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Beyond PSA: are new prostate cancer biomarkers of potential value to New Zealand doctors?

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Abstract

The widespread introduction of prostate-specific antigen (PSA) screening has enhanced the early detection of prostate cancer within New Zealand. However, uncertainties associated with the test make it difficult to confidently differentiate low-risk patients from those that require a definitive diagnostic biopsy. In consequence, the decisions surrounding prostate cancer treatment become extremely difficult. A number of new tests have become available which might have the potential to complement the current PSA screens. We review a number of the best validated of these which provide data that, although currently not available in clinical practice, some of these might have considerable potential to aid diagnosis, prognosis and therapeutic decisions for men with prostate cancer in New Zealand.

Prostate cancer is the most commonly registered male cancer in New Zealand making up 25.2% of all registrations, ahead of colorectal cancer and malignant melanoma of the skin, and the third most common cancer registration for both sexes.

Prostate cancer was also the third leading cause of male cancer deaths in 2006[1]. Although recent data might be interpreted as suggesting that there has been a decline in the incidence of prostate cancer since the year 2000[2], this may be an artefact of increased uptake of prostate-specific antigen (PSA) screening at that time. With increased PSA testing comes earlier diagnosis and registration of patients, which in turn will lead to an elevation of diagnosis in *younger* age groups (giving the pre-2000 increase).

The apparent post-2000 decline is thus a result of those patients already being picked up by the test who would have otherwise been diagnosed at that time. The likely result is a paradigm shift in the age distribution of patients with diagnosed prostate cancer, and a return to a steady gradual increase in diagnosed prostate cancer patients, as seen in the pre-PSA years[2].

PSA testing—the current method of prostate cancer risk and progression assessment *a prima facie*, falls well short of the performance required of a screen in an age of evidence-based medicine, with sensitivity and specificity of PSA testing being quoted as 74–84% and 90–94% respectively[3,4] and a positive predictive value of 21.9% (when using the traditional value of PSA 4.0 ng/mL as a threshold)[5].

Use of such a test as the basis of clinical decisions for prostate cancer patients renders *active surveillance* (a programme consisting of regular PSA and DRE (digital rectal examination) testing (in addition to regular biopsy of a patient's prostatic tissue) or *watchful waiting* (where treatment has a stronger palliative element and curative treatments are foregone)[116] as the most prudent course of action when a PSA level is shown to be in the *grey zone* of 2.5 ng/mL–10 ng/mL[6].

It should be noted, however, that active surveillance and watchful waiting, despite the implication of PSA values, are primarily indicated through key parameters of biopsy results, including Gleason score, clinical grade of disease, number of cores positive upon biopsy and volume of malignant tissue in each positive core. The current dependence on an invasive test for disease prognosis is reflective of the difficulty to differentiate between indolent and aggressive neoplasms with PSA, which is, in essence, a risk-stratification tool.

Indeed, this is further underpinned when one observes the high rate of false positive (95 in 1000 men aged 55–69 years who have the PSA test) and a substantial number of false negative results (23 per 1000 men aged 55–69 who have PSA testing and then biopsy)[3]. As a result, the decisions surrounding treatment become extremely difficult if the sole basis for the decision to treat was a non-invasive test such as PSA (in practice, just as for active surveillance, the decision to treat is primarily indicated through parameters of prostate biopsy).

Patients who do not need treatment may opt to be treated and suffer unnecessary side effects. Equally, those who do need treatment may choose not to be treated, and miss the opportunity for an early intervention. It is this dilemma which epitomises the experience of both patient and practitioner in dealing with the inherent uncertainty of PSA testing. Ideally, clinicians would be able to call on an accurate and reliable non-invasive risk-stratification system, whereby patients are empowered with precise knowledge to make more fully informed decisions on their health, and equally have a clearer understanding of the risk of recurrence[8].

This review discusses novel biomarkers in prostate cancer which have the potential to be incorporated in new risk-stratification systems, and their role in delivering the diagnostic and prognostic precision currently lacking in clinical prostate cancer treatment. We note that this list is not exhaustive, but covers several that would be potentially applicable to the New Zealand clinical situation.

PSA testing: the status quo

Current policy and practice

Screens for genetic susceptibility to breast cancer (BRCA1/2 screening[114]), or for the presence of early signs of cancer in the cervix (cervical cancer screening[113]) are both well established in Aotearoa/New Zealand. However, comparable well established methods are not available for screening genetic susceptibility to prostate cancer, despite the similarity in incidences of breast (2572 registrations, 2006) and prostate (2484 registrations, 2006) cancers[2].

The lack of a well substantiated and non-invasive screening test for early prostate cancer[3] (as compared with PAP smear testing in cervical cancer) requires a more aggressive and concerted effort from policymakers, clinicians and researchers to address the uncertainties and errors manifest in the PSA test, which defines the current status of prostate screening and on a more global level, the plight of men's health, in this country.

As a reflection of where the New Zealand healthcare system stands with its current prostate screening procedures—out of the eight criteria outlined by the New Zealand

National Health Committee (NHC) screening assessment, prostate cancer screening meets *only one criterion*—that prostate cancer is a condition which is a suitable candidate for screening[3]. Indeed, PSA and direct rectal examination (DRE) are described as unsuitable tests as:

> *"neither can be described as reliable, accurate, sensitive or specific enough for screening asymptomatic men."*
> National Health Committee (2004)

However, there exists a growing body of evidence which tentatively suggests that screening for prostate cancer is not without its benefits. Specifically, criterion three outlined by the NHC—that there is an effective and accessible treatment or intervention for the condition identified through early detection[3]—would seem to be supported by data presented from the Scandinavian Prostate Cancer Group-4 trial[144] demonstrating a reduction in metastatic disease incidence (RR=0.65; p=0.006) and disease-specific death (RR=0.82; p=0.09) for clinically localised prostate cancer specimens after a 12-year follow-up period with radical prostatectomy, as compared to watchful waiting.

Additionally, data extracted from a cohort of 7578 men in Sweden, randomised to screening, demonstrated a prostate cancer-specific mortality reduction of almost 50% (RR=0.56; p=0.002) over 14 years compared to non-screened controls[145], which would provide randomised controlled trial evidence demanded by the fourth criterion stipulated by the NHC—that a screening programme is effective in reducing morbidity and mortality.

Although the inevitable risk of overdiagnosis has been acknowledged by the study authors and elsewhere[145,146], these recent developments perhaps signal that it may be pertinent to once again review the current government policy on prostate cancer screening.

