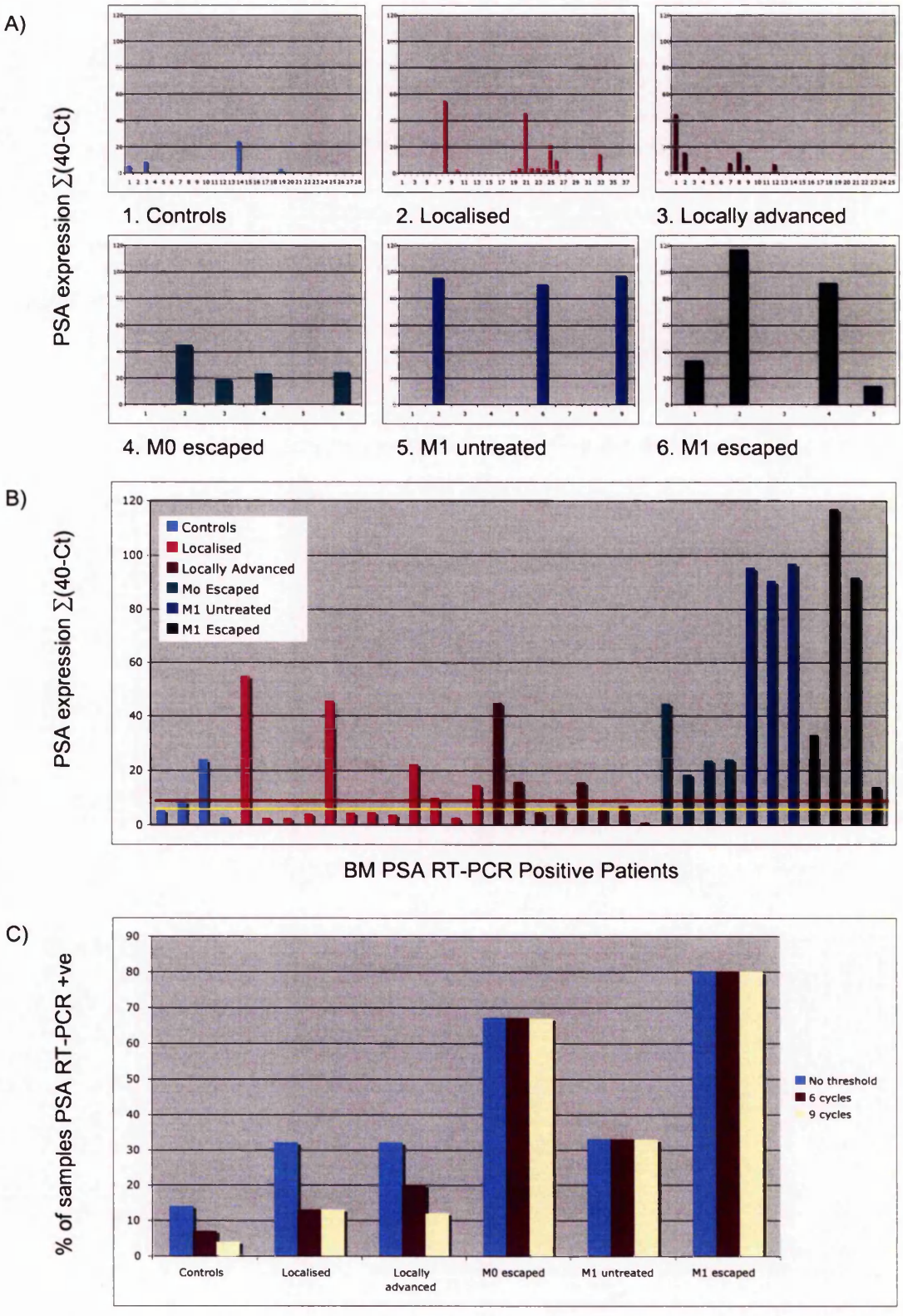



**Figure 4.10:** PSA RT-PCR evaluation of BM samples from the study population. A) Bar charts showing relative quantitative expression of PSA mRNA in all clinical samples standardised to the expression of the housekeeping genes GAPDH and ß-actin. Each clinical group is displayed in a separate bar chart. B) Bar chart displaying relative expression of PSA mRNA in all assay positive BM samples in clinical and control groups. Σ(40-Ct) thresholds of 6 cycles (yellow line) and 9 cycles (red line) are shown but have not been applied to the data. C) Bar chart illustrating the impact of threshold levels on the percentage of BM samples from control and patient groups that are PSA assay positive.

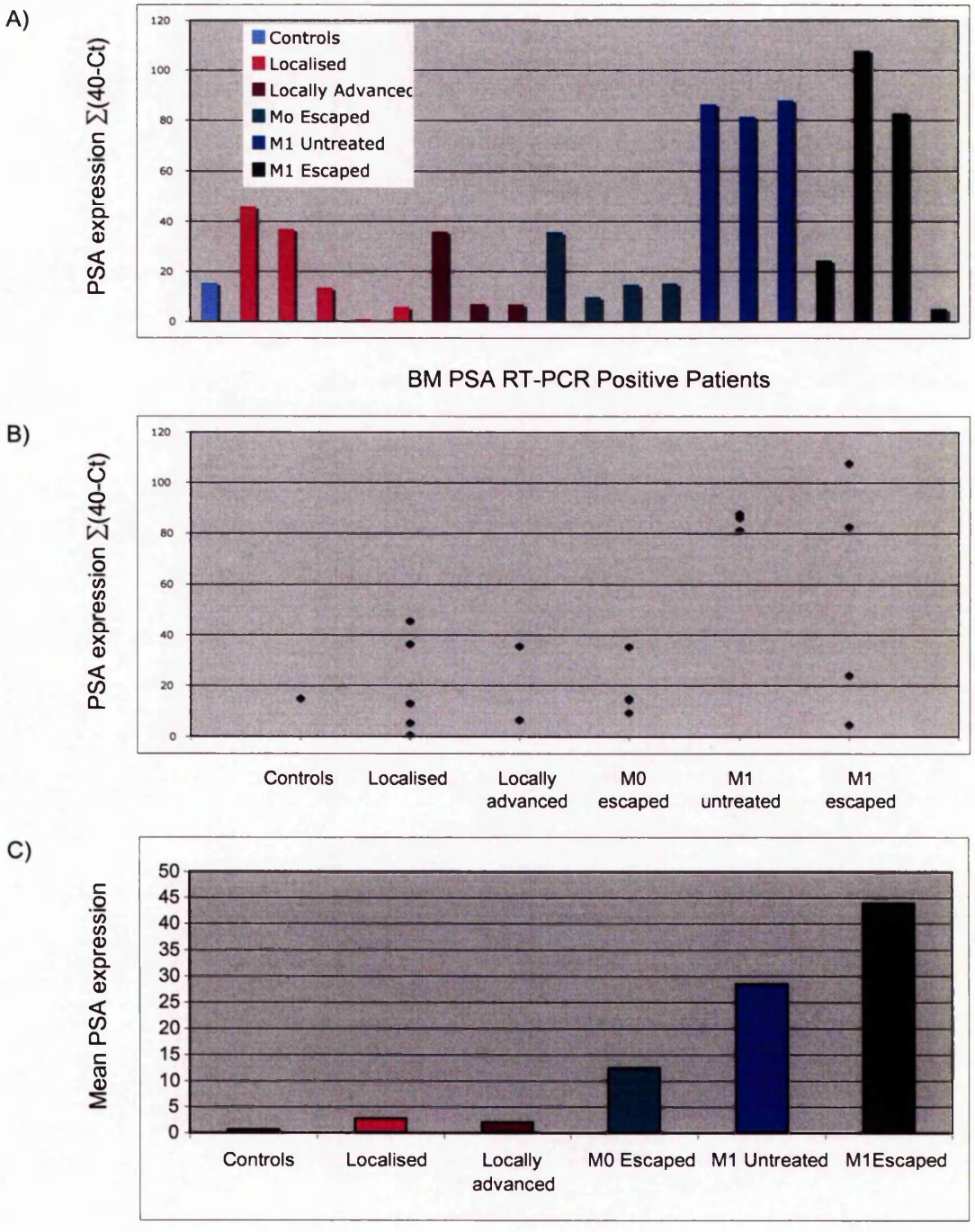



**Figure 4.11:** BM PSA RT-PCR assay with a Σ(40-Ct) threshold of 9 cycles applied. A) Bar chart showing relative PSA mRNA expression in all BM samples that were PSA assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. B) Scatter plot displaying the distribution of relative PSA mRNA expression within the study population. C) Bar chart displaying mean relative PSA mRNA expression in control and clinical groups.

| | % PSA RT-PCR +ve | Mean PSA mRNA expression (cycles) | Range of PSA mRNA expression (cycles) |
|---|---|---|---|
| Localised | 13 | 2.7 | 0 – 45.7 |
| Locally advanced | 12 | 2.0 | 0 – 35.8 |
| Non-metastatic hormone escaped | 67 | 12.4 | 0 – 35.6 |
| Untreated metastases | 33 | 28.4 | 0 – 87.7 |
| Metastatic hormone escaped | 80 | 43.9 | 0 – 107.7 |
| Controls | 4 | 0.5 | 0 – 15.0 |

**Table 4.14:** BM PSA RT-PCR assay – showing the proportions of PSA positive samples, mean PSA and ranges of PSA expression in each group with a Ct threshold of 9 cycles applied.

#### 4.2.3.2 Evaluation of HK2 RT-PCR in Bone Marrow

When HK2 mRNA was targeted in BM samples all control samples were marker free. Fourteen (16%) patients had detectable HK2 mRNA in their BM aspirates. These represented 8% of patients with localised disease, 16% with locally advanced disease, 44% with untreated metastases and 60% of hormone escaped metastases (figures 4.12a&b). The mean relative expression of HK2 for these groups was 0.6, 0.9, 24.7 and 33.5 respectively (table 4.15 & figure 4.13c). When HK2 RT-PCR positive, the relative HK2 mRNA expression was generally higher in men with metastatic cancer than other clinical stages (figures 4.13a&b). None of the BM samples from men with non-metastatic hormone escaped CaP were HK2 RT-PCR positive in BM. Overall, there was a significant positive correlation between HK2 expression and increasing clinical stage as is displayed in the scatter plot figure 4.13b (spearmans rho p=<0.0001).

In comparing HK2 expression between clinical groups within the population, significant differences were apparent (kruskall-wallis p=<0.0001). Specifically, HK2 expression was significantly higher in those patients with untreated metastatic (mann-whitney p=0.0002) and hormone escaped metastatic (mann-whitney p=<0.0001) disease than in controls. When BM HK2 expression was compared between patients with localised disease and more advanced CaP, marker levels were significantly higher in those with both untreated (mann-whitney p=0.001) and hormone escaped (mann-whitney p=0.0002) metastases (all data shown appendix 3).

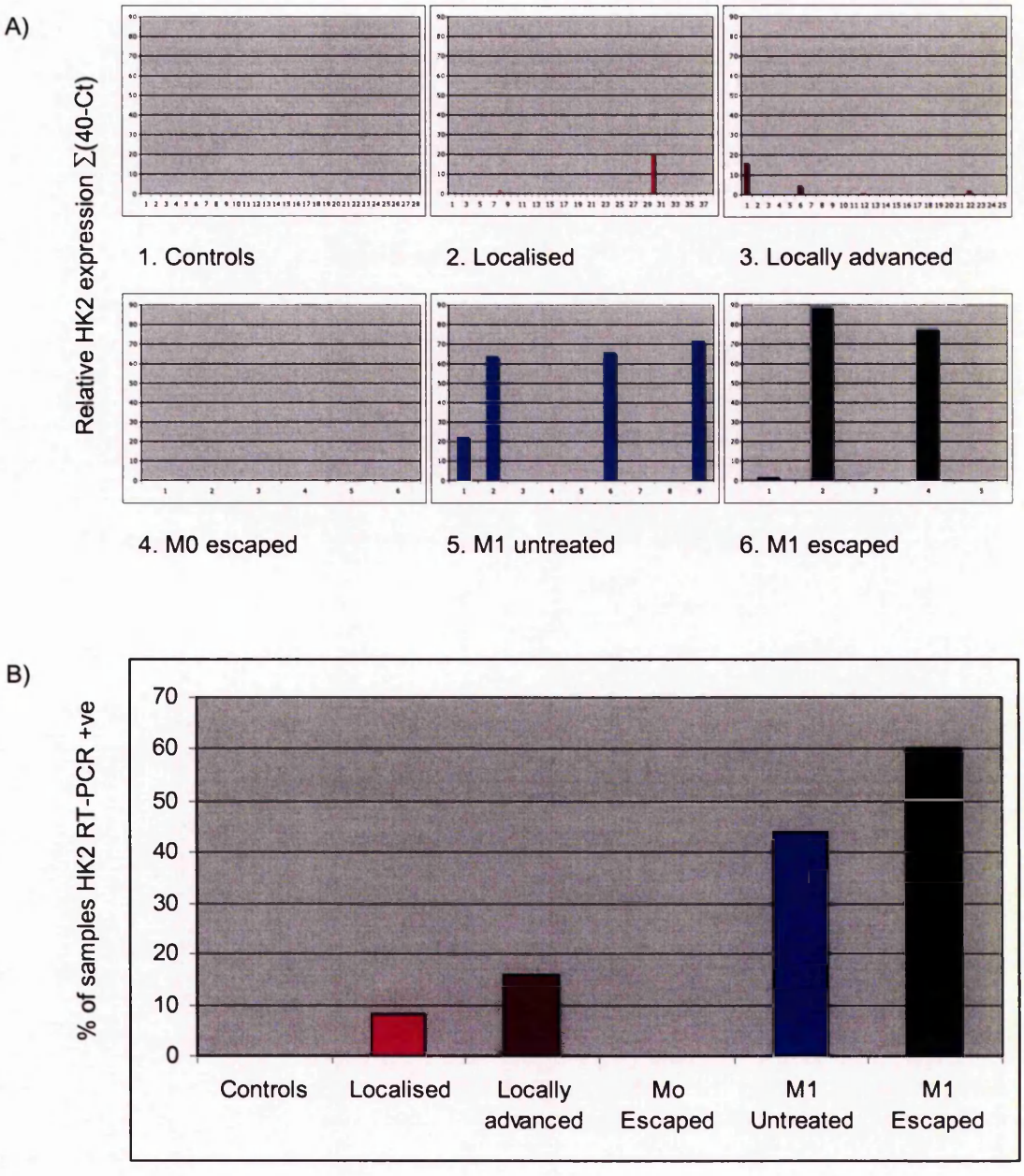



**Figure 4.12:** HK2 RT-PCR evaluation of BM samples from the study population. A) Bar charts showing relative quantitative expression of HK2 mRNA in all clinical samples standardised to the expression of the housekeeping genes GAPDH and ß-actin. Each clinical group is displayed in a separate bar chart. B) Bar chart showing the percentage of BM samples that were HK2 RT-PCR positive in the control and clinical groups.

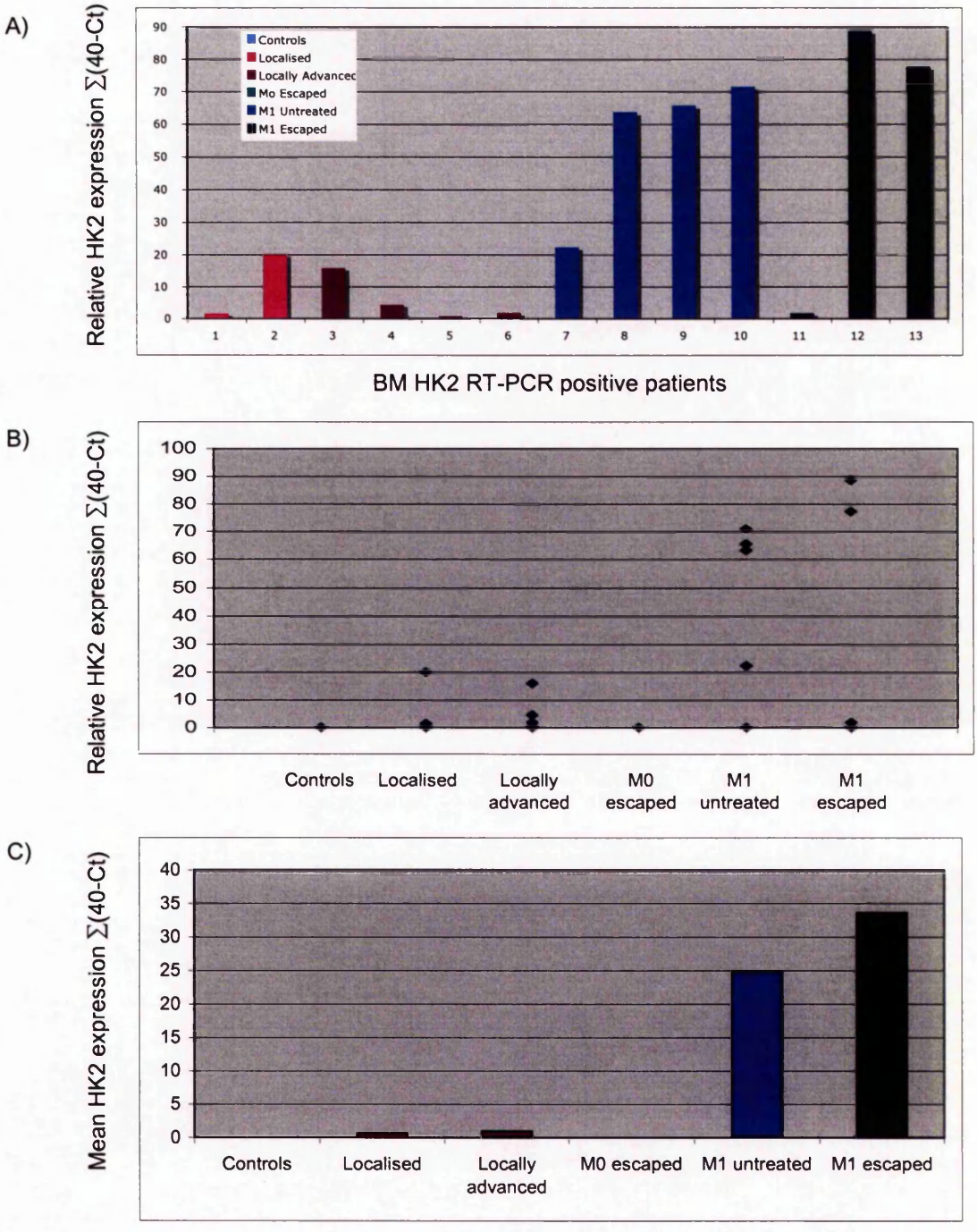



**Figure 4.13:** BM HK2 RT-PCR assay. A) Bar chart showing relative HK2 mRNA expression in all PB samples that were HK2 assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. B) Scatter plot displaying the distribution of relative HK2 mRNA expression within the study population. C) Bar chart displaying mean relative HK2 mRNA expression in control and clinical groups.

| | % HK2 RT-PCR +ve | Mean HK2 mRNA expression (cycles) | Range of HK2 mRNA expression (cycles) |
|---|---|---|---|
| Localised | 8 | 0.6 | 0 – 19.9 |
| Locally advanced | 16 | 0.9 | 0 – 15.7 |
| Non-metastatic hormone escaped | 0 | 0 | 0 |
| Untreated metastases | 44 | 24.7 | 0 – 71.2 |
| Metastatic hormone escaped | 60 | 33.5 | 0 – 88.6 |
| Controls | 0 | 0 | 0 |

**Table 4.15:** BM HK2 RT-PCR assay – showing the proportions of HK2 positive samples, mean HK2 and ranges of HK2 expression in each group, no Ct threshold applied.

#### 4.2.3.3 Evaluation of PSMA RT-PCR Assay in Bone Marrow

PSMA mRNA expression was detected in 85% of patient BM samples and 86% of controls. Between 78% and 100% of each clinical patient group were PSMA RT-PCR positive (Figure 4.14a & table 4.12). Relative PSMA expression in the control samples ranged between 5.3 and 71.2 cycles however 67% and 88% of the positive controls showed expression levels below 25 and 30 cycles respectively. A 25 cycle threshold reduced the percentage of positive controls to 29% while the percentages of each patient group with PSMA expression above this threshold were 13% localised, 32% locally advanced, 67% non-metastatic hormone escaped, 56% untreated metastases and 40% hormone escaped metastases (figure 4.14b). The mean PSMA expression for these marker positive control BM samples was 3.6 cycles. For the patient groups with non-metastatic CaP, it was 1.0 cycle for localised cancer, 5.5 cycles for locally advanced and 12.1 cycles in hormone escaped disease. Relative PSMA expression was higher still in samples from men with metastases being 23.4 cycles for untreated metastases and 28.3 cycles with hormone escape (figure 4.14c).

A 25 cycle PSMA threshold identified a marker positive patient population whose BM PSMA mRNA expression increased with advancing clinical disease status. Although improved, clinical specificity remained poor. The PSMA expression threshold was therefore increased to 30 cycles which excluded all but 3 (11%) control BM samples (figure 4.15a). 11% of localised disease BM were above this threshold as were 24% of locally advanced and 67% of non-metastatic hormone escaped cancers. There was no reduction in BM PSMA RT-PCR positive patients with metastases, 56% positive for untreated patients and 40% for hormone escaped. As is shown in Figure 5.15b, a significant positive correlation between PSMA expression in BM samples and increasing clinical stage was demonstrated (Spearman rho p=0.0001)

Mean PSMA expression remained high for men with untreated metastases, 20.6 cycles, and hormone escaped metastases, 26.3 cycles, compared with positive control samples, 2.8 cycles. For the non-metastatic cancers the mean expression was lower than in the controls for localised disease, 0.42 cycles, but slightly higher

for locally advanced, 4.1 cycles, and hormone escaped, 8.8 cycles (figure 4.15c & table 4.16). Despite these clear differences in PSMA expression between the more advanced patients and controls, only that between BM samples from those with untreated metastases and controls was statistically significantly (Mann-Whitney p=0.003). Despite high expression in the 40% of positive samples from men with hormone escaped CaP and the high proportion of PSMA positive samples from the non-metastatic hormone escaped group these did not quite reach significance compared to controls (Mann-Whitney p=0.059 & p=0.005 respectively).

Between clinical groups, PSMA expression was significantly higher in both the non-metastatic hormone escaped and untreated metastases groups than localised cancer (Mann-Whitney p=0.001 & p=0.001), while the observed difference between the localised and hormone escaped metastases groups did not quite reach significance at the reduced significance level of 0.003 (Mann-Whitney p=0.044) (all data shown in appendix 3).

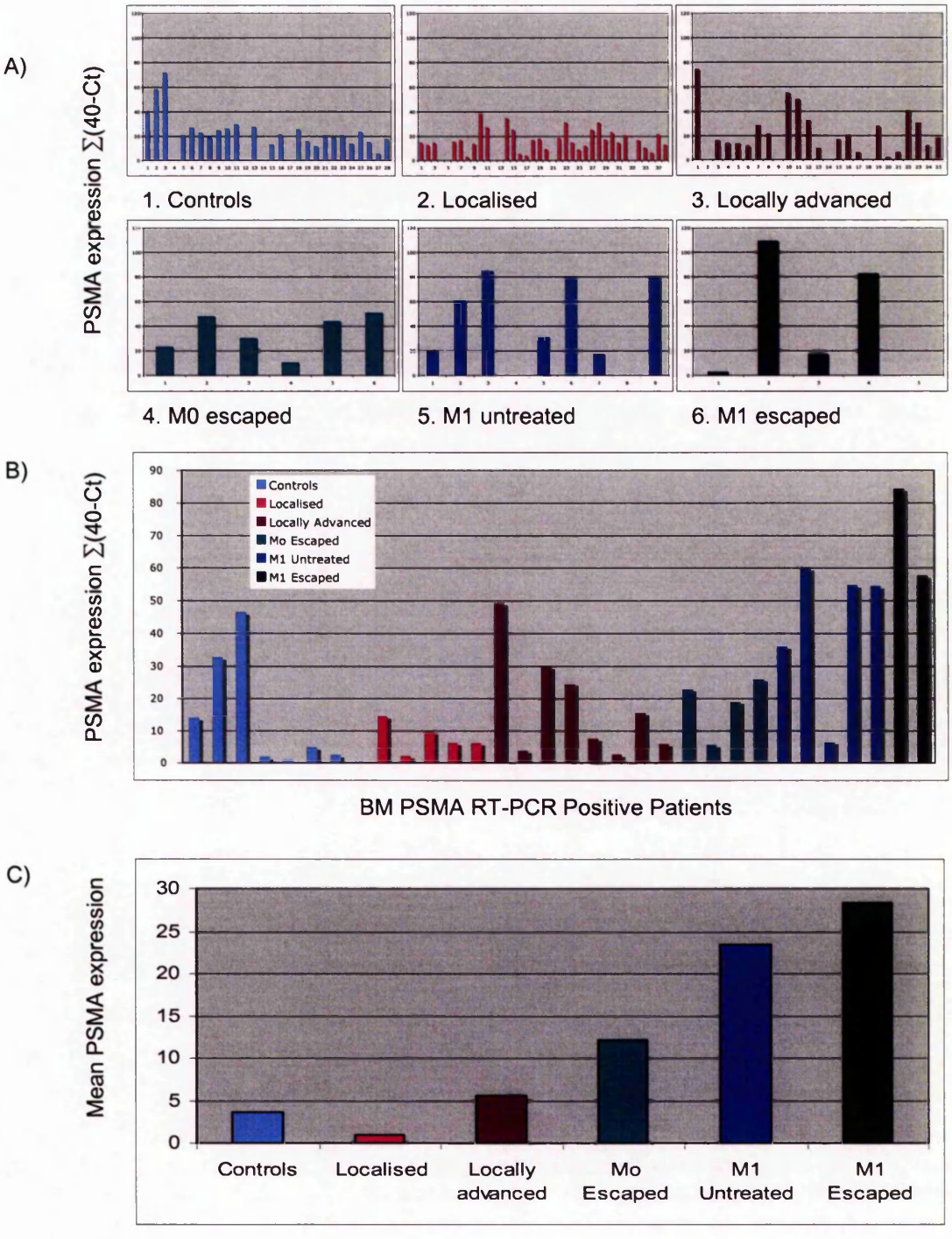



**Figure 4.14:** PSMA RT-PCR evaluation of BM samples from the study population. A) Bar charts showing relative quantitative expression of PSMA mRNA in all clinical samples standardised to the expression of the housekeeping genes GAPDH and ß-actin. Each clinical group is displayed in a separate bar chart. B) Bar chart showing PSMA mRNA expression with a threshold of 25 cycles applied. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. The number of marker positive samples is reduced yet clinical specificity improves. C) Bar chart displaying mean relative PSMA mRNA expression in control and clinical groups with a 25 cycles threshold applied.

