

# PROSTATE CANCER PROGNOSTIC TESTS: PRE AND POST DIAGNOSIS

A multiplicity of tests for the diagnosis and prognosis of PCa have been introduced and are slowly making their way into practice. This report details many of these tests and examines them in some detail. Copyright 2015 Terrence P. McGarty, all rights reserved.

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## 1 INTRODUCTION

There are a growing number of tests to enhance the diagnosis and prognosis of PCa. They fall into two groups.

The first group is what we call the Pre Tests, those used to ascertain the patient's risk of having PCa and performed before a definitive pathological diagnosis. The intent of these tests is to reduce the negative effects of unnecessary biopsies. PSA monitoring, through PSA, % Free PSA and PSA velocity, have shown some use but they can be confusing at times. They often result in unnecessary biopsies which the medical community is trying to reduce. On the other hand aggressive PCa can kill in a very short period of time and thus without better metrics one may see a dramatic increase in mortality, albeit in a smaller group.

The second group of tests is called the Post Tests, namely those examining actual PCa in a patient. They often focus on genetic markers which can be highly prognostic of aggressive development. The problem here of course is that identifying such an aggressive PCa does not result in reduce mortality since the approaches to dealing with metastasized PCa, especially to the bone, is still highly problematic.

There are many factors that pre-dispose to PCa. Family background is one. As Tao et al state:

*Besides age and racial background, family history of the disease is another major risk factor for prostate cancer, which has high probability of heritability Alberti et al. This is also supported by epidemiological studies in a Swedish cohort, which indicated about 11.6% of prostate cancer cases could be accounted by familial factors and that the risk is much greater for men with brothers suffering from prostate cancers.*

*Thus, it has been observed that first degree relatives of prostate cancer patients suffer almost double the risk as normal population for developing prostate cancer. This familial risk in first-degree relatives is more than 4-fold for early-onset cases occurring under 60 years of age.*

*Also studies on Nordic twin registries showed monozygotic twins suffer ~50% higher risk in than the dizygotic twins, which strongly indicates that genetic factors play a more important role in determining the risk rather than the lifestyle factors... Obesity was found to be associated with increased incidence of aggressive prostate cancer as well as prostate cancer recurrence. Prostate cancer-specific mortality is also likely to be elevated significantly by obesity....*

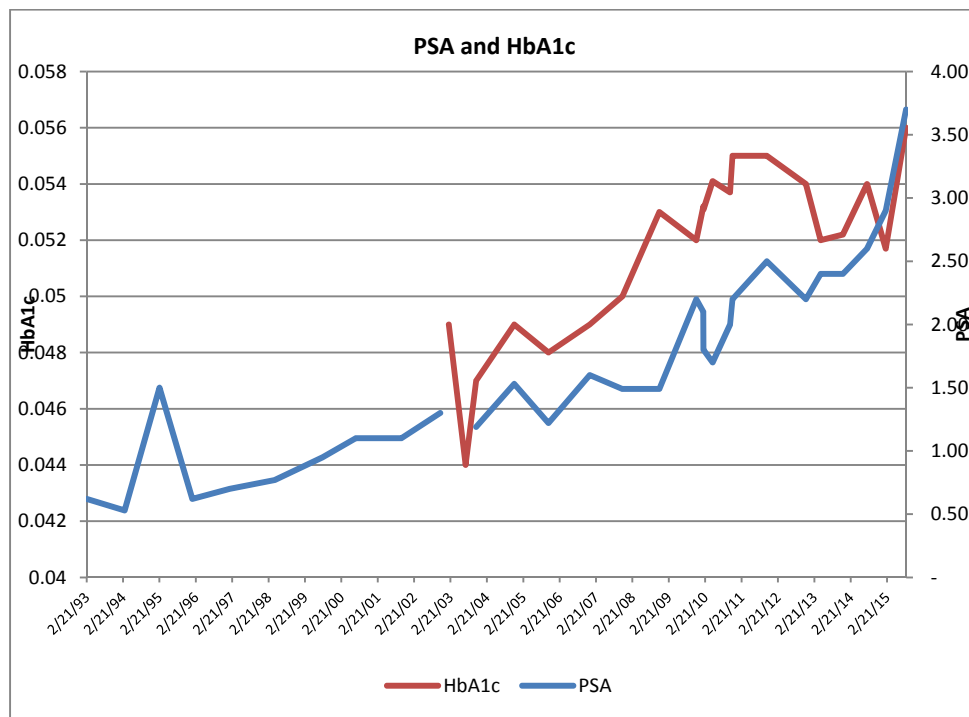
Thus a significant factor in ascertain PCa status is to have a detailed understanding of the family history. First degree relatives and their type of PCa, aggressive or indolent, may be one of the most significant factors. However the clear genetic nexus for such a suspicion is still wanting. Some studies have actually linked the heritability via the mother and not the father.

The key to monitoring for PCa has been the PSA test. However it faces an amount of uncertainty. Increasing PSA has many causes. As recently summarized by Akbas et al:

*It is noteworthy that high PSA level in men with prostate cancer is the result of disrupted basement membrane and ductal lumen architecture rather than an actual increase in PSA production. Therefore, prostatic pathology (prostatitis, benign prostatic hyperplasia – BPH or prostate cancer) is a crucial factor in determining PSA level. To a lesser extent, prostatic manipulation by DRE, biopsy or transurethral resection (TUR) might result in a slightly higher serum PSA.*

*However, it has been shown that DRE – related PSA rise is rarely clinically significant. Age, race, androgens and prostate volume are known PSA determining factors, with higher PSA expression in older, black men and in those with higher androgen levels and larger prostates. Also, significant decline in PSA level can be observed by using 5 $\alpha$ -Reductase inhibitors (type 2 isoenzyme inhibitors and dual type 1 and 2 isoenzyme inhibitors) for BPH treatment. Lastly, there is some evidence that other factors such as ejaculation, body weight, carbohydrate intake, and insulin resistance could influence serum PSA levels.*

Thus one may “normalize” PSA by other factors such as HbA1c, age, race, BMI, prostate size, as well. Thus measuring PSA may require a complex set of normalizing factors. The prognostic value of PSA alone can be problematic. In addition the temporal behavior of PSA provides a substantial clue to putative biological changes. For example we show below from a specific patient the variation of PSA with HbA1c. The patient saw a jump in HbA1c due to an increase in carbohydrate intake. This seems to have resulted in a highly correlated increase in PSA.



We demonstrate some of the driving factors below.



Thus there is no single or even a set of well understood factors which are exogenous to PCa that influence PSA.

