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Chapter IX

Integrating Molecular, Proteomic and Vaccine Development, Quality of Life and Psychometric Evaluations in a Comprehensive Research Program

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Abstract

Recent research in prostate cancer has involved recruitment of a wide range of professional expertise in implementing novel prostate tumor developments and treatments in the clinical setting. In addition, these collaborations have enabled more comprehensive evaluations to have been undertaken of the effects of established therapeutic strategies to reveal previously unrecognized adverse effects on patients. In this chapter, we will detail a number of recent research findings from the scientific literature together with our studies directed to addressing deficiencies perceived in current management by patients and local clinicians.

With reference to our early diagnosis and treatment programs, the cross-fertilization benefits from an integration of molecular, proteomic and vaccine development, quality of life and psychometric evaluations will be illustrated.

Introduction

Prostate cancer is a unique malignancy. Its epidemiology and high prevalence in the western world, clinically silent progression, unpredictable yet usually long natural history together with problematic treatment-related effects with current therapies, provide many opportunities, through interactions by seemingly disparate disciplines, to improve patient care.

Surgery and radiotherapy in their various forms (as open and laparoscopic radical prostatectomy, external beam radiotherapy and brachytherapy) constitute the most commonly used forms of therapeutic intervention for clinically localized disease. All are generally regarded as effective interventions for achieving local control but, in spite of continuing refinements, each continues to have its own significant morbidity profile manifested by serious consequences in subsets of patients. Since the most effective treatment for clinically localized disease remains to be determined, patients need to select the side-effect profile most acceptable to them in choosing a treatment from those available which, in the case of radiotherapy, now usually involves androgen suppression for a variable time. It is pertinent to appreciate that, if currently available treatments had minimal morbidity profiles, close observation would rarely be considered as an option and much of the prevailing decisional conflict associated with treatment selection would cease to exist.

Important aspects that we have focused on in our research endeavors relate to a more sensitive and specific means of detection, the impact of both diagnosis and treatments (including androgen suppression therapy) on patients psychologically as well as alternative forms of systemic therapy. The topics addressed below result from recognized deficiencies with contemporary management and a recognized potential to improve patient welfare by harnessing a variety of expertise in a cross-city-multidisciplinary-spirit of collaboration in an Australian environment.

Diagnosis

1. Current Clinical Status

(a) Disease Burden

Prostate cancer is the most common male internal malignancy diagnosed in the western world: in 2003, an estimated 220,900 American men were diagnosed with this disease [1]. Although death rates from prostate cancer are decreasing [2], it was estimated that 30,200 men in the United States died from this malignancy in 2002. The annual potential years of life lost due to prostate cancer in Australia have been estimated to be 6,660 [3].

However, disease-related mortality figures significantly underestimate the adverse impact of this condition on the well-being of patients. In particular, the oft quoted line that "more men die with this malignancy than from it" misleads, falsely implying an innocuous nature. Nevertheless, that many men with prostate cancer do not ultimately die from it, their demise attributed to another condition, raises other considerations. These include the importance of discriminating potential life-threatening from slowly growing or clinically insignificant prostate cancer, possible contributions of prostate cancer to other conditions and the ability/inability to more appropriately rank competing morbidities and causes of mortality in a changing environment in terms of therapeutic interventional successes and lengthening of patients' lives.

It is easy to underestimate the considerable morbidity that prostate cancer and its various treatments exact from middle aged and elderly men, the magnitude of unwanted effects and costs escalating with advancing stage of disease [2]. However, with our present state of clinical management, more aggressive attempts to diagnose prostate cancer indiscriminately

will expose many patients with clinically insignificant disease to treatment-related morbidities which they could otherwise very reasonably avoid. Thus, the need for research to address these issues is urgently required. Consequently, this malignancy, together with the inestimable morbidity from treatment and the interactive factors briefly alluded to above, constitute a major health problem [3].

(b) PSA/TRUS biopsy Approach

The widespread utilization of prostate specific antigen (**PSA**) and recognition that elevated levels in serum precede clinical evidence of prostatic disease has permitted earlier identification of prostate cancer, in particular. However, not all prostate cancers are associated with an elevated serum PSA with normal levels found, albeit uncommonly, in patients with more aggressive, dedifferentiated tumors [4].

It is well accepted that PSA is not a test for prostate cancer per se, with elevated levels also associated with prostatitis and benign prostate hyperplasia (**BPH**). The proportion of patients with prostate cancer varies depending on the population studied but, even though the large majority of men diagnosed currently present with serum PSA levels between 4-10 ng/ml [5] in the absence of any other discernable abnormality, most will not have cancer detected, even when multiple transrectal ultrasound (**TRUS**)-guided biopsies are taken [6-9], with biopsies repeated if suspicion of an undetected malignancy is high [5].

A dilemma facing clinicians not uncommonly is the patient whose biopsies fail to contain prostate cancer but for whom the level of suspicion that prostate cancer is present remains high. Djavan et al (2001) addressed the issue of repeat biopsies in this context, particularly in relation to when it is reasonable to stop. Cancer detection rates in 1051 biopsied men were 22%, 10%, 5% and 4%, respectively, with 1-4 TRUS biopsy sessions with 58%, 60.9%, 86.3% and 100%, respectively, having organ-confined disease [10].

Detecting prostate cancer when it is still localized is important as, currently, it is only curable when organ-confined. Diagnosis of this disease earlier as a consequence of introduction of the PSA blood test, acknowledged by the National Cancer Institute (NCI) as one of the factors contributing to lowering the mortality rate over the past few years [2, 11], has provided a diagnostic lead-time estimated by Gann et al (1992) in the Physicians' Health study [12] to be 5.5 years. In the mid to late 1980s only one third of prostate cancers were found at curable stages compared with today when 80% are organ confined-and potentially curable [12-14].

To improve the sensitivity of PSA in identifying prostate cancer, the use of PSA velocity, free/total PSA ratios, transition zone and total prostate volume/PSA relationships [15-19] have been employed. Even with the use of these additions to PSA testing, the level of certainty in predicting the causative pathology for elevated serum PSA levels remains unsatisfactorily low for individual patients. Furthermore, although the dreaded complication of life-threatening sepsis is fortunately uncommon, other complications do occur. These include rectal bleeding, hematuria, retention due to clots or exacerbation of pre-existing bladder outlet obstruction which, together hematospermia, often distress patients, in spite of careful forewarning. Another factor often overlooked is that of fiscal costs, which are not inconsequential especially in a hospital setting with the use of sedation/anesthesia with TRUS biopsies. All these factors serve to act as a disincentive to repeat biopsies. Indeed, patients

may be reticent to agree to further TRUS biopsy procedures to address fluctuations in serum PSA, even when these are proposed years after the initial biopsies were performed [20].