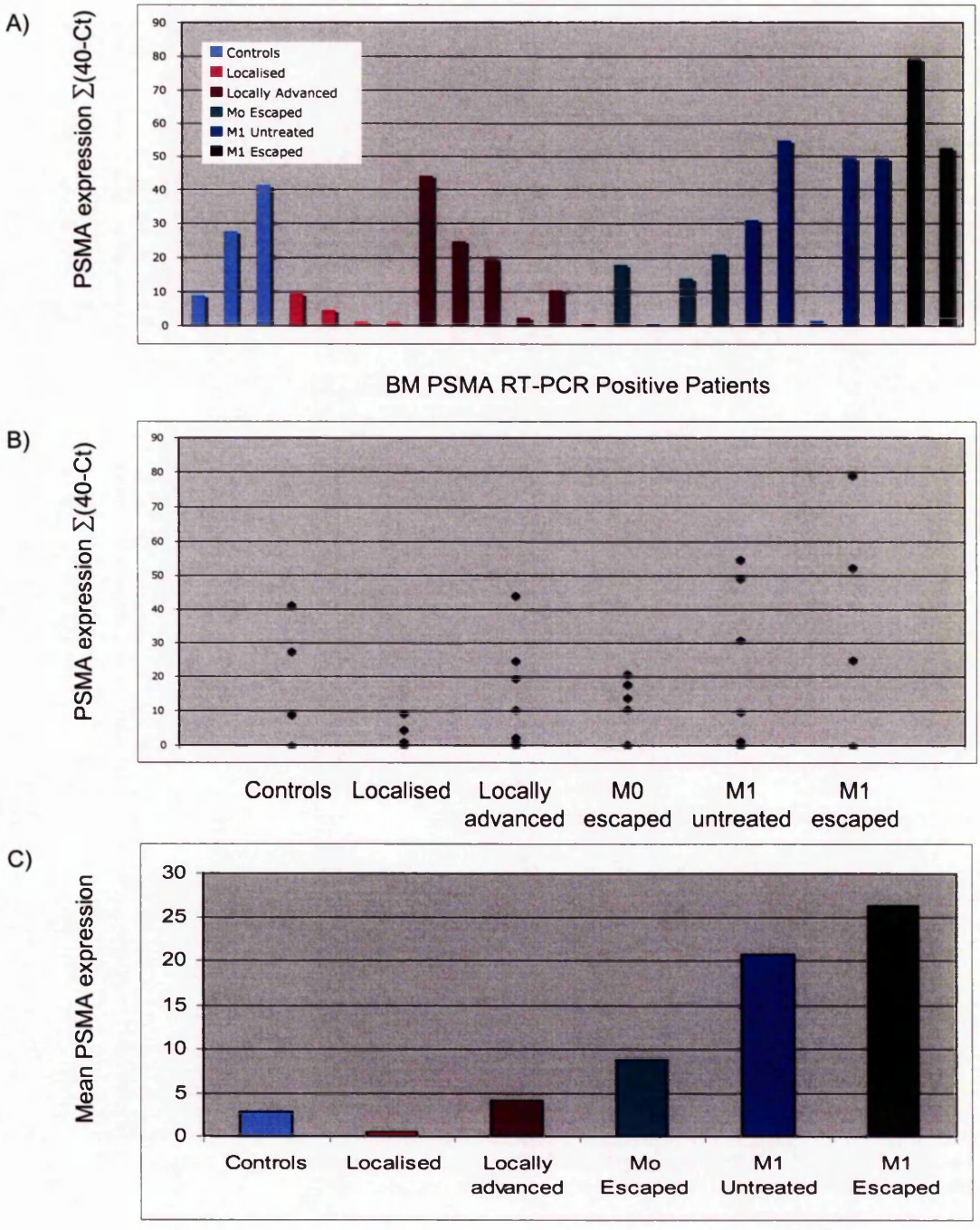



**Figure 4.15:** BM PSMA RT-PCR assay with a Σ(40-Ct) threshold of 30 cycles applied. A) Bar chart showing relative PSMA mRNA expression in all BM samples that were PSMA assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. B) Scatter plot displaying the distribution of relative PSMA mRNA expression within the study population. C) Bar chart displaying mean relative PSMA mRNA expression in control and clinical groups.

| | % PSMA RT-PCR +ve | Mean PSMA mRNA expression (cycles) | Range of PSMA mRNA expression (cycles) |
|---|---|---|---|
| Localised | 11 | 0.42 | 0 – 9.3 |
| Locally advanced | 24 | 4.1 | 0 – 44.1 |
| Non-metastatic hormone escaped | 67 | 8.8 | 0 – 20.8 |
| Untreated metastases | 56 | 20.6 | 0 – 54.7 |
| Metastatic hormone escaped | 40 | 26.3 | 0 – 79.2 |
| Controls | 11 | 2.8 | 0 – 41.2 |

**Table 4.16:** BM PSMA RT-PCR assay – showing the proportions of PSMA positive samples, mean PSMA and ranges of PSMA expression in each group with a Ct threshold of 30 cycles applied.

#### 4.2.3.4 Evaluation of PSCA RT-PCR in Bone Marrow

PSCA mRNA expression was detected in 93% of control BM samples and 74% of patient BM samples, 76% localised, 80% locally advanced, 67% non-metastatic hormone escaped, 67% untreated metastatic and 80% of hormone escaped metastatic CaP (figures 4.16a&b). Mean PSCA expression was 9.1, 10.6, 11.2, and 16.6 cycles for localised, locally advanced, non-metastatic hormone escaped, untreated metastatic and hormone escaped CaP respectively, none of which differed significantly from the 13.1 cycles found in the controls (figure 4.16c).

PSCA expression in all marker positive BM samples is displayed in figure 4.17a. Marker negative samples have been excluded. It is clear that PSCA expression was detected throughout the patient and control populations. Ct threshold were applied to the data at 10, 17 and 20 cycles. The impact of these on the proportions of BM samples that were PSCA RT-PCR positive and the mean PSCA expression for each clinical group is detailed in tables 4.17 and 4.18. The percentage of positive control samples was reduced from 93% to 57%, 25% and 18% respectively. At each threshold level the percentage of localised, 34%, 21% and 11%, and locally advanced, 48%, 28% and 12%, patient samples PSCA RT-PCR positive were lower than controls (figure 4.17b). Although the percentage of non-metastatic hormone escaped patients with marker positive BM was higher than controls, 50%, 33% and 33%, the mean PSCA expression was lower than that detected in controls, 4.7 vs 5.6, 2.3 vs 2.8 and 1.3 vs 2.1. The metastatic disease patient groups showed higher proportions of sample positivity, and mean PSCA expression using all thresholds (figure 4.17b&c). None of these observed differences in PSCA expression between groups reached statistical significance (Kruskal-Wallis for study population p=0.643), and no correlation between PSCA expression and clinical stage could be demonstrated.

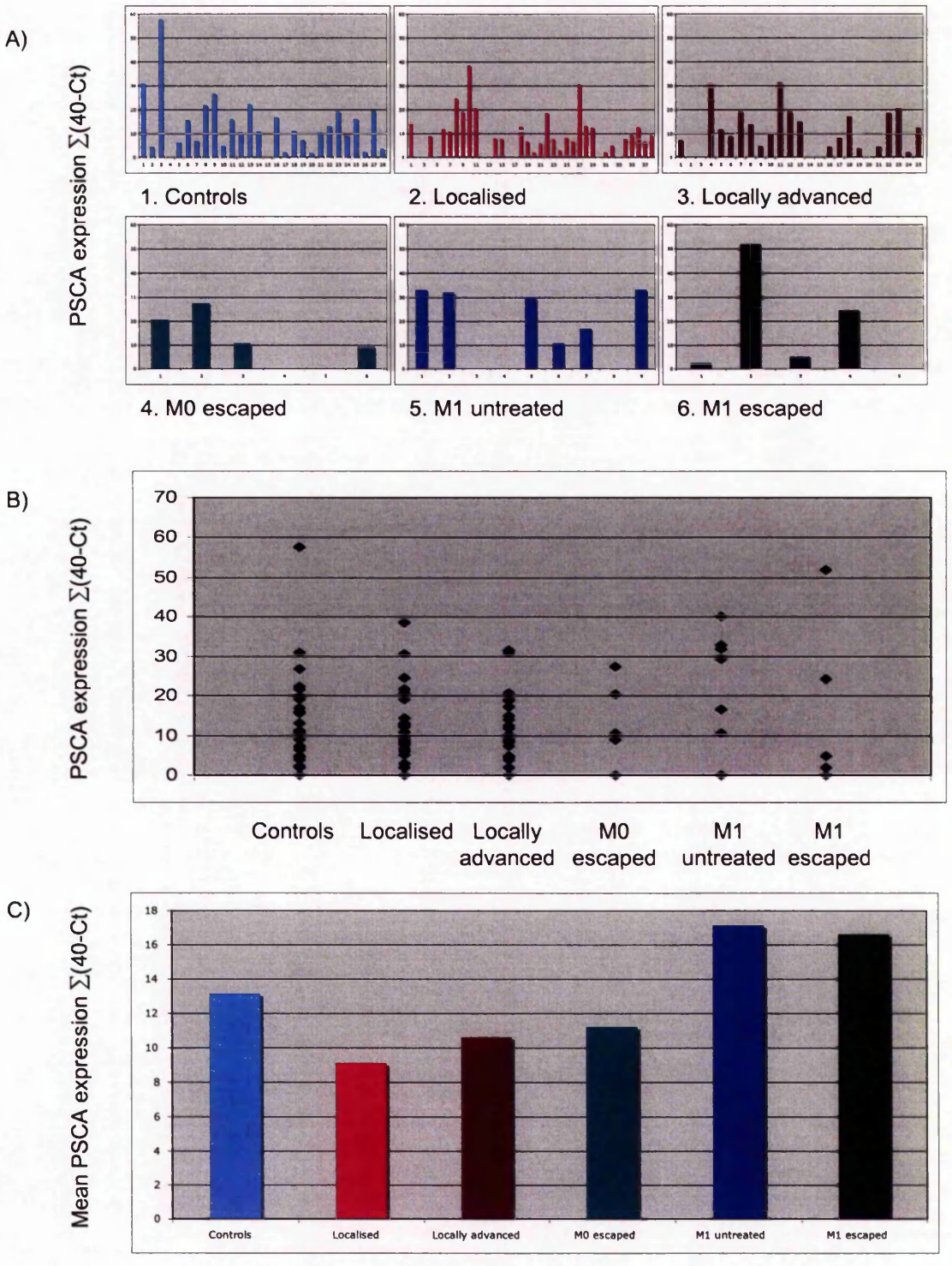



**Figure 4.16:** PSCA RT-PCR evaluation of PB samples from the study population. A) Bar charts showing relative quantitative expression of PSCA mRNA in all clinical samples standardised to the expression of the housekeeping genes GAPDH and ß-actin. Each clinical group is displayed in a separate bar chart. B) Scatter plot displaying distribution of relative PSCA mRNA expression within study population. C) Bar chart displaying mean relative PSCA mRNA expression in control and clinical groups.

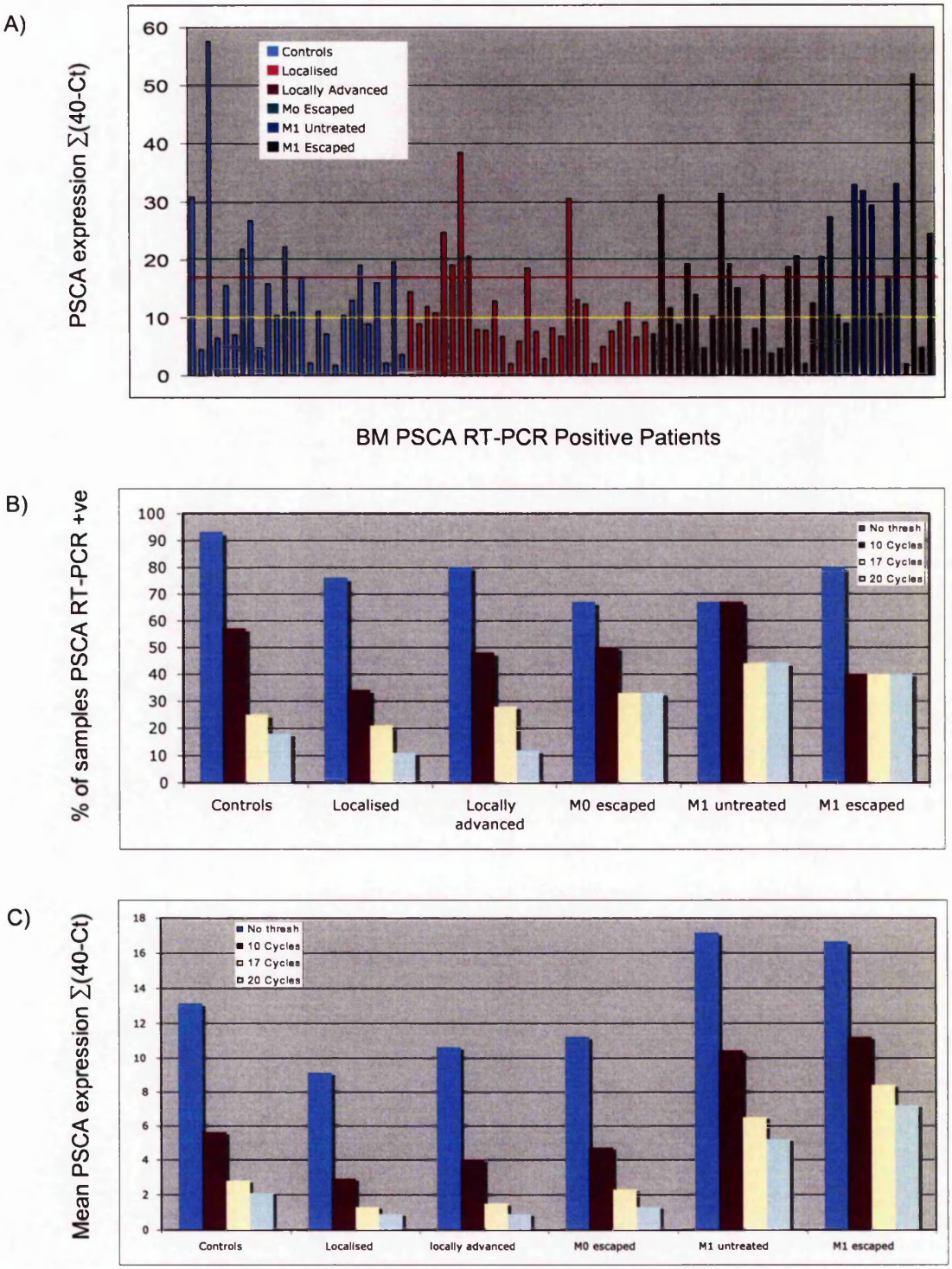



**Figure 4.17:** PSCA RT-PCR evaluation of BM samples from the study population. A) Bar chart showing relative PSCA mRNA expression in all BM samples that were PSCA assay positive, standardised to the expression of the housekeeping genes GAPDH and ß-actin. Thresholds of 10 cycles (yellow), 17 cycles (red) & 20 cycles (green) are shown but have not been applied to the data. Patients are displayed in clinical groups and all negative/sub-threshold samples have been excluded. B) Bar chart showing the percentages of control and patient samples that were PSCA assay positive without and following application of the 10, 17 and 20 cycle thresholds. C) Bar chart displaying the mean PSCA expression for control and clinical groups before and following the application of the 10, 17 and 20 cycle thresholds.

| Clinical Group | % PSCA RT-PCR +ve No Threshold | % PSCA RT-PCR +ve Threshold = 10 | % PSCA RT-PCR +ve Threshold = 17 | % PSCA RT-PCR +ve Threshold = 20 |
|---|---|---|---|---|
| Localised | 76 | 34 | 21 | 11 |
| Locally advanced | 80 | 48 | 28 | 12 |
| Non-metastatic hormone escaped | 67 | 50 | 33 | 33 |
| Untreated metastases | 67 | 67 | 44 | 44 |
| Metastatic hormone escaped | 80 | 40 | 40 | 40 |
| Control | 93 | 57 | 25 | 18 |

**Table 4.17:** Impact of $\Sigma$(40-Ct) threshold levels of 10, 17 and 20 cycles on the percentage of control and patient BM samples that were PSCA RT-PCR positive.

| Clinical Group | PSCA mean $\Sigma$(40-Ct) No threshold | PSCA mean $\Sigma$(40-Ct) Threshold = 10 | PSCA mean $\Sigma$(40-Ct) Threshold = 17 | PSCA mean $\Sigma$(40-Ct) Threshold = 20 |
|---|---|---|---|---|
| Localised | 9.1 | 2.9 | 1.3 | 0.9 |
| Locally advanced | 10.6 | 4 | 1.5 | 0.9 |
| Non-metastatic hormone escaped | 11.2 | 4.7 | 2.3 | 1.3 |
| Untreated Metastases | **17.1** | **10.4** | **6.5** | **5.2** |
| Metastatic hormone escaped | **16.6** | **11.2** | **8.4** | **7.2** |
| Controls | 13.1 | 5.6 | 2.8 | 2.1 |

**Table 4.18:** Mean BM PSCA expression in each clinical group before and with $\Sigma$(40-Ct) threshold levels of 10, 17 and 20 cycles applied.

#### 4.2.3.5 Evaluation of $DD3^{PCA3}$ RT-PCR in Bone Marrow

All control BM samples were $DD3^{PCA3}$ RT-PCR negative. As was found in PB very few patient BM samples were RT-PCR positive for this marker. Only five contained detectable $DD3^{PCA3}$ mRNA, one each from men with localised, locally advanced and hormone manipulated CaP as well as two from patients with untreated metastases. The $\Sigma$(40-Ct) values for each were 16.2, 7.1, 5.9, 66.5 and 86.0 respectively. All six replicate reactions were positive for the BM from the two men with metastases while target was detected in only one reaction for men with less advanced disease. None of the patients with detectable $DD3^{PCA3}$ in their BM samples had marker present in their PB samples. The $DD3^{PCA3}$ positive BM samples from the advanced patients were also strongly RT-PCR positive for all other markers. These patients both had serum PSA levels of over 200 ng/ml, generally higher than other men in this group. Their clinical outcomes remain to be determined.

Statistical analysis of the $DD3^{PCA3}$ data was limited by the small numbers of RT-PCR positive BM samples. Evaluation of differences between groups within the population only came close to significance (Kruskal-Wallis p=0.055), and therefore paired testing comparing the untreated metastatic group with controls and localised disease failed to show significant differences (Mann-Whitney p=0.01 & p=0.03 respectively).

### 4.2.4 Overview of Marker Expression in PB and BM

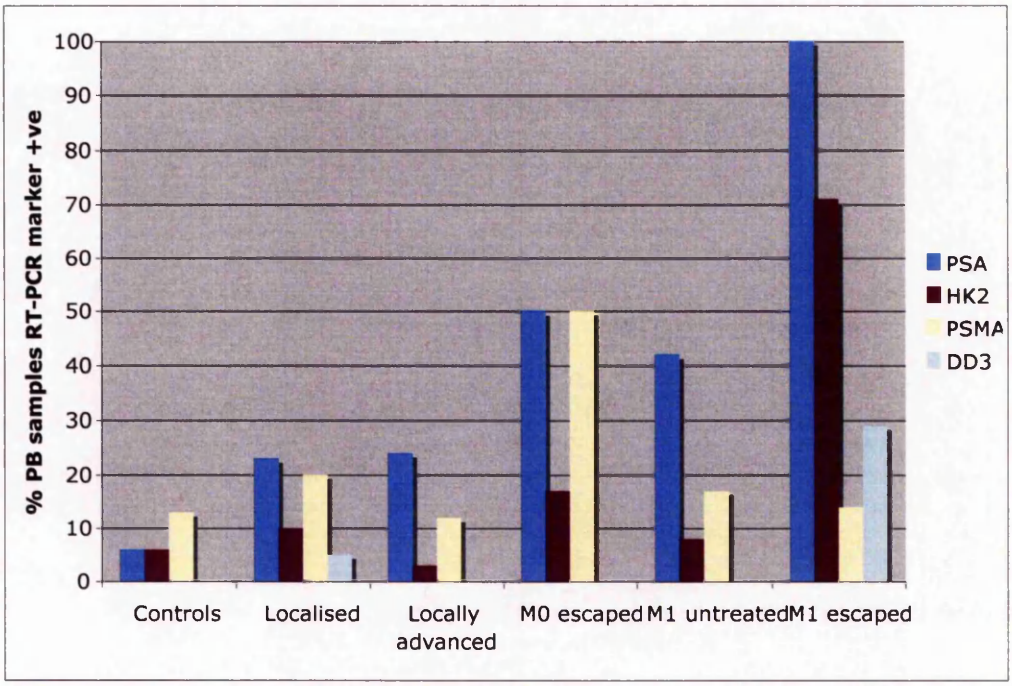
The detection of each marker has been described individually in some detail. It was also important to establish an overview of how their expression appeared together across the study population. This is illustrated in figures 4.18 and 4.19 for PB and BM respectively. Such patterns or profiles of marker expression for distinct clinical groups would subsequently be lost by multi-marker combination.

In PB, PSA was the most widely expressed marker (figure 4.18). PSA, PSMA and HK2 all followed similar patterns of expression with marker expressing PECs

generally being more commonly detected with increasing disease stage. Hormone escaped status inferred a higher probability of marker positivity and also higher levels of expression than hormone naïve metastases for all markers other than $DD3^{PCA3}$. Marker expression in untreated localised and locally advanced cancers was very similar with no clear differentiation between the two, while compared to advanced disease, particularly hormone escaped cancers, marker detection was less common and of a lesser magnitude. The most advanced cancer group, hormone escaped metastases, was identified by all markers in terms of frequency of detection and expression other than PSMA.

The patterns in BM were slightly different to those seen in PB although the distinction between localised/locally advanced CaP and advanced disease status remained clear (figure 4.19). PSA was again the most commonly detected marker with the highest expression levels in all groups. The presence of bone metastases appeared to have a greater influence on marker detection and expression levels than was seen in PB. Hormone escaped cancers again commonly produced circulating tumour cells although in the non-metastatic group PSA and PSMA mean expression levels were far lower than with metastases and HK2 positive cells were not seen. In advanced patients $DD3^{PCA3}$ was only seen in the presence of metastases although these were all untreated rather than the hormone escaped individuals seen in PB. The highest mean expression levels for PSA, PSMA and HK2 were seen with hormone escaped metastatic disease.

A)




B)

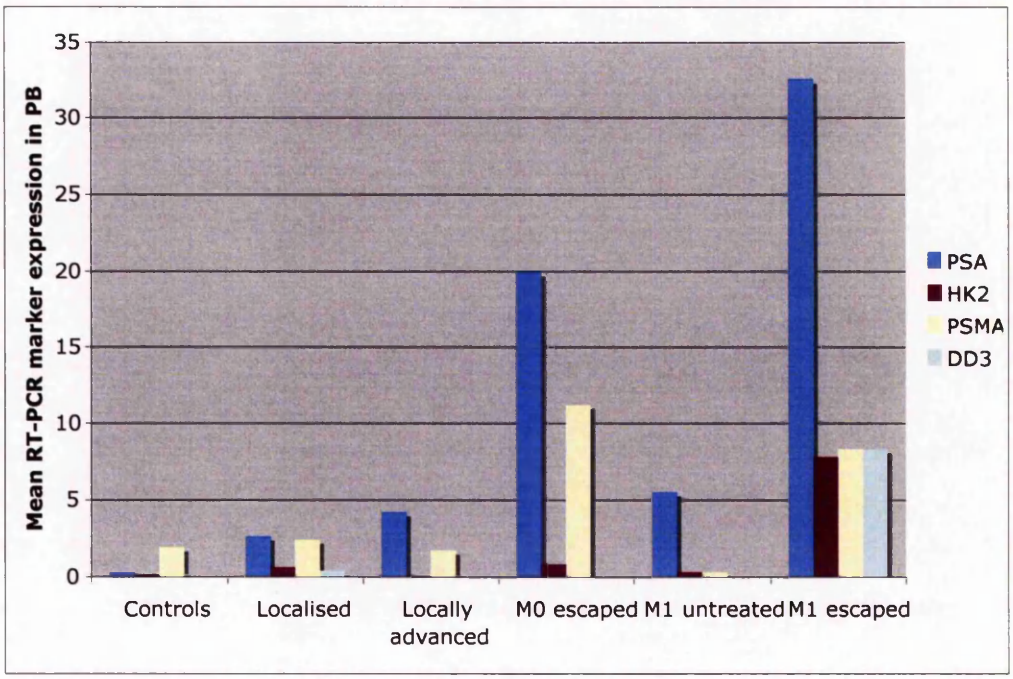
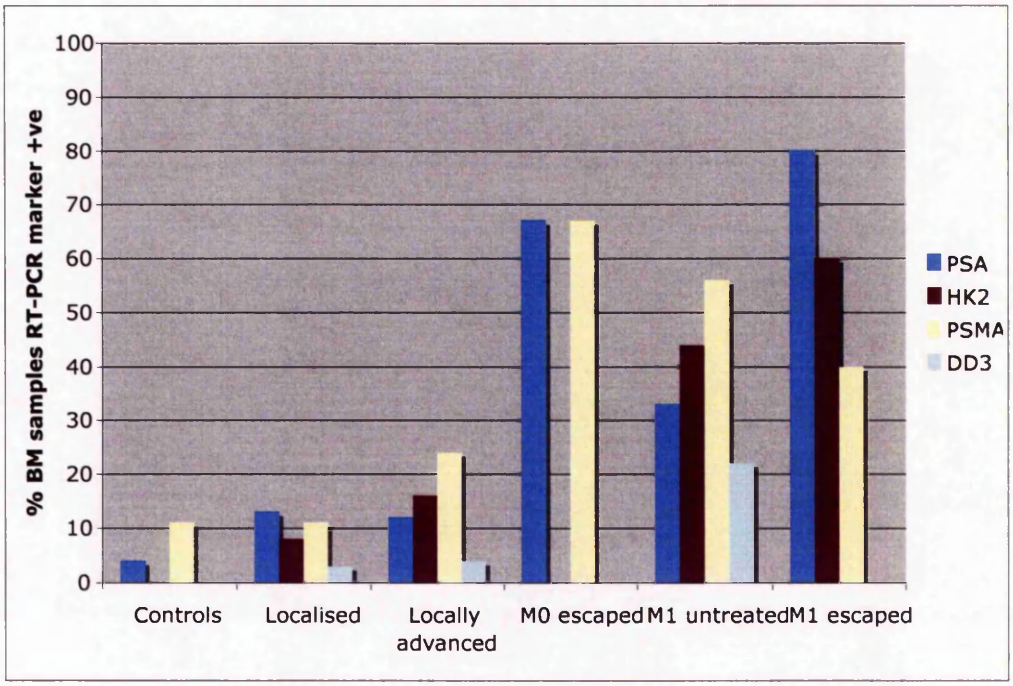



**Figure 4.18:** PB RT-PCR - PSA, PSMA, HK2 & DD3$^{PCA3}$. A) Bar chart showing the percentage of PB samples from controls and each clinical group that were RT-PCR positive for each of the four markers. B) Bar chart showing the mean expression of each of the four markers in each clinical group as determined by quantitative RT-PCR.