## 1.1 RISK FACTORS

In a paper by Louie et al the authors examine risks factors in PCa. They state:

*To our knowledge, this systematic review and meta-analysis is the first to evaluate the diagnostic performance of predicative models to identify participants at high risk for PCa.*

*Despite identifying 127 unique PCa predictive models, only six risk models for predicting any PCa and only one model for predicting clinically significant PCa were evaluated in  $\geq 5$  study populations.*

*This suggests that many poorly validated models exist. Aside from PCPT, our summary results suggest that the discriminative accuracy of five prediction models was better than PSA testing. Finne, Karakiewicz, Chun, ERSPC RC3 and Prostataclass models had a summary AUC > 0.74, suggesting their high discriminative ability compared with PSA testing (AUC = 0.66).*

*Among these validated models, Prostataclass and ERSPC RC3 have the highest discriminative value to predict any PCa (AUC = 0.79), suggesting them to be the best performing models. Our pooled AUC for PSA testing of 0.66 corresponds to a sensitivity of 21% for detecting any PCa, which is consistent with the pooled sensitivity estimate of PSA testing (21%) assuming a 91% specificity for men with a PSA cut-off of >4 ng/ml [54], the generally accepted abnormal threshold to prompt a man to undergo prostate biopsy for further PCa investigation [55]. Similarly, assuming no loss in specificity, an AUC of 0.80 corresponds to a sensitivity of 44%.*

*Thus, our results suggest that reported prediction models have the potential to double the sensitivity of PSA testing (44% versus 21%) to discriminate PCa and improve thresholds for biopsy.*

*Although our metaanalysis suggests that prediction risk models improve the performance of PSA testing for screening, the meta-analysis did not allow for performance comparison between models. Without applying and comparing all six prediction models in a cohort of men undergoing PCa screening, conclusions cannot be made about model superiority because the estimated predictive accuracy of AUC mainly reflects differences in population characteristics.*

## 1.2 GUIDELINES

Regarding guidelines the ACA guidelines as presented by Smith et al state:

*Men who have at least a 10-y life expectancy should have an opportunity to make an informed decision with their health care provider about whether to be screened for prostate cancer after receiving information about the potential benefits, risks, and uncertainties associated with prostate cancer screening; prostate cancer screening should not occur without an informed decision-making process.*

Clearly they do not consider any of the approaches we discuss herein. Although this guideline is better than the grossly punitive one of the USPTF, namely no PSA at all, it does not enlighten in the area. The NCCN Guidelines in 2015 state regarding PSA and testing<sup>1</sup>:

*The NCCN Guidelines incorporate a risk stratification scheme that uses a minimum of stage, grade, and PSA to assign patients to risk groups. These risk groups are used to select the appropriate options that should be considered for treatment and to predict the probability of biochemical failure after definitive local therapy.*

*Risk group stratification has been published widely and validated, and provides a better basis for treatment recommendations than clinical stage alone. The NCCN Guidelines Panel recognized that heterogeneity exists within each risk group. For example, an analysis of 12,821 patients reported that men assigned to the intermediate-risk group by clinical stage (T2b-T2c) had a lower risk of recurrence than men categorized according to Gleason score (7) or PSA level (10-20 ng/mL).*

*A similar trend of superior recurrence-free survival was observed in men placed in the high-risk group by clinical stage (T3a) compared to those assigned by Gleason score (8-10) or PSA level (>20 ng/mL), although it did not reach statistical significance.*

They continue:

*Personalized or precision medicine is a goal for many translational and clinical investigators. The Institute of Medicine has defined clearly lessons learned that should accelerate the development of useful biomarkers to inform men and their physicians about more proper choices for treatment of localized prostate cancer. Dr. Hayes has warned us that a “bad tumor marker is as bad as a bad drug”.*

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<sup>1</sup> [http://www.nccn.org/professionals/physician\\_gls/pdf/prostate.pdf](http://www.nccn.org/professionals/physician_gls/pdf/prostate.pdf)



*The Prostate Cancer Guidelines Panel takes pride in its leadership regarding the need for life expectancy estimation, use of nomograms and recommendations for active surveillance as the only option for men with low risk prostate cancer and life expectancy less than 10 years or very low risk prostate cancer and life expectancy less than 20 years.*

*American men continue to under select active surveillance for very low or low risk prostate cancer largely due to uncertainty about the risk of disease progression, an uncertainty that could be reduced by a molecular biomarker that can be measured accurately and reproducibly and provide prognostic or predictive information beyond risk group assignment and currently available tables and nomograms. Two tissue-based molecular assays appear further along in development and clinical use.*

*The Prolaris assay produces a cell cycle progression (CCP) score from RNA expression levels of 31 genes involved in CPP. The Oncotype DX Prostate Cancer assay produces a Genomic Prostate Score (GPS) from RNA expression levels of 17 genes from 4 different molecular pathways (stromal response, cellular organization, androgen signaling and cell proliferation). These tissue-based molecular assays can be performed on most formalin-fixed, paraffin-embedded prostate specimens.*

*For example, Prolaris has been successful in 93% of radical prostatectomy specimens<sup>37</sup> and 79% of diagnostic prostate biopsy specimens<sup>38</sup>). The Prolaris CCP score has been demonstrated predictive when applied in prospective-retrospective designs for biochemical recurrence or metastasis after radical prostatectomy, for survival when men were observed after diagnosis on transurethral resection of prostate or diagnostic needle biopsy, and for biochemical recurrence and survival after external beam radiation therapy.*

*The Oncotype DX GPS was developed from evaluation of a diagnostic prostate biopsy and radical prostatectomy series from Cleveland Clinic and validated in a diagnostic prostate biopsy and radical prostatectomy series from University of California, San Francisco.*

*GPS performed in the diagnostic prostate biopsy has provided information beyond usual clinical information that predict the likelihood of Gleason sum 7 or extraprostatic disease on radical prostatectomy.<sup>35</sup> Prolaris has changed treatment recommendations in 32% to 65% of cases and may enhance adherence to the treatment recommended.*

*Oncotype DX GPS improved upon risk group assignment, which may enhance rates of compliance with recommended active surveillance or diminish the number of surveillance prostate biopsies*

It should be noted that these Guidelines do expressly mention 4K<sup>2</sup>.

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<sup>2</sup> <http://clinical.opko.com/news/recommended-by-nccn-news> and [http://www.nccn.org/professionals/physician\\_gls/pdf/prostate\\_detection.pdf](http://www.nccn.org/professionals/physician_gls/pdf/prostate_detection.pdf)

### 1.3 OTHER METHODS

Other methods of detecting PCa include the biopsy, which itself has limitations. It samples at most 2-3% of the cells and unless guided appropriately can have poor results. As Brock states<sup>3</sup>:

*The current standard for the diagnosis of prostate cancer is the systematic transrectal ultrasound (TRUS)–guided biopsy with 10 to 12 biopsy cores. By including an additional targeted biopsy of suspicious hypoechoic areas, the detection rate of prostate cancer can be increased by 3.5% during the initial biopsy. However, the sensitivity of the conventional biopsy method is limited: autopsy studies that compare the prostate biopsy with whole-mount sections of the entire prostate gland place it at 53% .*

*Because of this diagnostic uncertainty, approximately one-third of patients with ongoing cancer suspicion must undergo a repeat biopsy within five years after the initial biopsy, which in turn gives a cancer diagnosis in 13% to 41% of cases. To reduce the rate of false-negative biopsies, the methods recommended for rebiopsies are the extended biopsy approach (such as saturation biopsy) or the modified access path approach (such as transperineal mapping). If prostate cancer is confirmed, the question arises as to whether the biopsy result can correctly identify the histological tumor stage and thus can be used for therapy planning or prognosis estimation. In fact, the risk of misclassification with conventional prostate biopsy ranges between 21% and 54% . ...*

*MRI/TRUS fusion–assisted targeted biopsy improves the detection rate of prostate cancer after a previous negative biopsy. Targeted biopsy is more likely to reveal clinically significant cancer than systematic biopsy; nevertheless, systematic biopsy should still be performed, even if the MRI findings are negative.*

### 1.4 CURRENT NCCN RECOMMENDATIONS TESTS

NCCN does provide a set of Pre and Post recommended tests. We will discuss both categories. The Pre Tests fall into two categories; those using PSA or Kallikreins and those examining methylation or epigenetic factors. The latter test requires a biopsy to determine methylation on the prostate cells.

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<sup>3</sup> See Egbers et al and also Barentsz, et al. for additional recommendations regarding this approach.

## 2 TESTS

We here provide a summary of some of the current tests which are available. We take no position as to the efficacy of any of these tests and at best we can rely upon the clinical trial that have been reported.