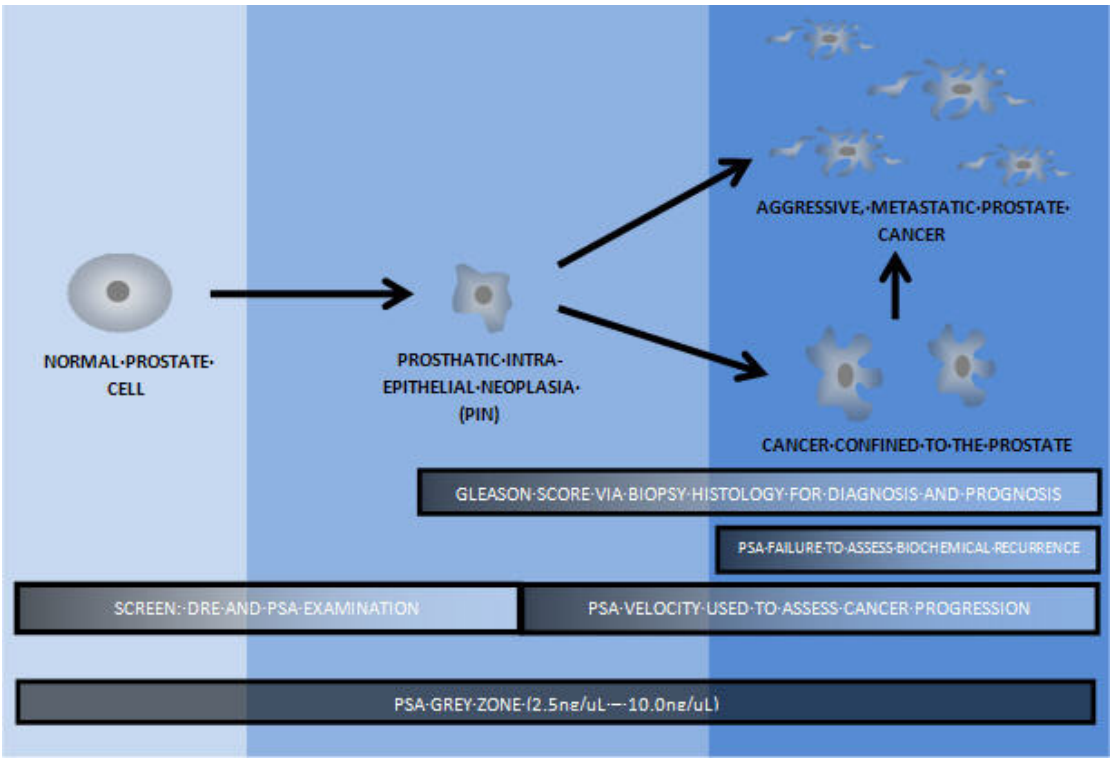
Strengths and limitations

PSA testing has demonstrable strengths. With 90% of new cases detected early enough for curative treatment[115] (where the treatments offer cure rates between 70%–90%) and changes in prostate cancer mortality ranging from 10%–39% in countries in Western Europe, North America and Australia[116] we can recognise that, although flawed, PSA is having a positive effect of the clinical treatment of prostate cancer.

In addition, when we consider that prostate cancer has a tendency to progress slower than other cancers (and even slower with androgen ablation therapy), the burden associated with the myriad of medical interventions such as radiotherapy, surgery and hospice care will often become more costly than an early, curative intervention administered on the basis of a routine PSA test[116].

Moreover, the natural course of prostate cancer means that if we were to forego PSA testing and diagnose on the appearance of symptoms, 70% of these cases will already have metastases. It must be acknowledged too, that PSA should only be seen as the initial step in prostate cancer assessment—TRUS (transrectal ultrasound) biopsy remains the gold standard in delivering diagnostic and prognostic data on prostate cancer.

Figure 1. Current use of PSA in monitoring progression, diagnosis and prognosis of disease



Note: The PSA *Grey Zone* (2.5ng/uL – 10ng/uL) [6] extends across the whole continuum of prostate cancer progression.

These recognised limitations of PSA testing have led to international initiatives towards developing and validating new biomarkers with higher sensitivity and specificity which alone, or in conjunction with current screening methods, are able to deliver more definitive results on the presence and nature of cancer in the prostate, in a fast, cost-effective and non-invasive manner.

Through the clinical application of novel biomarkers and effective implementation in the healthcare system, clinicians may aspire to deliver well informed and clear-cut decisions on the course of prostate cancer patients' treatments and prognoses, and ultimately deliver better health outcomes for men in Aotearoa/New Zealand.

Novel biomarkers: beyond PSA

As researchers delve further into the elements underlying sporadic prostate cancer, we begin to unearth increasing evidence of this being a heterogeneous disease[18]. Unlike the discovery of the Bcr-Abl gene in chronic myeloid leukaemia, it is unlikely that more research will reveal a single specific gene locus that is responsible for prostate cancer. Naturally, such a multifaceted disease demands an equally multifaceted approach to risk-stratification, screening and diagnosis.

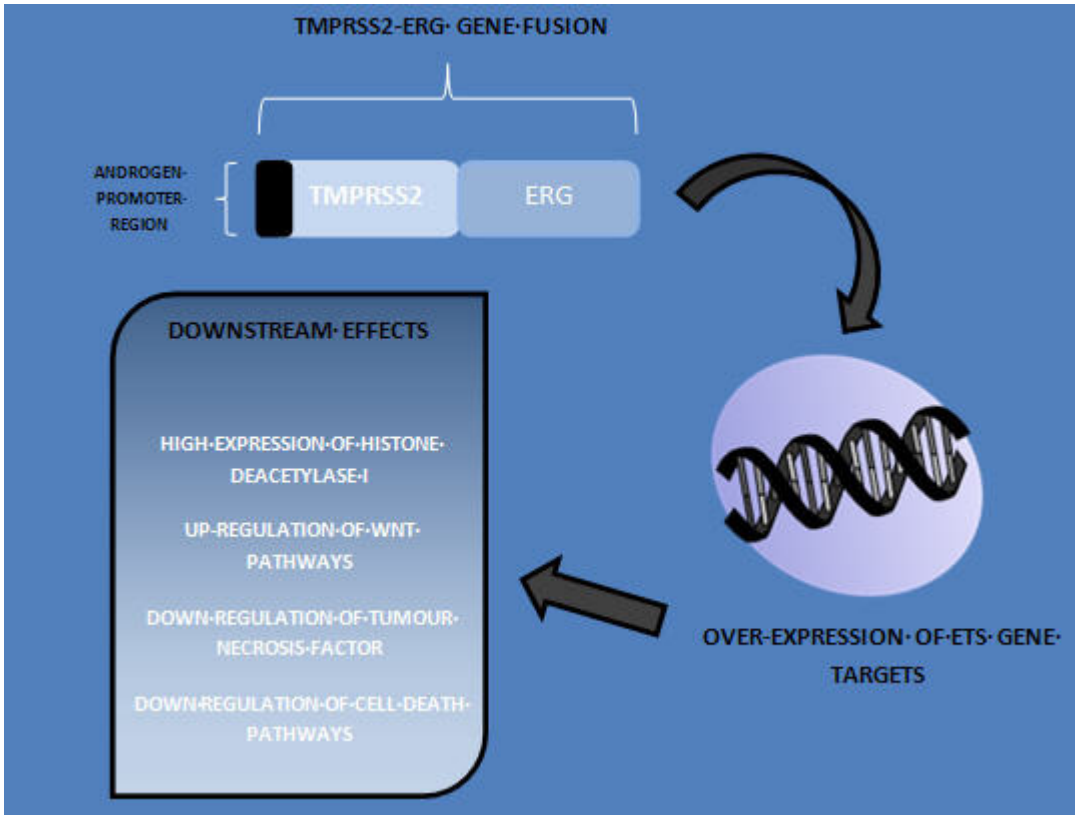
Novel biomarkers for sporadic prostate cancer have been found on many echelons of the central dogma of genetics: genetic (specifically DNA), epigenetic, transcriptomic,

proteomic and metabolomic approaches all show promise for use in clinical medicine in the future.