A)




B)

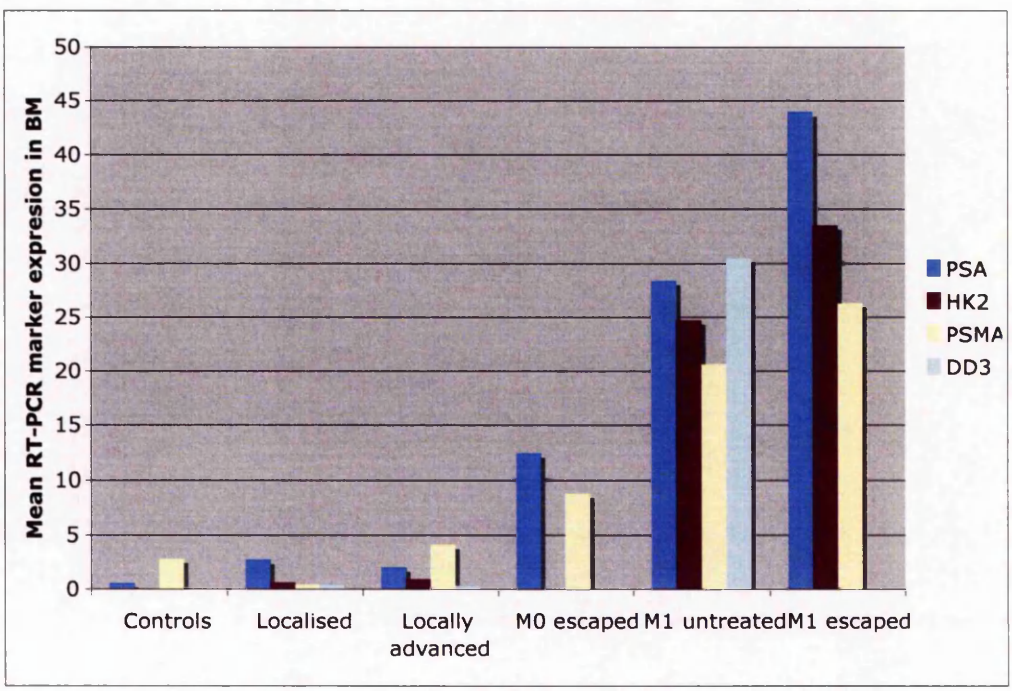



**Figure 4.19:** BM RT-PCR - PSA, PSMA, HK2 & DD3$^{PCA3}$. A) Bar chart showing the percentage of BM samples from controls and each clinical group that were RT-PCR positive for each of the four markers. B) Bar chart showing the mean expression of each of the four markers in each clinical group as determined by quantitative RT-PCR.

### 4.2.5 Combining PB and BM RT-PCR

When combined PB and BM data were considered, 15 patients were excluded as they had PB samples only. Additionally there was no paired control population.

For PSA, more samples were positive in PB than in BM for all groups other than non-metastatic hormone escaped disease. If positivity in either PB or BM was considered significant then PEC detection increased in all groups other than those with hormone escaped cancers. Few patients were assay positive in both PB and BM and this reduced the positivity rate for all groups although the impact was again less marked for men with hormone escaped cancer where PSA positive CTC detection rates were generally high (table 4.19 and figure 4.20a).

Combining mean PSA expression for both PB and BM showed a trend of increasing expression with increasing clinical stage. This cancelled out the opposing expression patterns seen for advanced patients when PB and BM were considered separately and overall expression levels for men with non-metastatic hormone escaped and untreated metastases were similar (table 4.20 and figure 4.20b).

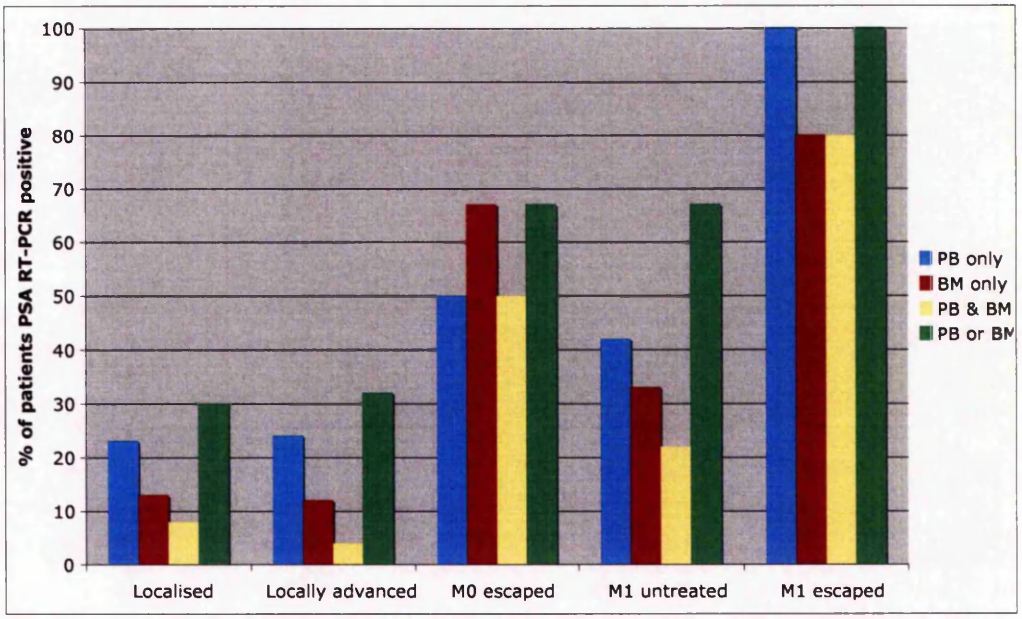
| | % of Patients PSA RT-PCR Positive | | | |
|---|---|---|---|---|
| | $PB^+$ | $BM^+$ | $PB^+$ & $PB^+$ | $PB^+$ or $BM^+$ |
| Localised | 23 | 13 | 8 | 30 |
| Locally Advanced | 24 | 12 | 4 | 32 |
| Mo Escaped | 50 | 67 | 50 | 67 |
| M1 Untreated | 42 | 33 | 22 | 67 |
| M1 Escaped | 100 | 80 | 80 | 100 |

**Table 4.19:** PSA RT-PCR Assay - Percentage of patients that were assay positive in PB or BM alone or in combination.

| Clinical Group | Combined PB & BM Mean PSA Expression |
|---|---|
| Localised | 5.6 |
| Locally Advanced | 6.4 |
| Mo Escaped | 32.3 |
| M1 Untreated | 35.7 |
| M1 Escaped | 74.9 |

**Table 4.20:** PSA RT-PCR Assay – mean PSA mRNA expression in each clinical group using combined PB and BM data.

A)




B)

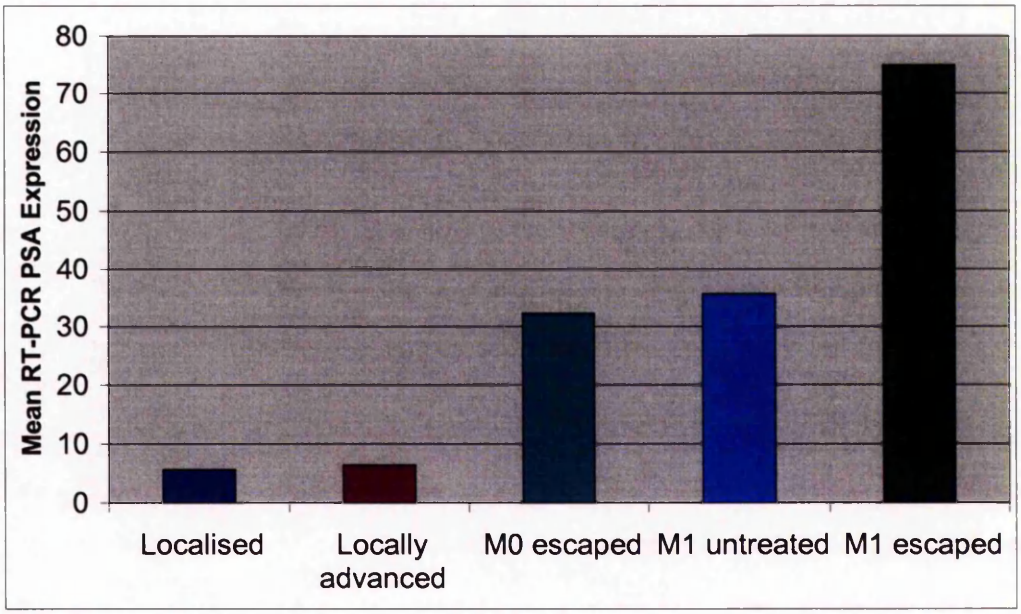



**Figure 4.20:** Combining PB and BM for PSA RT-PCR. A) Bar chart showing the percentage of PB or BM alone and in combination that were PSA RT-PCR positive for the 85 men with paired samples. B) Bar chart showing the mean PSA expression using combined PB and BM data for each clinical group.

For PSMA BM samples were more often positive than PB for all groups other than localised. Combining the PB and BM data generally improved yield while smaller numbers of patients were marker positive in both PB and BM. The patterns of marker detection between groups was maintained with the highest detection in men with non-metastatic hormone escaped disease (table 4.21 and figure 4.21a). Mean marker expression increased with increasing disease stage as it did for BM alone (table 4.22 and figure 4.21b). The expression pattern seen in PB alone of low expression in untreated metastatses was not evident using combined data.

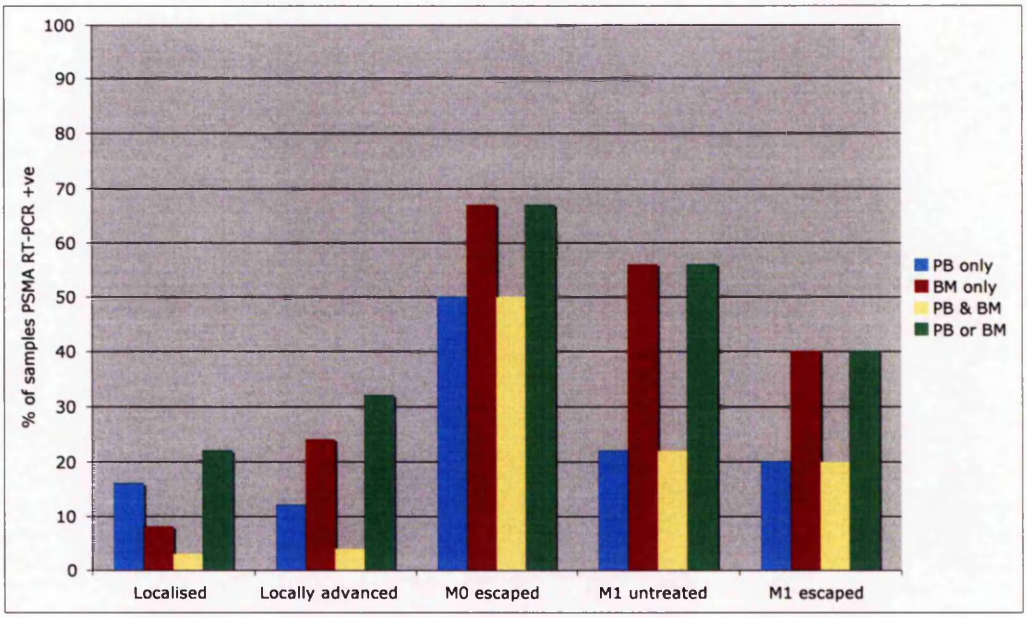
| | % of Patients PSMA RT-PCR Positive | | | |
|---|---|---|---|---|
| | $PB^+$ | $BM^+$ | $PB^+$ & $PB^+$ | $PB^+$ or $BM^+$ |
| Localised | 16 | 8 | 3 | 22 |
| Locally Advanced | 12 | 24 | 4 | 32 |
| Mo Escaped | 50 | 67 | 50 | 67 |
| M1 Untreated | 22 | 56 | 22 | 56 |
| M1 Escaped | 20 | 40 | 20 | 40 |

**Table 4.21:** PSMA RT-PCR Assay - Percentage of patients that were assay positive in PB or BM alone or in combination.

| Clinical Group | Combined PB & BM Mean PSMA Expression |
|---|---|
| Localised | 2.2 |
| Locally Advanced | 6.1 |
| Mo Escaped | 6.1 |
| M1 Untreated | 21 |
| M1 Escaped | 38 |

**Table 4.22:** PSMA RT-PCR Assay – mean PSMA mRNA expression in each clinical group using combined PB and BM data.

A)




B)

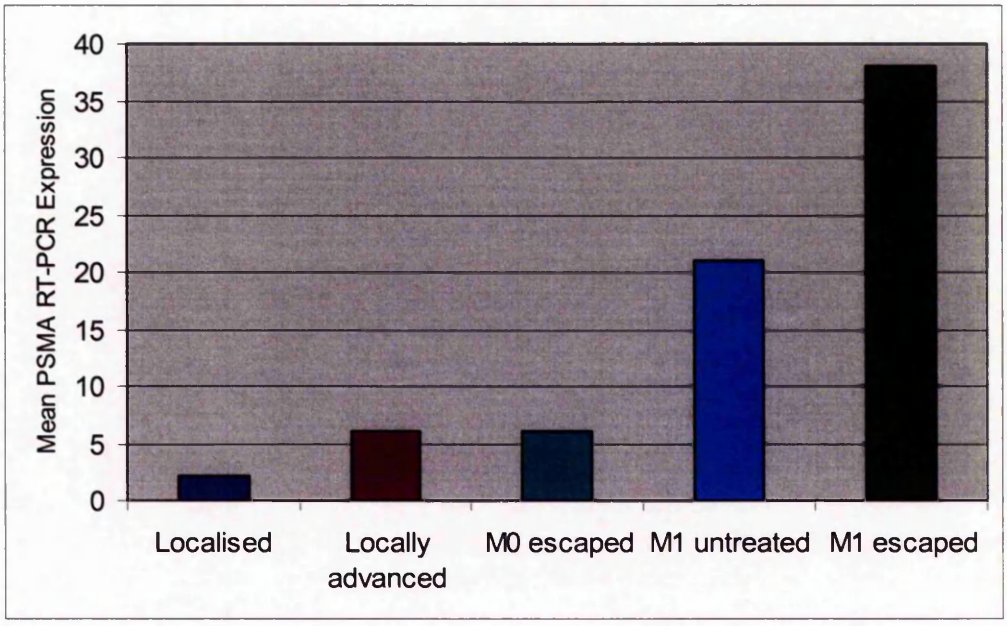
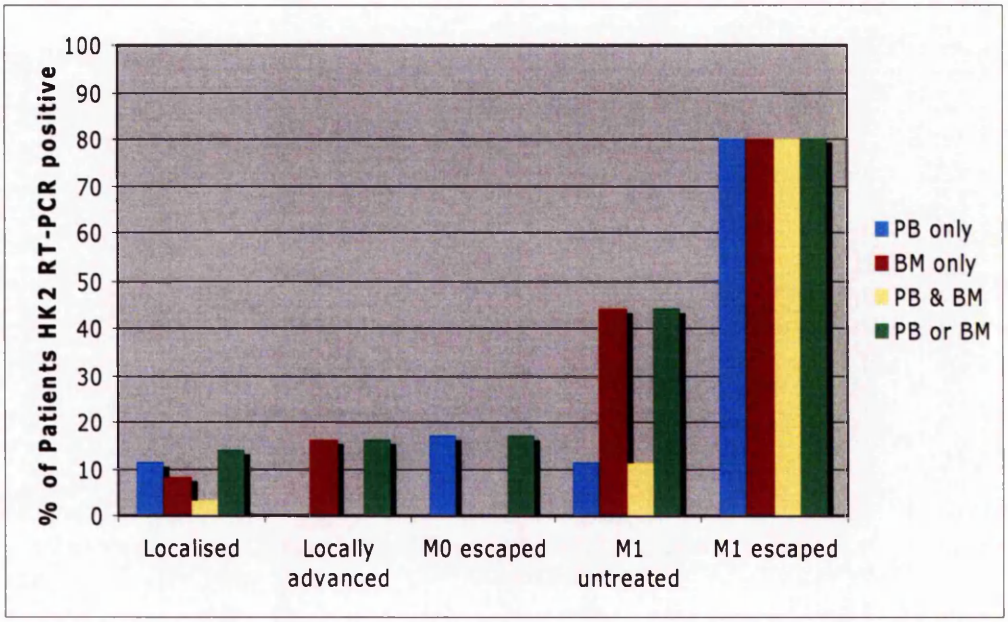



**Figure 4.21:** Combining PB and BM for PSMA RT-PCR. A) Bar chart showing the percentage of PB or BM alone and in combination that were PSMA RT-PCR positive for the 85 men with paired samples. B) Bar chart showing the mean PSMA expression using combined PB and BM data for each clinical group.

The majority of HK2 positive samples were from men with established bone metastases. For those with hormone escaped metastases 80% were HK2 positive in both PB and BM while the remaining 20% were negative in both. For those with untreated metastases 44% were BM positive and compared with 11% of PB samples all of which were also BM positive. Therefore combining PB and BM had no impact on overall yield, while $PB^{+}BM^{+}$ status decreased yield in the untreated group. PB was more commonly HK2 positive for localised and non-metastatic hormone escaped patients while HK2 positive BM samples were more common in locally advanced CaP. Combining PB and BM for these groups had little impact on mRNA yield (table 4.23 and figure 4.22a). Mean combined HK2 expression was high in all metastatic disease but low for all other patients, as it was in BM alone (table 4.24 and figure 4.22b).

Only 7 of the 85 men with paired samples were $DD3^{PCA3}$ positive and with such small numbers spread between the clinical groups, combining the PB and BM data made no significance to the utility of this marker. The data has been included in the multi-marker evaluation.

A)




B)

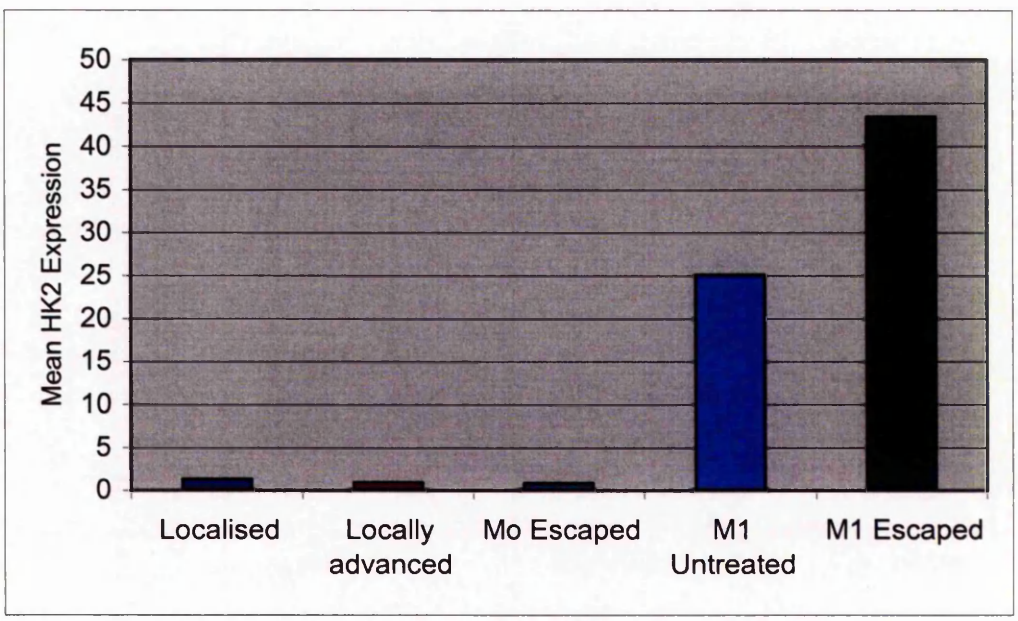



**Figure 4.22:** Combining PB and BM for HK2 RT-PCR. A) Percentage of PB or BM alone and in combination that were HK2 RT-PCR positive For the 85 men with paired samples. B) Mean HK2 expression using combined PB and BM data for each clinical group.

| | % of Patients HK2 RT-PCR Positive | | | |
|---|---|---|---|---|
| | $PB^+$ | $BM^+$ | $PB^+$ & $PB^+$ | $PB^+$ or $BM^+$ |
| Localised | 11 | 8 | 3 | 14 |
| Locally Advanced | 0 | 16 | 0 | 16 |
| Mo Escaped | 17 | 0 | 0 | 17 |
| M1 Untreated | 11 | 44 | 11 | 44 |
| M1 Escaped | 80 | 80 | 80 | 80 |

**Table 4.23:** HK2 RT-PCR assay – the impact of combining PB and BM data.

| Clinical Group | Combined PB & BM Mean HK2 Expression |
|---|---|
| Localised | 1.2 |
| Locally Advanced | 0.9 |
| Mo Escaped | 0.8 |
| M1 Untreated | 25 |
| M1 Escaped | 43 |

**Table 4.24:** HK2 RT-PCR Assay – mean HK2 mRNA expression in each clinical group using combined PB and BM data.

### 4.2.6 Multiple Marker RT-PCR

#### 4.2.6.1 PB Multiple Marker RT-PCR

Data for the four potentially useful markers PSA, PSMA, HK2 and $DD3^{PCA3}$ were utilised in combination by considering a positive assay result for any marker being indicative of CTCs. This approach increased mRNA detection compared to PSA alone from 23% to 35% for localised disease, 24% to 39% for locally advanced and 42% to 50% for untreated metastases. All men with hormone escaped CaP who were positive for any marker were also PSA positive, therefore the multi-marker approach had no impact on mRNA detection. Unfortunately this combined marker approach had a deleterious effect on false positive results with 24% of

control samples being assay positive for at least one of the four markers (table 4.25 and figure 4.23a).

Mean combined marker expression was higher in all patient groups compared to controls. Expression was the same for localised, locally advanced and untreated metastases. Both hormone escaped groups demonstrated high marker expression levels, maintaining the patterns seen with the individual markers of low expression levels in untreated metastases (table 4.26 and figure 4.23b).

#### 4.2.6.2 BM Multiple Marker RT-PCR

Combining the PSA, PSMA, HK2 and $DD3^{PCA3}$ assays for BM increased the false positive rate for controls to 14%. Compared to PSA alone there was no change for the most advanced clinical group as any patient who was positive for PSMA, HK2 or $DD3^{PCA3}$ was also PSA positive. PEC detection however increased compared to the best individual marker for all of the other groups, localised 13% to 29%, locally advanced 24% to 40%, Mo escaped 67% to 83% and untreated metastases 56% to 67% (table 4.27 and figure 4.24a). Mean marker combined marker expression was lowest in controls and increased sequentially with clinically determined disease load, from 4.1 for localised cancer to 104 in men with hormone escaped metastases (table 4.28 and figure 4.24b).