In a recent article in Prostate Conditions the author reports on a variety of pre and post tests. He states<sup>4</sup>:

*Biomarker tests continue to impact the world of prostate cancer from early detection through diagnosis, by helping men to better understand their risk of having the disease and identifying the aggressiveness of the disease if present.*

### 2.1 PRE DIAGNOSTIC TESTS

Let us begin with the Pre Tests. As we have noted before<sup>5</sup>

<i>Test</i>	<i>Description</i>	<i>Source</i>
4KScore	The 4Kscore Test is a blood test that provides a patient-specific probability for finding an aggressive (Gleason score 7 or higher) prostate cancer upon biopsy. The information can be used by the Urologist to have an informed discussion with the patient about whether or not to have a prostate biopsy. The 4Kscore Test measures four prostate-specific kallikreins in the blood: Total PSA, Free PSA, Intact PSA, and Human Kallikrein 2 (hK2). The blood test results are combined in an algorithm with patient age, digital rectal exam (nodule, no nodule), and prior negative biopsy (yes, no). The 4Kscore Test then provides a % probability on a scales from <1% to >95% for the patient having aggressive prostate cancer.	OPKO <a href="http://www.opko.com">www.opko.com</a>
ProgenesaPCA3	A FDA-approved urine test that detects the over-expression of the PCA3 gene, which is specific to prostate cancer and an accurate predictor of whether cancer may be present. The PCA3 score is used to determine if a repeat biopsy is needed in men who are used to determine a man's PCA3 score, which indicates the need for biopsy. Research is underway looking at PCA3 as prostate cancer screening population as well.	<a href="http://www.hologic.com">http://www.hologic.com</a>

<sup>4</sup> <https://www.prostateconditions.org/prostate-conditions/prostate-cancer/screening-a-prevention/12-prostate-conditions/111-biomarkers-in-prostate-cancer>

<sup>5</sup> See, *PSA Evaluation Methodologies: New Alternatives*, TWP No 80 December 2010. This was our first attempt to develop a dynamic model for PSA use in PCa detection. It relies upon a set of multi parameters and is in some ways putatively superior to some of the tests proposed herein. Yet no clinical trials have been performed with this approach. The problem is that it requires a decade long collection of at least annual PSA and %Free measures.

<i>Test</i>	<i>Description</i>	<i>Source</i>
Prostate Health Index	<p>phi is the only multi-analyte blood test listed as a marker of specificity for prostate cancer in the Guidelines. The Guidelines indicate that a phi value &gt; 35 is strongly suspicious for prostate cancer. phi is a powerful combination of three Beckman Coulter assays: (i) Access Hybritech PSA, (ii) Access Hybritech free PSA, (iii) Access Hybritech p2PSA3</p> <p>The new and novel p2PSA assay is specific to measuring [-2]proPSA. The [-2]proPSA biomarker is an isoform of free PSA that was identified as the most prostate cancer-specific form found in tumor extracts. The p2PSA results are combined with PSA and free PSA test results by an algorithm in the Beckman Coulter Access instrument, providing a probability of prostate cancer.</p>	<a href="http://prostatehealthindex.us/">http://prostatehealthindex.us/</a>
ERG Protein Tissue Marker	Development of an ERG protein assays to be utilized on prostate cancer biopsy tissue is also underway. Presence of the ERG protein in tissue helps to identify patients who have prostate cancer. Additionally, the presence of ERG in high grade PIN (pre-cancerous lesion) is indicative of a patient more likely to be diagnosed with cancer upon the next biopsy.	

## 2.2 POST DIAGNOSTIC TESTS

We now list a selection of the Post Diagnostic Tests. All of these are performed on pathologically identified PCa and are for the most part used in staging.

<i>Test</i>	<i>Description</i>	<i>Source</i>
NADiA ProsVue <sup>6</sup>	Used in conjunction with a clinical evaluation, the test can help identify patients at risk for recurrence of prostate cancer within an eight-year period following a prostatectomy. The test is an in-vitro diagnostic tool (takes place in a test tube), that determines the rate of change of serum tPSA (total PSA) over a period of time for further analysis. NADiA is a re-engineered IPCR assay that utilizes a non-native dsDNA label for analyte detection (IRIS International, Inc.). Details of the NADiA PSA assay procedure have been previously described. NADiA ProsVue is an in-vitro diagnostic assay for determining rate of change of serum total prostate specific antigen (tPSA) over a period of time. A retrospective clinical study of 304 patients evaluated the slope of three successive ProsVue tests over a period of at least ten months after a prostatectomy to identify prostate cancer patients with no evidence of disease or clinical progression. Recurrence of disease was determined by positive imaging, biopsy results or prostate cancer related death.	<a href="http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4452955/pdf/nihms687020.pdf">http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4452955/pdf/nihms687020.pdf</a> <a href="http://www.proiris.com/">http://www.proiris.com/</a>
InformMDx	The development of MDxHealth's second prostate cancer product (InformMDx) is on target. This test will provide prognostic assessment to distinguish between aggressive and non-aggressive prostate cancer. In Q1 2014 the company will start validation studies for the test.	<a href="http://mdxhealth.com">http://mdxhealth.com</a>
ConfirmMDx	By analyzing genes associated with prostate cancer, this test helps to prevent prostate-cancer-free men, who have already received a biopsy, from undergoing an unnecessary repeat biopsy. The analysis is performed on remaining prostate tissues from a previously negative biopsy. It also helps to identify high-risk patients for further tests or treatment.	<a href="http://mdxhealth.com">http://mdxhealth.com</a>
Prolaris Diagnostic Test	Prolaris provides a new measure of the aggressiveness of an individual's prostate cancer. Getting a Prolaris Score will give both the patient and physician additional information about the true nature of the cancer that no other test can. Prolaris is a measure of how fast a prostate cancer tumor is growing. Biopsy tissue samples can be used to determine a patient's personal Prolaris Score. Studies have shown that Prolaris provides an accurate assessment of cancer aggressiveness. Because every individual's prostate cancer is different, the result of each Prolaris test is unique to that patient.	<a href="http://www.prolaris.com/">http://www.prolaris.com/</a> <a href="https://www.myriad.com/products-services/prostate-cancer/prolaris/">https://www.myriad.com/products-services/prostate-cancer/prolaris/</a>
CCP, Cell Cycle Progression	The cell cycle progression (CCP) score, a prognostic RNA signature based on the average expression level of 31 CCP genes, has been shown to predict biochemical recurrence (BCR) after prostatectomy and prostate cancer specific mortality in men undergoing observation. However, the value of the CCP score in men who received primary external beam radiation therapy (EBRT) is untested	See TWP on CCP.