Genomics

TMPRSS2-ERG—This marker can be detected using RT-PCR methods, applied to urine samples from subjects whose prostate has been massaged. Discovery of this gene fusion is potentially the most significant advance in the last decade in the molecular pathology of prostate cancer.TMPRSS2 is a prostate specific gene[19,20] on chromosome 21 that codes for a transmembrane-bound serine protease[20]. The protease is predicted to react with a number of proteins on the cell surface, as well as extracellular matrix components, soluble proteins and proteins on nearby cells[21].ERG is a member of the ETS family of transcription factors which are able to activate or repress expression of genes involved in cellular proliferation, differentiation and apoptosis[22].

Figure 2. The potential significance of the TMPRSS2-ERG fusion



Note: The androgen-sensitive promoter region of the TMPRSS2 gene, through fusion to ETS family genes, could lead to androgen-driven overexpression of ETS family genes such as ERG. These in turn have been shown to cause downstream effects such as a high expression of the histone deacetylase I (HDAC I) gene, upregulation of Wnt pathways and downregulation of tumour necrosis factor and cell death pathways.[23]

Genes from the ETS family and TMPRSS2 lie nearby on chromosome 21, and hence fusions typically occur via rearrangements including deletion and translocation[24]. Cross et al[22] have suggested the possibility of certain sequences in TMPRSS2 and ERG which make some men more prone to these fusions that are seen in 49% of localised prostate cancers[22].

Furthermore, the timing of the occurrence of these fusions is particularly significant – TMPRSS2-ERG fusions have not been detected in morpohologically benign prostatic tissue but arise at a very specific point in the pathogenesis of prostate cancer, namely the high-grade prostatic intra-epithelial neoplastic stage (HGPIN) (essentially analogous to *carcinoma in situ*). In addition, in late-stage androgen receptor-negative cancers, TMPRSS2-ERG fusions were still present in the DNA but were not expressed[25], which aligns with the current understanding of the bypass mechanisms involved in androgen-independence and the fact that TMPRSS2 contains an androgen-dependent promoter region[22].

The clinical significance of these novel discoveries in the TMPRSS2-ERG fusion will be delineated more clearly as further studies are published.

In terms of prognostication, there have been groups who have looked at TMPRSS2-ERG fusions in comparison to measures such as Gleason Score, survival data and tumour recurrence. In general, TMPRSS2-ERG fusions were shown to be linked with worse prognoses[22]:

- 44% of Gleason pattern 5 contained TMPRSS2-ERG fusions compared with 7% of Gleason pattern 2 tumours[26]
- Non-fusion patients had a 90% survival at 8 years compared with 25% survival at 8 years in those identified having a particular pattern of TMPRSS2 fusion known as 2+ Edel (duplication of TMPRSS2-ERG fusion sequences and interstitial deletion of sequences 5' to ERG)[27]
- Tumours with TMPRSS2-ERG fusions had a higher recurrence rate after radical prostatectomy with an odds ratio of 7.1 (95% confidence interval 1.1-45)[28].

Despite their prostate specificity and their appearance in Prostatic Intra-epithelial Neoplasia (PIN), TMPRSS2-ERG fusions are unlikely to be suitable for screening as they have been found by Hessels et al[29] to show low sensitivity (37% in a cohort of 108). However, in the same study, the fusions were detected with a positive predictive value (PPV) of 94%[29], which suggests that it could be a useful risk-assessment tool whereby a clinician could request further biopsies in the cases where patients have a negative initial biopsy but persistently elevated PSA and positive test for the gene fusion product.

A similar pattern of *low sensitivity* but a *high positive predictive value* is seen in TMPRSS2-ERG fusions and their association with five key histological features[30]:

- Blue-tinged mucin
- Cribriform growth pattern
- Intraductal tumour spread

- Macronucleoli
- Signet-ring cell features

Ninety-three percent of cases in 253 prostate cancers with three of more of these features were TMPRSS2-ERG fusion positive (*high PPV*) but equally, 24% of TMPRSS2-ERG fusions did not show *any* of these features (*low sensitivity*) [30]. Its positive predictive value is comparable to the morphological features of HNPCC and BRCA-associated breast cancers, but the link between genotype and phenotype is not yet fully understood. Tumour morphology and association between TMPRSS2-ERG fusions thus stands as a potentially useful addition to the current armoury of diagnostic and risk-stratification methods, but further research is required in the field before we see collaboration between clinicians and histopathologic and cytogenetic services in New Zealand.

Urinary 8-hydroxydeoxyguanosine (8-OHdG)—It is widely agreed that reactive oxygen species (ROS) are direct causes of DNA damage. 8-hydroxydeoxyguanosine (8-OHdG), an oxidised nucleoside of DNA, is a frequently detected lesion where mismatch repair plays a key role[43].Upon DNA repair, 8-OHdG is excreted in the urine and thus can not only be a measure of DNA repair capacity, but *also* a biomarker for oxidative stress and potential carcinogenic initiation[44, 45].

Increased urinary DNA lesions were detected by Chiou et al[43] in both prostate and bladder cancer patients (58.5ng/mg creatinine of urinary DNA lesions in prostate cancer patients compared with 36.1ng/mg creatinine of Urinary DNA lesions in healthy patients) with a sensitivity of 31% and a specificity of 100%. Although their study population was small (and the fact that a biomarker of oxidative stress is not prostate-specific), the specificity of the test and the non-invasive nature of it suggests that with further investigation urinary 8-OHdG has potential as a biomarker which can allow for risk-stratification in those who have elevated serum PSA or a strong family history of prostate cancer.

8-OHdG is frequently detected in both non-malignant and malignant tissue. However, in non-malignant tissues extensive oxidative DNA damage drives cells to cell-cycle arrest (metabolic blockage), while in neoplastic prostate cancer cells it activates repair mechanisms favouring the escape from senescence and the expansion of DNA-damaged clones[133]. The combination of 8-OHdG in urine, measured along with cell-cycle check point evaluators such as CDKN1A, a cyclin-dependent kinase inhibitor and the product of the growth-arrested and DNA damage inducible gene Gadd45, from a parallel blood sample, may provide a greater understanding of the progression towards malignancy[134, 135].

Transcriptomics

Hepsin—Hepsin is a type II membrane associated serine protease whose structure and similarity to other serine proteases suggests that hepsin is involved in tumour growth, and hence hepsin stands as an attractive target in cancer biomarker development. Its prostate-specificity is best demonstrated through evidence of overexpression of hepsin (median 46.1-fold) in cancerous prostate tissue in 90% of prostate cancer samples (n=90)[46].