#### 4.2.6.3 Paired PB and BM Multiple Marker RT-PCR

When PSA, PSMA, HK2 and $DD3^{PCA3}$ were considered together for the 85 men with paired samples, 36 PB and 36 BM samples were positive for at least one of these markers while 49 men were marker positive in either PB or BM and 23 were positive in both. PB samples were more often marker positive than BM in localised and hormone escaped metastatic cancer while the opposite was found for the other groups. Considering either $PB^{+}$ or $BM^{+}$ as an overall positive result improved detection rates in the clinical groups with lower individual yield using PB or BM alone, while smaller numbers of patients were positive in both BM and PB in all groups (table 4.29 and figure 4.25a). Overall there was a trend of increasing marker detection with advancing disease status and mean combined PB and BM

multi-marker expression increased with increasing clinical disease status (table 4.30 and figure 4.25b).

| | Percentage of PB Samples Marker Positive | | | | |
|---|---|---|---|---|---|
| | PSA | PSMA | HK2 | $DD3^{PCA3}$ | **Any Marker** |
| Controls | 6 | 13 | 6 | 0 | **24** |
| Localised | 23 | 20 | 10 | 5 | **35** |
| Locally advanced | 24 | 12 | 3 | 0 | **39** |
| Mo Escaped | 50 | 50 | 17 | 0 | **50** |
| M1 Untreated | 42 | 17 | 8 | 0 | **50** |
| M1 Escaped | 100 | 14 | 71 | 29 | **100** |

**Table 4.25: PB multi-marker RT-PCR** – Percentage of controls and men in each clinical group that were RT-PCR for each of the markers PSA, PSMA, HK2 and $DD3^{PCA3}$ individually and in combination.

| Clinical Group | PB Mean Combined Marker Expression |
|---|---|
| Controls | 2 |
| Localised | 6 |
| Locally advanced | 6 |
| Mo Escaped | 34 |
| M1 Untreated | 6 |
| M1 Escaped | 63 |

**Table 4.26: Multi-marker RT-PCR in PB** – mean combined expression for the markers PSA, PSMA, HK2 and $DD3^{PCA3}$ for controls and each clinical group in PB.

| | Percentage of BM Samples Marker Positive | | | | |
|---|---|---|---|---|---|
| | PSA | PSMA | HK2 | $DD3^{PCA3}$ | **Any Marker** |
| Controls | 4 | 11 | 0 | 0 | **14** |
| Localised | 13 | 11 | 8 | 3 | **29** |
| Locally advanced | 12 | 24 | 16 | 4 | **40** |
| Mo Escaped | 67 | 67 | 0 | 0 | **83** |
| M1 Untreated | 33 | 56 | 44 | 22 | **67** |
| M1 Escaped | 80 | 40 | 60 | 0 | **80** |

**Table 4.27: BM multi-marker RT-PCR** – Percentage of controls and men in each clinical group that were RT-PCR for each of the markers PSA, PSMA, HK2 and $DD3^{PCA3}$ individually and in combination.

| Clinical Group | BM Mean Combined Marker Expression |
|---|---|
| Controls | 3.3 |
| Localised | 4.1 |
| Locally advanced | 7.2 |
| Mo Escaped | 21 |
| M1 Untreated | 91 |
| M1 Escaped | 104 |

**Table 4.28: Multi-marker RT-PCR in BM** – mean combined expression for the markers PSA, PSMA, HK2 and $DD3^{PCA3}$ for controls and each clinical group in BM.

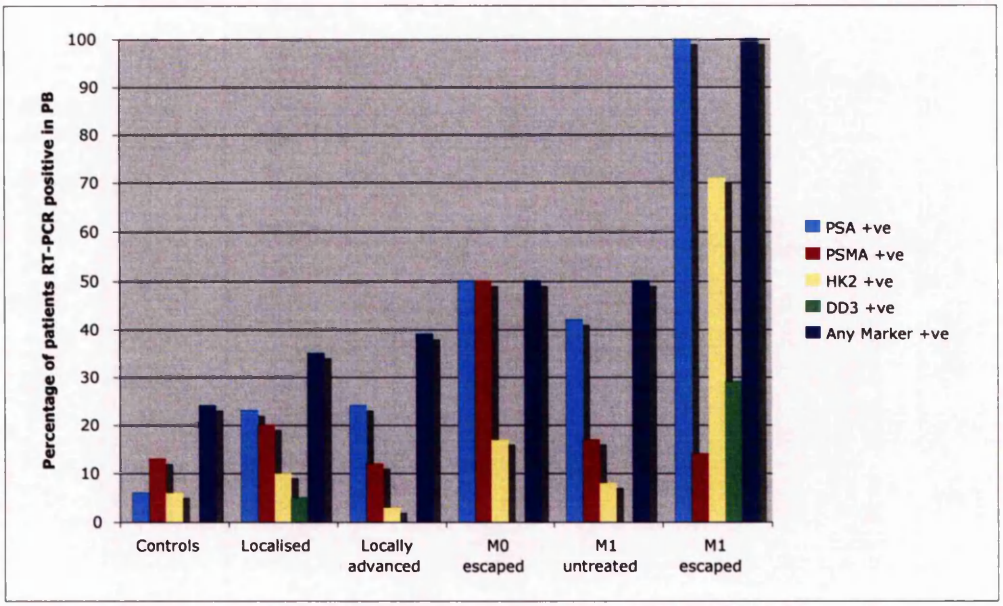
| | Percentage of Patients RT-PCR Positive | | | |
|---|---|---|---|---|
| | $PB^+$ | $BM^+$ | $PB^+$ & $PB^+$ | $PB^+$ or $BM^+$ |
| Localised | 32 | 27 | 16 | 43 |
| Locally Advanced | 36 | 40 | 20 | 56 |
| Mo Escaped | 50 | 83 | 50 | 83 |
| M1 Untreated | 67 | 67 | 56 | 78 |
| M1 Escaped | 100 | 80 | 80 | 100 |

**Table 4.29:** Combined PSA, PSMA, HK2 and $DD3^{PCA3}$ assays – the impact of combining PB and BM data.

| Clinical Group | Combined PB & BM Mean Marker Expression |
|---|---|
| Localised | 9.7 |
| Locally Advanced | 13 |
| Mo Escaped | 56 |
| M1 Untreated | 99 |
| M1 Escaped | 176 |

**Table 4.30:** Multi-Marker RT-PCR for PSA, PSMA, HK2 and $DD3^{PCA3}$ – mean marker mRNA expression in each clinical group using combined PB and BM data.

A)




B)

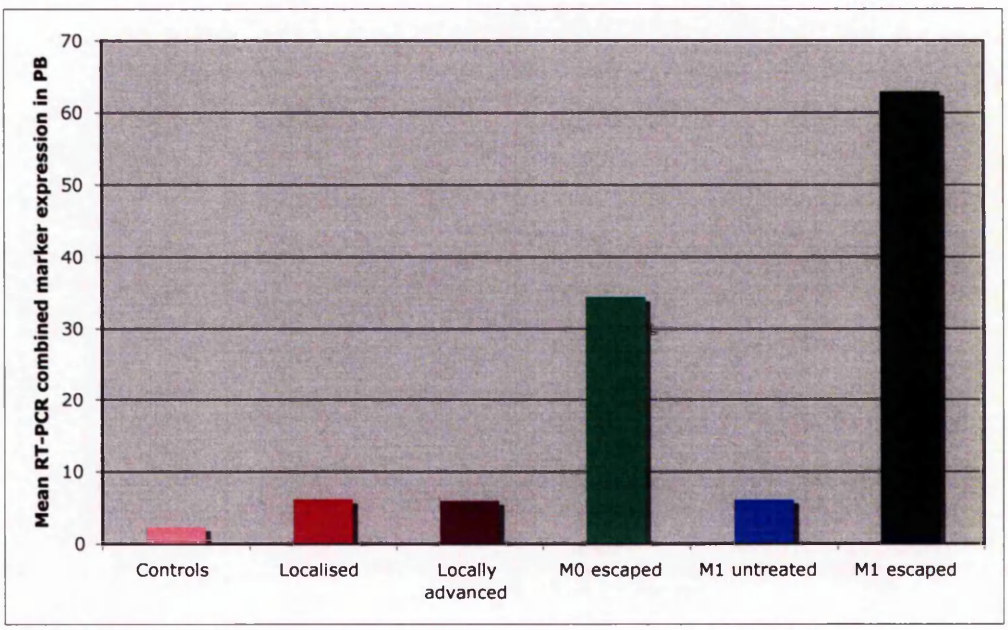
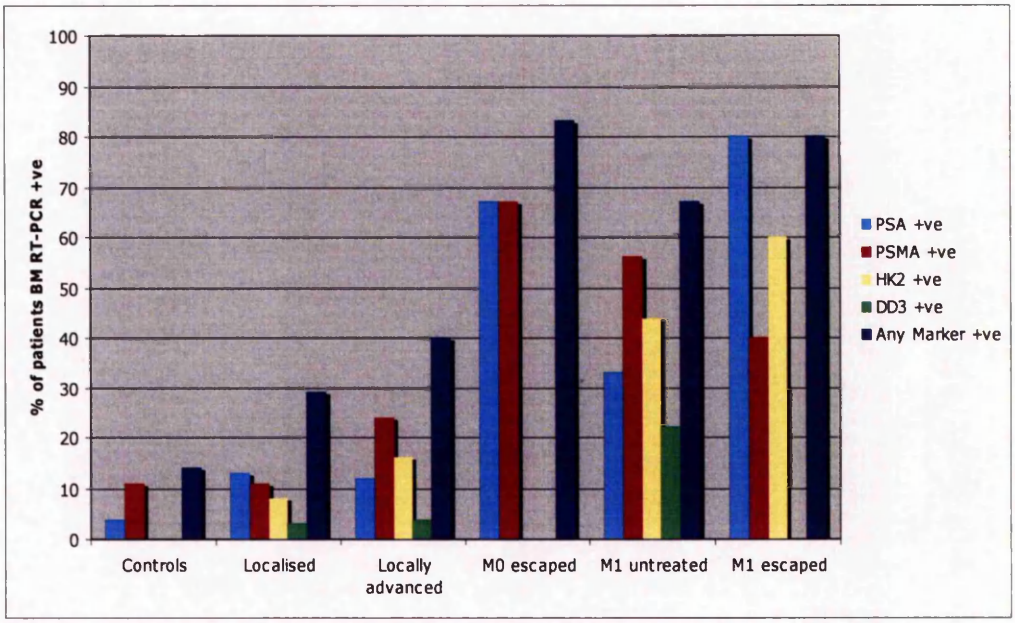



**Figure 4.23:** Multiple marker RT-PCR in PB for PSA, PSMA HK2 and $DD3^{PCA3}$. A) Bar chart showing the percentage of controls and patients in each clinical group that were RT-PCR positive in PB for each of the markers individually and in combination. B) Bar chart showing the mean combined marker mRNA expression in PB for controls and clinical groups.

A)




B)

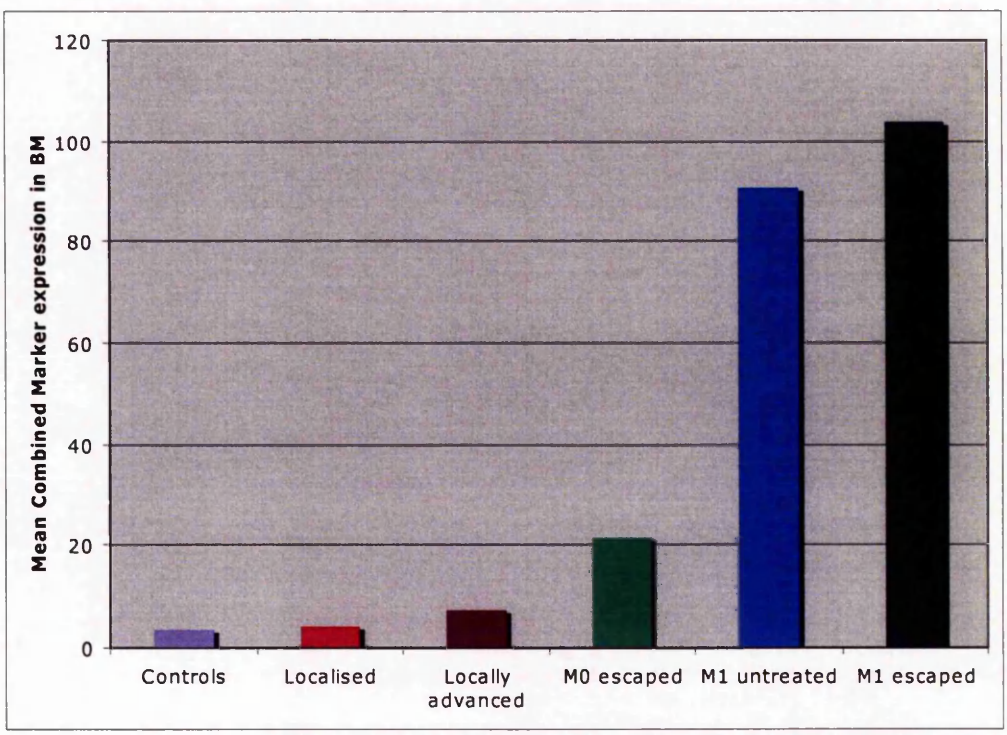
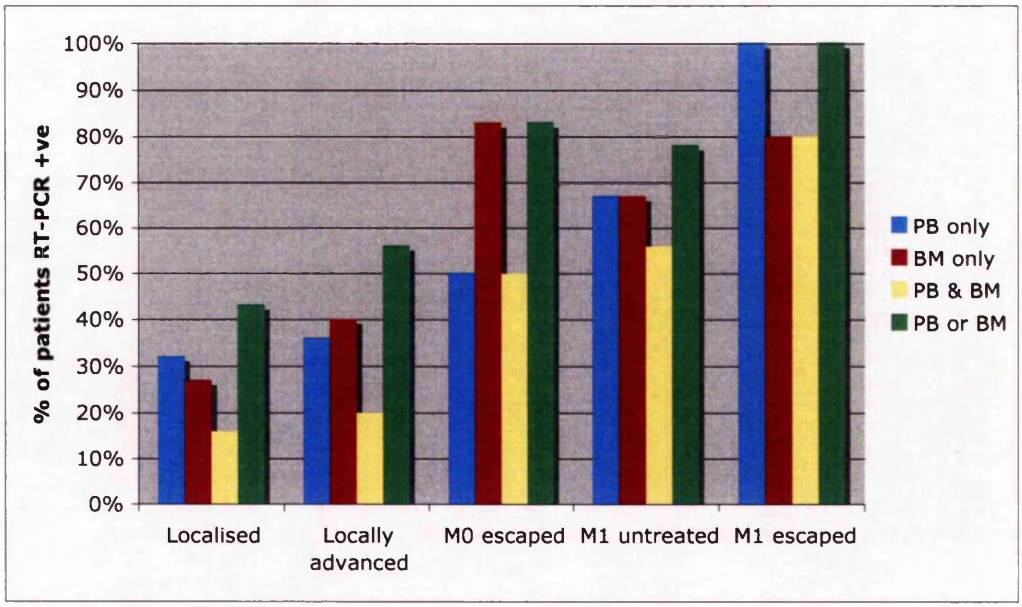



**Figure 4.24:** Multiple marker RT-PCR in BM for PSA, PSMA HK2 and DD3$^{PCA3}$. A) Bar chart showing the percentage of controls and patients in each clinical group that were RT-PCR positive in BM for each of the markers individually and in combination. B) Bar chart showing the mean combined marker mRNA expression in controls and clinical groups.

A)




B)

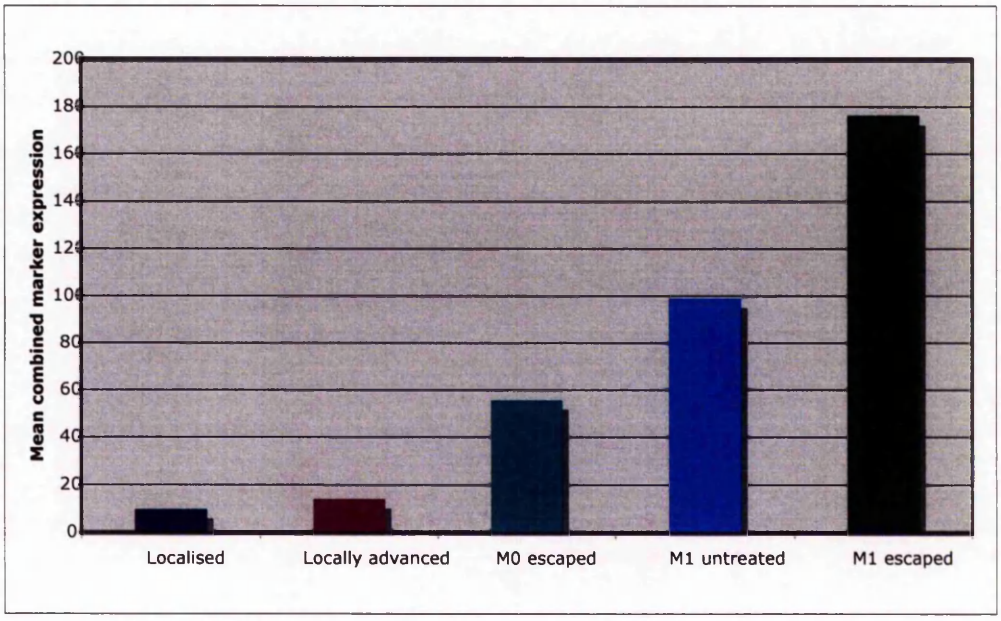



**Figure 4.25:** Combining PB and BM for multiple marker RT-PCR (PSA, PSMA, HK2 & $DD3^{PCA3}$). A) Bar chart showing the percentage of PB and BM samples that were positive individually or in combination for any of the four markers. B) Bar chart showing the mean combined PB and BM multi marker expression for each clinical group

## Chapter 5: Micro Fluidic RT-PCR Assay Results

### 5.1 Micro Fluidic Card Validation Using LNCaP Cell Line

During the period of research, ABI introduced custom made microfluidic cards, allowing the detection of up to 48 genes concurrently from a single patient sample. We therefore evaluated the sensitivity and specificity of this RT-PCR format and compared it with our own assays using the 96 well methodology.

Using 100ng of cDNA derived form LNCaP cells as target for the micro fluidic RT-PCR assay (method 2.5.2) both of the housekeeping genes GAPDH and β-actin were successfully amplified, confirming the integrity of the cDNA. The markers evaluated using the 96 well RT-PCR assay; PSA, PSMA, PSCA and HK2 were amplified using the micro fluidic assay with mean Ct values of 21.4, 23.1, 28.7 and 24.6 cycles respectively (figure 5.1). These Ct values compare with 19.5, 21.0, 29.6 and 22 cycles using the 96 well assay design (table 5.1).

Additionally, the micro fluidic assay was also used to assess the LNCaP derived cDNA for the expression of HK4, HK15, EZH2, prostasin, hepsin and u-PA. Positive assay results were obtained for all of these markers other than u-PA (figure 5.2). The Ct values for these markers were HK4 24.9 cycles, HK15 30.4, EZH2 25.2, prostasin 25.9 and hepsin 26.4.

RNA/DNA free water control reactions run simultaneously on the same microfluidic card were all negative, excluding any contamination of the assay components. Despite the exon-exon boundary assay design, a minus RT control assay was also run to ensure no genomic DNA contamination. This gave negative results for all markers in all replicate wells.

| Marker | Micro Fluidic Ct Value (cycles) | 96 Well Ct Value (cycles) |
|---|---|---|
| PSA | 21.4 | 19.5 |
| PSMA | 23.1 | 21.0 |
| PSCA | 28.7 | 29.6 |
| HK2 | 24.6 | 22.0 |

**Table 5.1:** Comparison of Ct values for the expression of PSA, PSMA, PSCA and HK2 in 100ng of cDNA derived from $10^6$ LNCaP cells obtained using micro fluidic and 96 well RT-PCR.

Ten fold dilutions containing 100ng, 10ng, 1ng and 0.1ng of cDNA derived from $10^6$ LNCaP cells were evaluated using the micro fluidic RT-PCR assay. The 2 housekeeping genes GAPDH and β-actin were detected using all of the amounts of cDNA. PSA, PSMA, HK2, HK4, EZH2, prostasin and hepsin were detected in all target dilutions. The mean Ct values for each marker using each dilution of cDNA are displayed in figure 5.3. The mean Ct values increase for each marker with decreasing amounts of cDNA target. PSCA and HK15 were detected in all but the assay using 0.1ng of input target cDNA. As the amounts of input LNCaP cDNA were decreased there was greater variability in the Ct value for same marker replicates, with over 3 cycle variability observed between replicates at 0.1ng (figure 5.4). Since 100ng of input cDNA gave good reproducibility between assay replicates as well as the lowest Ct values this was utilised for the subsequent assay development experiments.

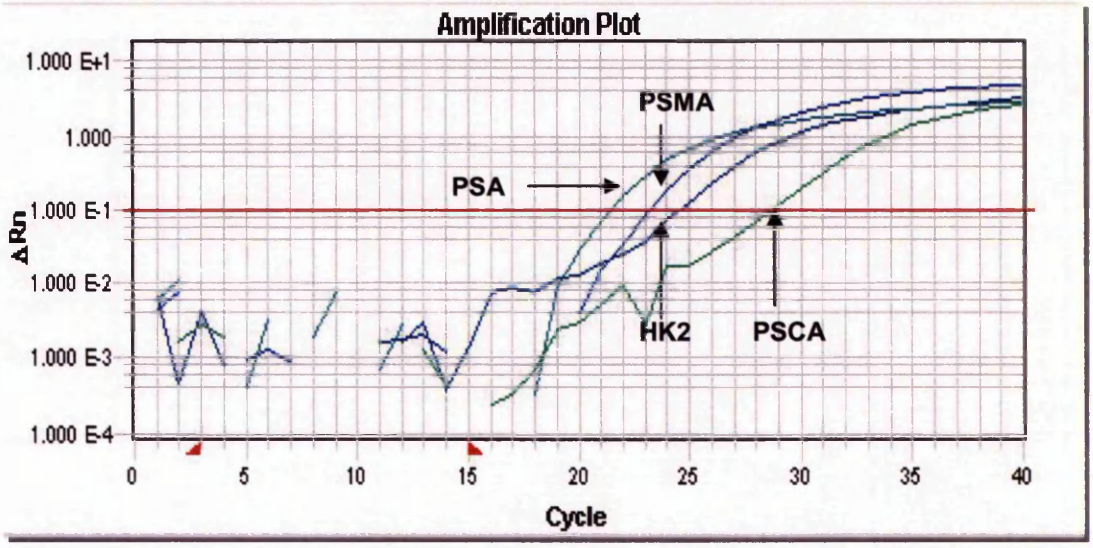



**Figure 5.1:** Microfluidic RT-PCR amplification plots displaying the relative expression of PSA, PSMA, PSCA and HK2 in 100ng of LNCaP derived cDNA. The Ct values for each marker were PSA 21.4 cycles, PSMA 23.1 cycles, PSCA 28.7 cycles and HK2 24.6 cycles.

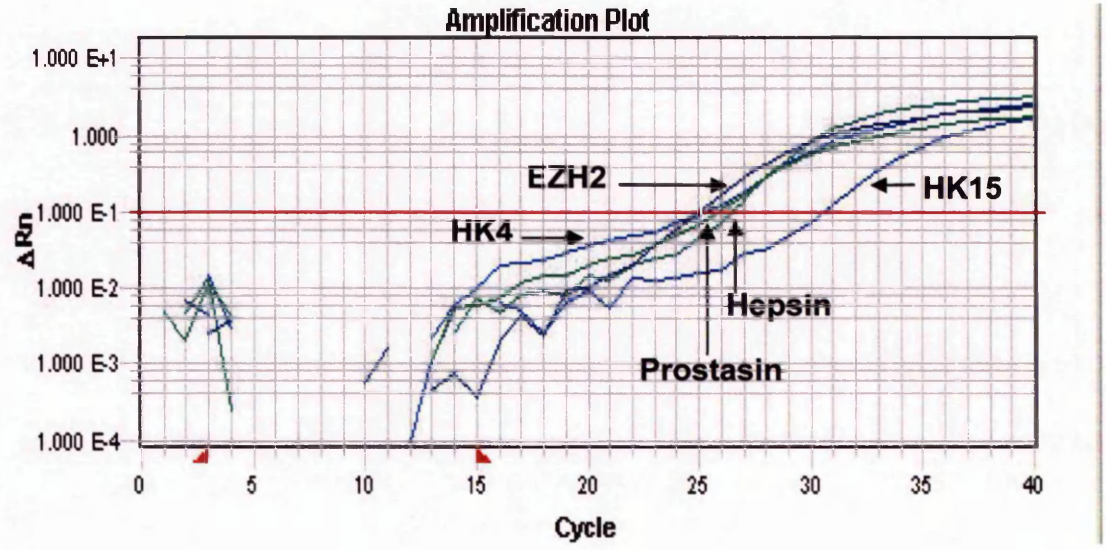



**Figure 5.2:** Micro fluidic RT-PCR amplification plots displaying the relative expression of the markers EZH2, Prostasin, Hepsin, HK4 and HK15 in 100ng of LNCaP derived cDNA. The Ct values for each marker were HK4 24.9 cycles, EZH2 25.2 cycles, Prostasin 25.9 cycles, Hepsin 26.4 cycles and HK15 30.4 cycles.

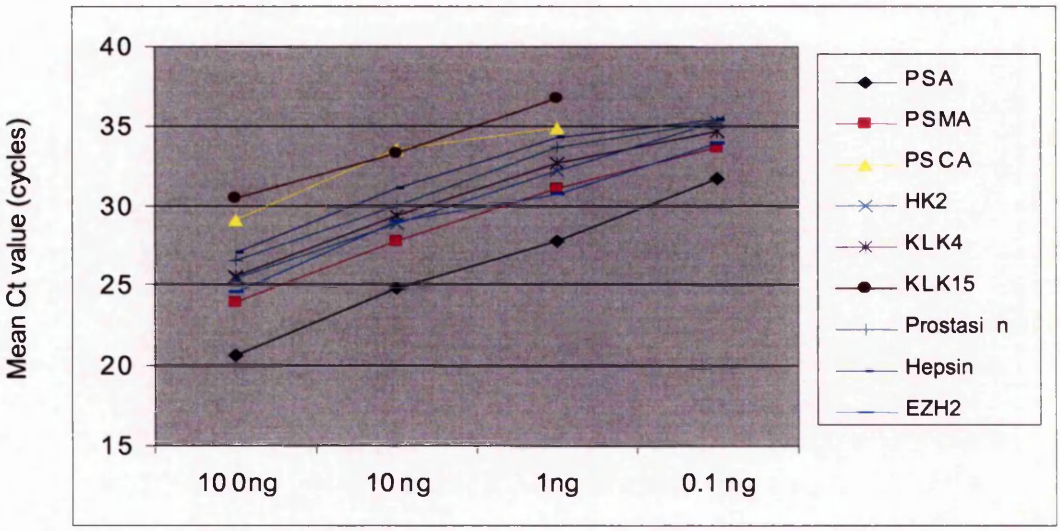



**Figure 5.3:** Micro fluidic RT-PCR - mean Ct value for each marker detected in serial dilutions of cDNA derived from $10^6$ LNCaP cells. Higher amounts of input target are detected sooner by RT-PCR as shown by lower Ct values. There is a linear relationship between the concentration of cDNA target and RT-PCR Ct value.