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<sup>6</sup> See Moui et al

<i>Test</i>	<i>Description</i>	<i>Source</i>
OncotypeDX	This test is a post biopsy test on a confirmed malignancy using a selection of the final 17-gene Oncotype DX panel, with several key features: (i) Predictive of clinical recurrence (local recurrence and/or metastatic disease) as well as other important endpoints. (ii) Genes in multiple biological pathways predictive of aggressive prostate cancer in the face of tumor heterogeneity and multifocality. (iii) Higher expression of the Stromal Response and Proliferation genes is associated with more aggressive prostate cancer. (iv) Higher expression of Cellular Organization and Androgen genes is associated with less aggressive prostate cancer.	<a href="http://prostate-cancer.oncotypedx.com">http://prostate-cancer.oncotypedx.com</a>

As we stated in WP 98

*The recent report on such a cancer prognostic model such as Oncotype DX by Knezvic et al is a putatively prognostic method used in prostate cancer. Fundamentally what they do is examine cancer cells for the expression of various genes and examine three sets; baseline expressions, excess expressions and reduced expression. They use the baseline to set levels for excess and reduced. They then use the excess or reduced in a one dimension expression to determine a prognostic measure. This seems to be in contrast with work we reported on a few months ago .*

*Like PSA measures, CA125, CEA, and the like, they try to reduce everything to a single number. We argue here that such an approach is problematic at best. Furthermore they fail totally to demonstrate any internal pathway influence. There is no predictive basis for their approach predicated upon the actual dynamics of the cell. It is purely correlative and there may be substantial confounders involved. This approach is an example of what we feel to be the poorer aspects of genomics applied to cancer prognostics.*

## 2.3 OTHER POSSIBLE TARGETS

Biomarkers will be increasingly valuable for patients and physicians in the decision making process. Biomarkers in prostate cancer will one day help determine the best treatment option upon diagnosis. Prostate cancer comes in varying aggression levels, on which effective treatment is dependent. Emerging biomarkers include:

**TMPRSS2:ERG:** This urine test under development helps to identify a subset of aggressive prostate cancers with high specificity and may play a role in monitoring the response to hormonal or other therapies in an individual patient. TMPRSS2:ERG gene fusions are prostate cancer specific DNA arrangements found in half of prostate cancers.

**ERG Gene Tissue Marker:** Development of ERG gene assays, which measures the fusion of TMPRSS2 to ERG, to be utilized on prostate cancer biopsy tissues is also underway. This tissue assay will also help to identify prostate cancer aggressiveness and may lead to personalization of

treatment options. ERG rearrangement in prostate cancer at the time of diagnosis, are markers for predicting subsequent tumor behavior and can help in better predicting the clinical outcome.

Phosphatase and tensin homolog (PTEN): Research on the PTEN gene is aimed at helping to reduce the likelihood of false-positive tests by distinguishing between localized prostate cancer and non-cancerous enlargement of the prostate. The PTEN gene is a four-protein signature that is commonly altered in men with prostate cancer.

Emerging Biomarkers: RAF, BRAF, SPOP, EZH2, and Spink1 are biomarkers under analysis that provide great promise for future prostate cancer diagnosis, risk, aggressiveness and individualized treatment options.

### 3 KALLIKREINS

Kallikreins are a collection of genes located on Chromosome 19 which are reflective of possible cancer status. One of these specifically, KLK3 or PSA is a well-known marker of PCa albeit with possible high false alarm rates. From NCBI we have the following description:

*Kallikreins are a subgroup of serine proteases having diverse physiological functions. Growing evidence suggests that many kallikreins are implicated in carcinogenesis and some have potential as novel cancer and other disease biomarkers. This gene is one of the fifteen kallikrein subfamily members located in a cluster on chromosome 19. Its protein product is a protease present in seminal plasma. It is thought to function normally in the liquefaction of seminal coagulum, presumably by hydrolysis of the high molecular mass seminal vesicle protein. Serum level of this protein, called PSA in the clinical setting, is useful in the diagnosis and monitoring of prostatic carcinoma. Alternate splicing of this gene generates several transcript variants encoding different isoforms.*

We will now examine these genes and their proteins at a high level. Several of them relate to the prostate while most at this time do not. The details of the pathways that they impact are open to discussion. We have examined this for KLK3 (PSA) in other reports but those of the other Kallikreins are still a work in progress.

Kallikreins are a subgroup of serine proteases having diverse physiological functions. Growing evidence suggests that many kallikreins are implicated in carcinogenesis and some have potential as novel cancer and other disease biomarkers. These genes are in a family of fifteen kallikrein subfamily members located in a cluster on chromosome 19.

#### 3.1 VARIOUS KALLIKREINS

The Table below is from Lawrence et al as well as from Diamandis and Yousef.

<i>Kallikrein</i>	<i>Alternative Name: Function</i>	<i>Organ</i>
KLK1	hK1; KLKR; Klk6  This gene is one of the fifteen kallikrein subfamily members located in a cluster on chromosome 19. This protein is functionally conserved in its capacity to release the vasoactive peptide, Lys-bradykinin, from low molecular weight kininogen.	Vasculature



<i>Kallikrein</i>	<i>Alternative Name: Function</i>	<i>Organ</i>
KLK2	hK2; hGK-1; KLK2A2  The protein encoded by this gene is a highly active trypsin-like serine protease that selectively cleaves at arginine residues. This protein is primarily expressed in prostatic tissue and is responsible for cleaving pro-prostate-specific antigen into its enzymatically active form. This gene is highly expressed in prostate tumor cells and may be a prognostic maker for prostate cancer risk. Alternate splicing results in both coding and non-coding transcript variants.	Prostate
KLK3	KLK3 kallikrein-related peptidase 3 (APS; PSA; hK3; KLK2A1)  This gene's protein product is a protease present in seminal plasma. It is thought to function normally in the liquefaction of seminal coagulum, presumably by hydrolysis of the high molecular mass seminal vesicle protein. Serum level of this protein, called PSA in the clinical setting, is useful in the diagnosis and monitoring of prostatic carcinoma. Alternate splicing of this gene generates several transcript variants encoding different isoforms.	Prostate
KLK4	ARM1; EMSP; PSTS; AI2A1; EMSP1; KLK-L1; PRSS17; kallikrein  Growing evidence suggests that many kallikreins are implicated in carcinogenesis and some have potential as novel cancer and other disease biomarkers. In some tissues its expression is hormonally regulated. The expression pattern of a similar mouse protein in murine developing teeth supports a role for the protein in the degradation of enamel proteins. Several transcript variants encoding different proteins have been found for this gene.	Teeth Ovary
KLK5	SCTE; KLKL2; KLK-L2  This gene is one of the fifteen kallikrein subfamily members located in a cluster on chromosome 19. Its expression is up-regulated by estrogens and progestins. The encoded protein is secreted and may be involved in desquamation in the epidermis. Alternative splicing results in multiple transcript variants encoding the same protein	Endocrine Skin Ovary Breast Prostate Testicular
KLK6	hK6; Bssp; Klk7; SP59; PRSS9; PRSS18  The encoded enzyme is regulated by steroid hormones. In tissue culture, the enzyme has been found to generate amyloidogenic fragments from the amyloid precursor protein, suggesting a potential for involvement in Alzheimer's disease. Multiple alternatively spliced transcript variants that encode different isoforms have been identified for this gene.	Brain Ovary

<i>Kallikrein</i>	<i>Alternative Name: Function</i>	<i>Organ</i>
KLK7	hK7; SCCE; PRSS6  The encoded protein has chymotrypsin-like activity and plays a role in the proteolysis of intercellular cohesive structures that precedes desquamation, the shedding of the outermost layer of the epidermis. The encoded protein may play a role in cancer invasion and metastasis, and increased expression of this gene is associated with unfavorable prognosis and progression of several types of cancer. Polymorphisms in this gene may play a role in the development of atopic dermatitis. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene, which is one of fifteen kallikrein subfamily members located in a gene cluster on chromosome 19.	Skin Ovary
KLK8	NP; HNP; NRPN; PRSS19; TADG14  The encoded protein may be involved in proteolytic cascade in the skin and may serve as a biomarker for ovarian cancer. Alternate splicing of this gene results in multiple transcript variants encoding different isoforms.	Skin Ovary
KLK9	KLKL3; KLK-L3  The protein encoded by this gene is a kallikrein-related serine protease. This gene is activated by steroid hormones in a human breast cancer cell line, making it a good marker for cancer detection. The encoded protein is found primarily in the cytoplasm	Breast Ovary
KLK10	NES1; PRSSL1  Its encoded protein is secreted and may play a role in suppression of tumorigenesis in breast and prostate cancers. Alternate splicing of this gene results in multiple transcript variants encoding the same protein.	Prostate Breast Ovary
KLK11	TLSP; PRSS20  Alternate splicing of this gene results in multiple transcript variants encoding distinct isoforms which are differentially expressed.	Prostate Ovary
KLK12	KLKL5; KLK-L5  Alternate splicing of this gene results in three transcript variants encoding different isoforms.	Breast
KLK13	KLKL4; KLK-L4  Expression of this gene is regulated by steroid hormones and may be useful as a marker for breast cancer. An additional transcript variant has been identified, but its full length sequence has not been determined.	Breast