These findings have been confirmed through the work of Magee et al[47] in an analysis of 4712 genes. In the same analysis, Hepsin was found to be over-expressed in prostatic intra-epithelial neoplasia in comparison to BPH which points to a relationship between Hepsin and neoplastic transformation. In addition, one can propose that such a biomarker can aid in the prognostication of Gleason 4 and 5 tumours with the discovery of a correlation between increased Hepsin expression and higher Gleason score[46].

The major shortcoming of the use of Hepsin is the fact that it can only be detected in tissue specimens and, despite attempts to use RNA extracted from urine for quantitating hepsin[136] is not currently detectable from urine or serum samples[48]. Thus, the arrival of Hepsin as a prognostic tool for differentiation of indolent from aggressive tumours depends firmly on the discovery of novel methods of detection that will render it more accessible to clinical practice.

Prostate cancer antigen 3 ($DD3^{PCA3}$)—$DD3^{PCA3}$ is a novel, prostate-specific gene found to be up-regulated in cancerous prostate cells and over-expressed in >95% of clinical specimens[31,33]. PCA3 is more specific for prostate cancer than serum prostate-specific antigen (PSA), which is prostate-specific but not cancer-specific[41].

The proof of its prostate specificity has been shown through RT-PCR methodologies, in which PCA3 mRNA expression was low but quantifiable in benign prostatic tissue, but undetectable in normal and malignant tissue from other organs[32]. Equally, proof of over-expression of $DD3^{PCA3}$ in malignant prostate tissue with a median 66-fold up-regulation (compared to expression in benign tissue) has been demonstrated by Northern Blot analyses[31].

$DD3^{PCA3}$ has been concluded to express non-coding mRNA (defined through the presence of alternative splicing, polyadenylation, lack of an extended open reading frame and numerous stop codons) for which there is no discrete cytoplasmic protein product—despite overexpression of the mRNA transcript[31]. The function of the $DD3^{PCA3}$ gene and its non-coding mRNA transcript are currently undefined; hence, there is equally little known about the role of the $DD3^{PCA3}$ gene in pathogenesis of prostate cancer.

The magnitude of overexpression of the $DD3^{PCA3}$ gene in malignant specimens when compared to the near-negligible amounts of $DD3^{PCA3}$ expression in benign prostatic tissue confirms that the ultimate cause of the lack of a cytoplasmic protein product from PCA3 mRNA expression lies in the transcription as opposed to translation of other processing steps[31].

Although conflicting literature does exist on the subject of the $DD3^{PCA3}$ gene's clinical utility, the majority pertaining to the matter confirm that $DD3^{PCA3}$ has strong diagnostic value, particularly in differentiating early-stage prostate cancer from benign prostatic hyperplasia (BPH)[34,35,36]. PPV of 52.2% in men with PCA3 ≥100 is reported by Roobol et al 2010a and Robool et al 2010b.

This marker stands as one of the most attractive risk-stratification tools to detect early prostate cancer for a gamut of reasons:

- The $DD3^{PCA3}$ test does not require a biopsy– the mRNA is collected from urine after DRE and prostatic massage[34].
- $DD3^{PCA3}$ levels are directly reflective of tumour burden (as it is mRNA from cancer cells) and are *not* affected by prostate size, unlike PSA (which is a surrogate serum marker). This reduces the number of false positives detected in BPH cases and hence *increases* overall specificity[32].
- $DD3^{PCA3}$ mRNA expression adds the most value to current diagnostic tools at PSA values between 2.5ng/ml and 4.0ng/ml[34].
- The quantitative PCA3 score has been found to correlate to the frequency of prostate cancer-positive biopsy—thus it can act as a means to stratify patients into categories of prostate cancer risk[32].

In theory, it has all the hallmarks of a test which can deliver the much sought after specificity that is currently lacking in determining whether to biopsy or not. However, current validation studies have struggled to produce definitive results confirming $DD3^{PCA3}$ mRNA as a clinically applicable biomarker.

Five studies which look the performance of $DD3^{PCA3}$ which use ≥2.5ng/ml or ≥3.0ng/ml as PSA cut-off values gave the following values (as an average across the five studies)[37, 38,39,40,41]:

PPV:	**28.3%**
Sensitivity:	**62.6%**
Specificity:	**74.8%**
(Sample Size [average]: 303)	

Values for sensitivity have been quoted as high as 82% at 2.5ng/ml PSA cut-off[42] and for specificity. Mearini et al[34] claim 100% sensitivity (when PSA and $DD3^{PCA3}$ are combined) in a tPSA range <4ng/ml. It must also be noted that PCA3 scores and PSA cut-offs can be varied to change the specificity and sensitivity, whereby a higher PCA3/PSA cut-off will produce very high specificity (i.e. very few false positive results) but much compromised sensitivity (high number of false negative results) and vice versa with lowered cut-off values.

In addition, the means by which PCA3 is assayed for (i.e. the technology used) can also alter these results. What these values demonstrate is a classic teething issue of a novel biomarker; the lack of consistency in the type of assay used to identify the marker as well as small sample sizes hampers the production of consistent results and ultimately prevents the attainment of a definitive answer on the applicability of $DD3^{PCA3}$ as a prostate cancer biomarker.

This being said, its prostate-specificity and its potential to differentiate between indolent neoplasms and early malignant tumours ensures that further extensive

research will be conducted into the utility of DD3^{PCA3} as a biomarker aiding clinicians in early diagnosis of prostate cancer.

Epigenomics

Glutathione-S-transferase P1 (GSTP1)—From the family of Glutathione-S-transferases, GSTP1 conjugates chemically reactive electrophiles with glutathione, thus preventing DNA damage from reactive oxygen species and carcinogens which release reactive electrophilic metabolites[49]. Promoter hypermethylation of the region expressing GSTP1 has been directly linked to the loss of GSTP1 expression in prostate cancer[50,51,52]; indeed, this somatic genomic alteration is manifest in over 90% of prostate cancers—making it the most frequent epigenetic event reported in prostate cancer[51,52,53].

With respect to its role in cancer pathogenesis, GSTP1 hypermethylation and the resulting loss of expression is a process presently considered as a *promoter* of cancer (as opposed to an *initiator*), with loss of GSTP1 increasing susceptibility of DNA to oxidants and free radicals[54].

GSTP1 hypermethylation is an attractive target for more intensive investigation into its role as a prostate cancer biomarker for many reasons:

- Its role in the pathogenesis of prostate cancer has been elucidated and the mechanism is well understood.
- GSTP1 hypermethylation is not frequently observed in normal prostate tissue[50,53] (although there have been reports of GSTP1 hypermethylation in high grade prostatic intra-epithelial neoplasia).
- GSTP1 hypermethylation is less frequent in non-prostate genitourinary malignancies (e.g. renal and bladder cancer) [54].
- GSTP1 is not limited by the accessibility of sample collection; it can be identified in a range of body fluids: urine, serum, and ejaculate [54].

Although non-invasive procedures including collection of urine and ejaculate are held as the ideal means of attaining diagnostic information, there are key shortcomings with the use of these tissues. It has been shown that GSTP1 methylation levels are higher in plasma compared to urine, suggesting that prostate cancer is preferentially disseminated into the bloodstream rather than the prostatic ductal system[54].