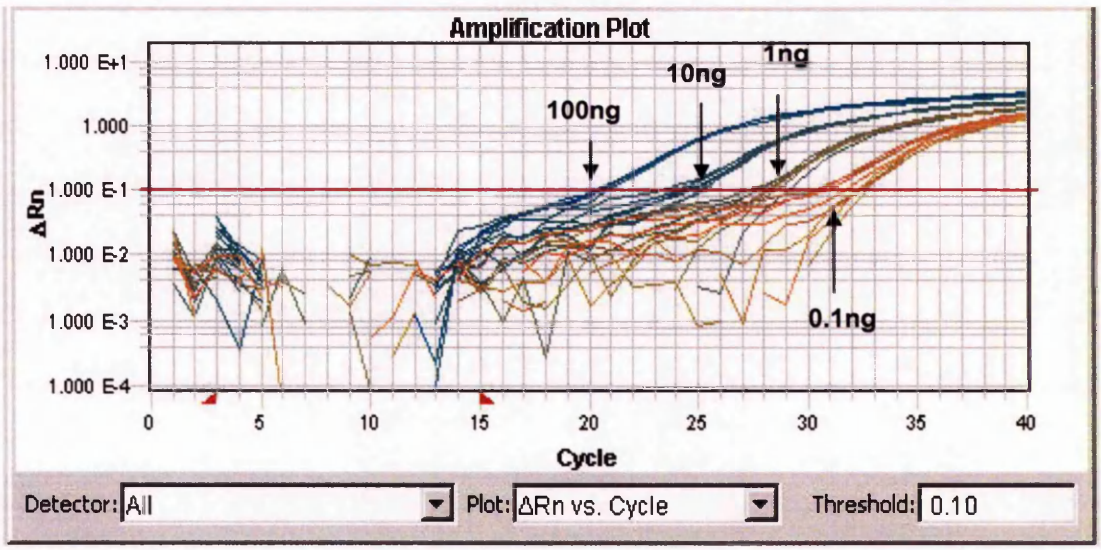



**Figure 5.4:** Micro fluidic RT-PCR amplification plots for PSA assay replicate reactions using decreasing amounts of input cDNA. With 100ng of cDNA the assay demonstrates good replicate reproducibility with only a small variation on Ct values, while with 0.1ng the Ct values may vary by almost 3 cycles.

## 5.2 Defining Micro Fluidic Card RT-PCR *in Vitro* Sensitivity

The potential of the micro fluidic cards for spiked sample analysis was determined by loading the cards with 100ng aliquots of cDNA derived from 2.5ml samples of female PB spiked with decreasing numbers of LNCaP cells ($10^6$ to 1 cell/ml of blood). The housekeeping genes GAPDH and β-actin were amplified in all samples. No prostate markers were detected in PB samples spiked with 1 or 10 LNCaP cells per 1ml PB. PSMA was detected at 36.7 cycles in one replicate assay and EZH2 at 32.1 cycles, again in only 1 in 4 replicates, of the $10^2$ LNCaP spike sample. PSA, PSMA, HK2, EZH2, prostasin and hepsin were detected in the $10^3$ cells per ml spike. In addition, PSCA, HK4 and HK15 were detected when $10^4$ LNCaP cells were present. All markers except uPA were detected in the $10^5$ and $10^6$ LNCaP cell spike PB samples (table 5.2).

| Marker | Assay Limit of Detection (LNCaP cells spiked /1ml female PB) |
|---|---|
| PSA | $10^3$ |
| PSMA | $10^2$ |
| PSCA | $10^4$ |
| HK2 | $10^3$ |
| HK4 | $10^4$ |
| HK15 | $10^4$ |
| EZH2 | $10^2$ |
| Prostasin | $10^3$ |
| Hepsin | $10^3$ |
| uPA | Not detected |

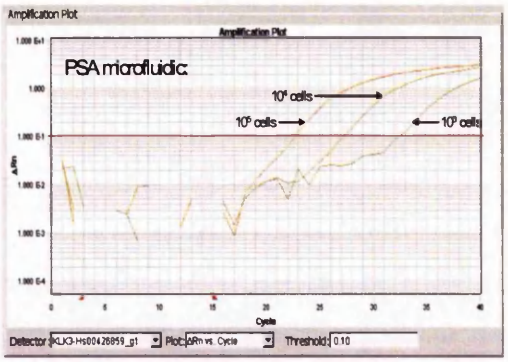
**Table 5.2:** *In vitro* sensitivity for all markers using micro fluidic RT-PCR. The limits of detection (LNCaP cell per 1ml of PB) of serial dilutions of LNCaP cells spiked into female PB for each of the 10 markers of interest specific assays is shown.

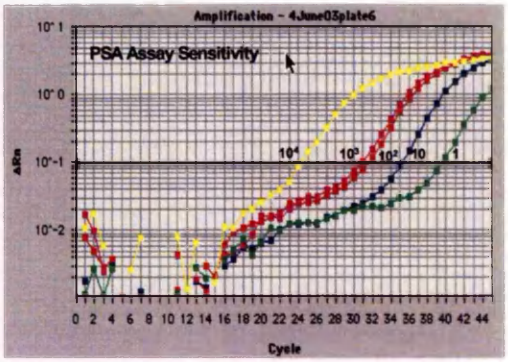
## 5.3 Comparison of *in Vitro* Sensitivity of Micro Fluidic and 96 Well RT-PCR Assays

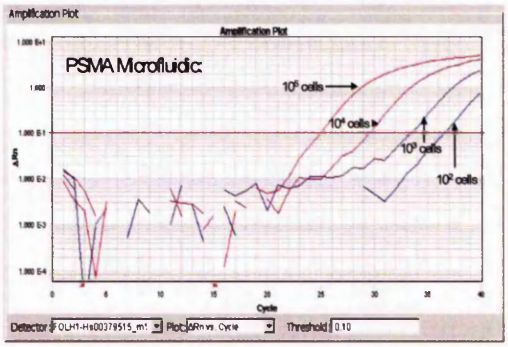
The limits of detection for PSA, PSMA, PSCA and HK2 using the micro fluidic RT-PCR assays are detailed in section 5.2, and for the 96 well format in section 3.7. Table 5.3 details the *in vitro* sensitivities using the two different methodologies. The 96 well PSA assay was 1000 fold more sensitive than the commercially produced micro fluidic PSA assay with limits of detection of 1 cell and $10^3$ cells respectively. For PSMA, HK2 and PSCA the 96 well format was more sensitive by a factor of 10. Using the 96 well assay, the detection limits were 10 cells for PSMA, $10^2$ cells for HK2 and $10^3$ cells for PSCA compared with $10^2$, $10^3$ and $10^4$ cells for the 3 markers respectively using micro fluidic RT-PCR. The comparable amplification plots are shown in figure 5.5.

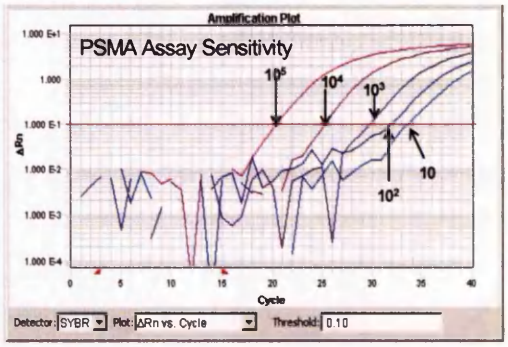
| Marker | Microfluidic Card Sensitivity (PECs/1ml female PB) | 96 Well Plate Sensitivity (PECs/1ml female PB) |
|---|---|---|
| PSA | $10^3$ cells | 1 cell |
| PSMA | $10^2$ cells | 10 cells |
| HK2 | $10^3$ cells | $10^2$ cells |
| PSCA | $10^4$ cells | $10^3$ cells |

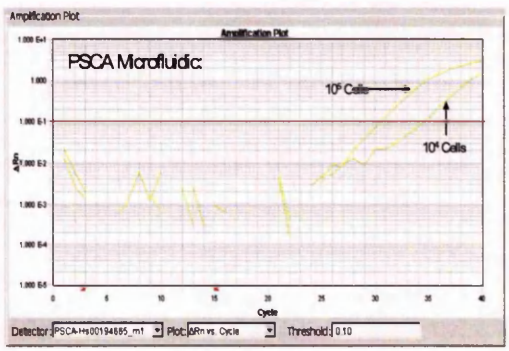
**Table 5.3:** Comparison of *in vitro* sensitivity for the four markers of interest using microfluidic card and 96 well plate formats. In testing their abilities to detect serial dilutions of LNCaP cells spikes into female PB, the microfluidic card is 10 fold less sensitive for the PSMA, HK2 and PSCA assays and 1000 fold less sensitive for PSA.

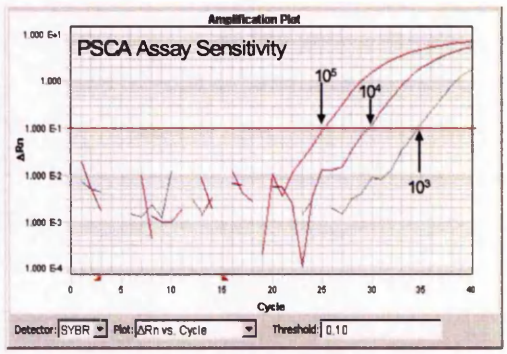


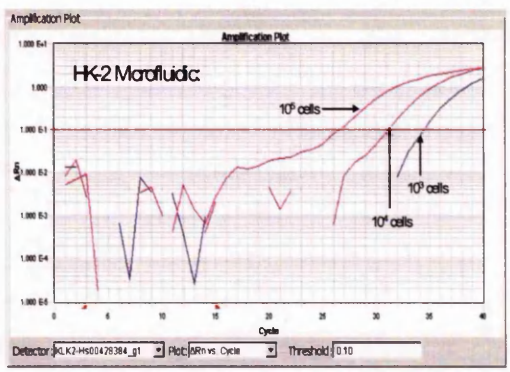



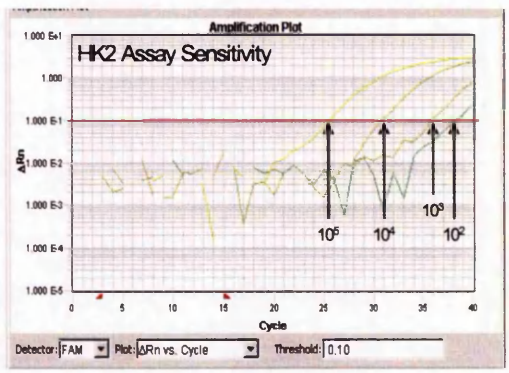




Micro Fluidic RT-PCR

96 Well RT-PCR

**Figure 5.5:** Comparing micro fluidic and 96 well RT-PCR assays. Amplification plots for cell spike experiments for the markers PSA, PSMA, PSCA and HK2 for both formats. The 96 well format is 100 fold more sensitive for PSA and 10 fold for PSMA, PSCA and HK2 than the micro fluidic assay.

### 5.4 Prostate Specificity of Markers Targeted by Micro Fluidic RT-PCR Assays

The prostate specificity of each marker assay was established by evaluating cDNA derived from 4 samples of female PB and BM. House keeping genes were successfully amplified for all samples. For PB, EZH2 was detected in 3 of the 4 control samples with a mean Ct of 32.6 cycles and uPA was detected in 2 of the 4 samples with a mean Ct of 33.9 cycles. All other marker assays were negative. In BM, EZH2 and uPA were positive in all controls with mean Ct values of 26 and 29.1 cycles respectively. The assays for PSMA, prostasin and hepsin were positive in 2 of the BM controls. The mean Ct values for these markers were PSMA 36.1 cycles, prostasin 34.4 cycles and hepsin 34.3 cycles.

### 5.5 Evaluation of Clinical Samples Using Micro Fluidic RT-PCR Assays

To evaluate the potential of micro fluidic cards as a multi marker assay, samples from 24 men with CaP, 20 paired PB and BM and 4 PB only, were evaluated using both the micro fluidic and 96 well RT-PCR formats. Seven patients had metastatic hormone escaped CaP, 7 untreated metastases, 6 hormone escaped cancer with a negative bone scan, 2 locally advanced and 2 clinically localised disease. The clinical staging details for these patients is shown in table 5.4. All of the comparative micro fluidic and 96 well RT-PCR data is displayed in appendix 4.

When BM samples were evaluated, more samples were RT-PCR marker positive using the 96 well format than the micro fluidic technique, for PSA, PSMA and PSCA. For PSA 55% and 35% of samples were RT-PCR positive for the 96 well and micro fluidic assays respectively, for PSMA 50% and 40% and for PSCA 35% compared with 10%. For HK2 35% of BM samples were RT-PCR positive using each method (table 5.5). For PSA and PSCA all samples which were micro fluidic assay positive were also positive using the 96 well assay. Three samples were PSMA assay positive and 1 sample HK2 positive solely using micro fluidic RT-PCR. For all of these samples only 1 of the 4 replicate reactions was marker positive.

| Sample ID | Clinical Group | Clinical Stage | Serum PSA | Gleason Score |
|---|---|---|---|---|
| 101 | Localised | T1c Nx Mo | 4.1 | 3+3=6 |
| 106 | Localised | T2b Nx Mo | 5.7 | 3+3=6 |
| 012 | Locally advanced | T3 N1 Mo | 9.2 | 3+4=7 |
| 071 | Locally advanced | T2b Nx Mo | 32.9 | 3+3=6 |
| 032 | Mo Escaped | T2b Nx Mo | 6.9 | 2+3=5 |
| 049 | Mo Escaped | T4 Nx Mo | 10 | 5+4=9 |
| 050 | Mo Escaped | T3 Nx Mo | 29 | 3+3=6 |
| 057 | Mo Escaped | T1b Nx Mo | 25 | 2+2=4 |
| 060 | Mo Escaped | T4 Nx Mo | 46 | 3+4=7 |
| 081 | Mo Escaped | T2 Nx Mo | 33 | 3+3=6 |
| 044 | M1 Untreated | T3 Nx M1 | 23 | 4+5=9 |
| 046 | M1 Untreated | T1c Nx M1 | 53 | 3+4=7 |
| 083 | M1 Untreated | T2 Nx M1 | 42 | 3+3=6 |
| 097 | M1 Untreated | T4 Nx M1 | 184 | 4+4=8 |
| 098 | M1 Untreated | T3 Nx M1 | 230 | 3+3=6 |
| 104 | M1 Untreated | T3 Nx M1 | 100 | 5+5=10 |
| 105 | M1 Untreated | Tx Nx M1 | 19.5 | 3+3=6 |
| 010 | M1 Escaped | Tx Nx M1 | 55.2 | 5+4=9 |
| 021 | M1 Escaped | T4 Nx M1 | 656 | 4+3=7 |
| 039 | M1 Escaped | Tx Nx M1 | 298 | 4+5=9 |
| 040 | M1 Escaped | T4 Nx M1 | 431 | 3+3=6 |
| 043 | M1 Escaped | Tx Nx M1 | 117 | 3+3=6 |
| 063 | M1 Escaped | Tx Nx M1 | 1230 | 2+3=5 |
| 065 | M1 Escaped | T4 Nx M1 | 35 | 4+3=7 |

**Table 5.4:** Clinical staging data for patients evaluated by micro fluidic RT-PCR assay.

With regards the novel markers targeted using the micro fluidic assay only, 95% of BM samples were assay positive for EZH2, 90% for u-PA, 45% for Hepsin, 35% for Prostasin, 35% for HK4 and 10% for HK15. Both HK15 positive samples and 6 of the 7 HK4 positive samples were from patients with bone metastases. The 2 HK15 positive samples were also positive for all the other 9 target markers. All of the samples positive for HK4 were also RT-PCR positive for either PSA or HK2, while 5 were positive for both. For prostasin, 6 of the 7 positive samples were from men with advanced disease, while hepsin positivity was demonstrated in all clinical groups.

Quantitative marker expression could not be directly compared between the two assay formats as the internal housekeeping control assay, to which marker of interest expression was standardised, differed in each technique. Although the target gene was the same in each, assay efficiency was not comparable as different primers and modes of target detection were used in the 96 well and micro fluidic methods.

| Target Marker | Micro Fluidic RT-PCR % BM samples +ve | 96 Well RT-PCR % BM samples +ve |
|---|---|---|
| PSA | 35 | 55 |
| PSMA | 40 | 50 |
| PSCA | 10 | 35 |
| HK2 | 35 | 35 |

**Table 5.5:** Comparison of the percentage of the 20 BM samples collected from men with CaP which were RT-PCR positive for PSA, PSMA, PSCA and HK2 using the micro fluidic and 96 well assays.

For PB, fewer samples were RT-PCR positive using the micro fluidic assay than the 96 well format for PSA, PSMA, PSCA and HK2. The percentages of RT-PCR positive samples when comparing micro fluidic with 96 well RT-PCR were 8% and 63% for PSA, 8% and 21% for PSMA, 4% and 8% for PSCA and 0 and 29% for HK2 (Table 5.6).

| Target Marker | Micro Fluidic RT-PCR % PB samples +ve | 96 Well RT-PCR % PB samples +ve |
|---|---|---|
| PSA | 8 | 63 |
| PSMA | 8 | 21 |
| PSCA | 4 | 8 |
| HK2 | 0 | 29 |

**Table 5.6:** Comparison of the percentage of the 24 PB samples collected from men with CaP which were RT-PCR positive for PSA, PSMA, PSCA and HK2 using the micro fluidic and 96 well assays.

Overall only 5 PB samples were positive for any of the 4 markers PSA, PSMA, PSCA or HK2 using the micro fluidic format. None of these samples were micro fluidic RT-PCR positive for more than one of these markers.

The 2 PB samples that were PSA RT-PCR positive using the micro fluidic assay were from men with hormone escaped metastatic disease, were also PSA positive using the 96 well assay and had microfluidic PSA positive paired BM samples.

For PSMA, again only 2 PB samples were micro fluidic RT-PCR positive. Both were from men with metastatic disease, only one sample had a paired BM sample which was also PSMA micro fluidic RT-PCR positive.

For the novel markers, 63% of PB samples were RT-PCR positive for EZH2, 67% for uPA, 21% for Hepsin, 4% for prostasin and 4% for HK4. No patients were HK15 RT-PCR positive. As for BM EZH2 and uPA were detected in the majority of PB samples. The 5 patients positive for Hepsin all had advanced CaP. Four of

these had paired BM samples, 2 of which were Hepsin RT-PCR positive. Only 1 PB sample was positive for prostasin and 1 sample positive for HK4. Each was from a patient with hormone escaped metastatic CaP and both samples were also PSA RT-PCR positive. Their paired BM samples were positive for the same respective marker.

### 5.5 Practical Aspects of Micro Fluidic RT-PCR

The time required to run paired PB and BM samples from 4 patients using the micro fluidic assay was 75 minutes which includes 15 minutes for loading samples onto the card and 60 minutes for PCR cycling and product detection.
For the same number of clinical samples using the 96 well format the total time was over 8 hours.

The cost per patient for RT-PCR evaluation of paired PB and BM samples was estimated at £164 using the 96 well format compared with £52 using micro fluidic RT-PCR. These values did not include labour costs where clearly micro fluidic RT-PCR has a significant advantage. Additionally the micro fluidic card offered the potential to evaluate a far broader set of markers without any increase in cost or labour.

## Chapter 6: Discussion

### 6.1 Overview

The increasing incidence of CaP and its detection both in younger men and at an earlier stage has changed the demographics and treatment needs of this patient population. In contemporary urological practice more patients are potential candidates for radical curative treatments yet appropriate patient selection is bound both by the limitations of current disease staging modalities and our inability to predict the disease course accurately. The role of watchful waiting in the management of CaP is also unclear; the variable and currently unpredictable nature of the disease's natural history limiting this modality's more extensive implementation. The relevance of longitudinal natural history studies from the pre-PSA era to contemporary CaP populations is unclear. There is a clear need for a clinical tool capable of accurate staging and prediction of patient outcome.

The aim of this work was to establish the utility of RT-PCR in prostate cancer staging and prediction of natural history using an optimised methodology incorporating real time quantitation, a multiple target assay and two site patient sampling.

Our understanding of metastasis formation suggests that circulating tumour cells represent an early stage in the metastatic process. The detection of circulating cells would potentially enable the metastases to be identified at a far earlier stage than current staging tools allow.

The ability of RT-PCR to detect circulating PECs has been extensively demonstrated. However, its clinical utility remains unclear as the results of studies have been conflicting [238]. There are a number of reasons for this, one major factor being the lack of standardisation of technique. This reflects the fact that the use of RT-PCR is extremely dependent on meticulous development of the many assay components. Variations in clinical sensitivity may simply reflect limitations in assay *in vitro* sensitivity yet the impact on positive yield of marker choice and sample source must also be considered. As highlighted in section 1.9.6, the choice of

assay target is essential not only in maximising cell detection but also in avoiding false positive results.

Errors in sampling also play their part. Single point patient sampling only reflects a single time point in a dynamic process that may well involve intermittent cell shedding. Single site sampling similarly may not reflect the process as a whole given that disseminated tumour cells are not distributed homogenously [184]. Indeed, higher yields of tumour cells have been reported when using multiple blood sampling and paired bilateral iliac crest bone marrow samples [239,240]. We have shown that generally, combining PB and BM data increased molecular CTC detection for individual patients.

Iatrogenic cell shedding may also be a source of false positive results. Prostate manipulation during transurethral resection of the prostate (TURP) [223,241], radical prostatectomy [242], prostate biopsy [243] and brachytherapy [244] have all been shown to disseminate prostate epithelial cells. Price *et al* demonstrated circulating cell clearance within 4 weeks in 91% of patients who had detectable PECs in PB samples following prostate biopsy [243]. This, as well as the high CTC detection rates in patients with pathologically localised disease seen in some studies, highlights the fact that not all circulating PECs have the potential to form secondary cancers and of those with such metastatic potential a significant proportion will be cleared by the immune system.

Sampling or pipetting errors and cross contamination occurring during sample processing and reaction set up can have a significant impact on a process as sensitive as RT-PCR. Careful sample handling and the use of duplicate or triplicate reactions can minimise but not entirely exclude this.

The uncertainty stemming from the conflicting evidence in the literature is compounded by our lack of understanding of other processes impacting on tumour progression. These include tumour cell dormancy [245,246] and many other intricacies of the metastatic process, as well as inherent limitations of RT-PCR in circulating cell detection. Beyond detecting the presence of circulating cells, RT-PCR can tell us little else directly regarding the cell or its potential to cause progressive distant

disease, although the unstable nature of RNA may make its presence likely to be indicative of viable cells. Quantification of the number of mRNA molecules in a sample cannot differentiate between a large number of circulating cells each expressing a small number of mRNA replicates and a few cells each containing many messages. As the detected cells cannot be isolated their phenotype, viability and hence 'pathogenicity' remains unknown.

### 6.2 Assay Development

The selection of a suitable cell line model was dependent upon a requirement for multiple marker expression. The four selected prostate cell lines were well characterised and routinely cultured in our laboratory. Preliminary assay development utilised PSA and PSMA, the two markers most widely studied in prostate RT-PCR. RNA extraction and reverse transcription protocols were well established and the integrity of these methods and the cDNA target was confirmed by successful housekeeping gene co-amplification and clean minus RT and negative control reactions for all four cell lines. Only LNCaP derived RNA was found to include mRNA for PSA and PSMA. Primer sets for PSCA, HK2 and DD3 also successfully amplified specific target mRNA in LNCaP derived total RNA, confirming LNCaP as an appropriate model for all five prostate specific markers. These findings reflect published cell line data [62,247] [65,70] and the almost exclusive use of LNCaP in RT-PCR assay development by other groups [238]. All subsequent assay development therefore used the LNCaP cell line model.

During assay development and optimisation experiments using LNCaP derived RNA it was noted that there was considerable variation in Ct values for markers between experiments using identical amounts of target and similar assay components. For example in initial experiments evaluating cell line marker expression compared with primer optimisation and cell spike experiments which were performed using different target samples. There are a number of potential sources of error which could account for such variation and lack of reproducibility. The LNCaP cells from culture were harvested at confluence yet there was some variation regarding the exact time since last passage. The local environment including nutrient availability and cell-cell contact has a significant impact on cell cycling and therefore mRNA and protein expression. The use of synchronised

cells would address this issue yet would also add considerable complexity. In cell spike experiments the use of haemocytometer cell counting and subsequent pipetting of small cell numbers is likely to have resulted in some variablity between experiments and indeed, throughout the methodology there was significant potential for pipetting and solution sampling errors.

This does therefore question the significance of the small variations in Ct values seen in experiments performed for cell line and assay optimisation, which would potentially be effected by such sources of error. More importantly, such inter-experiment variability could have a significant impact on assay clinical performance, the impact of which cannot be determined. As is discussed later (section 6.4.1), performing replicate reactions improves representative sampling of dilute solutions and would also be expected to reduce the significance of assay component pipetting errors [248]. However, far better *in vitro* reproducibility was seen with micro fluidic RT-PCR where a far simpler methodology removed many of the stages which might be susceptible to sampling error.

In optimising the LNCaP cell line, prolonged culture and the presence of additional DHT in the growth medium had no significant effect on gene expression for PSA, PSMA, DD3 and HK2. Androgenic stimulation of the androgen receptor within the cell nuclei has been shown to regulate the expression of PSA [249], PSMA [250] and HK2 [251]. It would therefore be expected, given the androgen dependence of LNCaP, that culture in the presence of DHT would increase PSA and HK2 expression and decrease PSMA expression. However, Igawa *et al* [252] demonstrated that serial passage of LNCaP cells selects cells which are androgen independent for growth although Denemeade *et al* [253] showed that gene expression for PSA and HK2 remain androgen responsive in late passage cells. The observed lack of effect of DHT on marker expression may therefore reflect androgen independence in passage 93 LNCaP cells.