After a diagnosis of prostate cancer is made, an aspect often overshadowed by the intense focusing on the associated unwanted effects of the various management options for clinically localized disease, is that of occult metastases. However, the fact remains that in this era of earlier detection of prostate cancer through the use of PSA testing, even when the tumor is thought to be localized after staging tests, up to 25% of men have non-localized disease [21]. This has prompted suggestions of a PSA cut-off of <3 ng/ml, a level well below both PSA and/or digital rectal examination (DRE) being useful discriminators of malignant compared with non-malignant pathology. Apart from a lowered PSA cut-off resulting in many more men having TRUS biopsies, some patients will continue to have non-localized disease. Lodding et al (1998) [22] reported that approximately 15% of prostate cancers identified using a PSA cut-off between 3 and 4 ng/ml had extracapsular growth.

Although the introduction of PSA and TRUS biopsies have resulted in prostate cancer being diagnosed much earlier and at a lower stage than ever before, a point of limiting returns appears to have been reached with respect the probability of further significant advances in lead-time detection of organ-confined disease being achieved, as a result of further refinements and adaptations to the PSA/TRUS biopsy approach. TRUS biopsies remain unpleasant, invasive and imprecise. As a consequence, the need for a more accurate test for prostate cancer, which PSA alone, and in all its forms and guises is not, is warranted now more than ever.

2. Potential Roles for Ejaculate and Urethral Washings

(a) Ejaculate Cytology

As an alternative to refining blood markers, which inevitably require biopsy as the second step, we chose to pursue a more direct assessment of the prostate through the non-invasive approach of studying ejaculate. Initially we examined ejaculate specimens cytologically from 37 men, suspected of having prostate cancer and who underwent TRUS biopsies subsequently. Frankly malignant and atypical cells were identified in ejaculate specimens from 14 of 37 patients. Of 12 patients with TRUS biopsies positive for malignancy, 9 (75%) had abnormal cells in their ejaculates. Five of 15 men with negative biopsies for adenocarcinoma also had abnormal ejaculate cytology. As a result of increasing our numbers by studying further patients (unpublished), we were able to show that abnormal prostatic cells were present in approximately $^2/_3$ of specimens from men diagnosed with prostate cancer [23, 24]. For patients apparently with false positive cytology, this finding was found to precede a subsequent TRUS biopsy diagnosis of cancer in most of these cases by a lead-time of up to 2 years, highlighting the potential of this new direction.

Morphologically, the prostatic cells were unmistakable, being quite different to those of urothelial and seminal vesical lineage. In addition, prostatic cells stained positively for both PSA and prostatic acid phosphatase (**PAP**). These cells were present in much greater numbers and with greater regularity than those retrieved from voided urine or following prostatic massage, both these methods having provided consistently modest numbers of prostatic cells for examination in the past [23]. Both microacinar formation and 3-

dimensional clumps of cells were a feature of prostate cancer cells in ejaculate [23, 24], a finding which we considered augured favorably for our ambition to employ prostatic cells for evaluating the potential of this body fluid in the diagnosis prostate cancer. Our findings have attracted considerable interest with the diagnostic potential of this approach now having been confirmed [26, 27]. Because cytology is subjective and unsuited to adaptation in inexpensive and automated diagnostic assays, we focused on molecular analyses, with the promises of enhanced sensitivity and quantitative assessment, to overcome these deficiencies.

(b) Molecular Confirmation of Prostatic Epithelial Cell Detection in Ejaculate

The next step was to validate our cytological findings by molecular studies. Ejaculate and voided urine immediately following ejaculation (**urethral washings**) were examined from patients prior to TRUS biopsies and from volunteers <30 years without a family history of prostate cancer, using reverse transcriptase-polymerase chain reaction (**RT-PCR**) with primers for **PSA** [28]. As anticipated, PSA mRNA was not detected in urine from men who had undergone radical retropubic prostatectomy (**RRP**) or from women. With this approach however, as few as 10 LNCaP cells could be detected by PSA RT-PCR when added to female urine. Thus, we established a sensitive method for detecting prostatic cells in ejaculate and urethral washings and have shown that PSA RT-PCR is a reliable indicator of prostatic cells in these samples [28].

(c) Optimising Prostate Cell Retrieval

We then proceeded to maximize selective retrieval of prostate cells from ejaculate and urethral washings. Over a period of several years, we developed and refined an efficient process for provision of a "pure" population of prostate cells for our studies. Although our priority has been to focus on molecular profiling as the preferred form of analysis of this enriched population of prostate cells, other forms of analysis, such as proteomic spectrometry may be used in providing a multifactorial approach to diagnosis. However, with all such strategies, a rate-limiting step is employment of an efficient prostate cell retrieval process as described briefly below.

(d) Prostate Epithelial Cell Enrichment

Both ejaculate and urethral washings provide a heterogeneous aggregate, necessitating isolation and enrichment techniques to optimise prostate cell isolation. These have been described recently elsewhere [29]. Briefly, following centrifugation of ejaculate samples and urethral washings (with ejaculate supernatant stored separately at -80°C for proteomic and other studies), the resulting pellet is re-suspended in a Percoll gradient column which separates mononuclear (especially non-prostatic epithelial) cells in a band separate from and above sperm and other ejaculate constituents which accumulate in a pellet at the bottom of the column [29]. A subsequent incubation stage involves the use of antibodies to a prostate cell-surface antigen and Protein G coated magnetic beads. Since we have confirmed that prostate specific membrane antigen (**PSMA**) is over-expressed in prostate cancer cells [30, 31], we use an anti-PMSA antibody to a cell-surface epitope of this molecule. By quantitative analysis, we have shown that the combined approach of a Percoll gradient column and the use of PSMA coated magnetic beads increases prostate epithelial cell purity 2-fold compared with Percoll gradient column alone.

Due to the expense of commercially available PSMA antibodies, we manufactured our own sheep anti-PSMA IgG antibodies against a GST-fusion protein which contained an extracellular epitope displayed on this transmembrane antigen. These antibodies are particularly effective for a number of laboratory methodologies including Western Blotting, immuno-precipitation, immunofluorescence and the most relevant application to our project, immunocapture of PSMA-expressing cells onto protein-G coated magnetic beads. Initial experiments, with these antibodies have shown that the magnetic beads captured 94-98% of LNCaP (PSMA +ve) cells and 8-18% of DU145 (PSMA -ve) cells: 5-10% of LNCaP cells were attached to the beads when no antibody was present (unpublished data). This antibody has also been used in FACS for prostate epithelial cells from a mixed cell type population (unpublished data).