With ejaculate, the inherent nature of such a collection procedure, particularly with older men, renders this avenue as one unlikely to see significant clinical exposure. Solutions such as prostatic massage to release cancer cells into the prostatic urethra before collection have so far delivered mixed results[48,58,59]. The difficulties faced in attaining clinically applicable detection rates through non-invasive methods remains a barrier yet to be surmounted.

Currently, the most promising results portraying GSTP1 hypermethylation have been produced from tissue samples. The use of quantitative methylation specific PCR (QMSP) in screening for GSTP1 methylation has been reported to deliver 85.5% sensitivity and 96.8% specificity (n=128)[56].

When further tests were conducted on the same set of tissue specimens to assess the capacity for differentiation between non-cancerous tissue and histologically-proven adenocarcinoma (n=21), the QMSP assay correctly diagnosed the specimens with 90.9% sensitivity and 100% specificity and 100% positive predictive value.

In addition, Harden et al[57] demonstrate a 15% increase in specificity of the gold-standard of prostate diagnosis—histopathologic assessment—through combining histopathologic assessment with QMSP for GSTP1. Furthermore, there is evidence that this method may be complemented with a measure of ENT SCTR methylation[137].

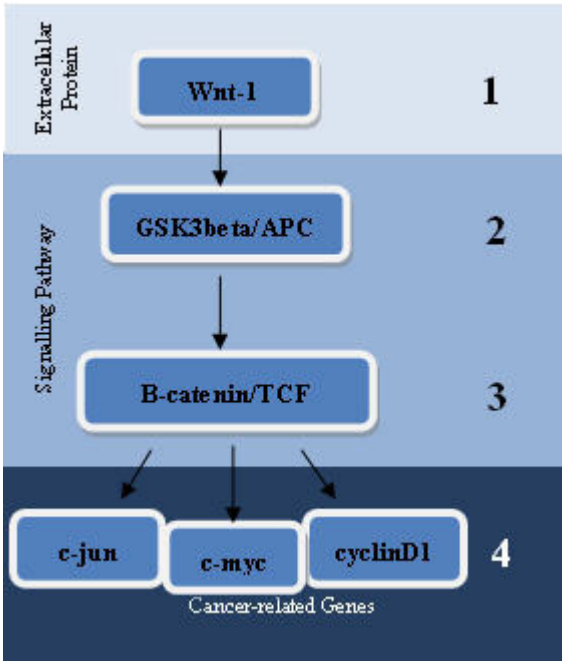
These results highlight the potential for GSTP1 hypermethylation as a means of complementing histopathological diagnosis of prostate samples and furthermore, a means of differentiating indolent and malignant neoplasms in cases where PSA levels alone are unable to discriminate[56].

Wnt signalling and methylation—Wnt signalling and its subsequent pathways are known to be crucial in mammalian and embryonic development[60, 61].

Its role in the pathogenesis of cancer can be summarised by the following diagram (modified from van der Poel HG[60]):

Figure 3. Potential involvement of the Wnt pathway in the development of malignancy. The steps portrayed are:

- **Binding of Wnt ligand to the frizzled transmembrane receptor.**
- **Decreased phosphorylation of B-catenin by GSK3-B. Therefore, stabilised B-catenin now accumulates in the nucleus.**
- **Nuclear B-catenin converts the TCF/LEF DNA binding complex from a transcriptional repressor into a transcriptional activator.**
- **Transcriptional activation of many cancer-related genes[61].**



In the case of prostate cancer, there are a handful of epigenetic changes which are thought to alter the Wnt signalling pathway:

- Hypermethylation of the APC (adenomatous polyposis coli) gene is increased 8-fold in prostate tumours relative to samples of benign prostatic hypertrophy[61]. It has been proposed that DNA hypermethylation of the APC gene, an important component of the B-catenin degradation complex, may lead to the nuclear accumulation of B-catenin and hence the activation of the Wnt signalling pathway activating various oncogenes[61].
- Equally, E-cadherin is a cell-membrane protein, which is known to both interact with B-catenin and be involved in the process of epithelial-to-mesenchymal transmission (EMT), a key step in the development of malignancy[63]. When the promoter for the E-cadherin gene is silenced by methylation, it not only promotes EMT but also the release of B-catenin away from the cell membrane and into the cytoplasmic and nuclear compartments. The presence of B-catenin in the nucleus will hence activate Wnt signalling[61,62].
- Secreted-frizzled related proteins (SFRPs) and Wnt inhibitory factor-1 (Wif-1) are antagonists for Wnt signalling. Thus, silencing of genes which express SFRPs and Wif-1 through hypermethylation will lead to aberrant Wnt signalling and cancer progression. Although silencing of genes encoding SFRPs and Wif-1 has been identified in many cancers, including colorectal, lung, and bladder cancers and lymphocytic leukaemia[64], there is insufficient evidence to definitively claim that Wnt antagonist genes play a key role in prostate cancer development.

Despite the extensive elucidation of the Wnt signalling pathway, there remain questions over its relevance to prostate cancer and whether assays for hypermethylation of any of the aforementioned genes will aid the delineation of a diagnostic landscape. However, the role of potential cancer promoters, exemplified by Wnt signalling, should be investigated further, as their presence may well be of use in risk-stratification processes in future. For example, Wnt pathway factors also promote osteoblastic lesions[138,139].

Xenobiotic metabolism and methylation—Xenobiotics (chemical compounds that are foreign to the body) have been widely studied as potential initiators for cancer. An extensively researched xenobiotic is the family of polycyclic aromatic hydrocarbons (PAHs): particularly prevalent in automobile exhausts and cigarette smoke, these compounds are known to be both toxic and carcinogenic[61].

The two cytochrome P450 enzymes responsible for initiating PAH metabolism through oxidation, CYP1A1 and CYP1B1, have been shown to be subject to alterations in expression in human prostate cancer specimens and prostate cancer cell lines through epigenetic activity[61]. In knock-down mice studies, there has been proof demonstrating that:

- Loss of CYP1A1 induction acutely *increases* sensitivity to PAH toxicity.
- Loss of CYP1B1 *protects* against PAH toxicity.

With this in mind, when observing results of experiments on prostate cancer specimens and cell lines which reveal both suppression of CYP1A1 induction and overexpression of CYP1B1 through respective hypermethylation and hypomethylation, we can ascertain that:

- A gene which protects against a carcinogen (PAH) is suppressed.
- A gene which positively mediates carcinogenic toxicity is over-expressed.

Thus, the epigenetic effects on these two genes synergise to have the combined effect of increasing sensitivity to PAH toxicity[61].Furthermore, in the context of GSTP1 promoter hypermethylation and hence GSTP1 suppression, there is not only down-regulated oxidation of PAHs but additionally, down-regulated glutathione conjugation, which ultimately renders both phases of xenobiotic metabolism adversely suppressed.