PSCA expression was increased in late passage cells, compared to early passage cells but to a lesser degree when cultured in the presence of DHT. This corresponds with Tran *et al* [66] who suggest that PSCA expression marks a more dedifferentiated cell population.

Assay specificity is essential in RT-PCR techniques, therefore nucleotide marker specific probes were incorporated into the PSA, PSMA and PSCA assays. However, positive results were recorded in the minus RT reactions when probes were used, something that had never been observed when using SYBR green for product detection. The fluorescence traces from these reactions was different to those recorded from a normally amplified product. The significance of these traces is unclear with no similar problems being reported either in the literature or by the manufacturers of the light cycler. Exclusion of these traces by raising the Ct value above their uppermost extent had a significant deleterious effect on assay sensitivity. Reaction Ct values were increased by around 2 cycles, a particular issue at the limits of assay sensitivity. Probe use also tended towards the assay being less sensitive (section 3.6.2.2). The specificity advantage of probes must be balanced against a loss of sensitivity. This, combined with the uncertainty surrounding the minus RT reactions resulted in probe use being abandoned in favour of SYBR green for PSA, PSMA, PSCA and DD3 product detection.

The exception to SYBR Green use was for the HK2 assay. Significant amounts of primer dimer were produced in all reactions including minus RT controls. This primer dimer gave a positive result as SYBR Green is non-product specific, binding to any double stranded DNA. In control samples this was not a significant problem as long as the product dissociation confirmed primer dimer only. However in the target samples there would often be two product peaks, one in keeping with expected product and the other with primer dimer. The detected fluorescence recorded on the amplification trace would be due to a combination of these products not wholly the marker of interest. Therefore a qualitative result would be valid, however a quantitative Ct value would not as it was impossible to separate fluorescence from the two products. Primer dimer production was not eliminated by altering primer concentrations. Introducing a nucleotide probe to the assay allowed specific identification of HK2 product and excluded the detection of primer dimer. The primer dimer would of course still be produced, which could impact on reaction efficiency by reducing the amounts of free primer to anneal to target. However, probe incorporation improved the HK2 assay *in vitro* sensitivity from $10^3$ to $10^2$ LNCaP cells per 1ml of female PB. This is interesting since quite the

opposite was found when probes were used with the other markers. The only possible explanation is that there were different amounts of primer used in the final HK2 plus probe set up than in the original SYBR green assay. This may have resulted in less primer dimer and thus more free primer available for specific annealing resulting in more effective PCR amplification.

### 6.3 Assay Sensitivity

Fidler [182] suggested that $10^5$ tumour cells need to be present in the circulation for metastasis to occur, thus the target assay sensitivity was 10 cells per ml of blood. One ml of blood contains between 4 and 11 x $10^6$ nucleated cells. The limits of detection of the assay varied depending on the target of interest, being 1 cell per ml of blood for PSA, 10 cells for PSMA and 100 cells for PSCA and HK2. The majority of reported PSA assays have limits of detection of 1 cell in a background of $10^6$ negative cells although sensitivities as low as 1 in $10^7$ [254] or $10^8$ [255] have been reported. For PSMA assays the literature suggests sensitivities between 1 cell in $10^4$ [256] and $10^7$ [254] marker negative cells. Thus the PSA and PSMA assays had sensitivities within the ranges successfully achieved by others.

The only group to use PSCA as a target for RT-PCR achieved a limit of detection of 1 cell in 1 ml of blood using a nested assay [221]. This superior sensitivity is likely to reflect the use of a nested technique. The advantages in terms of sensitivity of this technique must be balanced against both its increased complexity and susceptibility to false positive results from amplification of contaminants or illegitimately transcribed RNA. Despite this the specificity of our PSCA assay has proved to be unreliable, with target of interest being amplified in RNA extracted from female blood. The reason for this remains unclear as a source of contamination has not been identified and the primer sequences are solely PSCA specific. Similarly it appears unlikely that at the sensitivity achieved by this assay that these results represent illegitimately transcribed RNA amplification. Assay specificity is discussed in detail in section 6.5.

The HK2 assay could detect 100 cells per 1 ml of PB, 10 fold less sensitive than the target level. Previous assays targeting HK2 report levels of detection of between 1 cell in $10^6$ and 1 cell in $10^9$ leucocytes [257,258,259]. Two of these assays

have involved nested techniques, and Slawin *et al*, in achieving the greatest sensitivity reported a false positive rate of 36% [259].

The $DD3^{PCA3}$ based assay could not detect $10^5$ LNCaP cells in 1 ml of female blood. In the initial cell line work (section 3.2) the relative gene expression of $DD3^{PCA3}$ in LNCaP cells was lower than all the other four markers which may explain this result. As $DD3^{PCA3}$ expression has not been detected in any other prostate cancer cell lines [70,247] there is no other prostate cell alternative for use in an *in vitro* model. Other workers in this field have experienced similar problems when using prostate cancer cell lines to establish sensitivity for $DD3^{PCA3}$ based RT-PCR assays [70,71]. In the limited number of studies targeting $DD3^{PCA3}$ mRNA through RT-PCR or nucleic acid based amplification assay (NASBA) techniques there have been no published LNCaP cell spike sensitivity data [260,71]. One approach to this hurdle has been to insert a $DD3^{PCA3}$ target sequence into a plasmid, facilitating known numbers of target mRNA replicates to be used in serial dilutions spiked into a suitable RNA background (see section 2.3.10) [71]. Utilising this method, 1 plasmid copy could be detected in 100ng of female blood derived cDNA while 100 copies of $DD3^{PCA3}$ RNA could be detected per 2.5ml of female blood when $DD3^{PCA3}$ RNA transcribed from the plasmid construct was spiked into female blood samples. This level of sensitivity is not directly comparable to cell spike data however it has been suggested that a single LNCaP cell contains between 100 and 1000 PSA mRNA molecules [261]. Therefore the $DD3^{PCA3}$ sensitivity achieved here in terms of mRNA copies would equate to 1 cell per ml of blood in a PSA based assay.

## 6.4 Technical Considerations at the Limits of Assay Performance

### 6.4.1 Reproducibility & representative sampling of dilute solutions

It is clear from the above discussions of sensitivity that all of the assays would be required to perform at the limits of their *in vitro* sensitivity to maximise PEC detection *in vivo*. One of the problems experienced in this RT-PCR work was reproducibility of results between replicate samples. During assay development the impact that this would have in the clinical setting was not immediately

apparent. *In vitro* work evaluated the finite limit of cell detection using each marker and it was only at this limit that significant variability between replicate results would occur. For example, the PSA assay could detect 1 LNCaP cell per 1ml of female PB but in only one of three replicate reactions. At 10 cells per ml all replicates were PSA RT-PCR positive. Similar variability in marker quantification was demonstrated with micro fluidic RT-PCR, where in evaluating serial dilutions of LNCaP cDNA there was significant variation in the Ct values from 'identical' replicate reactions in the most dilute samples (figure 5.4).

The principle of RT-PCR molecular staging is the ability to detect viable tumour cells within a background of non-tumour cells. Inevitably the target cells are greatly out numbered by background and this situation is amplified considerably at the level of mRNA target. Assays must therefore work reliably at their limits of performance to provide a robust clinical tool. As discussed above, a clinically significant circulating cell yield is in the region of 10 tumour cells per ml of PB. The *in vitro* performance of our markers show that only the PSA assay could detect cell concentrations beyond this and the other markers would be at or beyond their limits of performance of detection in identifying patients with this concentration of circulating cells. It would be predicted therefore, assuming good specificity, that clinically the assays would reliably detect patients with established advanced metastatic disease and high tumour cell loads while only PSA might be expected to consistently perform in those with micrometastatic disease. The performance of the other markers in these less clinically advanced groups would be far more dependent on total cell load and levels of marker expression by these cells. Clinical performance in BM would be expected to be better as higher relative tumour cell numbers are expected although the background cellular environment remains a far less evaluated entity with no *in vitro* studies being performed here. In actual fact there was considerable variation in cell yields between PB and BM, and this was dependent on the individual assay and disease status. This is discussed further in section 6.6.9 (combining PB and BM).

In optimising the methodology for use with clinical samples, there are important technical issues relating to the use of RT-PCR with dilute solutions. Representative sampling is essential at each step of molecular staging and is a

potential source of error at each of these. Patient sampling is discussed above, but assuming that the clinical samples contain PECs these will be greatly diluted by leucocytes and following total RNA extraction, the target messages will be diluted further. McIntyre *et al* highlighted the importance of representative sampling of dilute solutions when, in their PSA/PSMA RT-PCR study, significant and persistent variability was seen in assay results between duplicate reactions [248].

RT-PCR methodology typically uses only small aliquots of total RNA for marker specific PCR amplification. Around 1% of the total RNA was evaluated in each reaction for the 96 well format and around 0.02% for micro fluidic RT-PCR. When sampling from a dilute solution the probability of that sample being representative of the solution can be estimated using the Poisson distribution. Figure 6.1 shows a Poisson curve. The x-axis shows the number of target mRNA molecules in an aliquot taken from a given dilute solution while the y-axis is the probability of this event occurring. $\lambda$ is the most likely number of molecules to be sampled and would be a true representation of the mRNA content. The tails of the distribution demonstrate that there is a small chance of sampling a very large or very small number of molecules and in the latter case this may be beyond the sensitivity limit of the assay represented by the x-axis intersect 'S'. The probability of a positive result is the area under the curve to the right of 'S' divided by the total area under the curve. The higher the number of cells in the initial sample the greater the value of $\lambda$ and the greater the probability of a positive RT-PCR assay result. The probability of a positive test result can be improved by increasing target concentration in that solution or through repetitive solution sampling. Jung *et el* showed that the positivity rate for a typical RT-PCR assay at the limit of its sensitivity was 60% [261]. A ten fold increase in target concentration improved detection rates to close to 100%. The number of replicate reactions necessary to ensure a positive result and thus exclude false negatives can be calculated using a poisson table and this shows that 5 replicate reactions must be performed to give a probability of a positive result of 98%. It must be appreciated that this applies to each individual assay at the limit of its sensitivity. Therefore, unless the patient specimen is processed and analysed in its entirety for each assay then a number of test repetitions will have to be performed to exclude false negatives,

within the performance limits of the particular assay. Six replicate reactions were used to evaluate all clinical samples for all markers. This is an important concept as when the raw RT-PCR data is studied, it can be seen that few samples had six positive assay results for any given marker. This suggests that the majority of the marker positive samples had circulating cell concentrations in the region of the limit of assay sensitivity.

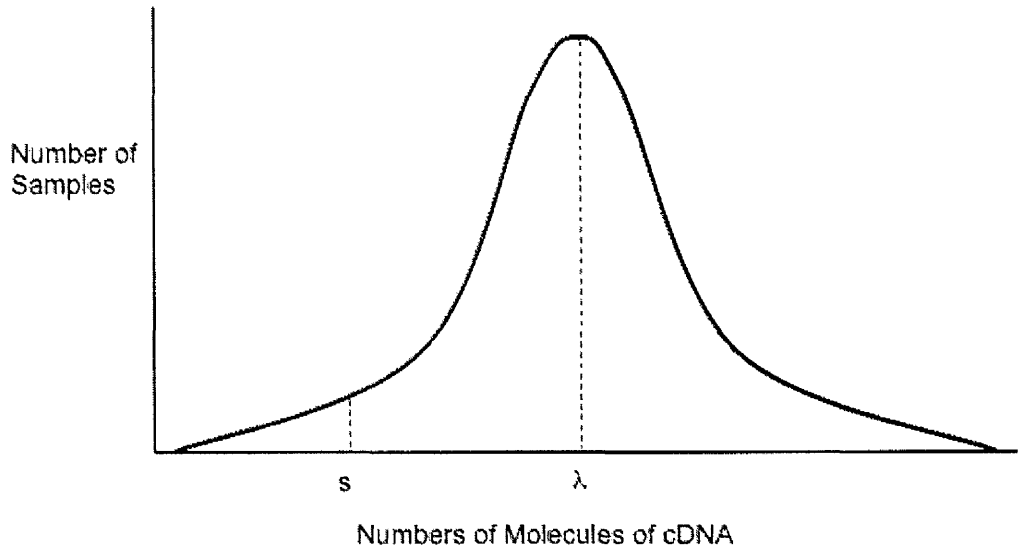



**Figure 6.1: Graph showing the probability of sampling a certain number of cDNA molecules in a dilute solution.** λ is the mean number of molecules and is the most likely sampling event. The limit of assay sensitivity is represented by 'S'.

### 6.4.2 Reproducibility at the limits of detection - the Monte Carlo Effect

The second important consideration is PCR amplification reproducibility at the limits of assay detection. PCR efficiency in dilute solutions is limited by the number of target molecules. There are random differences in amplification efficiencies depending on whether the primers are able to bind to the target and, with exponential PCR amplification, at what stage in the cycling primer annealing occurred. This can have a significant impact on the final assay result. Karrer *et al* described the limitations of PCR amplification of dilute target within a complex mixture and termed this non-reproducibility the Monte Carlo effect [262]. The lower the concentration of target the more likely the reaction is to be affected by the Monte Carlo effect and thus there is greater potential for variation between

replicates. This again highlights the importance of using replicate assays and is an additional explanation as to why only a proportion of replicate samples were assay positive. The random nature of the variability however makes quantitative comparisons of PCR product at the limits of assay performance more difficult.

### 6.5 Assay Specificity *in Vitro*

During assay development samples of female PB free from CaP cells were evaluated to establish the specificity of each molecular marker. The RT-PCR assays for PSA, PSMA, HK2 and $DD3^{PCA3}$ were negative for all replicate reactions using mRNA from female PB. The specificity problems that would be experienced when evaluating large numbers of clinical samples in the main body of the study were therefore not identified for these markers. The possible reason for this is that only samples from two female volunteers were evaluated during these validation studies and as was subsequently seen, only a proportion of control samples would contain detectable target.

In contrast, PSCA was detected in female control PB samples, however at higher Ct values and thus in smaller amounts than in cell spike samples. It was expected that this would also occur in the larger study population and therefore low levels of expression in controls could be excluded by applying Ct threshold levels.

The use of BM was not evaluated in the assay validation stages and in retrospect this was a flaw in the study design, as there is no cell spike validation data, and this might have identified specificity issues at an earlier stage. The collection of BM samples, particularly from healthy individuals, was however far more difficult both practically and ethically than for PB. The establishment of a bank of BM samples with an optimised technique for their collection and processing was not achieved until the PB assay validation work was complete. Time constraints for completion of the study resulted in this BM validation being excluded.

## 6.6 RT-PCR Evaluation of Clinical Samples

### 6.6.1 Issues Regarding Data Analysis and Interpretation

The real time RT-PCR data collected during assay validation provided a simple representation of the limits of cell detection for each assay and convincingly displayed the potential of quantitative RT-PCR, demonstrating the technique's ability to reliably detect different amounts of input mRNA target. The demands of the clinical setting were far higher and quantitative data analysis for the study population was far more complex. Issues of specificity had not been identified. Clinical sensitivity would only be established during the clinical study and thus the importance of assay performance at its limits of sensitivity would only be appreciated at that stage. Validation work gave no impression as to the reliability of the assays in detecting small differences in input target, a factor that would be important in differentiating between clinical groups.

#### 6.6.1.1 Multiple Replicate RT-PCR Assay Reactions

The use of multiple replicate assay reactions for each clinical sample was a necessity of the technique. This produced a large amount of data for final analysis in a methodology involving quantitative data for five markers from two clinical samples per patient. To allow comparison between individuals it was essential to have a simple numeric value for each marker for each sample. There is no reported modelling for utilising this type of quantitative RT-PCR data and some issues remain undetermined. For example, can two samples be directly compared using a simple sum or mean of their replicate results if one has a single positive replicate containing a large amount of target while the other has five positive reactions each with a small amount of target. Based on the theory described in section 6.4 regarding sampling dilute solutions and reaction replicates, it was concluded that since six replicates were being employed, then a positive result in any replicate reaction was an overall positive result for that sample and the sum of the replicate results would be reasonable to allow comparison of target mRNA expression.

### 6.6.1.2 Expression of quantitative data

The raw value (Ct) for each positive RT-PCR reaction was recorded as a number of cycles from 0 to 40. Since an increasing Ct value was inversely proportional to the amount of input mRNA then using (40-Ct value) produced an assay result with a direct relationship to the amount of input mRNA. All clinical samples therefore produced six (40-Ct value) results for each marker which were added together to give a single $\Sigma$(40-Ct) value.

At the outset of the study it had been anticipated that the assay data for all markers from both blood and bone marrow for each individual would be analysed to produce a test result that was clinically relevant and easy to utilise. No modelling of this type of data has previously been described and therefore various approaches were considered. Expressing results as a single numeric value accounting for all elements of the test or in terms of the number of circulating cells detected were appealing concepts. Each would allow simple comparison with clinical data and disease course.

Manipulating the data in this way would depend on assay factors, sample factors and PEC factors. All assays would have to be robust and reproducible at the levels of sensitivity required to detect clinically relevant circulating PECs and in addition assay performance would have to be identical for each marker. Combining PB and BM data might overlook clinically important patterns; could the presence of CTCs in BM be more significant in subsequent clinical course than PB? If the cell number approach was used then these would be estimated from standard curves derived from LNCaP cell spike experiments. Such a model however would assume that all circulating PECs and cell line cells express identical numbers of mRNA molecules for each marker which is in direct conflict with the evidence demonstrating the heterogenous nature of tumour cells [185,263,264].

Producing patient group marker profiles would identify the significance of each marker and the pattern of marker expression in patient outcome. It was not possible to produce these from the data collected from this study because the assays were not sensitive or specific enough, the clinical groups were small and

the follow up short. Therefore only small amounts of positive data were available for numerous small groups which were best displayed in graphs to show general trends rather than significant marker profiles.

#### 6.6.1.3 Issues of Assay Specificity

The majority of published RT-PCR studies report excellent clinical specificity. A few studies are however exceptions, with false positive rates of up to 66% for PSA [265], 92% for PSMA [266] and 14% for HK2 [220]. This is discussed in detail for each marker in sections 6.6.2 to 6.6.6. We recorded positive assay results in significant numbers of disease free control PB and BM samples for PSA, PSMA and PSCA as well as to a lesser degree for HK2 in PB only. Where this has been reported previously in qualitative RT-PCR studies it has not been possible to utilise the data any further and conclusions of poor marker specificity have been drawn [212,213,259,265,266]. One of the potential advantages of quantitative RT-PCR is the ability to differentiate between levels of marker expression, and for example, when Patel *et al* detected PSA mRNA in 66% of control PB samples by real time PCR they noted that expression levels were on average 7 fold lower in the control compared to CaP samples [265]. Beyond this there is little else published in the literature to suggest a solution to this problem, yet it was envisaged that a similar approach could be applied to our real time data. On evaluating the expression profiles for each marker for the entire study and control populations it was clear that significant proportions of positive control samples could be excluded by setting expression thresholds, levels above which would be considered indicative of significant marker expression. These levels were different for each marker and were selected purely based on minimising positive results in the controls which had a variable impact on the CaP populations for different markers and still did not exclude all false positive results. This approach was most successful for PSA. For PSMA where false positive rates and expression levels were higher, larger numbers of patient samples were also excluded and for PSCA this approach could not demonstrate CaP specificity for this marker. The specificity problems encountered with all of these markers limited the amount of useful RT-PCR data available for analysis.

#### 6.6.1.4 Issues of statistical analysis

Statistical analysis of the RT-PCR data was complicated by a number of issues. The study population was composed of a heterogenous patient population, divided into a number of relatively small groups as determined by clinical disease status. This, as well as the fact that there were relatively small numbers of positive assay results, made it difficult to identify significant patterns and trends. General trends within the entire population, such as increasing marker expression with advancing disease status, could be evaluated using Spearman's rho, a measure of the linear relationship between two variables.

The quantitative data was not normally distributed. Ideally, the median rather than the mean would have been the most appropriate descriptor to give an overview of marker expression within a group to allow comparison. The small numbers of positive samples within the groups, particularly those with localised and locally advanced CaP, meant that the median would often be zero. The mean was therefore used for this purpose, however, standard deviations were not appropriate given the data distribution. Additionally, it was necessary to use non-parametric statistical tests, comparing ranks. The Kruskal-Wallis test was used to identify significant differences between any groups within the study population at a significance level of 5% ($p=0.05$). However, to compare each group to every other group using the Mann-Whitney U, required that the Bonferroni correction be applied. This correction looks to reduce the likelihood of finding a significant difference by chance when multiple tests are carried out. In comparing the 6 study groups with one another, 15 tests were performed thus a reduced significance level of $<0.003$ was applied. This correction is very conservative and indeed real differences may be overlooked, type II errors may be increased in an attempt to minimise type I errors.

#### 6.6.1.5 Issues Regarding Multiple Marker RT-PCR Assays

One of the novel approaches being evaluated in this study was to concurrently evaluate samples for the quantitative expression of five molecular markers. To make this a practical proposition for a study population of over 100 patients all of the assays had to work efficiently within the same reaction conditions using

standard reaction components. As detailed in section 1.9 many factors impact on assay sensitivity and specificity and this is reflected in the variation of protocols seen in the literature. Additionally, quantitative RT-PCR necessitated the use of the ABI PRISM sequence detection light cycler and there are specific rules in primer/assay design which should if possible be adhered to in order that the assays are as efficient as possible. Specific Primer Express software was initially used to develop appropriate real time primers, however for HK2 and $DD3^{PCA3}$ these were not successful. Therefore alternatives designed by other groups were used which were optimised for different reaction conditions to those previously described [71,194]. The potential impact on assay performance in a real time reaction was appreciated but unquantified at the outset of the clinical study. For example, the $DD3^{PCA3}$ assay amplicon was large at 381bp and therefore it would be expected that amplification would be less efficient than the 85bp PSA product. Therefore concurrently running five RT-PCR assays and the resulting limits on individual assay optimisation may explain the poor sensitivity for some markers.

#### 6.6.1.6 Issues Regarding Study Patient Population

At the outset of this study it had been envisaged that within the allotted time of two years it would be possible to collect paired PB and BM samples from 200 men with prostate cancer and 30 disease free controls. The majority of patient samples would be from men with clinically localised CaP while a smaller group of men with metastatic disease would be evaluated to confirm clinical sensitivity.

Time constraints meant that assay development and clinical sample collection would proceed concurrently. Thus, a number of assumptions regarding the assays, the targets and the patient population were made. Each assay should show a high degree of clinical specificity and should be reliable and robust at the required sensitivity. Generally, there would be clear patterns of marker expression, increasing with advancing disease stage and clearly identifying those with a poor clinical prognosis. Identifying patients who fitted into rigid clinical grouping criteria and would consent to sampling would be achievable in a finite time period.

Bone marrow aspiration is clearly an invasive procedure, particularly when performed under local anaesthetic and in the trial setting offered no clinical

advantage. The target patient population were already having come to terms with a diagnosis of cancer, an uncertain or even bleak future and the prospect of aggressive oncological therapy. Since recruitment was slow, the sampling criteria were widened to include patients with a variety of stages of disease. In retrospect this weakened the study's potential considerably particularly since the clinical sensitivity of the assays were lower than expected. Therefore overall there were fewer patients with fewer positive results distributed between a number of smaller groups. Identifying significant results would be far more difficult and statistical testing had to allow for these groups by using reduced significance levels (section 6.6.1.4).

As is discussed in section 6.3, based on the *in vitro* performance, only the PSA assay would be expected to perform reliably with the small PEC numbers that would typically be circulating in patients with micro metastases. Additionally, variability in assay performance between markers meant that cell detection by each marker depended not only on the presence of cells and their marker expression but also on the individual assay capability. Given these constraints, the sampled study population was not perfectly suited to the final multi-marker assay model and a target population of only men with advanced disease might have been more informative since they would be expected to have higher CTC loads.

### 6.6.2 PSA RT-PCR Assay

#### 6.6.2.1 PSA RT-PCR assay *in vitro* specificity

The majority of PSA RT-PCR studies show good CaP specificity with 0% to 7% controls being assay positive, thus the 6% described here compares well with these (table 6.1). A few workers have described less prostate specific assays, Thiounn *et al* detecting PSA mRNA in 9% of men with BPH and 18% non prostate controls [267], Gala *et al* in 19.4% of controls [212], Henke *et al* in 30% of healthy controls and 55% of men with BPH [213]. More recently, Patel *et al* used a real time RT-PCR assay with comparable *in vitro* sensitivity to ours and reported that 67% of control PB samples were assay positive [265].

### 6.6.2.2 PB PSA RT-PCR assay - clinical sensitivity

Based on its *in vitro* performance, the PSA RT-PCR assay was expected to reliably detect men with advanced disease through the presence of CTCs. This of course assumed that clinical sampling would be representative and CTCs PSA expressing. All men with hormone escaped metastatic disease and 63% of all those with bone metastases had detectable PSA mRNA in their PB samples.

PSA in PB is the most widely evaluated target in CaP molecular staging and reports of clinical sensitivity are varied (Table 6.1). Between 35% and 100% of patients with advanced disease have been found to be PSA RT-PCR positive [62,192,208,210,214,218,220,221,240,257,265,268-277]. As discussed previously the *in vitro* sensitivities of the reported assays are varied and this will be reflected in their clinical sensitivity however the majority of assays report limits of detection of $1:10^6$ and despite this there remains significant variability in clinical sensitivity [62,192,220,221,257,265,270,277]. This is therefore likely to reflect the heterogeneity of methodologies and patient cohorts. Patient groups with advanced disease evaluated in RT-PCR studies are often small and poorly described or defined in that they may contain a heterogenous mix of locally advanced, nodal and distant metastatic disease which may be untreated, hormone manipulated or hormone escaped. Direct comparison between studies and with the clearly characterised groups described here must be considered in this context.