As a small proportion of prostate cancer cells do not express the PSMA antigen, we are examining the use of a second prostate cancer-specific monoclonal antibody bound to magnetic beads in a second incubation stage to enhance our yield even further, although we estimate that our cellular yield is in the vicinity of 1000 prostate epithelial cells from prostate cancer patients' ejaculates compared with approximately 400 prostate epithelial cells from patients whose TRUS biopsies do not demonstrate cancer.

(e) Identification of Relevant Cancer Epithelial Cell Markers

A major priority for exploiting the enriched population of prostatic cells has been to identify a panel of discriminating molecular markers for profiling, to provide an earlier, and non-invasive method for diagnosis and prognosis. A number of candidate markers from the scientific literature, supplemented with findings from our own laboratories, have been selected to provide a discriminating panel of molecular markers.

In our (ongoing) quest to identify the most discriminating markers, we examined patterns of gene expression by microarray profiling of prostate cancer and BPH tissues then related our findings to the published literature. Real-time PCR was used to confirm the microarray findings and to quantify the level of over-expression of our candidate genes together with 13 other genes selected from the literature against a panel of 17 tumors and 11 BPH samples [30]: We identified 3 candidates, δ-catenin (*CTNND2*), Gal-3 (*GALNT3*) and PSMA (*FOLH1;*), which were over-expressed in prostate cancer compared with BPH (>3-fold). When tumors were compared as a group with BPH, there was a significant difference in the number of PSMA ($p = 0.01$), Gal-3 ($p < 0.00$) and δ-catenin ($p < 0.00$) transcripts. Immunohistochemistry with PSMA and Gal-3 antibodies and *in-situ* hybridization with δ-catenin specific riboprobes identified localization was predominately to prostatic epithelial cells, with greater expression in prostate cancer tissues than BPH, which was essentially negative for all 3 markers.

- **PSMA:** PSMA is a trans-membrane protein which, unlike PSA, is over-expressed at a cellular level in prostate cancer. With our polyclonal IgG antibody (mentioned previously) directed to a surface epitope, PSMA is targeted for quantitative RT-PCR. Reported by others and confirmed by ourselves, PSMA has been detected in both benign and prostatic carcinoma epithelial cells and in clinical terms is generally regarded as prostate-specific [30-32].

- **Gal-3 (*GALNT3*):** The gene product of Gal-3 (*GALNT3*) functions in initiating mucin-type O-glycosylation by catalyzing the formation of an O-glycosidic link between Gal*N*Ac and serine or threonine residues. Previous studies have revealed Gal-3 (*GALNT3*) is only expressed in organs that contain secretory epithelia and is highly expressed in human tumor cell lines arising from epithelial glands [33, 34]. Early studies showed Gal-3 (*GALNT3*) expression was increased in well-differentiated adenocarcinoma cell lines [35], while recent immunohistochemical analyses of pancreatic, colon, and gastric tumor tissues demonstrated a similar increase in Gal-3 (*GALNT3*) expression, suggesting a role as an indicator of differentiated adenocarcinomas [36-38]. In contrast, however, increased Gal-3 expression was associated with moderately and poorly differentiated tumors in small cell lung carcinomas and with poorer prognostic outcomes [39], similar to our findings with prostate cancer [30].
- **δ-catenin:** δ-catenin is an adhesive junction-associated protein that promotes cell scattering [40]. Both Northern blot and *in situ* hybridisation techniques have indicated that δ-catenin is almost exclusively expressed in the nervous system normally with highest levels of expression occurring during brain development [41, 42]. We recently reported detection of δ-catenin over-expression in prostate cancer compared with BPH using Real time RT-PCR and Northern blot techniques with localization to epithelial cells confirmed by *in-situ* hybridization with specific riboprobes [30]. As most intense staining has been reported in neuro-epithelial precursor cell populations of mice [40], it was conjectured that δ-catenin expression may be associated with neuroendocrine cells in the prostate. However, our *in-situ* analysis did not support this suggestion with localization being in the glandular secretory epithelial cells [30].

Thus, our results have confirmed previous work with PSMA [31, 32], and have identified a number of new markers. Both Gal-3 (*GALNT3*) and δ-catenin appear to be differentiating markers of prostate cancer in tissue samples. We have flagged these genes, together with others noted below, as potential differentiating markers for prostate cancer in ejaculate [29]. In further differentiating malignant from non-malignant prostatic epithelial cells, a number of other genetic changes have been reported, but many of these are not over-expressed sufficiently (>3 fold) so are unlikely to be useful for our diagnostic purpose using cells isolated from ejaculate and urethral washings.

(f) Other Relevant Cancer-Discriminatory Genes or Cell Surface Markers

Other genes, cited in the scientific literature for potential use in our assays, include the transmembrane protein, Hepsin [43], DD3(PCA3) [44], and hypermethylation of the Glutathione S-transferase P1 (GSTP1) gene promoter [45].

- **Hepsin:** Hepsin, a membrane-bound serine protease, was identified by gene expression analysis of a large cohort of prostate tumors using microarray technology by 3 independent groups. Significantly increased expression was displayed in malignant tissues compared with normal and BPH tissues [43]. Hepsin expression

was highest in organ-confined, lower Gleason grade disease [43], making it a good candidate for early detection. Our analysis of Hepsin mRNA transcripts in 19 prostate cancer tissues and 17 BPH tissues by RT-PCR confirms these findings with a 12-fold increase in Hepsin expression in prostate cancer tissues ($p=0.01$)

- **DD3/PCA3:** This novel prostate-specific non-coding mRNA, was shown by Northern blot analysis to be highly prostate specific and markedly over-expressed (on average 54-fold) in prostate cancer tissues [44, 46]. The diagnostic value of DD3/PCA3 has been determined in 2 separate studies, utilising tumor tissue and prostatic washings [44]. More recently, Hessels et al (2003) examined DD3(PCA3), following prostatic massage with a detection rate of 67% (16/24 biopsy-proven prostate cancer of 108 patients studied). DD3/PCA3 is not translated so quantitation is performed by Real time RT-PCR and, as shown in the table below, we found a highly significant difference in expression of RNA from this gene in prostate cancer compared with BPH [47].
- **GSTP1:** Hypermethylation within the GSTP1 promoter has a very strong correlation with prostate cancer [45] but a very low sensitivity for BPH. While GSTP1 hypermethylation was not detected in serum, ejaculate or urine from patients with BPH, it was present in 94 % of tumors, 72% of plasma or serum samples, 50% of ejaculate samples and 36 % of urine samples from patients with PCa [45]. Similarly, Cairns et al (2001) found hypermethylation of the promoter region of GSTP1 in urine specimens from 6 out of 22 patients with prostate cancer (27%) who had localized tumor which was clinically amenable to cure [48].