<i>Kallikrein</i>	<i>Alternative Name: Function</i>	<i>Organ</i>
KLK14	KLK-L6  The altered expression of this gene is implicated in the progression of different cancers including breast and prostate tumors. The encoded protein is a precursor that is proteolytically processed to generate the functional enzyme. Alternative splicing results in multiple transcript variants	Prostate Breast Testicular
KLK15	ACO; HSRNASPH  In prostate cancer, this gene has increased expression, which indicates its possible use as a diagnostic or prognostic marker for prostate cancer. The gene contains multiple polyadenylation sites and alternative splicing results in multiple transcript variants encoding distinct isoforms.	Prostate Ovary Breast

From Waltering<sup>7</sup>:

*Kallikrein related peptidase 3 (KLK3), better known as prostate specific antigen (PSA), is located in chromosome 19q13.41. KLK3 encodes a single chain glycoprotein with a molecular mass of 33 kDa and functions as a serine protease. It belongs to the family of the fifteen kallikrein members located in a cluster in the same chromosomal region.*

*All kallikrein genes encode five exons of similar size and have high sequence homology with other family members. Many of these peptidases also have several alternative splice variants and are known to be regulated by androgens. KLK3 was cloned in 1987.*

*KLK3 expression has been shown to be elevated in BPH and in highly differentiated PCs, but it is decreased during PC progression. The use of KLK3 as a PC biomarker (the so called PSA test) began in the mid-1980s. In a recent European study, which included more than 160,000 men aged 55 to 69, it was found that PSA based screening reduced PC mortality by 20%.*

*However, there was a high risk of overdiagnosis. Androgen regulation of KLK3 includes both the proximal promoter and the enhancer ARE located 4 kb upstream from the TSS. Recruitment of AR and its coregulators create a chromosomal loop from the enhancer to the core promoter. Kallikrein family members have also been suggested to play a putative role in PC progression. For example, KLK3 has been suggested to directly degrade extracellular matrix glycoproteins and facilitate cell migration.*

### 3.2 KALLIKREINS AND PCA

From a Eureka report on this work they state<sup>8</sup>:

<sup>7</sup> Waltering, K., Androgen Receptor Signaling Pathway in Prostate Cancer, PhD Thesis, Univ Tampere, Sept 2010.

<sup>8</sup> [http://www.eurekaalert.org/pub\\_releases/2014-10/uotm-rdg100214.php](http://www.eurekaalert.org/pub_releases/2014-10/uotm-rdg100214.php) also see <http://medicalxpress.com/news/2014-10-gene-aggressive-prostate-cancer-diagnosis.html>

*Researchers at The University of Texas MD Anderson Cancer Center have identified a biomarker living next door to the KLK3 gene that can predict which GS7 prostate cancer patients will have a more aggressive form of cancer.*

*The results reported in the journal of Clinical Cancer Research, a publication of the American Association of Cancer Research, indicate the KLK3 gene – a gene on chromosome 19 responsible for encoding the prostate-specific antigen (PSA) – is not only associated with prostate cancer aggression, but a single nucleotide polymorphism (SNP) on it is more apparent in cancer patients with GS7.*

*Researchers have linked Gleason score, an important predictor of prostate cancer outcomes, to several clinical end points, including clinical stage, cancer aggression and survival. There has been much research associated with prostate cancer outcomes as well as GS7 prostate cancers, which is an intermediate grade of cancer accounting for 30 to 40 percent of all prostate cancers.*

*"This is the first report that I am aware of that indicates a genetic variant can stratify GS7 prostate cancer patients," said Jian Gu, Ph.D., associate professor at MD Anderson, and a key investigator on the study. "This is important because this group with heterogeneous prognosis is difficult to predict and there are no reliable biomarkers to stratify this group."*

*In this study, researchers investigated inherited genetic variants to see if there would be any promising biomarkers for prostate cancer patients. The investigators studied the genetic makeup of 72 SNPs identified from the genome-wide association studies (GWAS) in 1,827 prostate cancer patients. They analyzed associations of these SNPs with disease aggression, comparing them in clinically defined high and low aggressive cases. They found a SNP on the KLK3 gene that can predict an aggressive form of GS7 disease.*

*"Treatment options for the GS7 disease are controversial because the burden of combined treatment modalities may outweigh the potential benefit in some patients," said Xifeng Wu, M.D., Ph.D., professor and chair of Epidemiology, and lead investigator on the study. "It is critical that we develop personalized treatments based on additional biomarkers to stratify GS7 prostate cancers. Additional biomarkers may help us achieve personalized clinical management of low and intermediate risk prostate cancer patients."*

*Wu also said her team are expanding the study and taking a pathway-based approach to systemically investigate genetic variants in microRNA regulatory pathways as biomarkers for the prognosis of prostate cancer patients. "We are also working on circulating biomarkers. Eventually, we will incorporate all biomarkers, epidemiological and clinical variants into nomograms to best predict the prognosis of prostate cancer patients at diagnosis."*

In a paper by Mikropoulos et al on Medscape<sup>9</sup>:

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<sup>9</sup> <http://www.medscape.com/viewarticle/830689>

*Several SNPs associated with PrCa risk in the 8q24 locus were among the earliest identified. The 8q24 region is a gene-poor region located upstream of the MYC proto-oncogene and this suggested an association with its expression, which was later proven to occur in a tissue-specific manner. Another important SNP is rs10993994 in the region containing the MSMB gene on chromosome 10. This risk allele associates with reduced MSMB protein expression. MSMB expression is high in normal and benign prostate tissue and low in PrCa. MSMB regulates cell growth and when lost, tumor cells grow in an uncontrolled manner. The odds ratio (OR) for this SNP's association to PrCa was established as 1.61. This is a potential biomarker since urine MSMB assays have been developed and their role in screening is being evaluated...*

*SNP rs2735839 was identified between the KLK2 and KLK3 genes on chromosome 19 where there is a kallikrein gene cluster. Kallikreins are serum proteases and the most well-known member of this group is the prostate-specific antigen (PSA), which is widely used for screening and monitoring PrCa. SNPs were also identified in the intronic region areas of the LMTK2 gene, which codes for cdk5, the SLC22A3 gene, which codes for an organic cation transporter and NUDT10, which regulates DNA phosphorylation.*

*In proximity to the TERT gene (encoding TERT) on 5p15, a further susceptibility SNP was identified (rs2242652). Telomerase is important in counterbalancing telomere-dependent replicative aging. SNPs in this region have been associated with numerous cancers, such as basal cell carcinoma, lung cancer, bladder cancer, glioma and testicular cancer.*

*This SNP showed an association with high PSA levels, as well as increased risk of developing PrCa. Fine-mapping analysis identified a total of four loci independently associated with PrCa risk in the TERT region, one of which was associated with changes in gene expression.*

*rs2121875 is a SNP located at 5p12 within the FGF10 locus associated with an increased risk of PrCa. FGF10 is often overexpressed in breast carcinomas, and encodes a FGF essential for a range of developmental processes, which also has an important role in the growth of normal prostatic epithelial cells.*