Where advanced CaP groups have been sub-analysed, Seiden *et al* [275] showed, as in our study, that patients with hormone escaped metastatic cancer were more likely to be assay positive compared to the metastatic group as a whole, while Israeli *et al* [256] showed lower expression in treated metastases than hormone escaped cancer which is in keeping with CTC theory. In contrast, Ellis *et al* found that only 45% of patients with hormone escaped metastases were assay positive while 75% of those with untreated metastases had detectable CTCs [240]. This observation is more difficult to explain. However, Halabi *et al* also detected PSA target in only 44% of patients with HRPC using a very sensitive RT-PCR assay. Targeting PSA alone may under estimate CTC number, heterogenous marker expression is well recognised. However, these may be true negative results since rates of disease progression even in this advanced stage are variable and some

individuals may not have significant numbers of circulating PECs. Interestingly, we detected PECs more often in men with non-metastatic hormone escaped CaP than those with untreated metastases. There have been no other reports of this observation although it may have gone unrecognised within advanced patient groups. The hormone escaped state is indicative of disease progression whether there are established detectable secondaries or not and the presence of CTCs would be in keeping with this. The question of interest is whether hormone escape enhances the ability of tumour cells to enter the circulation.

| Author | Year | Controls % PSA +ve | Advanced % PSA +ve | Limit of Detection |
|---|---|---|---|---|
| Current Study | - | 6% | 100% M1 escaped<br>42% M1 untreated<br>50% Mo escaped | $1:10^6$ |
| Moreno [206] | 1992 | 0 | 33% | - |
| Katz [214] | 1994 | 0 | 78% | $1:10^5$ |
| Seiden [275] | 1994 | 0 | 35% All M1<br>50% M1 escaped | - |
| Israeli [62] | 1994 | 3% | 86% M1 escaped<br>25% M1 manipulated | $1:10^6$ |
| Ghossein [278] | 1995 | 0 | 35% | $1:10^6$ |
| Cama [268] | 1995 | 0 | 80% | $1:10^5$ |
| Jaakkola [272] | 1995 | 0 | 50% | - |
| Sokoloff [192] | 1996 | 11% | 88% | $1:10^6$ |
| Melchior [274] | 1997 | 0 | 64% N1/M1/recurrent | $1:10^8$ |
| Ignatoff [210] | 1997 | - | 100% | - |
| Zhang [277] | 1997 | 0 | 64% | $1:10^6$ |
| Corey [208] | 1997 | 0 | 46% | $1:10^8$ |
| Ellis [240] | 1998 | 2% | 75% M1 untreated<br>46% Escaped | $1:10^8$ |
| Gao [269] | 1999 | 0 | 42% LA & advanced | $1:10^7$ |
| Kantoff [218] | 2001 | - | 48% | - |
| Sourla [276] | 2001 | - | 75% | - |
| Halabi [271] | 2003 | - | 44% HRPC | - |
| Kurek [273] | 2003 | 7% | 80% | $1:10^6$ |
| Shariat [220] | 2002 | 7% | 88% | $1:10^6$ |
| Hara [221] | 2002 | 1.4% | 50% N1 or M1 | $1:10^6$ |
| Kurek [257] | 2004 | - | 80% | $1:10^6$ |
| Patel [265] | 2004 | 66% | 80% | $1:10^6$ |

**Table 6.1:** PSA RT-PCR in advanced CaP - clinical RT-PCR studies targeting PSA in PB.

### 6.6.2.3 PB PSA RT-PCR assay - clinically localised CaP

As expected, higher proportions of men with advanced disease (M1 or M0 hormone escaped) were found to have CTCs compared to those with clinically localised or locally advanced CaP. Similarily, mean PSA expression levels increased with advancing disease status. Twenty three percent of those with clinically localised CaP were PSA RT-PCR assay positive which was similar to the 24% in the locally advanced group, although the latter demonstrated higher mean PSA expression.

Previous clinical studies of PSA RT-PCR in PB reported a great deal of variation in CTC detection with assay positivity between 6% and 54% for patients with clinically localised disease (table 6.2). As discussed previously this reflects a lack of standardisation of methodology resulting in variable *in vitro* and clinical sensitivities. The clinical groups described for localised cancers are also somewhat heterogenous; for example Thiounn *et al* included patients with serum PSA levels of up to 39 ng/ml [267], Martinez-Pineiro *et al* patients with clinically staged T3 cancers [279] and Kurek *et al* sampled all of their localised patients after 3 months of hormone manipulation therapy [273]. Many of the other studies do not define their clinical staging criteria. The patient groups used in the current study are clearly defined and may exclude many patients from the clinically localised group that would be included by other workers. This, along with the reductions in detection rates resulting from assay specificity optimisation, may explain why only 23% of our clinically localised cancers were assay positive, lower than some other reports. Similarly, this variability in patient classification may also explain why there was little difference in RT-PCR positivity between clinically localised and locally advanced patients. Many in the latter group were classified based purely on a serum PSA >10ng/ml some of whom would have had pathologically localised cancer. There are few reports specifically concerning patients with clinically locally advanced cancer and many of the patients allocated to this group in our study would have been allocated to either the localised or advanced groups by other workers.

| Author | Year | Clinically Localised | Clinically Locally Advanced | Pathologically Localised | Pathologically Locally Advanced |
|---|---|---|---|---|---|
| Current study | - | 23% | 24% | - | - |
| Katz[214] | 1994 | 39% | - | - | 68% |
| Seiden[275] | 1994 | 6% | - | - | - |
| Cama[268] | 1995 | 34% | - | 69% | - |
| Ghossein[270] | 1995 | 16% | 30% | - | - |
| Israeli[62] | 1995 | - | - | 0 | 7% |
| Loric[280] | 1995 | 11% | - | 6% | 20% |
| Sokoloff[192] | 1996 | - | - | 59% | 72% |
| Corey[208] | 1997 | 19% | - | 22% | 16% |
| De Cremoux[281] | 1997 | 21% | - | 21% | 20% |
| Melchior[274] | 1997 | 20% | - | 16% | 27% |
| Thiounn[267] | 1997 | 22% | - | 14% | 35% |
| Zhang[277] | 1997 | 13% | - | - | - |
| Ellis[240] | 1998 | 20% | - | 17% | 22% |
| De la Taille[215] | 1999 | 27% | - | 16% | 51% |
| Gao[282] | 1999 | 32% | - | 38% | 26% |
| Llanes[283] | 2000 | 28% | - | 33% | 22% |
| McIntyre[217] | 2001 | 27% | - | - | - |
| Straub[223] | 2001 | 54% | - | 40% | 72% |
| Adsan[284] | 2002 | 43% | - | 37% | 42% |
| Hara[221] | 2002 | - | - | 2% | 0 |
| Kurek[273] * | 2003 | - | - | 34% | 32% |
| Martinez-Pinero[279] | 2003 | 43% | - | 45% | 40% |
| Patel[265] | 2004 | 37% | - | - | - |

**Table 6.2:** PSA RT-PCR in localised CaP – clinical RT-PCR studies targeting PSA in PB

(* = samples taken after 3 months of hormone manipulation)

#### 6.6.2.4 PB PSA RT-PCR - clinical correlation

For patients with clinically localised CaP no correlation was demonstrated between PSA RT-PCR status and clinical outcome or pathological stage. Most studies of RT-PCR in clinically localised CaP report on CTC against pathological staging. In our study the clinically localised group contained only 24 patients who underwent RRP and therefore had pathological staging, which limited the data available for analysis. Of these patients, only 5 had pT3 CaP, all RT-PCR negative, 11 had positive margins, 2 RT-PCR positive and 4 had experienced PSA failure, all RT-PCR negative.

As can be seen in table 6.3 very few studies have shown a clear clinical utility for PSA RT-PCR in localised CaP. The Columbia university group have generally had the greatest success, consistently showing PSA RT-PCR to be superior to other staging modalities in predicting pathological stage [214,268,285] and additionally that pre-operative assay status predicted PSA recurrence following RRP [215]. The only other groups to demonstrate a significant relationship between PSA RT-PCR and pathological stage are Thiounn *et al* [267] and Straub *et al* [223] although the former study was limited by poor assay specificity. For Localised CaP managed expectantly McIntyre *et al* reported that positive PSA RT-PCR predicted disease progression [217]. Other groups reporting positive results with PSA RT-PCR in localised cancers have combined it with other molecular markers such as PSMA [277].

For patients with advanced CaP the groups described in the current study were small and were primarily included to establish clinical sensitivity. No clear relationship between PSA RT-PCR status and outcome was demonstrated for these patients although follow up data for these groups is incomplete. In studies evaluating larger cohorts of such patients, the results have been promising. Kantoff *et al* observed improved survival for men with HRPC who were PSA assay negative [218]. Using the same assay, Halabi *et al* sampled 162 men with HRPC for PSA RT-PCR and found that survival was significantly shorter for the RT-PCR positive patients, 11 months versus 21 months [271]. For hormone naïve patients, Sourla *et al* showed that 43% of patients become RT-PCR negative following complete androgen blockage, and that this was a predictor of longer progression

free survival [276]. These findings confirm the clinical importance of CTCs in cancer progression but suggest that RT-PCR techniques may be more suited to evaluating patients with advanced disease. Although an interesting prognostic tool, this is unlikely to significantly alter patient management.

| Author | Year | Clinical Correlation | Details |
|---|---|---|---|
| Katz[214] | 1994 | Yes | Capsular penetration<br>Positive margins |
| Seiden[275] | 1994 | No | - |
| Cama[268] | 1995 | Yes | Capsular penetration<br>Positive margins |
| Ghossein[270] | 1995 | No | Trend with increasing serum PSA |
| Israeli[62] | 1995 | No | - |
| Loric[280] | 1995 | No | - |
| Sokoloff[192] | 1996 | No | - |
| Corey[208] | 1997 | No | - |
| De Cremoux[281] | 1997 | No | - |
| Melchior[274] | 1997 | No | - |
| Thiounn[267] | 1997 | Yes | Pathological stage<br>Positive margins |
| Zhang[277] | 1997 | Yes | Pathological stage (PSA/PSMA combined) |
| Ellis[240] | 1998 | No | |
| De la Taille[215] | 1999 | Yes | Pathological Stage<br>PSA recurrence |
| Gao[282] | 1999 | No | - |
| Llanes[283] | 2000 | No | - |
| Straub[223] | 2001 | Yes qRT-PCR | Pathological stage<br>Serum PSA<br>Gleason score |
| McIntyre[217] | 2001 | Yes | PSA velocity in expectant management |
| Adsan[284] | 2002 | No | - |
| Hara[221] | 2002 | No | - |
| Kurek[273] * | 2003 | No | - |
| Martinez-Pinero[279] | 2003 | No | - |
| Patel[265] | 2004 | No | - |

**Table 6.3:** Studies evaluating the clinical utility of PSA RT-PCR in PB.

### 6.6.2.5 BM PSA RT-PCR assay – clinically localised CaP

There are relatively few studies targeting PSA in BM. For patients with clinically localised CaP PSA RT-PCR positivity has been reported between 28% and 71% (table 6.4). We detected marker in 13% of our localised cohort. The studies with the highest detection rates used assays with *in vitro* sensitivities between 10 and 100 fold greater than our PSA assay [208,274,282]. Highly sensitive assays are likely to overstate marker expression and the implied presence of circulating cells. Corey for example detected CTCs in 68% of men with pathologically localised disease using PSA RT-PCR pre RRP [208]. This would predict a poor outcome for 68% of men with localised cancers, assuming all these CTCs are indicative of subsequent clinical treatment failure. This is clearly not the case in clinical practice.

Overdetection by such sensitive assays may be either through amplification of illegitimately transcribed mRNA, unlikely since in both series disease free controls were uniformly RT-PCR negative, or the detection of very small numbers of circulating PECs that are not of any clinical significance. It is well recognised that a significant proportion of CTCs are not viable [286] and of those that are few will form metastases [182]. This limits the utility of such sensitive qualitative assays for molecular staging and prediction of clinical outcome. Indeed, this would suggest that an *in vitro* sensitivity of 1 tumour cell in $10^6$ PBMCs is optimal for RT-PCR molecular staging. However both Wood[207] and Zippelius[287] described assays with comparable sensitivities to ours, yet reported positive RT-PCR results in 45% and 28% of clinically localised cancers which are far higher than our 13%. Of note is that neither of these groups report on disease free BM controls. Prior to setting threshold values to reduce false positives from 14% to 4%, 32% of patients with localised disease in our study were BM PSA RT-PCR positive. Optimising specificity for our assay may have limited its utility in localised disease staging as recurrence rates following RRP are up to 35%, significantly higher than the 13% predicted by this assay.

#### 6.6.2.6 BM PSA RT-PCR assay – advanced CaP

With regard to patients with advanced CaP, our BM assay showed good clinical sensitivity with 80% of men with hormone escaped metastastic disease being PSA RT-PCR positive which compares well with the 56% to 100% reported for metastastic disease [207,208,211,274]. However, only 33% of men with untreated metastases were assay positive compared with 67% of those with hormone escaped CaP but a negative isotope bone scan. A similar but less marked pattern was shown for PB. There are no previous reports of this finding yet the heterogenous clinical status of advanced patient groups in other studies would overlook this. It may be that once CaP cell populations become hormone independent PEC shedding from both primary and metastatic sites becomes a more frequent event and thus single point in time sampling is more likely to detect cells. Although a lower proportion of patients with untreated metastases were RT-PCR positive, the mean PSA mRNA expression when detected was far higher than for non-metastastic CaP. This is difficult to reliably interpret since it may reflect high cell numbers or high PSA mRNA expression in a few cells. The former may in fact be the case since PSA RT-PCR PB status in advanced CaP has been shown to have a significant relationship with disease course and survival [218,271,278]. Thus of those patients with metastases some may have far higher numbers of CTCs and as a result are RT-PCR positive hence their disease would be predicted to run a more accelerated course. Of course it would be expected that all patients with established metastases would have detectable PECs in their BM and it is likely that with repeated or multiple site sampling the proportions of assay positivity for metastatic patients would increase. Melchior *et al* showed that two site BM sampling increased PSA RT-PCR positivity from 57% to 74% compared with a single site [274]. This suggests that smaller numbers of cells may not be detected by our assay but the significance in molecular staging is in identifying the CTCs or CTC load that will make a difference in clinical disease behaviour. Differentiating between those with high and low CTC numbers is probably more significant than identifying every circulating PEC. Long term follow up will clarify matters for this study cohort.

| Reference | Controls | Clinically Localised | Path T1/T2 | Path T3 | Metastatic | Sensitivity |
|---|---|---|---|---|---|---|
| Wood[207] | 0 | 44% | 20% | 65% | 100% | $1:10^6$ |
| Wood[219] | No data | 45% | 29% | 30% | - | $1:10^6$ |
| Melchior[274] | 0 | 56% | 56% | 73% | 86% | $1:10^8$ |
| Corey[208] | 0 | 71% | 68% | 76% | 77% | $1:10^8$ |
| Zippelius[287] | 0 | 28% | - | - | - | $1:10^6$ |
| Deguchi[211] | No data | - | 11% | 57% | 56% | $1:10^6$ |
| Gao[282] | No data | 44% | 49% | 51% | - | $1:10^7$ |

**Table 6.4:** Studies detecting CTCs by PSA RT-PCR in BM.

### 6.6.2.7 BM PSA RT-PCR assay – clinical correlation

Our assay showed increasing rates of BM PSA assay positivity and higher relative PSA expression with advancing clinical stage and could differentiate between the extremes of disease, yet it failed to demonstrate any correlation with established prognostic criteria, pathological stage or margin status in those undergoing radical prostatectomy or disease outcome in the short term. This may reflect insufficient patient numbers within the study groups and reduced CTC detection issues as discussed above. Additionally, prediction of disease outcome and survival is yet to be established through longer term follow up.

The evidence for defining a clinical role for BM RT-PCR in the literature has been, as for PB, conflicting. Wood *et al* in the first BM PSA RT-PCR study demonstrated a correlation between RT-PCR assay status and pathological stage following RRP, 20% of T2 and 65% of T3 cancers being assay positive [207]. They also showed shorter disease free survival following surgery for BM PSA RT-PCR positive patients [219]. Using more sensitive PSA assays, other groups failed to demonstrate a role for BM PSA RT-PCR in predicting pathological stage [208,274,282], although Melchior *et al* did show that positive BM RT-PCR taken at a mean of 4.6 months post RRP was correlated with pathological stage, in that 67% of RT-PCR positive patients had extracapsular disease while this was only 22% for assay negative individuals. This implies that tumour cell shedding is only of significance if the cells

persist once the primary tumour has been excised, which again highlights the need to identify the subpopulation of CTCs that have truly metastatic potential.

None of the previous reports of BM RT-PCR used quantitative techniques. We showed that mean PSA BM expression increased with advancing disease status and also that quantitative data can be used to minimise false positive results. Applying this principle of marker load to the over sensitive yet highly CaP specific assays described above would be one approach to differentiating insignificant PECS from true micrometastases.

### 6.6.3 HK2 RT-PCR Assay

#### 6.6.3.1 HK2 RT-PCR assay – clinical specificity

The HK2 assay recorded positive results in 6% of control PB samples which compares with between 0 and 14% in published series [208,220,222,257-259]. The poorest reported specificity was seen with a very sensitive nested assay displaying *in vitro* LoD of 1 in $10^9$ [259]. Only small numbers of men with clinically localised, locally advanced and untreated metastases were HK2 positive; 10%, 3% and 8% respectively. Higher proportions of patients in the hormone escaped groups were assay positive, being greatest, 71%, as expected in those with established metastases.

#### 6.6.3.2 PB HK2 RT-PCR assay – clinically localised CaP

In localised CaP 10% of men were PB HK2 positive, while reported HK2 positive PEC detection ranges between 13% and 60%. The larger pre-RRP molecular staging studies showed no significant difference in HK2 expression between pT1/2 and >pT2 CaP [257,259], although Slavin *et al* showed a significant relationship between RT-PCR status and node positive cancer (pT1/2 = 23%, >pT2No = 25%, N1=55%) [259].

#### 6.6.3.3 PB HK2 RT-PCR assay – advanced CaP

Our HK2 assay only performed well with high levels of target mRNA. Kawakami *et al* reported similar findings in that HK2 could only be detected in metastatic CaP despite the presence of PSA positive cells in PB from less advanced patients, although they do not report the sensitivities of their assays [258]. However, other studies found that for men with metastatic CaP between 33% and 70% of patients were HK2 RT-PCR positive[222,257,258]. The low positivity rates seen in the current study probably reflect the limited sensitivity of the optimised assay which compares poorly with the 1 in $10^6$ to $10^9$ achieved by others.

The large difference in detection rates between hormone naïve and escaped metastatic cancer is interesting especially since HK2 was also more commonly seen in the hormone escaped bone scan negative group than in those with newly diagnosed metastases. This is similar to the pattern seen with the PSA and PSMA assays. The confirmation of patterns of PEC detection seen using these other markers adds weight to the conclusions drawn with regards CTCs in disease progression. As CaP progresses to hormone independence, CTC numbers increase.

#### 6.6.3.4 PB HK2 RT-PCR assay – overview

As an independent marker in PB, the HK2 assay does not appear to have a great deal to offer over and above the PSA assay. For example, in advanced patients, where the bulk of HK2 positive cells were detected, all HK2 positive samples were also PSA positive. Compared to PSA RT-PCR, the clinical sensitivity of HK2 was lower as would be expected from their respective *in vitro* sensitivities and overall rates of marker detection were far lower. A number of other studies have evaluated PB samples with both PSA and HK2 RT-PCR [220,222,257,258]. In metastatic cancer PSA was detected more often than HK2 by all but Ylikoski *et al* who found similar results using both markers [222]. Similar findings were reported for clinically localised cancer with the exception of Shariat *et al* who found HK2 to be a better predictor of occult lymph node involvement than PSA [220].

In this study the only lack of concordance between PSA and HK2 results was in three patients with localised or locally advanced CaP that were $HK2^+/PSA^-$. This

was unexpected given both the greater sensitivity of the PSA assay and the evidence that HK2 expression is generally lower than PSA expression in prostate cells [288]. This pattern of mRNA detection may however simply reflect the phenotype of the CTCs in these patients which may be basal or endocrine and thus not PSA expressing. Others have reported this finding but its relevance to clinical outcome is yet to be established [208,222].

#### 6.6.3.5 BM HK2 RT-PCR assay – overview

In BM, the HK2 RT-PCR assay was highly specific, all disease free controls being negative. However, its reduced *in vitro* sensitivity compared with PSA was reflected in only 60% of hormone escaped metastatic cancers being HK2 assay positive. For these most advanced patients all HK2 positive samples were also PSA positive and the one patient that was $PSA^{+}/HK2^{-}$ had very low PSA expression. Low detection rates were apparent in assaying BM from patients with localised and locally advanced cancers with only 8% and 16% being positive in each respective group. The majority of these HK2 positive BM samples were also PSA assay positive but as in PB three samples from localised and locally advanced patients were $HK2^{+}/PSA^{-}$.

Of note is that no patients with hormone escaped non-metastatic cancer were HK2 positive in their BM samples while 67% of these were PSA positive and also 17% of patients in this group were HK2 positive in PB. This may have been a sensitivity issue since the mean PSA expression in this group was far lower than for patients with metastases and it would be expected that HK2 expression would also be lower and conceivably below the limit if detection of the HK2 assay. Sensitivity would also explain the low CTC yields in the less advanced cancers but the reasons for differences between PB and BM HK2 results remain unclear. Additionally, 44% of men with untreated metastases were HK2 BM positive. This is a pattern not seen with the other markers or with HK2 in PB, where fewer patients were assay positive in this group than in the hormone escaped non-metastatic cancers.

Corey *et al* are the only other group to target HK2 in BM using an assay which was significantly more sensitive *in vitro* than this assay, LoD 1 PEC in $10^7$ background

cells [208]. They detected HK2 positive cells in 41% of clinically localised CaP (38% pT2, 48% pT3) and 39% of advanced disease showing limited clinical sensitivity although the advanced group was heterogenous and not purely metastatic cancer. Fifty percent of men with metastases were HK2 BM RT-PCR positive. As in our study significantly more BM samples were assay positive in each clinical group using a more sensitive PSA targeted assay, and when considering both markers concurrently only 13% of assay positive patients were positive for HK2 alone.

Additionally, they compared HK2 in PB with BM, showing assay positivity for localised patients of 13% PB and 41% BM and in advanced cancer 31% and 39% respectively. We found that cell yield was lower in BM than PB for localised and all hormone escaped CaP while the converse was seen in locally advanced and untreated metastases.

Therefore the utility of our HK2 BM assay in localised disease was clearly limited by its poor sensitivity with only small numbers of positive assay results. HK2 does appear a reliable specific marker in BM yet as in PB it is questionable as to whether independently it adds significantly to PSA RT-PCR staging as few patients were $PSA^{-}/HK2^{+}$. Larger studies will be required to study this sub-population.

### 6.6.4 PSMA RT-PCR Assay

#### 6.6.4.1 PSMA RT-PCR assay – clinical specificity

The PSMA assay showed acceptable sensitivity *in vitro* however its clinical performance was marred by poor specificity and the need to apply exclusion limits to the data which was particularly high for BM analysis at 30 cycles. The specificity issue of this PSMA assay is difficult to explain in an assay with an *in vitro* sensitivity of $1:10^5$ which is at best comparable with the $1:10^4$ to $1:10^6$ achieved by other workers who have not reported so many false positive results [192,277,289]. The detection of large amounts of illegitimately transcribed mRNA is unlikely given the LoD of the PSMA assay and thus the prostate specific nature of the marker is brought into question. The majority of published PB PSMA studies show good prostate specificity [62,192,221,257,268,277,280,290,291], yet there are significant exceptions,

Gala *et al* detected PSMA in 84% of controls [212] and Lintula *et al* in 92% [266]. Both explain these findings as the result of illegitimate transcription detected by nested assays although these assays were no more sensitive than those which were CaP specific.

Only two other groups have targeted PSMA in BM, Zippelius *et al* [287] detected PSMA in 44% of controls while Koutsilieris *et al* [292] reported no false positive results in 20 BM controls. It is recognised that PSMA is produced by non-prostate tissues (section 1.3.2.2) and it has also been detected in $CD34^+$ leucocytes [293] which is particularly relevant for CTC protocols. Why there is such variability in specificity between assays is unclear but the large amount of variation in methods and assay design are strong contenders.

#### 6.6.4.2 PB PSMA RT-PCR assay – clinical performance

Once satisfactory specificity was established, 20% of PB samples from the localised group remained assay positive. In patients with more advanced disease PSMA expression was high in those with non-metastatic hormone escaped cancer, far more so than in untreated metastases, a pattern that was also seen when targeting PSA and HK2 in PB. This may again reflect active cell shedding in the hormone escaped state although it might be expected that patients with hormone escaped metastases would also have significant numbers of PSMA positive CTCs as was seen with PSA and HK2.

A number of groups have targeted PSMA in PB (table 6.5). Detection of PSMA in patients with advanced disease was higher in all of these studies, ranging between 39% and 91%, than the 24% of assay positive PB samples from our entire advanced cohort. As noted above the non-metastatic hormone escaped subgroup showed PSMA expression in 50% of samples. Despite this limited clinical sensitivity, the 20% of PSMA positive patients with clinically localised CaP falls within the large range, 5% to 72%, reported previously. No correlation with clinical outcome was shown with the short term follow up of these patients. Only three previous studies have shown any correlation between PSMA PB status and outcome. Interestingly all three also found PSMA to have greater clinical sensitivity than PSA. Okegawa *et al* [291] found PSMA RT-PCR status to be a predictor of

disease recurrence following RRP, while the two publications from the Cleveland Clinic, Zhang *et al* [277] and Grasso *et al* [290] correlated combined PSMA/PSA RT-PCR status with pathological staging.

#### 6.6.4.3 BM PSMA RT-PCR assay – clinical performance

For BM, 11% of samples from the localised group remained positive. The data was more informative for advanced disease. Fifty percent of metastatic patient BM samples were positive while the group with the highest proportion of assay positive samples was hormone escaped non-metastatic cancer, although the relative expression in this group was lower than for those with metastases. There was a strong correlation between PSA and PSMA expression in BM from these patients with 73% of the PSMA positive samples also being PSA positive.