This information suggests that some prostate cancers may display acute sensitivity to PAH exposure. Such a finding has strong potential for clinical utility in New Zealand, and might be included in risk-stratification for prostate cancer given that:

- The 2006 Census shows 23.0% of all New Zealanders aged 15-64 are regular or current smokers[65] (smoking being a known behavioural exposure to high PAH levels).
- Smokers have been associated with higher prostate-cancer associated mortality in large epidemiologic studies[66] (although the strength of this association has varied between studies and meta-analyses[67, 68]).

The strong epidemiologic facet to the issue, particularly in an Aotearoa/New Zealand context with a high prevalence of regular tobacco use, demands further investigation into the epigenetic alterations to xenobiotic metabolism, in the hope of uncovering further putative biomarkers for prostate cancer.

Proteomics

α-methyl-acyl-coenzyme A-racemase (AMACR)—AMACR is an isomerase which is involved in both R-stereoisomer to S-stereoisomer conversion and peroxisomal B-oxidation of branched-chain fatty acids[69,70].It is currently in clinical use as an immunohistochemical marker for prostate cancer (autoantibodies to AMACR have been detected in serum more readily than the AMACR protein itself)[48], aiding in diagnosis of biopsy specimens, in which it delivers impressive sensitivities and specificities of over 90%[71,72].

Although androgen ablation therapy has been shown to down-regulate AMACR expression[73], it is widely agreed that AMACR is a major improvement on serum PSA testing with biopsy specimens, when differentiating between benign and malignant neoplasms[74].

The success of AMACR in biopsy specimens of prostate cancer however has not yet been reproduced in urine or serum. Rogers et al.[75] report 100% sensitivity and 58% specificity (n=26) when performing Western blot analyses on urine specimens and Zielie et al.[76] produced sensitivity and specificity values over 85% (n=21). However, this was only through use of normalised AMACR transcript levels relative to PSA

level for each prostatic secretion sample, whereby these levels were then compared to an experimentally-defined diagnostic cut-off value determined by a control group.

The small sample sizes and lack of long follow-up periods in such studies leave scope for further, larger-scale studies, to be conducted on the clinical utility of AMACR as a non-invasive biomarker. Furthermore, development of a standardised, reproducible protein-based assay. such as an ELISA (Enzyme-linked immunosorbent assay) with a standardised cut-off value for differentiating positive and negative results, would go a long way in validating such a biomarker as one able to distinguish indolent from aggressive tumours.

Human kallikrein 2 (hK2 or KLK2)—Homologous to PSA in 80% of its amino acid sequence identity, hK2 is a serine protease that is prostate-specific, with expression regulated by androgens on an androgen receptor. As a result, there is extensive immunologic cross-reaction between hK2 and PSA rendering comparisons between hK2 and PSA expression difficult. Despite the paucity of studies in the field, it has been identified that hK2 tissue expression is higher in malignant compared with benign prostate tissue—moreover, cells expressing PSA tend to be less frequent in poorly differentiated malignant tissue compared to benign tissue[49, 77, 78]. This lends hK2 prognostic capability and predictive value in monitoring the course of disease more robust than what is currently delivered through PSA testing.

hK2 is a biomarker which is limited through the variability in assay configuration and antibody specificity in particular, in addition to other atypical issues with biomarkers which include diagnostic and sampling criteria and age of samples. Furthermore, one must note that, as with PCA3/PSA ratios, the sensitivity and specificity of such a test is completely dependent on the diagnostic cut-off value chosen. One can produce a 95% sensitivity, that is *detect* 95% of all cancers, but at the same time, have a specificity of 24% (meaning 76% of men will have to undergo an unnecessary biopsy) at a given hK2/free PSA ratio[79].

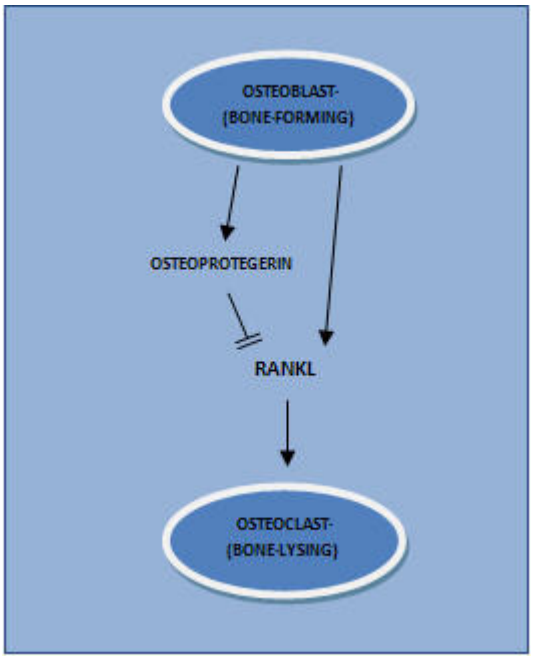
The greatest strength of this potential biomarker lies perhaps in its predictive value for biochemical recurrence in patients with PSA $\leq$ 10.0ng/mL (AUC for extra-capsular extension and seminal vesicle invasion were 0.662 and 0.719 respectively for hK2 compared with 0.654 and 0.663 respectively for tPSA). Additionally hK2 is able to maintain its prognostic value for biochemical recurrence of disease when corrected for clinical variables[80, 81]. This is clinically pertinent as hK2 performs comparatively well in the "grey zone" of PSA 2.5 – 10.0ng/ml - the area of greatest weakness of PSA testing.

Furthermore, the "grey zone" of PSA 2.5 – 10.0ng/ml is a category with burgeoning numbers of patients as a result of a drive for early diagnosis ultimately culminating in more men being diagnosed with prostate cancer whilst having a PSA level in the "grey zone". Thus, hK2 may play a synergistic role with PSA testing, to deliver more accurate prognoses for patients with low-PSA level cases of disease.

The significance of the improvement with hK2 testing in diagnostic and prognostic strength on current methods is insufficient to see it replace PSA testing outright, but rather, with further validation, provide adjuvant diagnostic and prognostic value in serum testing.

Osteoprotegerin—As prostate cancer advances, it has the ability to induce the formation of osteoblastic lesions, which in turn manifest themselves as osteosclerotic (abnormally hardened or dense bone) lesions, initially forming in the axial, but later in the appendicular skeleton[82]. Osteoprotegerin (OPG) is a cytokine produced by osteoblasts (bone-forming cells) which inhibits RANKL (also produced by osteoblasts), an activating cytokine of bone-lysing osteoclasts[82,83]:

Figure 4: A simplified schematic representation of the role of osteoprotegerin (OPG) in the inhibition of osteoclastic activity and hence formation of osteoblastic lesions



Thus, the possibility of metastatic prostate tumour cells secreting OPG and potentially causing osteoblastic changes in the architecture of bone is of interest in monitoring the progression of advanced prostate cancer cases. Moreover, bone is known to be the most common site of prostate cancer metastases[18], further underpinning the importance of OPG as a potential biomarker in advanced prostate cancer.

Indeed the data produced from current studies highlight OPG as a promising serum-based marker which, unlike PSA, is specific for detection of bone metastases:

- Serum levels of OPG were found to be significantly higher in advanced prostate cancer patients than those at other stages of prostate cancer[84, 85].
- Serum OPG identified patients with bone metastases at a sensitivity of 88% and specificity of 93%[86].
- Elevation of serum OPG not observed in bone metastases of any other malignancies[87].