*In 2013, we reported on 23 new susceptibility alleles associated with PrCa, 16 of which were also associated with aggressive disease.. A SNP located at 1q32 (rs4245739) in proximity to the MDM4 gene is of potential clinical significance. MDM4 inhibits cell cycle arrest and apoptosis, via p53 downregulation. Another SNP (rs11568818) with a potential prognostic value is situated at 11q22 within a region containing the gene MMP7. MMP7 encodes for a matrix metalloproteinase, which is pivotal for tumor metastasis and overexpression of MMP7 is a potential biomarker for PrCa aggressiveness and risk of metastatic disease. Finally, SNP (rs7141529) at 14q24 is an intronic SNP within the RAD51B gene, which is an important DNA repair gene involved in homologous recombination, also associated with PrCa risk.*

### **3.3 COMPLEXITY OF KALLIKREINS**

From Lawrence et al we have the following Table which adds to the complexity of naming for this gene family:

<i>KLK</i>	<i>Alternative Names</i>
KLK1	Tissue/renal/pancreatic kallikrein, hK1, mGK-6 (mouse), pMAK3 (mouse), rGK-1 (rat), PS (rat), RSK1105 (rat)
KLK2	Glandular kallikrein, hGK1, hK2
KLK3	Prostate-specific antigen (PSA), APS, KLK2A1, hK3
KLK4	PRSS17, KLK-like 1, enamel matrix serine protease 1 (EMSP1), androgen-regulated message 1 (ARM1), PSTS, prostase, pemB, enamel serine proteinase (pEMS), hK4
KLK5	KLK-like 2, stratum corneum tryptic-like enzyme (SCTE), hK5
KLK6	PRSS9, PRSS18, brain and skin serine protease (BSSP), protease M, zyme, neurosin, myelencephalon specific protease (MSP), hK6
KLK7	PRSS6, stratum corneum chymotrypsin-like enzyme (SCCE), hK7
KLK8	PRSS19, neuropsin, HNP, ovasin, tumor-associated differentially expressed gene-14 (TADG-14), brain serine protease 1 (BSP1), hK8
KLK9	KLK-like 3, hK9
KLK10	PRSSL1, normal epithelial-Specific 1 (NES1), hK10
KLK11	PRSS20, trypsin-like serine protease (TLSP), hippostasin, hK11
KLK12	KLK-like 5, hK12
KLK13	KLK-like 4, hK13
KLK14	KLK-like 6, hK14
KLK15	Prostinogen, ACO protease, HSRNASPH, hK15

Again, from Lawrence et al we have the sequence on Chromosome 19 of the 15 KLK genes:



The authors also state:

*Once activated, kallikreins function as endopeptidases to cleave bonds within polypeptide chains. Like all members of the PA clan, the proteolytic activity of kallikreins depends on the catalytic triad of histidine57, aspartate102, and serine195 residues (standard bovine chymotrypsin numbering) that span the active site.... Androgens regulate the prostatic expression of several human kallikreins, in particular KLK2 and KLK3. The earliest evidence for androgen-regulated KLK3 expression came from immunohistochemistry experiments showing that prostatic KLK3 levels mirror serum testosterone concentrations: low in prenatal development and childhood, greater in puberty, and highest in adulthood. Soon after the KLK2 and KLK3*

genes were cloned, their androgen responsiveness was confirmed at the mRNA level using Northern blots of androgen-treated LNCaP prostate cancer cells.

*These observations were verified with a range of in vitro and in vivo experiments. Numerous studies have since used KLK2 and KLK3 as prototypical AR target genes to investigate different aspects of androgen signaling in prostate cells. KLK3 levels are also monitored in patients undergoing androgen ablation therapy for prostate cancer because KLK3 is re-expressed when AR signaling is reactivated in castrate-resistant tumors. KLK3 levels, however, are highly heterogeneous in castrate-resistant prostate cancer and do not directly correlate with tumor growth. This variability may be due to the different ways that tumors adapt to castrate androgen levels including overexpression and mutation of the AR, up-regulation of transcriptional coactivators, and intratumoral steroidogenesis.*

As Diamandis and Yousef have noted regarding all Kallikreins:

*Kallikreins are a subgroup of the serine protease enzyme family. Until recently, it was thought that the human kallikrein gene family contained only three members. In the past 3 years, the entire human kallikrein gene locus was discovered and found to contain 15 kallikrein genes. Kallikreins are expressed in many tissues, including steroid hormone-producing or hormone-dependent tissues such as the prostate, breast, ovary, and testis. Most, if not all, kallikreins are regulated by steroid hormones in cancer cell lines. There is strong but circumstantial evidence linking kallikreins and cancer.*

*Prostate-specific antigen (PSA; hK3) and, more recently, human glandular kallikrein (hK2) are widely used tumor markers for prostate cancer. Three other kallikreins, hK6, hK10, and hK11, are emerging new serum biomarkers for ovarian and prostate cancer diagnosis and prognosis. Several other kallikreins are differentially expressed at both the mRNA and protein levels in various endocrine-related malignancies, and they have prognostic value. The coexpression of many kallikreins in the same tissues (healthy and malignant) points to the possible involvement of kallikreins in cascade enzymatic pathways. In addition to their diagnostic/prognostic potential, kallikreins may also emerge as attractive targets for therapeutics.*

They further make note of common features of the kallikrein:

*Table 2. Common structural features of the human kallikrein genes and proteins.*

- 1. All genes are formed of five coding exons, and most of them have one or more extra 5 untranslated exons. The first coding exon always contains a 5 untranslated region, followed by the methionine start codon, located 50 bp away from the end of the exon. The stop codon is always located 156 bp from the beginning of the last coding exon.*
- 2. Exon sizes are very similar or identical.*
- 3. The intron phases of the coding exons (i.e., the position where the intron starts in relation to the last codon of the previous exon) are conserved in all genes. The pattern of the intron phase is always I-II-I-0.*
- 4. The positions of the residues of the catalytic triad of serine proteases are conserved, with the histidine always occurring near the end of the second coding exon, the aspartate in the*

*middle of the third coding exon, and the serine residue at the beginning of the fifth coding exon.*

- 5. All kallikrein proteins are synthesized as pre/propeptides with a signal peptide of 17–20 amino acids at the amino terminus, followed by an activation peptide of 4–9 amino acids (with the exception of hK5), followed by the mature (enzymatically active) protein.*
- 6. The amino acid of the substrate-binding pocket is either aspartate, indicating trypsin-like specificity (11 enzymes), or another amino acid [probably conferring chymotryptic (PSA) or other activity].*
- 7. Most, if not all, genes are under steroid hormone regulation.*
- 8. All proteins contain 10–12 cysteine residues that will form five to six disulfide bonds. The positions of the cysteine residues are also fully conserved.*

Debela et al have noted regarding the structure of Kallikreins:

*Human tissue kallikreins (hKs) form a family of 15 closely related (chymo)trypsin-like serine proteinases. These tissue kallikreins are expressed in a wide range of tissues including the central nervous system, the salivary gland, and endocrine-regulated tissues, such as prostate, breast, or testis, and may have diverse physiological functions. For several tissue kallikreins, a clear correlation has been established between expression and different types of cancer. For example, the prostate-specific antigen (PSA or hK3) serves as tumor marker and is used to monitor therapy response. Using a novel strategy, we have cloned, expressed in *Escherichia coli* or in insect cells, refolded, activated, and purified the seven humantissue kallikreins hK3/PSA, hK4, hK5, hK6, hK7, hK10, and hK11.*