(g) Combinations of Markers in Molecular Detection of Prostate Cancer in Tissues

We then proceeded to examine combinations of markers to compare molecular detection rates of prostate cancer compared with BPH in tissue sections since previous experience using single gene markers has not been sufficiently sensitive [49-51]. As is our practice, sections with comparable numbers of epithelial cells were chosen for comparison to avoid the potential confounding factor of a predominance of stromal cells in BPH. Real-time RT-PCR was used to verify differences in gene expression determined by microarray profiling (above) and this methodology was also employed to compare expression levels for combinations of the 4 most promising candidate genes. The level of expression of Gal-3 (*GALNT3*), PSMA Hepsin and DD3(PCA3) were determined. A logistic regression model was used to obtain a predictive index using all 4 biomarkers, which predicted the classification of 100% of prostate cancer and BPH tissues correctly.

Figure 1. Graph o
■ = Gal*N*Ac-T3;
Gal*N*Ac-T3; ◇ =
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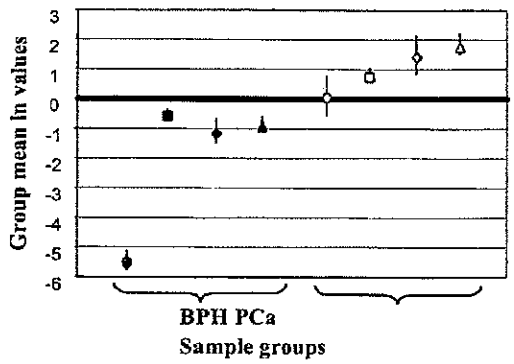

Figure 1. Graph of the sample group means (samples expressed to ln) for each biomarker where ● = DD3;. ■ = Gal*N*Ac-T3; ◆ = Hepsin; ▲ = PSMA as measured in the BPH sample group and ○ = DD3; □ = Gal*N*Ac-T3; ◇ = Hepsin; △ = PSMA as measured in the Cancer samples (Error bars indicate plus and minus Standard Error for each biomarker).

The logarithmic regression analysis of all 4 markers and sub-combinations of the markers is summarized in Table 1.

Table 1. Statistical Analysis of Biomarkers

Marker Combinations	Groups	Numbers	% samples correctly categorised
MULTVARIATE ANALYSIS			
GalNAc-T3	PCa	18	100.0%
PSMA	BPH	32	100.0%
Hepsin			
DD3	Overall	50	100.0%
Hepsin	PCa	18	100.0%
PSMA	BPH	32	100.0%
DD3	Overall	50	100.0%
GalNAc-T3	PCa	18	94.4%
Hepsin	BPH	34	97.1%
DD3	Overall	52	96.2%
GalNAc-T3	PCa	18	94.4%
PSMA	BPH	33	97.0%
DD3	Overall	51	96.1%
GalNAc-T3	PCa	21	81.0%
Hepsin	BPH	33	96.9%
PSMA	Overall	54	90.6%

A combination of all four markers resulted in a detection rate with an accuracy of 100% for both prostate cancer and BPH tissue sections. This 100% accuracy was maintained when three of these four, PSMA, DD3(PCA3) and Hepsin, were examined. These findings are heartening, indicating that a combination of markers greatly enhances sensitivity of molecular

diagnosis of prostate cancer compared with single marker identification using histopathologic characterization of tissues as a reference. The next step will be to determine whether use of this combination of markers extrapolates to provide comparable predictability in the molecular analysis of enriched prostate epithelial cells obtained non-invasively from enriched specimens of ejaculate and urethral washings [52].

Consequently, we have identified a panel of discriminating genetic markers so that a molecular profile is able to be developed for individual patients for use progressively in detection and, ultimately, for determining prognosis. By combining several selected markers to examine RNA quantitatively and for detection of GSTP1 DNA methylation, we expect to be able to detect prostate cancer much more reliably and sensitively than the current PSA immunoassay/TRUS biopsy approach, regardless of the level of PSA cut-off and the adjuncts to PSA employed. We anticipate that this strategy will advance the lead-time for diagnosis even further, with an accompanying reduction in the proportion of patients with non-localized prostate cancer at presentation. Hopefully, this will translate ultimately better rates of patient survival.

(h) Spectometric Analysis: Recent Developments

Although multiple molecular cellular markers are likely to be more than adequate for accurate, earlier diagnosis, the predictability of the ejaculate approach can be strengthened even further if analysis includes results from other assessment methods from the same patient samples in a multivariate analysis. Consequently, we have examined the prospect of seminal fluid spectrometry (using the non-cellular ejaculate supernatant) as a complementary strategy.

A variety of spectrometric techniques are available for examining tissues [53-59]. Until recently, Magnetic Resonance (**MR**) spectrometry has been the form of analysis evaluated most extensively for prostate cancer [60]. Lynch and Nicholson used MR spectrometry to examine citrate to spermine ratios in prostatic fluid obtained by prostatic massage from anaesthetized patients and found significantly higher levels of spermine in men with prostate cancer [61], illustrating the potential of ejaculate analysis for differentiating between malignant and benign prostatic disease. However, the main limitations of MRS are that it does not generate clearly resolved signals from molecules >10 000 daltons, thus limiting evaluation of many proteins, and volumes of at least 500 µl are required for processing [61].

Recent advances in mass spectrometry permit high-throughput separation and analysis of proteins. Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (**MALDI-TOF**) spectrometry can analyze many proteins in a sample simultaneously. The technique creates a "profile" of proteins, distinguished by molecular weight. The related technique of Surface-Enhanced Laser Desorption/Ionisation(**SELDI**)-TOF [62] allows further discrimination and classification of the types of differences that contribute to the mass spectrometry profile by incorporating *in situ* selection of biomolecules on different adsorptive surfaces, from which surfaces the biomolecules can be directly analyzed in the spectrometer. A large number of studies have now employed both these techniques to analyze protein profiles in diseased tissues and biological fluids, especially in cancer [62-65].

Petricoin *et al* (2002) [66] and Adam *et al.* (2002) [67] employed SELDI mass spectrometry on archival serum specimens in ovarian and prostate cancer patients, respectively. In each case they used a "training" set of samples to define the parameters for

malignant and benign samples and analyzed a second set of samples blind. Adam *et al* reported a sensitivity of 83%, a specificity of 97% and a positive predictive value of 96% for their study population [67]. Although Adam *et al.* detected a large number of peaks (124) after clustering and disease group discrimination, which was then refined to 9 definitive peaks following further bio-statistical analysis, it is significant that no single peak was identified that could completely discriminate samples from patients with prostate cancer in comparison with those with non-malignant disease [67].