Although there is much promise in the potential of OPG to provide prognostic information post-androgen ablation, one must be aware of a key caveat in the interpretation of serum OPG levels. OPG levels, although not elevated through bone metastases of other malignancies, are increased in cases of rheumatoid arthritis and vascular diseases[88, 89].

Given that these pathologies, as well as prostate cancer, generally occur in older populations, it would be appropriate to interpret serum OPG levels based on age-stratified values in a clinical setting, normalised for the presence of "background" OPG sources such as vascular disease.

With a commercial serum OPG ELISA now available[18], the progress of randomised, controlled studies of serum OPG as a marker for prostatic bone metastases now have the reproducibility required for clinically robust diagnostic and prognostic assays. Ultimately, such studies can produce further data on a biomarker which may aid clinicians in determining the course of disease for advanced, metastatic prostate cancer.

Telomerase—Telomeres are sequences of DNA which stabilize and protect the ends of chromosomes, and their maintenance is regulated by telomerases, which in turn are encoded for by the telomerase reverse transcriptase (*TERT)* gene. Loss of telomeres is associated with the processes of chronic inflammation, oxidative stress and cell division. Whether telomeric loss in such processes is causally linked to the finding that telomerase activity is expressed in at least 90% of prostate cancers[90, 91], remains to be seen.

Telomerase has been successfully detected in prostate biopsy specimens, prostatic fluid and urine[18]. However, the variability of results produced by various studies, suggests techniques such as prostatic massage, as well as the sensitivity of differing assays, plays a role, particularly with urine samples, in the qualitative analysis of telomerase in prostate cancer urinary specimens[49].

Sensitivity and specificity value ranges of 58%, 90%, 100% and 100%, 76%, 88%[92, 93, 94], respectively, are testament to the inconsistency that currently stands in relation to telomerase assays and testing.

Further evaluation of telomerase assays through multi-centre investigations with large cohort numbers is required before we can ascertain its true value in the discernment of malignancy in the prostate.

Metabolomics

The field of metabolomics is perhaps the most underexploited pathway in the search for novel cancer biomarkers. Analysis of metabolic alterations in prostate cancer may be of use in tracking the progression of malignancy. A selection of the well-studied metabolites and their relationship to prostate cancer are summarised in the table below:

Table 1. Associations of prominent metabolites with prostate cancer

Metabolite	Association with prostate cancer
Lactate	High levels in prostate cancer compared to normal prostate and BPH tissue[95, 96, 97, 98, 99, 100]. Associated with increased glucose metabolism is a characteristic feature of tumour cell metabolism[107] as glucose is converted to lactate via glycolysis[103].
Citrate	Low levels in prostate cancer compared to normal prostate and BPH tissue[95, 96, 97, 98, 99, 100]. Loss of citrate has strong correlation with tumour grade (determined through Gleason Score[105, 106])—low levels in early stage prostate cancer and absent in poorly differentiated tumours[101]. Citrate oxidation and hence lower levels of intracellular citrate occurs due to loss of ability to accumulate and hence lower levels of intracellular zinc in malignant cells[102, 103].
Choline/Creatine	Elevated in prostate cancer[95, 96, 97, 98, 99, 100]; increased levels correlate to Gleason Score[105, 106].
Polyamines	Higher levels in healthy tissues[104] compared with lower levels in prostate cancer[95, 96, 97, 98, 99, 100]. Absent in 80% of high grade tumours—thus loss of polyamine metabolites are potentially a marker for both stage and grade of prostate cancer[104].
Sarcosine	Despite considerable interest in a paper in the journal Nature reporting a potential role for sarcosine in prostate cancer, as delineated by metabolomic profiles[142], the relevance of this metabolomic marker is widely debated[140,141]. However, there may be utility in its inclusion as a component in multiplex modelling with other prostate cancer biomarkers[143].
Others	Taurine, glutamine, glutamate, and alanine have been found to be associated with malignancy, but have not correlated directly with tumour grades[103].

The elucidation of the link that exists between prostate cancer and metabolites of tumour cells continues. The early data published on the significance of the association of metabolites, particularly citrate and choline (indeed a low citrate/choline ratio is indicative of a high-grade tumour, when measured with Magnetic Resonance Spectroscopy (MRS) [104, 108]) stipulates that further studies are warranted in the quest to uncover metabolomic tests which are able to accurately map the progression of prostate cancer tumours through clinically feasible and robust biomarker assays.

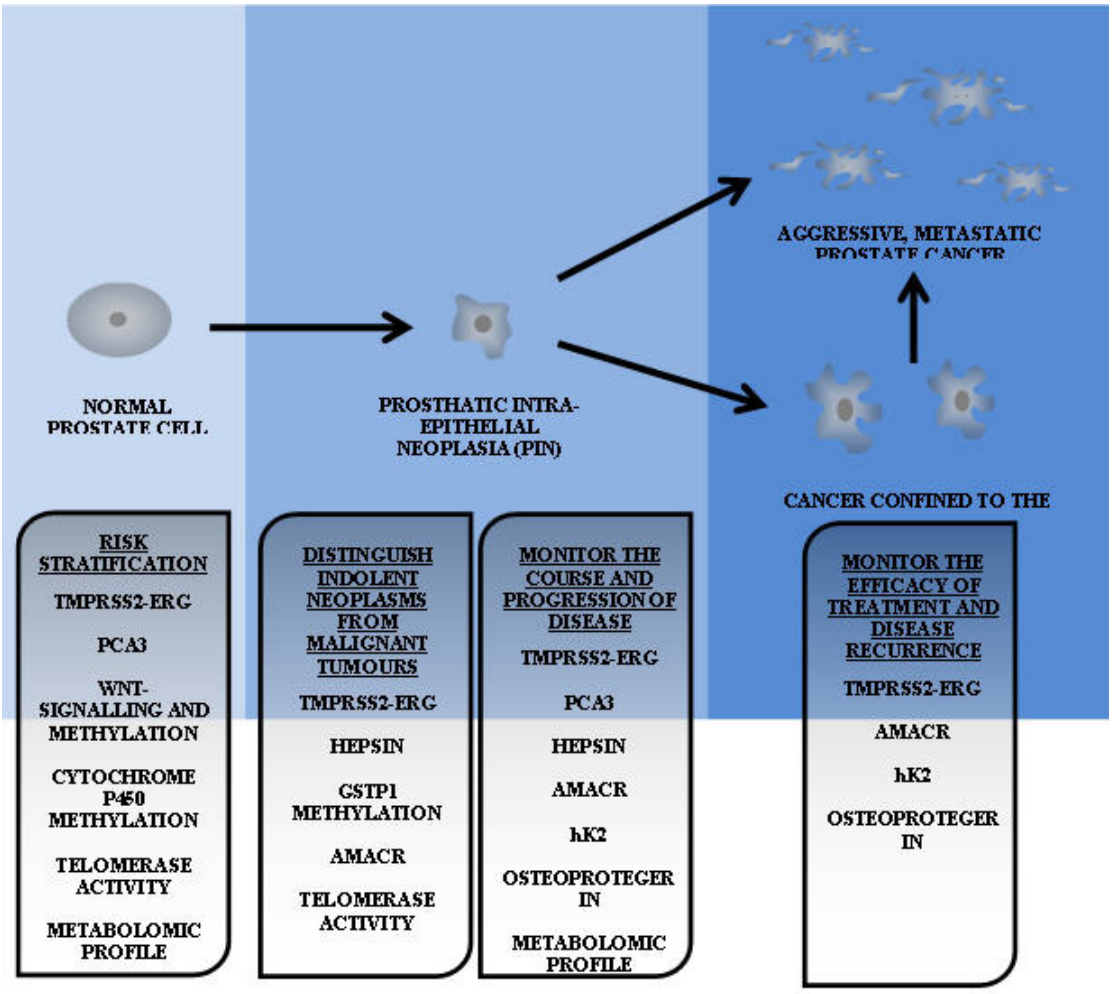
Summary of the potential clinical applications of novel prostate cancer biomarkers