Only Koutsilieris *et al* [292] have evaluated PSMA RT-PCR BM samples from CaP patients, with positive assay results in all patients with untreated metastases. For clinically localised disease at least 29% were PSMA RT-PCR positive although this may have been higher since any patient that was not also PSA RT-PCT positive was excluded (details not reported).

Overall, our PSMA RT-PCR assay showed some interesting trends in the advanced patient groups in both PB and BM, while for molecular staging of clinically localised disease its utility was more limited particularly in BM. Long term follow up data is awaited but at this time little else beyond these trends can be implied or concluded from this assay.

| Author | Year | Controls | Clinically Localised | Path Localised | Path Locally Advanced | Advanced CaP |
|---|---|---|---|---|---|---|
| Current study | 2004 | 13% | 20% | - | - | 24% |
| Israeli [62] | 1994 | 5% | - | 72% | 60% | 86% |
| Cama [268] | 1995 | 0 | - | 24% | 33% | 50% |
| Eschwege[242] | 1995 | NR | 21% | - | - | - |
| Loric [280] | 1995 | 0 | 44% | 35% | 60% | 85% |
| Sokoloff [192] | 1996 | 11% | - | 16% | 22% | 39% |
| Lintula [266] | 1997 | 92% | 57% | - | - | 82% |
| Zhang [277] | 1997 | 0 | 23% | - | - | 91% |
| Gala [212] | 1998 | 84% | - | - | - | 84% |
| Grasso [290] | 1998 | 0 | - | 35% | 78% | - |
| Okegawa[291] | 1998 | 0 | - | 33% | 66% | 91% |
| Hara [221] | 2002 | 3% | - | 5% | - | 64% |
| Kurek [257] | 2004 | 5% | | 59% | 63% | 90% |

**Table 6.5:** RT-PCR studies targeting PSMA in PB. (NR = not reported)

### 6.6.5 PSCA RT-PCR Assay

Despite its promising reported expression profile in CaP [68,294], we found no role for PSCA in RT-PCR molecular staging as its expression was not prostate specific. PSCA mRNA was detected in high proportions of all patient and control PB and BM samples and expression levels were comparable throughout the groups.

The only reported experience of PSCA RT-PCR for CTCs in CaP showed complete CaP specificity, while detecting PECs in 50% of patients with advanced cancers but not in patients with localised or locally advanced disease [221]. Their PSCA assay was nested which generally improves specificity and may explain the discrepancy with our results. One non-prostate CTC study targeted PSCA in gastrointestinal tumours again using nested RT-PCR. No positive assays were

seen in PB from healthy volunteers although 20% of samples from patients with non-malignant conditions were positive [295].

PSCA expression is well recognised in other cancers including renal, bladder, ovarian, pancreatic and gastrointestinal. Additionally, it is expressed in normal prostate, urothelium and kidney indicating that it is not entirely prostate specific. Since the nature of our amplified assay product was confirmed through sequencing, and blast searches found no candidates for cross reaction it can only be concluded that PSCA mRNA must be expressed at detectable levels by the cellular elements of PB and BM thus limiting its utility as a target for molecular staging.

### 6.6.6 $DD3^{PCA3}$ RT-PCR Assay

$DD3^{PCA3}$ was entirely CaP specific. However, we were unable to demonstrate adequate clinical sensitivity for it to be used as a stand alone marker for RT-PCR based molecular staging using PB and/or BM. *In vitro*, its sensitivity was 1000 fold less than that of the PSA RT-PCR assay, while *in vivo* only 8 individuals had detectable $DD3^{PCA3}$ expressing cells in either PB or BM samples.

There are a number of possible explanations for these findings. The expression of $DD3^{PCA3}$ and its upregulation in primary cancers is clearly established. However there is only sparse data relating to its expression in metastases. Although Bussemakers *et al*, in their initial description of $DD3^{PCA3}$, demonstrated marker expression in CaP metastases, they evaluated only 4 samples, and the numbers of prostate cancer cells present in such samples are likely to be far higher and in greater concentrations than can be expected in circulating cell studies [69]. Indeed in metastases, the relative expression of $DD3^{PCA3}$ compared to other prostate markers and the average expression per cell remains unknown. This may have a significant bearing on $DD3^{PCA3}$ detection in the small dilute cell populations targeted in molecular staging protocols.

Additionally, we demonstrated that the relative expression of $DD3^{PCA3}$ was significantly lower than that of PSA in LNCaP cells and this may also be similar in circulating PECs. This may reflect sensitivity limitations of the assay design/setup,

since the $DD3^{PCA3}$ amplicon is significantly larger than that for PSA and therefore PCR amplification will be less efficient. Alternative assay designs incorporating exons 1 to 3 lacked prostate specificity. If however $DD3^{PCA3}$ is only expressed at extremely low levels by tumour cells then improving marker detection and thus the utility $DD3^{PCA3}$ will require alternative methodological approaches.

The only other study utilising $DD3^{PCA3}$ as a target for circulating cell detection in CaP evaluated blood samples from 63 men with CaP and 22 with BPH [260]. Prior to prostate manipulation they could detect no $DD3^{PCA3}$ target in any samples, but following treatment (Prostatectomy, brachytherapy, thermoradiotherapy, TURP) blood samples from 46% of CaP and 9% of BPH patients were $DD3^{PCA3}$ positive. Thus in this patient cohort, the only detectable circulating $DD3^{PCA3}$ expressing cells were shed iatrogenically, not through the metastatic process.

The utility of $DD3^{PCA3}$ targeted RT-PCR techniques in urine sediments is promising, primarily in the diagnostic setting [71,296]. Moreover, Mulders *et al* have recently suggested its potential as a prognostic predictor, demonstrating a correlation between urinary $DD3^{PCA3}$/PSA mRNA ratios and Gleason score of subsequently biopsy diagnosed CaP [297]. However, prostate cells present in the urine following prostatic massage or in the circulation following prostatic manipulation have been shed mechanically or iatrogenically and not by the stepwise metastatic process [298]. These are likely to represent an entirely different cell population, with different characteristics, metastatic potential and molecular marker expressions compared to true micrometastases. The cells of interest in molecular staging are locally invasive, have altered adhesion and motility characteristics and have undergone migration into the circulation. Further, only a small proportion of cells that have undergone intravasation will ultimately go on to form metastases [182]. It is the $DD3^{PCA3}$ expression in these cells which is the key to the potential of this marker.

### 6.6.7 Quantitative RT-PCR

Quantitative RT-PCR has only been used in a small minority of RT-PCR studies. The first report by Sokoloff *et al* using $^{32}P$ labelled PCR hinted at its potential by differentiating quantitatively between established metastatic and clinically localised

CaP [192]. It was envisaged that quantitative data would allow identification of clinically significant CTCs thus addressing the over-detection previously reported using very sensitive assays. However we found that even with modest assay *in vitro* performance, specificity was a problem and it was here that qRT-PCR was essential. Excluding low levels of marker expression allowed establishment of acceptable assay specificity. In addition, qRT-PCR showed higher expression of the markers PSA, PSMA and HK2 in advanced CaP compared to controls and men with localised and locally advanced disease, yet the quantitative data was not able to differentiate more accurately between or within groups. Similar findings were reported by Patel *et al* using a PSA qRT-PCR assay with a LoD of $1:10^6$ [265]. They experienced poor assay specificity with 67% of control PB samples being RT-PCR positive and although PSA expression was slightly higher in patients with localised CaP they could not statistically differentiate between these groups. In metastatic cancer, as in our study PSA expression was significantly higher than in PB samples from both controls and localised cancer.

In the largest quantitative RT-PCR study Kurek *et al* evaluated 115 men with clinically localised CaP prior to RRP and concluded that quantifying PSA mRNA could not differentiate between different pathological disease stages [273]. Unfortunately controls and patients with metastases were only evaluated qualitatively thus it is not possible to fully assess this quantitative assay's potential. Only one group have been able to use qRT-PCR to successfully predict pathological stage [223]. Using PSA qRT-PCR in a real time assay the mean number of PSA mRNA copies detected were 16 for controls, 293 for pT2 CaP and 2221 for >pT2 CaP. This was the only qRT-PCR study to use real time PCR for quantitation and is therefore methodologically similar to our assay. Despite this we were unable to replicate this molecular staging which again highlights the impact of the details of primer and assay design on clinical performance.

### 6.6.8 The Utility of Multiple Marker RT-PCR

The rationale for multiple marker sample evaluation was to maximise CTC yield and to identify patterns of marker expression predictive of clinical outcome. Since this had not been described previously there were a number of issues in achieving these aims that only became apparent on analysing the data. The problems

encountered in each individual assay would be multiplied when attempts were made to combine them, particularly assay specificity and sensitivity. Based on the literature it had been assumed that each marker assay would be highly CaP specific and that each assay would achieve a LoD of at least 1 tumour cell in a background of $10^6$ cells. However, the PSCA assay data had to be excluded due to poor marker specificity and thresholds were applied to PSA, PSMA and HK2 data to minimise marker detection in the control groups.

The next issue was how best to combine the markers. If using purely the qualitative data there were two simplistic approaches that have been used in other multi-marker studies. A patient could be considered to have CTCs present either if any of the four marker assays was positive thus maximising cell detection [277] or only if they were positive for all four markers, optimising specificity [208,257,299]. The former approach in PB made little difference to cell yield for patients with advanced CaP as would be expected since this depended upon the clinical sensitivity of the single best performing assay, i.e. PSA. However, compared to targeting PSA alone, CTC positivity increased from 23% to 35% and 24% to 39% for localised and locally advanced cancers respectively. Unfortunately 24% of control samples were RT-PCR positive when the same calculation was applied. In BM, again CTC detection in control samples increased yet only to 14%. Small improvements in cell yield were seen in advanced patients while it increased from 13% to 29% for localised and from 12% to 40% for locally advanced CaP when compared to PSA alone. Zhang *et al* improved CTC detection in clinically localised CaP from 13% and 23% with PSA or PSMA alone to 29% when combining the two assays [277]. Their assays were completely CaP specific hence they did not have to consider the impact of combining markers on false positive results.

The second approach of marker combination was not of any practical benefit for our data as it depended upon significant cell yields for all marker assays and it was clear that the HK2 and DD3 assays detected CTCs in very few patients. This would exclude large numbers of PSA and PSMA positive patients. Therefore it would only be applicable to a data set with significant over-detection in a localised disease cohort. Additionally it assumes that all significant CTCs will express all prostate molecular markers. It is well recognised that marker expression in CaP

cell populations is heterogenous and this has also been shown in CTCs [226,300], thus significant micro metastases may be excluded if only a subpopulation of the circulating PECs are appreciated.

When the requirement for both PSA and PSMA to be positive was applied to our data, all controls were negative. Presumably, it is very unlikely to have illegitimate transcription of two mRNA species in a single sample. In the clinical groups CTC detection in PB fell, compared to PSA alone, in all groups other than non-metastatic hormone escaped, to 13% localised, 0% locally advanced, 8% untreated metastases and 14% metastatic hormone escaped. In BM, detection fell in all groups to 0% localised, 4% locally advanced, 50% non-metastatic hormone escaped, 33% untreated metastases and 40% metastatic hormone escaped. Far better clinical sensitivity would be required by all markers to practically utilise this approach.

Mitsiades *et al* successfully used this approach when targeting PSA and PSMA, and maintained CTC detection in 35% of PB and 26% of BM samples in men undergoing RRP [299]. Combined PSA and PSMA RT-PCR positive status was shown to be an independent predictor of disease free survival. Both marker assays were nested and were therefore likely to be highly sensitive, although the exact *in vitro* sensitivities are not reported. Since, the individual assay positivity rates are not published, any overdetection using the single marker formats and subsequent reduction in this achieved by the combined marker approach cannot be evaluated.

The advantage of this approach to marker combination was also demonstrated by Corey *et al*. Their individual assays were clearly detecting false positive results in localised CaP BM samples since, 71% were PSA positive and 41% HK2 positive [208]. Requiring both to be positive reduced significant marker detection to 30% and improved sensitivity in detecting extracapsular disease.

Conversely, Kurek *et al* found when targeting PSA, PSMA and HK2 in PB, that none of the markers either individually or in any combination could predict

pathological stage [257]. Positive assay yields in men with clinically localised disease fell from 35% for PSA alone to 7% when combining all three markers.

When considering quantitative data the combination of markers becomes more complex. Quantitative values for each marker cannot simply be added together to produce a single global figure as patterns of individual marker up and down regulation would be lost, hence overlooking one of the potential benefits of multimarker RT-PCR. Marker expression profiles may be the key to useful RT-PCR molecular modelling and therefore all the elements of the data must be maintained during analysis. There is to our knowledge no appropriate mathematical model to apply to this type of data. Developing such a complex statistical tool would require large amounts of reliable data from extensive well characterised clinical groups all with long term clinical follow up. Additionally, each assay would have to be entirely reliable and robust. Even if these were achieved, issues of sampling and CTC dynamics would remain, for example, differentiating between cells with metastatic potential and those that will be cleared in the same individual. The data presented here could not be successfully used in this regard since the clinical groups were small, there were only small amounts of positive data and there were issues of both sensitivity and specificity of the assays.

### 6.6.9 Combining PB and BM RT-PCR

In designing this study, the use of combined PB and BM sampling had been expected to improve CTC yield compared to either medium alone. Combined data could not be compared directly to the results for PB or BM individually since paired samples were only available from 85 patients in the study group. Additionally, the majority of the control BM samples did not have a corresponding PB sample which made the control data unreliable in this setting considering the specificity issues highlighted previously.

When the four markers PSA, PSMA, HK2 and DD3 were combined BM was more often positive than PB in the locally advanced, non-metastatic hormone escaped and the untreated metastases while the converse was true for localised and hormone escaped metastatic CaP. For PSA alone, PB was more often RT-PCR positive than BM for most groups but not every BM positive patient was also PB

positive. Indeed, only small numbers were $PB^+/BM^+$ although this was the case in 80% of men with metastatic hormone escaped CaP. This suggests that the presence of PSA positive cells in both PB and BM is indicative of more advanced/aggressive disease. Since the overall numbers of such patients were small, no significant conclusion could be drawn in this regard, although it is an issue worthy of further study.

As expected, regarding a positive assay in either PB or BM as an overall positive result generally increased positivity rates when considering a single marker alone or when combining the four useful markers, although the changes were small and made no impact on the clinical significance of the RT-PCR assays.

It would be expected that if a patient has tumour cells in their BM then these should also be present in PB since metastasis is a stepwise process. Only three RT-PCR studies have used paired sampling [208,274,299]. Mitsiades *et al* found in men with clinically localised CaP that PB was more often combined PSA-PSMA positive than BM and that if CTCs were present in BM then they were always also detectable in PB [299]. Conversely Melchior *et al* [274] detected PSA in 56% of BM but only 16% of PB in men with pathologically localised CaP and similarly Corey *et al* [208] detected both PSA and HK2 more often in BM than PB samples. It has been suggested that this high cell yield from BM reflects the propensity of CaP to preferentially metastasise to bone and similar findings have been seen in breast tumours and neuroblastoma, tumours which, like CaP, typically form bone metastases [301,302].

The variability in RT-PCR PEC detection in PB and BM described here may reflect heterogenous tumour cell distribution, different cell marker expression in PB and BM CTCs and sampling issues in a dynamic system. Therefore combined PB and BM sampling increased overall CTC yield. However, the lack of reliable comparative controls and the problems described previously relating to the individual assays and their potential in a multimarker analysis prevented any further conclusions being drawn.

## 6.7 Micro Fluidic RT-PCR Discussion

RT-PCR molecular staging techniques have not become established in the clinical setting. The reasons for this have been discussed previously (section 6.1) and reflect the complex methodologies utilised by such molecular tools which are typically labour intensive, expensive and show poor reproducibility. Micro fluidic RT-PCR in principle addresses some of these issues. The micro fluidic RT-PCR cards used here provided a high throughput system to concurrently evaluate multiple samples for the expression of multiple molecular markers.

Many of the potential sources of error present in standard assays were eliminated by using industry designed and optimised assays which were commercially embedded onto a carrier card. A single aliquot of the clinical sample in mastermix was the only manual step with potential for error in setting up a reaction plate. All markers were evaluated from the same input target sample aliquot thus minimising inter-well variability that is a potential problem when using a 96 well format. Good reproducibility between wells was generally seen using the microfluidic cards, although at low target concentrations only a proportion of the wells targeting each marker would be RT-PCR positive.

Assay sensitivity and its performance at the limits of detection are of critical importance when RT-PCR is used to detect the presence of circulating tumour cells since the target mRNA molecules are inevitably in a vast background of non-target mRNA. When compared directly to the 96 well RT-PCR assay designed in the laboratory, using four markers that were common to both, the micro fluidic assay was at least 10 fold less sensitive in detecting LNCaP cells spiked into female PB. The 96 well format was also more clinically sensitive, detecting circulating cells in far more PB and BM samples than the micro fluidic assay. Differences in sensitivity reflect assay design and the reaction efficiency of the primers and probes used. The micro fluidic assay used probes for product detection for all targets while SYBR Green was used for PSA, PSMA and PSCA in the 96 well assay. During the development of the latter, probe incorporation was found to have a significant impact on assay sensitivity and this may well have played a part here. Interestingly, the HK2 assays used probes in both formats yet

the 96 well format was still 10 fold more sensitive *in vitro* and also clinically more sensitive for PB although they were comparable for BM samples.

Clinically, there was a closer correlation in results when BM samples were evaluated compared to PB. The reasons for this are somewhat unclear and this is confounded by the fact that cell spike sensitivity experiments were not performed using BM. Since CaP preferentially metastasises to bone then there are likely to be higher concentrations of target PECs within the BM, particularly in patients with established bone metastases. Therefore the patient samples evaluated here, which came mainly from a population of patients with clinically advanced or metastatic CaP, may have simply had more target present than in their paired PB samples. The amounts of target mRNA in the PB samples being lower, would have been closer to or beyond the clinical sensitivity of the micro fluidic assay, which was shown to be less sensitive than the 96 well format *in vitro*.

There are technical considerations which could potentially improve micro fluidic sensitivity. 100ng of input cDNA were used in the micro fluidic experiments for all 48 reactions fed by each loading reservoir, while in the 96 well assay 100ng was used for each reaction. The 96 well technique sampled the cDNA solution separately for each reaction while the micro fluidic assay sampled it once per patient sample for all markers and all replicate reactions. The theory of such sampling problems are discussed in section 6.4.1. Improvements in circulating cell detection could also be achieved through non-target mRNA depletion of the samples, for example globin depletion, which removes a significant proportion of the background mRNA 'noise' [303].

Typically, RT-PCR is a balance of sensitivity against specificity as was discussed for the development of the 96 well assay. The micro fluidic assay generally appeared to have better specificity than the 96 well assay although far fewer control samples were evaluated and therefore its specificity was not assessed as stringently. Good clinical specificity allows far easier and clearer interpretation of positive assay results yet if this is at the expense of sensitivity then the clinical utility of the technique may be limited since the desired role for RT-PCR is in

detecting clinically significant micro-metastatic disease in patients with clinically localised cancer.

A potential role for the micro fluidic RT-PCR format in the research setting is in the evaluation of novel markers as the technique allows fast set up and simultaneous multimarker assay analysis. Here we studied the six novel markers EZH2, uPA, HK4, HK15, prostasin and hepsin. EZH2 and uPA showed poor prostate specificity being detected at similar levels in control and clinical samples. Thus the high frequency of expression in clinical samples cannot be reliably interpreted in terms of circulating cell status. Although prostate tissue expression of both of these markers has been shown to correlate with a more aggressive disease course, we have shown that the proteins coded for by the target mRNA are not specific to prostate cells and thus limit their utility in circulating cell studies.

Prostasin was more prostate specific in keeping with previous reports showing no expression in leukocytes [78]. Generally, prostasin was detected in BM in men with advanced cancer, rather than those with clinically localised disease. It has been shown by other workers that prostasin is down regulated in more advanced CaP cell lines and in metastatic and hormone escaped cancers, and its expression is associated with a less aggressive phenotype. Our data suggests quite the opposite although the study group was limited.

Hepsin also showed reasonable clinical specificity. When detected, it did not appear to preferentially select a particular clinical group, however it was only seen in a small number of samples. Work with primary prostate tumours shows higher hepsin expression with high tumour Gleason grade and thus potentially an association with aggressive disease behaviour [74,76]. There are no reports of its expression in metastases nor in the role it plays in the metastatic pathway thus little can be predicted with regards its expression in micro-metastases.

Human Kallikreins 2 and 3 have been extensively studied in prostate cancer and of the novel markers targeted here HK4 and HK15 may be the most interesting for further evaluation. Firstly their expression was entirely prostate specific. Both appeared to identify BM samples from men with the most advanced CaP. These

samples were also all RT-PCR positive for other targets suggesting the presence of high numbers of circulating cells. Neither of these markers has been studied in CaP circulating cell studies. Prostate cell line and tissue work suggests that HK4 is downregulated while HK15 is upregulated with more aggressive phenotype [58-60]. Expression in metastases remains to be determined. The data presented here suggests that both are expressed in circulating cells in advanced CaP.

This preliminary evaluation of micro fluidic RT-PCR used a small group of patients of whom a significant proportion had advanced disease as well as a limited number of controls. Defining the role of prostasin, hepsin, HK4 and HK15 in circulating cell molecular staging of prostate cancer requires a far larger study population and long term clinical outcomes. These four markers, particularly the kallikreins with their prostate specific nature, do warrant further investigation.

Despite the limitations of its sensitivity, micro fluidic RT-PCR technology does have significant practical advantages over both traditional and real time techniques. The simple assay set up minimises sources of methodological error and greatly reduces labour, time and cost. All of these elements are important considerations in the development of a technique and in defining its utility.

### 6.8 Limitations of RT-PCR

From the above discussions it is clear that RT-PCR can be used to detect circulating prostate epithelial cells and that generally, higher rates of assay positivity and higher levels of marker expression are seen with advancing disease stage. Where the uncertainty lies is in predicting the metastatic potential of these cells. Circulating tumour cells display favourable characteristics with regards to loss of cellular adhesion, motility, invasion and intravasation yet metastasis formation at a distant secondary site will only be achieved by a tiny proportion of these cells. Recent reviews of our understanding of metastasis highlight the complexity of the process [304-307]. Additionally, significant numbers of detected CTCs may not be viable. Larson *et al* found that in PB from men with metastatic CaP only 17% of CTCs detected by flow cytometry and fluorescence microscopy

were intact cells [286], and Mehes *et al* reported similar findings in PB for breast cancer patients [308].

Therefore it remains unclear whether the presence of circulating cells alone can be predictive of clinical outcome and metastasis formation. A larger population of CTCs would be more likely to contain cells with all the required characteristics for colonisation of a distant site and the association of higher marker expression with advancing disease stage is suggestive of larger CTC numbers. Using RT-PCR this is implied rather than unequivocally determined. Even quantitative RT-PCR methodology is unable to accurately determine circulating cell numbers or identify their nature as it simply measures the overall marker expression within a population of cells. Additionally, the selection of molecular markers suitable for RT-PCR CTC detection are not necessarily those which reflect the gene expression patterns which are important in cell survival and metastasis formation. Hence the sensitivity advantage of RT-PCR over other methods of CTC detection may be outweighed by its limitations in determining the nature of these cells.

### 6.9 Conclusions and Future Directions

In evaluating quantitative multi-marker RT-PCR in PB and BM to detect CTCs in men with CaP we have demonstrated that this is a potentially valuable approach to molecular staging. The complex nature of RT-PCR methodology has been highlighted and the utility of our technique has been limited by technical challenges both novel and those previously reported.

Issues of assay sensitivity and specificity limited CTC yield particularly in patients with clinically localised CaP who potentially have the most to gain from accurate disease staging. We have been able to show trends of increasing marker expression with advancing disease stage and interesting patterns of expression in patients with advanced disease relating to hormone responsiveness yet small patient numbers limited more conclusive results. In retrospect, given the limited clinical sensitivities of many of the assays more might have been gained from concentrating on men with advanced CaP and increasing the size of this cohort. However, only the long term follow up of our current patient cohort will identify any

correlation between RT-PCR profile, disease progression and clinical outcome for each patient group.

The novel elements utilised in this RT-PCR study inevitably produced large amounts of data and identified problems relating to data manipulation and analysis. It is clear that if such a complex methodology is to be definitively evaluated then highly robust assays using large patient cohorts will be required to produce reliable data for statistical modelling. Only then will it be possible to conclusively determine the technique's clinical utility.

Our results again confirm the potential of PSA and HK2 as molecular targets. PSMA and $DD3^{PCA3}$ marker performance was limited but this may well reflect assay design rather than unsuitable molecular marker characteristics of these genes and they both warrant further investigation. We cannot recommend PSCA as a target for RT-PCR staging as it was not CaP specific. Additionally we have identified hepsin, prostasin and human kallikreins 4 and 15 as suitable markers for RT-PCR CTC detection and their potential should be evaluated further in larger studies.

We have also demonstrated the advantages of micro fluidic RT-PCR for reliable high throughput molecular staging. Although there are issues of sensitivity with the assays studied, the technique is suited to high volume investigational work and more importantly is easily transferable to commercial and healthcare laboratories if a clinical role is identified for RT-PCR staging.

RT-PCR remains a promising technique for studying CTCs in CaP yet defining its role in clinical practice has been hampered by methodological complexities and inherent technical limitations. Advancing RT-PCR technologies combined with novel molecular targets have identified potential solutions. Future directions are likely to target multiple established and novel markers and evaluate samples from large study populations necessitating high throughput reliable techniques. Additional steps such as globin depletion or epithelial cell enrichment may improve clinical performance [303,309], while the concurrent use of whole CTC extraction techniques may allow further studies, such as cytogenetics, to detail the nature of

detected cells [310,311]. The approaches to molecular staging described here including quantitative techniques, multiple marker targeting, multiple site sampling and micro fluidic RT-PCR may all play a part in developing RT-PCR into a useful clinical tool with the ability to predict the metastatic potential of circulating tumour cells.

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