More recently, Bañez et al (2003) [68] employed a decision tree algorithm approach with serum analyzed by SELDI-TOF spectrometry. They studied specimens from a total of 106 patients with prostate cancer and 56 controls, all of whom had serum PSA levels <4 ng/ml with 33 having had prostatic biopsies negative for cancer. Serum from 44 prostate cancer men and from 30 controls was used to create a reference training set. Combined spectral data from a weak cation exchange array and a copper metal affinity capture array were then evaluated diagnostically for the test set, correctly classifying 53 of 62 cancer cases as prostate cancer and 22 of 26 controls as controls with an 85% sensitivity and 85% specificity for the detection of prostate cancer [68].

(i) Spectrometric Analysis: Our Preliminary Data with Ejaculate Supernatant

Concomitant with our cell-based studies, we have analyzed seminal fluid for changes in prostatic secreted proteins. Initially, we measured free/total PSA [69] then kallikrein protein-2/PSA [70] relationships, on the premise that there would be ejaculate changes similar to those detected in serum. Conclusive differences between prostate cancer and TRUS biopsy-negative patient specimens were unable to be demonstrated with these specific assays. However, by contrast, our preliminary studies with MALDI-TOF analysis of seminal fluid have been most promising.

In our pilot study, MALDI-TOF analysis was performed on 28 seminal fluid samples from 19 prostate cancer patients, 9 men with negative TRUS biopsies for cancer and 13 men <30 years and without a family history of prostate cancer. Discriminant analysis (SPSS Inc, USA) was applied to the selected peaks in the spectrometric profile of proteins between 5-100kDa. Peaks were selected on the basis of visual inspection for presence/absence and relative intensity (when present in more than one sample type). Three distinct "cluster" patterns were obtained for the three groups of men in this "training" data set (**Fig 2**).

Utilizing the paradigm obtained for prostate cancer and non-prostate cancer specimens (above), a further 99 archive seminal fluids from 91 patients with TRUS-referenced findings were analyzed by MALDI-TOF and categorized as prostate cancer or non-prostate cancer based on the selected peaks in their spectra. Clear discrimination between the 2 groups (prostate cancer: mean –1.32; Non-prostate cancer mean 1.91) was again seen (**Fig 3**). A sensitivity of 92% (51/55 patients) and a specificity of 89% (32/36 patients) were observed. However, 2 of the men whose tumors were not detected by the MALDI-based program had extensive local disease which was unmistakable as prostate cancer on digital rectal examination (**DRE**) and 2 had small foci of Gleason 3+2 and 3+3, respectively, raising the possibility that these two men have clinically clinically insignificant prostate cancer.

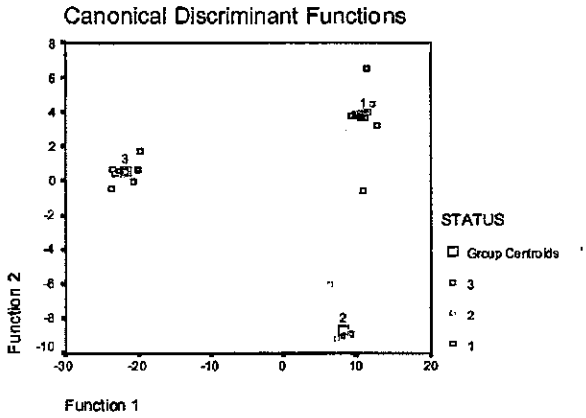

Fig 2: Cluster pattern distributions for prostate cancer (1) non-prostate cancer (3) and controls (2) – men <30 yr without a family history of prostate cancer

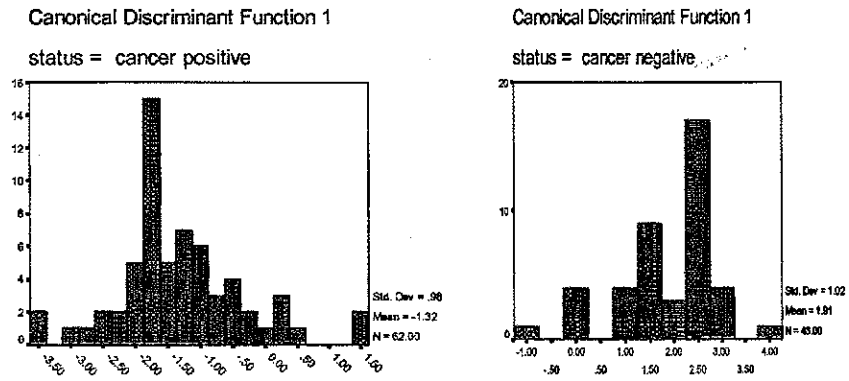

Fig 3: Histogram of the discriminatory analysis of MALDI-TOF spectra from PCa-positive and PCa-negative seminal fluid specimens.

We have initially concentrated on MALDI-TOF for proteomic analysis because it has the potential to provide a more complete picture in a single assay than might be available from the SELDI system since the latter is selective for different groups of proteins. Additionally, with a spectral range of 5-100 kDa, there is a lesser likelihood of confounding effects from smaller peptide fragments that may have been produced in long-term storage specimens as might be the case with SELDI. In addition, seminal fluid rather than serum, which we are collecting to study subsequently, was selected because the range and concentration of potentially relevant proteins in this fluid is expected to be greater than in serum, and 'background noise' from other proteins would be lower.

These decisions have been vindicated in that our analyses show at least 5 spectral peaks as potential conjoint differentiating markers. Another approach for proteomic spectrometry is to use the population of prostatic cells obtained from seminal fluid enrichment strategies we

have developed, this technique having been reported recently for archival cytological material from slide preparations [71].

Psychological Factors with Prostate Cancer

The increased and less formal patient contacts resulting from our early diagnosis studies have made us even more aware of the many uncertainties inherent in the diagnostic and treatment processes. The imprecise information on which management choices have to be made by patients coupled with uncertainty regarding the presence or absence of metastases and, subsequently, the timing and nature of palliative treatment for prostate cancer patients provide many issues for consternation. These uncertainties and the need to address them take a psychological toll on both patients and their families so, as a collaborative group, we have undertaken studies to address deficiencies in the scientific literature to provide a sound basis for evidence-based strategies to lessen adverse psychological impacts at various stages in the natural history of this disease.

1. Patient Support Requirements

Our collaborative studies into the psychosocial aspects of prostate cancer in relation to patient support requirements have been undertaken with two guiding principles in mind. First, research topics were identified from needs identified from clinical experience, and second our research efforts were aimed ultimately to improve supportive care for men with prostate cancer in *our* community.