Table 2. Applications of promising novel biomarkers for prostate cancer

Application	Biomarker	Specimen
Risk-stratification for prostate cancer	TMPRSS2-ERG[22]	Urine[22]
	PCA3[32]	Urine[34]
	8-OHdG[43]	Urine[43]
	Wnt-Signalling and Methylation[61]	Biopsy[61]
	Cytochrome P450 Methylation (Altered Xenobiotic Metabolism)[61]	Biopsy[61]
	Telomerase Activity[90, 91]	Urine[49]
	Metabolomic Profile[104, 108]	– (*In situ* Endorectal Magnetic Resonance Spectroscopy Imaging [MRSI])[98]
Distinguish indolent neoplasms from malignant tumours	TMPRSS2-ERG[22]	Urine[22]
	Hepsin[46, 47]	Biopsy[46]
	GSTP1 Methylation[50, 53, 56, 57]	Biopsy, Serum, Urine, Ejaculate[54]
	AMACR[71, 72]	Biopsy, Urine, Serum[74, 75, 76]
	Telomerase Activity[90, 91]	Urine[49]
Monitor the course and progression of disease	TMPRSS2-ERG[30]	Urine[22]
	PCA3[18]	Urine[34]
	Hepsin[46]	Biopsy[46]
	AMACR[73]	Biopsy, Urine, Serum[74, 75, 76]
	hK2[49, 77, 78]	Serum[80, 81]
	Osteoprotegerin[84, 85]	Serum[18]
	Metabolomic Profile[104]	– (*In situ* Endorectal Magnetic Resonance Spectroscopy Imaging [MRSI])[98]
Monitor the efficacy of treatment and disease recurrence	TMPRSS2-ERG[28]	Urine[22]
	AMACR[73]	Biopsy, Urine, Serum[74, 75, 76]
	hK2[80, 81]	Serum[80, 81]
	Osteoprotegerin[86]	Serum[18]

Adapted from Hessels et al.[18]

Figure 5. Potential application of new biomarkers in prostate cancer diagnosis and assessment of status



Implementation of novel biomarkers into clinical practice: a strategy unique to Aotearoa/New Zealand

"Research is to see what everybody else has seen, and to think what nobody else has thought." (Albert Szent-Gyorgyi, 1893–1986; 1937 Nobel Prize for Medicine)

Such an adage epitomises the ethos of biomedical research and undoubtedly encapsulates the modern approach to discovery and development of novel biomarkers in prostate cancer. However, the voyage of such scientific idealism from theory to practice will ultimately always be dictated by a plethora of guidelines and regulations as well as financial and practical limitations.

Indeed, Pepe et al[110] have delineated five phases with which researchers are able to stratify biomarkers into stages of development.

Table 3. Phases of biomarker development

Phase	Aims
Phase 1—Preclinical Exploratory Studies	Identify and prioritise potentially useful biomarkers.
Phase 2—Clinical Assay Development for Clinical Disease	Assess true positive and false positive rates in the assay. Assess the ability of the assay to differentiate specimens with and without cancer. Compare biomarker measurements in tissue specimens and non-invasive specimens. Optimise the reproducibility of the assay. Assess factors such as age, gender and ethnicity with relation to biomarker measurements. Assess correlation between biomarker measurements and the stage, grade, histology and prognosis of tumours.
Phase 3—Retrospective Longitudinal Repository Studies	Assess ability of biomarker to detect preclinical disease. Define criteria for a positive screening test. Compare multiple biomarkers and develop a combination-biomarker algorithm for screen positivity.
Phase 4—Prospective Screening Studies	Assess of the sensitivity and specificity of the biomarker-based test in a population. Assess the feasibility of implementation of such a screening programme. Assess patient compliance and the factors governing patient compliance. Assess speculatively effect of screening on costs and cancer-associated mortality. Monitor character and progression of tumours not detected by screen (the false negative results).
Phase 5—Cancer Control Studies	Estimate the reduction in burden of cancer and cancer mortality in the population resulting from biomarker. Analyse costs of screening and treatment in comparison to alternative screening methods.

Although few biomarkers will progress linearly through each phase[110], the significance of such a framework lies within the depth with which a biomarker must be analysed and rigorously assessed before the decision is made to impart sparse resources into a novel development. The lengthy wait for novel biomarkers in the clinical assessment of prostate cancer is testament to the stringency of the processes and regulations required.

What has transpired is a delicate balance between the production of biomarkers that are accurate, non-invasive, inexpensive and clinically-robust, and the demand for having such biomarkers available in the near future for clinical use, given the progressive increase in cancer burden in New Zealand over the last 15 years (due to a 7% increase in cancer incidence in males between 1996-2011 and a 20% decrease in cancer mortality in males over the same time period[111]).

The reversal of this upward trend in cancer burden in men will not only occur with the more immediate introduction of novel prostate cancer biomarkers, but also through integration of novel discoveries into primary health care. The primary healthcare system in New Zealand stands as the crucial interface between the healthcare system and the population in which many biomarkers through risk-stratification methods will potentially be able to diagnose pre-clinical prostatic disease and differentiate indolent from aggressive phenotypes, ultimately leading to potential substantial improvements in current clinical practice.

It is also important that, despite statistics portraying more New Zealand European men being diagnosed with prostate cancer it has been shown, through mortality data, that more Pacific Islands and Maori men die of the disease[126]. Whether the disease is of a fundamentally different nature in this group and requires a different approach to treatment, or whether it is being diagnosed at a later stage, may also become far clearer with more systematic use of a panel of biomarkers which may become available in future as more biomarkers become validated through evidence manifest in large-scale clinical trials.

The realisation of a comprehensive prostate cancer screening programme depends primarily on the work of researchers and their capacity to "*think like nobody else has thought*", unearthing one or many biomarkers which may provide evidence-based, compelling and definitive diagnostic and prognostic information in the field of prostate cancer, which clinicians will ultimately be able to utilise in bringing about better health outcomes for men in Aotearoa/New Zealand.

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