*Moreover, we have determined their extended substrate specificity for the nonprime side using a positional scanning combinatorial library of tetrapeptide substrates. hK3/PSA and hK7 exhibited a chymotrypsin-like specificity preferring large hydrophobic or polar residues at the P1 position. In contrast, hK4, hK5, and less stringent hK6 displayed a trypsin-like specificity with strong preference for P1-Arg, whereas hK10 and hK11 showed an ambivalent specificity, accepting both basic and large aliphatic P1 residues. The extended substrate specificity profiles are in good agreement with known substrate cleavage sites but also in accord with experimentally solved (hK4, hK6, and hK7) or modeled structures. The specificity profiles may lead to a better understanding of human tissue kallikrein functions and assist in identifying their physiological protein substrates as well as in designing more selective inhibitors.*



## 4 PRE PROGNOSTIC TESTS

Pre Prognostic Tests are performed before a biopsy or before prostatectomy. The objective is to have some less invasive metric of the potential for aggressive PCa without complete removal of the prostate. Currently PSA, % Free PSA, and PSA velocity are used. The problem is that with PSA alone the AUC of the sensitivity/specificity curve is low, near 0.6.

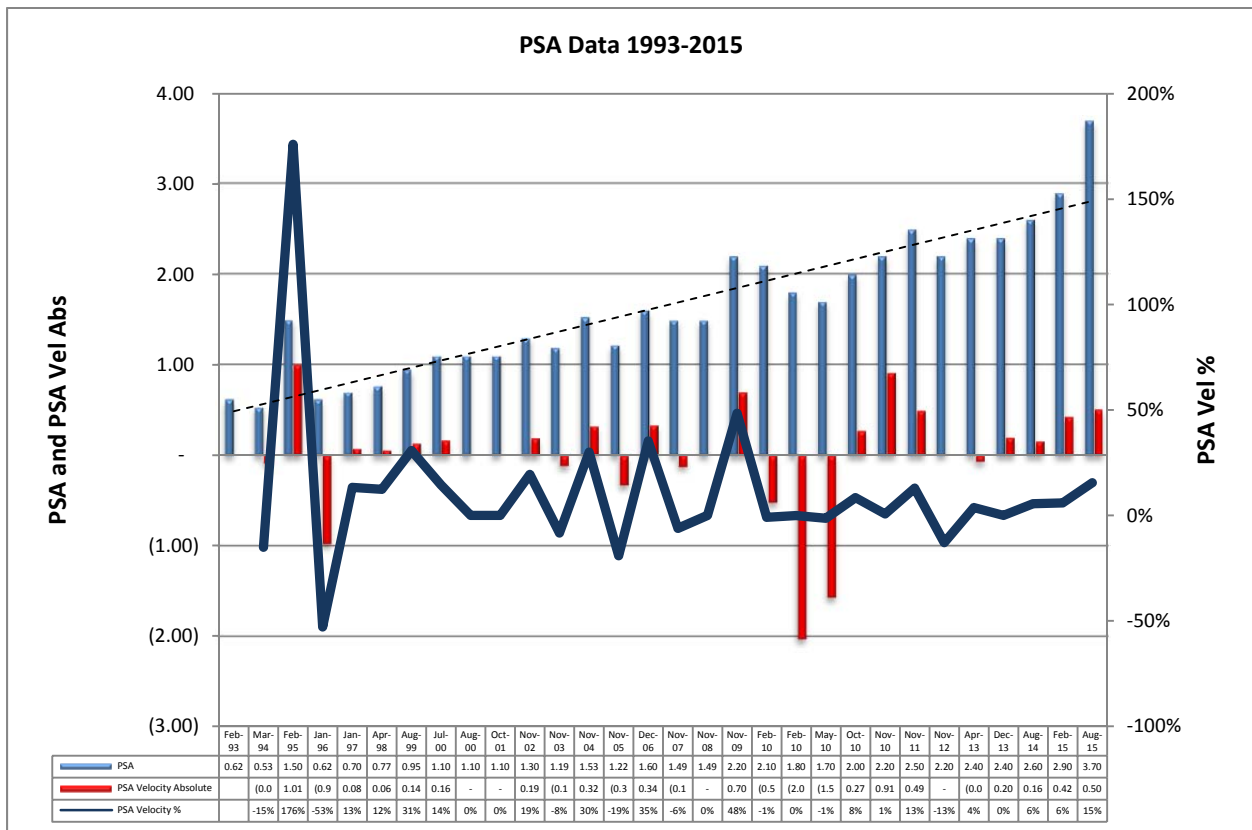
We will examine the 4K test as a specific example. Many others appear to function in a similar manner. Namely they are non-invasive and use blood or urine markers. We have previously discussed exosome and oncosome tests as well but will not reiterate them here.

The key question for Pre Prognostic Tests is; What can we determine given a non-invasive method that will reduce the risk of unnecessary biopsies yet minimize the risk of missed aggressive malignancies? We know that PSA alone is not strong enough. We also know that we do not understand the genomic complexity of an aggressive PCa. Thus given what we can obtain from blood borne markers, or even using urine borne markers, and other factors, can we attain a measure that satisfies the desired end point?

### 4.1 NCCN APPROVED TESTS

The NCCN has provided Guideline in 2015 for the Detection of PCa. We summarize them below. We have separately considered other multiple factors including temporal factors. These temporal factors do not seem to play a role in any of these tests. It is suspected that the reason for such is that the data is generally not available.

If one looks at the patient below we are asked if this sudden change is a concern.



Clearly this is a 23 year pattern of PSA measurements and as such demonstrates a trend. The velocity may be a problem. However the % Free number is still high.

We now examine the many current NCCN indicate Pre Tests which are available. We quote from NCCN where appropriate.

#### 4.1.1 Age- and Race-Specific PSA Reference Ranges

PSA measurements have been a standard for two decades until the USPTF report in 2012. As we have noted the USPTF report relies upon an American and European set of studies which in our opinion are equally flawed. The US for using solely a 4.0 cutoff and never revising it to reflect better understanding and second the European for both that flaw as well as there being excessively long periods between tests. Now NCCN states:

*Age-specific PSA reference ranges were introduced by Oesterling and colleagues as a method to increase cancer detection (ie, increase sensitivity) in younger men by lowering PSA cutoffs for biopsy and to decrease unnecessary biopsies (ie, improve specificity) in older men by increasing PSA cutoffs. Several groups have investigated these age-specific ranges with equivocal results. Others have suggested race-specific reference ranges. However, the exact roles of these age- and race-specific PSA cutoffs in the early detection of prostate cancer remain unclear. The panel has no recommendations regarding routine use of these ranges.*

#### 4.1.2 PSAV

PSA velocity, PSAV, is a time averaged rate of change. It is not a change between just two measures but a weighted average over several. It is a classic medical measure; namely did something change. It does not indicate the cause of the change. As NCCN states:

*The rate of change in PSA over time is broadly termed PSA velocity (PSAV), determined by at least 3 separate PSA values calculated over at least an 18-month period. Carter and colleagues first showed that PSAV is greater in men eventually diagnosed with prostate cancer than in men not diagnosed with the disease and suggested its use as a screening tool.*

*In a subsequent study of 980 men enrolled in the Baltimore Longitudinal Study of Aging (BLSA), Carter and colleagues explicitly linked PSAV with the risk of prostate cancer death by observing that PSAV recorded 10 to 15 years before cancer diagnosis (commonly with PSA <4 ng/mL) was associated with disease-specific survival up to 25 years later: the relative risk of prostate cancer death was higher in men with PSAV >0.35 ng/mL/year compared to those with PSAV <0.35 ng/mL/y (RR, 4.7; 95% CI, 1.3-16.5; P = .02). These data provide support that PSAV may help identify lethal cases. However, the small number of deaths from prostate cancer precludes definitive conclusions.*