Preliminary studies were undertaken to ascertain men's preferences for information and support for cancer amongst 1,461 well men from urban and rural areas across Queensland [72]. Three sources of support were identified that included the man's close social support network, external emotional support, and external informational support. Men reported preferring to receive support from their close social network, that included their families and their clinicians. Men also indicated that the source they would be most likely to turn to for information about cancer was their clinician. Less educated men were less likely to seek external information about cancer. We also assessed prostate cancer screening behaviors for men in this group who were over 40 years of age [73]. In all, 39% of these men had undergone PSA testing for the purpose of the detection of prostate cancer. Men with urologic symptoms who had undergone PSA testing were more worried about prostate cancer and perceived themselves as more vulnerable to prostate cancer compared with both asymptomatic tested and untested men. We concluded that urologic symptoms act as a risk cue to men for prostate cancer and as such should be included as a group variable in future research in this area.

Further community based research has involved working closely on both a state and a national basis with peer-led prostate cancer support groups. Prostate cancer support groups have become more prominent over the past decade in North America, Canada, the United Kingdom and Australia [74-77]. These groups provide a mechanism for community based peer support and are a source of advice about the unmet needs of men with prostate cancer at

all disease stages. Accordingly, we initially undertook an assessment of the unmet supportive care needs of 206 men with prostate cancer who were members of these groups in our state (Queensland) [78]. One third of the men in these groups reported moderate to high unmet needs in the sexuality, psychological, and health system and information domains. Men who were closer in time to diagnosis had higher needs for help with physical and daily living. Younger men reported higher need in the sexuality domain; greater need for help in patient care and support was requested by men with advanced prostate cancer and by men who had lower levels of education or who had received radiation therapy. Those who lived in major urban centers had greater psychological support needs. These findings provided a basis for the development of a range of patient education materials, both written and audiovisual.

Subsequently, we have been working in collaboration with others on a three-phase study to assess the effectiveness of peer support groups for men with prostate cancer across Australia. This study adopted a multilevel approach to ascertain the activities of these bodies nationally and to assess the perceptions of group leaders, group participants and finally clinicians in relation to the mechanisms by which these bodies support men. The first phase of this study found a broad range of activities ranging from support of men to advocacy and fundraising, with the primary method of support being group discussion and education meetings [79]. Data about group level variables such as group size and leadership that was collected in phase 1 was then able to be entered in analyses in subsequent study phases. Thus in Phase 2, we have surveyed over 1200 men from these groups nationally and undertaken multilevel analyses to assess predictors of both positive and negative support received. This phase of the study included development of a new measure, the Prostate Cancer Peer Support Inventory, to assess support received. Data from this study is currently under review. The third and final phase of this research will assess clinicians' perceptions of the utility of peer support groups for prostate cancer and will make recommendations for the future development of these programs.

In addition, we have undertaken a prospective descriptive study of adjustment of 111 men diagnosed with localized prostate cancer, assessing them first at the time of diagnosis, and subsequently two and twelve months after treatment. The data collated for evaluation included decision analysis at diagnosis, tracking of the use of alternative therapies and a description of psychological and decision-related adjustment over time using previously validated measures. First, we used Verbal Protocol Analysis to systematically elicit men's decision making processes at the time of diagnosis and before treatment [80]. We found that non-systematic decision processes, such as deferral to the doctor's opinion, recollections of other peoples' cancer experiences, and lay beliefs about health, were used by 91% of men. Despite information-seeking about prostate cancer being high, 73% of men had sought information about prostate cancer from external sources, although systematic processing of the medical and treatment aspects of the cancer was limited. On average men considered four or fewer medical and treatment aspects in their decision-making. We concluded from this study that prostate cancer patients did not use information about medical treatment comprehensively when making treatment decisions and that decisional support needs to be sensitive to both this and the pervasive influence of lay beliefs and heuristics in men's decision-making. Accordingly we are currently developing a decision support program along these lines.

In addition, we have described the use of alternative therapies over the first twelve months of the illness experience in this cohort of men, and identified psychological predictors of therapy use [81]. Use of alternative therapies at diagnosis was found to be related to greater uncertainty about prostate cancer treatment, while men who were using alternative therapies twelve months after treatment had lower levels of psychological distress. Finally, we described men's levels of cancer-related traumatic psychological distress over this trajectory and, to our knowledge, are the first to document decision-related distress using a previously validated measure, the Decisional Conflict Scale [82, 83]. We found that in general men with localized prostate cancer adjusted well psychologically to their treatment with regards to both psychological distress and life satisfaction. However, by contrast, decision related distress was high and persisted over time. This information has been used in the development of further patient education materials focused on helping men to cope effectively with a diagnosis of prostate cancer, as well as a group based psycho-educational program.

2. Randomised Controlled Studies of Cognition and Quality of Life with Androgen Suppression Chemotherapies

(a) Current Practice

One of the most contentious issues in prostate oncology is the timing of commencement of androgen suppression as palliative therapy for patients with non-localized disease. On the basis of the indisputable endpoint of survival, the advantages of early introduction of various forms of hormonal therapy appear very modest at best [84]. Nevertheless, even minor elevations in serum PSA continue to serve as the trigger for commencing androgen suppression [85]. The substantial change in the profile of patients receiving chemical castration (in preference to the less popular reference treatment of bilateral orchiectomy) is expected to continue to change over time [85]. Increasingly, these men will be "PSA failures" after local treatments, with many destined to receive androgen suppression therapy for many years.

This preoccupation with perceived cancer control based on the erroneous notion that this equates with lowering of serum PSA values, might be justified if the various forms of palliative hormonal therapy were not associated with significant unwanted effects and if all recipients benefited in terms of a durable response in clinical regression of tumor. However, significant problems accompany all forms of hormonal manipulation [86], many of which are not reversible with intermittent androgen blockade (**IAB**) [87].

Despite an absence of level-one evidence, advocates of early commencement of hormonal manipulation contend that survival is enhanced and "quality of life" is improved with this approach. The issue of improved survival with IAB remains unresolved and the second contention also is uncertain with "quality of life" often used in a vague and most general sense. Since previous studies which used specific quality of life measures but non-random assignment reported mixed results, we undertook the first randomized controlled study of patients for whom androgen suppression was optional, to address this relevant and acknowledged deficiency [88, 89].

Another issue regularly dismissed in prostate cancer patients has been that of potential adverse cognitive effects with androgen suppression therapies although it is well accepted that Luteinising hormone releasing hormone (LHRH) analogues and the menopause are associated with adverse cognitive findings in women [90-93].

(b) Psychometric Evaluation

Using well validated and established instruments, we examined Health-Related Quality of Life (HRQoL) concurrent with neurocognitive evaluations of patients with non-localized prostate cancer randomized to goserelin (Zoladex) leuprorelin (Lucrin) (LHRH agonists), cyproterone acetate (Androcur) (progestational anti-androgen), and close observation. Men of similar age and health who did not have clinical signs of prostate cancer completed the same assessments concurrently. In a repeated measures design, participants were assessed at pre-treatment baseline and after 6 and 12 months of treatment [94-96].

Eighty-two men were randomized, 77 were assessed at baseline, 65 at 6 months and 62 completed the 12 month study. The drop-out rate was greatest for the patients randomized to receive cyproterone acetate (including one man who died from massive hepatic necrosis) with 11 of 19 participants continuing at 12 months compared with 14 of 19 for controls: all but two of the 39 men randomized to LHRH agonists continued treatment uninterrupted at 12 months. Of 20 community participants, 15 continued for the duration of the assessment period. Questionnaires on emotional distress, existential satisfaction, physical function and symptoms, social and role function, subjective cognitive function, and sexual function, were combined with standard neuropsychological tests of memory, attention and executive functions.

A number of well established and validated instruments were used in order to measure specific dimensions of HRQoL, appraisal and coping. HRQoL dimensions were emotional distress (Depression Anxiety Stress Scales), existential satisfaction (Satisfaction With Life Scale), physical/urinary function (European Organization for Research and Treatment of Cancer Core Quality of Life Questionnaire [EORTC-QLQ-C30]), social/role function (EORTC-QLQ-C30), subjective cognitive function (EORTC-QLQ-C30), and sexual function (supplementary module to EORTC-QLQ-C30). Threat appraisal and self-efficacy were measured by questionnaires modeled on those from previous studies. Coping was measured with the COPE questionnaire [94].

Intelligence quotient (IQ) was estimated with a four-subtest short form of the Wechsler Adult Intelligence Scale – Revised. The National Adult Reading Test (NART) supplemented with the Schonell Graded Word Reading Test for fewer than 10/50 items correct on the NART was also used. *Memory* was tested with Visual Memory and Verbal Memory Indices from the Wechsler Memory Scale – Revised (WMS-R), the sum of words recalled from 5 list-learning trials of the Auditory Verbal Learning Test (AVLT) and 30-minute delayed recall in the Rey-Osterrieth Complex Figure Test. Attention measures were the Attention and Concentration Index from WMS-R, Digit Symbol from the Wechsler Adult Intelligence Scale – Revised, time on the Trail Making Test Parts A and B and the sum of three one-minute trials of the Controlled Oral Word Association Test (COWAT). *Executive Functions* were evaluated by participants completing the "Victoria" version of the Stroop Test. Visuospatial perception and organization were tested with the copy trial of the Rey-Osterrieth Complex

Figure Test. In order to rule out an indirect effect on cognition through depression, participants completed the Depression Anxiety Stress Scales (DASS-21). The total score on this scale was used to measure emotional distress.

(c) Findings

Overall, patients randomized to hormonal treatment arms failed to exhibit a benefit in HRQoL parameters compared with those assigned close observation and community controls. Sexual dysfunction, particularly rated as important in adversely affecting HRQoL, was increased for patients on androgen-suppression therapies in comparison with close observation and volunteers in the community group. It is notable that most patients recruited and randomized had PSA values between 30 and 50 ng/ml at baseline. Emotional distress was increased in patients assigned to cyproterone acetate or close clinical monitoring. Furthermore, compared with pre-treatment, there was evidence of an adverse effect of leuprorelin, goserelin and cyproterone acetate on cognitive function, in approximately 50% of these patients) but this was not observed in any man randomized to close observation (Green et al, in press 2004) [96]. This adverse effect on cognitive function had not been reported previously.

Our conclusion from this study is that, in addition to considering the adverse physical effects of hormonal deprivation, the importance placed on sexual activity, memory, attention and executive functions by individual patients should also be taken into account before commencing chemical castration.

Were we to plan this study today we would not include cyproterone acetate, consistent with the recommendation of the UK Committee on Safety of Medicines that, because of hepatotoxicity, cyproterone acetate in prostate cancer should be restricted to short courses unless patients are unresponsive to, or intolerant of, other treatments. Whether adverse cognitive effects are associated with the use of other antiandrogens, such as flutamide and bicalutamide in men with prostate cancer, remains unknown and unstudied to the best of our knowledge. Currently, we are undertaking a trial comparing patients randomized to bilateral orchiectomy or goserelin using the same parameters as above.

Vaccines in Prostate Cancer

The studies described above highlight just some of the problems associated with established interventions in the management of patients with prostate cancer underscoring the importance of adhering to the principle that unwanted effects of our treatments should not be worse than the problem(s) for which they are given. In prostate cancer, which commonly afflicts older men with significant co-morbidities, the quest for effective, minimally disruptive therapies assumes particular relevance.

1. Principles and Background

Immune-based therapies for cancer can potentially target tumor cells specifically, by inducing a cellular or an antibody response against mutated proteins, or cancer-associated

antigens [97, 98]. Proteins peculiar to the tissue of tumor origin, for which the prostate is a rich source with PSA, PSMA and PAP prominent amongst others [99], represent another potential target for specific immune therapies. Although the aim of these therapies is to induce a sustained response to "self", in practice the side effect profile of active cell-based immunotherapies has been very acceptable with a notable absence of serious problems. Thus, the early promise reported with dendritic cell (**DC**) –based vaccines for prostate cancer [100, 101] was greeted with considerable enthusiasm, even more so because of local experience with DC-vaccines in the pioneering work with Melanoma conducted in Brisbane at the Queensland Institute of Medical Research (**QIMR**) and the Mater Hospital [102]. A further inducement for us to embark upon DC-vaccine studies for prostate cancer was the recent appointment of a world-recognized authority on DCs to the directorship of the Mater Medical Research Institute (**MMRI**).

The adaptive and non-adaptive arms of the immune system possess inbuilt checks against overt autoimmunity and strong homeostatic mechanisms to limit the activation of responses against antigens which apparently pose no threat to the host. Further, a variety of immune response defects have been described in men with prostate cancer [103]. The challenge is therefore to induce a sustained immune response to tumor-associated antigens in the face of existing disease, which may have metastasized to a variety of tissues such as bone, despite a demonstrable measure of immune compromise. Tantalizing reports of complete remissions following DC therapy in patients with a variety of cancers [101, 104, 105] indicate these obstacles, highlighted by Lopez and Hart (2003) [106], can be overcome in at least a subset of patients. Indeed, specific immunotherapies may ameliorate the immune defects observed in patients with prostate cancer [107]. An excellent review by Fong and Small (2003) [99] summarizes other work in the field of prostate cancer immunotherapy, including DC-based vaccines, which we will not attempt to replicate. In the text below, we concentrate on how we have implemented measures to address some unresolved general principles in our clinical trials.

Our experience with a DC-based therapy for melanoma [102] has suggested that

- Sustained treatment may be necessary for complete, durable remissions. This statement can be understood in the context of Matzinger's "danger" model [108]. The rationale behind tumor immunotherapy is that, in the absence of appropriate cues from an immune system which has evolved to recognize pathogens, tumors generally do not induce fully activated immune responses. Therefore, they would not be expected to sustain such responses over the long period that might be needed to clear disease. Because DC-based therapies are very well tolerated, continued vaccination at intervals is acceptable.
- Patients with bulky disease are less likely to respond to DC-based vaccine therapies. It has been considered for some time self-evident that lower levels of disease are more likely to respond to immune therapy and patients with minimal residual disease have a demonstrated survival benefit following immunotherapy [109]. We found that tumor bulk, measured radiologically or indirectly with a circulating protein marker, was negatively correlated with objective clinical response.

2. Prostate Cancer Immunotherapy with PSMA Peptides

Murphy et al reported their findings from a phase I trial of PSMA vaccination in 51 patients with advanced, hormone refractory prostate cancer in 1996. Patients were divided into five groups, two of which received injection of one of two HLA-A2 restricted PSMA peptides, one group received autologous DC alone and two groups were administered autologous DCs pulsed with one of two PSMA peptides. A T-cell response was observed in some patients infused with DC pulsed with either peptide. Clinically, seven men had a partial response, based on National Prostate Cancer Project (**NPCP**) criteria and >50% PSA decrease, and 11 demonstrated stable disease. Five of the seven clinical responses were present in the cohort receiving DCs pulsed with PSMA peptide. Subsequent follow-up demonstrated four durable partial responses with an average response duration of 232 days.

Based on the principles above and results from Murphy's group, we formulated a clinical trial of patients' own monocyte-derived DCs primed with two PSMA peptides, in conjunction with fellow members of the Northern Section of the Urological Society of Australia and oncological colleagues with immunological and molecular expertise provided by scientists at QIMR, University of Queensland, Queensland University of Technology and MMRI. We concentrated on patients with early hormone escape manifested by 2 successive rising serum PSA values >4 ng/ml in the absence of demonstrable metastases on bone and computerized tomography (**CT**) scans, since this population was considered most likely to have minimal tumor burden. Because of the expected inexorable rise in PSA accompanying disease progression in these men, changes in PSA doubling time and/or PSA slope, PSA stabilisation or PSA reduction were chosen to provide valid intermediate clinical endpoints together with continuing absence of demonstrable metastases on bone and CT scans [99, 110].

In this study, which is ongoing, immunotherapy is given at 4 weekly intervals, with the offer of maintenance DC-vaccine treatment for an indefinite period if signs of clinical response are observed after 28 weeks. This trial, with patients randomised to receive vaccine intravenously (**IV**) (with placebo intradermally [**ID**]), vaccine ID (with placebo IV) or placebo IV *and* ID every 4 weeks for 28 weeks, has been designed to determine which of the 2 routes of vaccine delivery is more effective and whether the vaccine has an effect on the primary tumor.

Although the medical literature indicated at the time of planning that PSMA peptides were the most suitable antigens for priming DC cells [14, 15], limitations associated with use of these peptides together and the secondary aim have imposed considerable constraints on recruitment [111]. Only men who are HLA-A2 positive are likely to have a T-cell response to a DC-based vaccine using peptides as the antigen and, consequently, only men with this phenotype are eligible: men who have had previous pelvic radiotherapy or a radical prostatectomy (**RP**) are ineligible. Patients have transrectal ultrasound (**TRUS**)-guided biopsies of their prostates at entry then again at 6 months. In addition to the requirements of bone scan and CT-scan negativity, there is a control arm, which has dissuaded a number of potential patients from enrolling although men randomised to receive placebo both IV *and* ID are offered vaccine (IV *or* ID after re-randomization) at 28 weeks if they are still eligible.

Despite these extremely strict criteria, 41 men with negative bone and CT scans have been referred for consideration for the study. Ten men have been randomised (with 2 of these

re-randomised at 28 weeks): Two men have yet not reached the first milestone of 28 weeks follow-up so that ten six-month-periods of treatment (2 with placebo) are available for analysis. Two of the 4 men who received vaccine IV demonstrated evidence of tumor progression both radiologically and radioisotopically at 6 months. Two of the 4 men given vaccine ID remained clear radiologically and radioisotopically at 28 weeks (one has stable disease at 28 months and the other has just had lymph node metastases demonstrated at 35.5 months): Two men who received placebo initially and were re-randomized to ID had demonstrable metastases and local progression, respectively, at 28 weeks. The patient with local progression had an indwelling catheter with repeated hematuria and infections. Three men receiving active treatment have achieved disease stabilization well beyond 12 months and up to 2.5 years, results we consider to be particularly heartening given the otherwise very poor prognosis of these patients.

3. Immunotherapy Using Autologous Tumor

In addition, we have recently commenced a second DC-vaccine study, also targeting patients escaping hormonal control but with a much larger tumor burden, some of which is harvested as the source of antigen. To date, 3 men have been recruited to this study with tumor from metastatic neck lymph nodes obtained from 2 patients and from a large pelvic lymph node retrieved laparoscopically from the third. Unlike our first study, MHC status and previous definitive treatment of the primary lesion do not influence eligibility for entry.

Our prior experience with lymph node specimens of at least 5 g indicates sufficient tumor cells will be available from selected patients for preparing antigens for priming immature DCs. At the time of lymph node harvesting, we are also obtaining tissue from both secondary tumor deposits and the primary cancer to identify patterns of gene overexpression using microarray analyses. These findings will be related to patients' clinical and immunological progress, in particular responsiveness to the vaccines. A further aspect of this second study is that we are combining HRQoL assessments at baseline and at six months to provide objective evidence of the patients' perceptions of this vaccine regimen in relation to their disease and its treatment.

Conclusion

In this chapter, we have attempted to demonstrate not just interactions by contributors from a variety of backgrounds to a number of prostate cancer projects but also how such expertise enhances these seemingly unrelated studies in a manner that would otherwise be unlikely to happen. We consider that benefits of our widespread collaborations are that research effectiveness is maximized and that this translates to better patient care. By integrating our efforts, our limited physical and fiscal resources are optimized. It is our sincere hope that this approach of working together through a spirit of cooperation rather than dissipating energies in competition with each other in this city, will pay dividends in the longer term with respect to advancing knowledge and improving the management of prostate cancer patients.

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