#### 4.1.3 %f PSA

Percent Free PSA is a measure of good PSA. We have examined this in detail elsewhere. NCCN states:

*Unbound or free PSA (fPSA), expressed as a ratio of tPSA, is a clinically useful molecular form of PSA, with the potential to improve early detection, staging, and monitoring of prostate cancer. Several molecular forms of PSA are known to circulate in the blood. In most men, the majority (60%-90%) of circulating PSA is covalently bound to endogenous protease inhibitors. Most immunoreactive PSA is bound to the protease inhibitor alpha-1-antichymotrypsin. Other immunoreactive PSA-protease inhibitor complexes, such as alpha-1-antitrypsin and protease C inhibitor, exist at such low serum concentrations that their clinical significance has not been determined. In addition, a large proportion of PSA is complexed with alpha-2-macroglobulin (AMG). Unfortunately, this PSA-AMG complex cannot be measured by conventional assays because of the shielding (or "caging") of PSA antigenic epitopes by AMG.*

#### 4.1.4 cPSA<sup>10</sup>

*PSA exists in free and several complexed forms. Direct measurement of the complexed form with alpha-1-antichymotrypsin is now available. For practical purposes, tPSA consists essentially of fPSA and the alpha-1-antichymotrypsin complexed form (cPSA). The threshold levels are therefore not equivalent: cPSA levels of 2.2 ng/mL and 3.4 ng/mL are equivalent to tPSA levels of 2.5 ng/mL and 4.0 ng/mL, respectively. In a multicenter trial of 831 men, of whom 313 had*

<sup>10</sup> <http://www.ncbi.nlm.nih.gov/pubmed/12384160>

prostate cancer, researchers found that cPSA in the range of 80% to 95% sensitivity thresholds increased specificity compared with tPSA.<sup>126</sup> Results were similar for percent cPSA and percent fPSA.

As Hominger et al note:

*Complexed PSA (cPSA) has been shown to improve specificity in the detection of prostate cancer over that of total PSA (tPSA) testing in men with tPSA values greater than the cutoff value of 4.0 ng/mL.*

*However, recent studies have reported a 25% incidence of prostate cancer in men with tPSA values in the 2.5- to 4.0-ng/mL range. We performed a multicenter study of cPSA in a population of men who underwent prostate biopsies because of elevated PSA levels or abnormal digital rectal examination (DRE).*

*As part of this study, we sought to assess the clinical value of cPSA in comparison to tPSA, the free/tPSA ratio (f/tPSA) and the complexed/tPSA ratio (c/tPSA) in early detection of prostate cancer in men with tPSA values in the range of 2 to 4 ng/mL. The study was performed at 7 centers. Sera were drawn from men who underwent biopsy procedures consisting of >10 prostate tissue cores. Receiver operating characteristic (ROC) analysis was performed from the results of patients with tPSA values in the range of 2 to 4 ng/mL, including men with suspicious as well as unremarkable findings on DRE. Sera were collected and tested with the Bayer tPSA and cPSA assay and the Beckman free PSA and tPSA assays.*

*ROC analysis was performed for all samples in the 2- to 4-ng/mL PSA range. At biopsy, 158 men had no evidence of malignancy and 57 (26.5%) were diagnosed with prostate cancer. ROC analysis indicated that the area under the curve (AUC) for cPSA was 0.64, which was statistically significantly greater than that achieved for tPSA (AUC, 0.57;  $P < 0.0001$ ). The AUC for f/tPSA and c/tPSA were 0.60 and 0.63, respectively, which was not statistically significantly different from that of tPSA or cPSA ( $P \geq 0.252$ ).*

*A cutpoint of 2.5 ng/mL for tPSA and 2.1 ng/mL for cPSA provided a specificity of 20.3% and 34.2%, respectively, and sensitivity levels of 86%. Using cutpoints of 25% for f/tPSA and 74% for c/tPSA provided a specificity of 11.0% and 21.5%, respectively, and sensitivity levels of 97%.*

*In all, >92% of the cancers treated with radical prostatectomy were organ confined, and the histologic grading of the tumors ranged from moderately to poorly differentiated with Gleason scores from 5 to 9. These data confirm that there is a high incidence of clinically significant prostate cancer in men with tPSA levels <4.0 ng/mL. Measurement of cPSA proved useful in stratifying men with tPSA values in the 2- to 4-ng/mL range into high- and low-risk groups for prostate cancer. The use of cPSA as a single test was found to enhance detection of prostate cancer over that of testing with tPSA and PSA ratios in men with tPSA values in the range of 2 to 4 ng/mL.*

#### 4.1.5 PSAD

We have also examined PSA normalized by prostate density. To do so however requires a possible invasive approach. Prostate volume is measured in biopsy but is difficult to measure non-invasively. NCCN states:

*PSA density (PSAD) requires the measurement of prostate volume by TRUS and is expressed as the PSA value (in ng/mL) divided by prostate volume (in cc). PSAD is a means of discriminating prostate cancer from BPH: the lower the PSAD, the greater the probability of BPH.<sup>131</sup> <sup>132</sup> Thus, PSAD potentially identifies men who do not have prostate cancer but have high PSA secondary to large-volume prostates. A PSAD cutoff of 0.15 ng/mL/cc was recommended in earlier studies, which spared as many as 50% of men from unnecessary biopsies. However, some subsequent studies have reported that the 0.15 cutoff has insufficient sensitivity.*

#### 4.1.6 PCA3

*PCA3 is a noncoding, prostate tissue-specific RNA that is overexpressed in prostate cancer. Current assays quantify PCA3 overexpression in post-DRE urine specimens. PCA3 appears useful in predicting biopsy outcomes at both initial and repeat biopsies. However, it appears most useful in determining which patients should undergo a repeat biopsy.<sup>140</sup><sup>143</sup>*

The following are some of the Pre Test proprietary tests currently available and provided by NCCN.

#### 4.1.7 phi<sup>11</sup>

*Development of novel biomarkers continues. The phi is a combination of existing tests (ie, tPSA, fPSA, proPSA). In a multi-center study, it was noted to have approximately double the sensitivity of fPSA/tPSA for cancer detection in those with serum PSA concentrations between 2 and 10 ng/mL. In addition, the phi correlated with cancer grade and had an AUC of 0.72 for discrimination of high-grade (Gleason >7) cancer from low-grade cancer or negative biopsy. The phi was approved by the FDA for use in 2012 in those with serum PSA values between 4 and 10 ng/mL.*

#### 4.1.8 4Kscore<sup>12</sup>

*The 4Kscore test is another combination test that measures free and tPSA, human kallikrein 2 (hK2), and intact PSA and also considers age, DRE results, and prior biopsy status.<sup>150,151</sup> This test reports the percent likelihood of finding high-grade (Gleason >7) cancer on biopsy. A prospective multi-institutional U.S. trial of 1012 patients showed that 4Kscore results have a high discrimination value (AUC, 0.82).<sup>152</sup> In this study, using a threshold for biopsy of >15% risk allowed for 591 biopsies to be avoided (58%), while 183 high-grade tumors were detected and 48 high-grade tumors (4.7% of the 1012 participants) were missed. When 4Kscore was*

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<sup>11</sup> <http://prostatehealthindex.us/>

<sup>12</sup> <http://4kscore.opko.com/>

examined in 6129 men in another prospective study, the AUC was also 0.82 (95% CI, 0.80-0.84).<sup>153</sup> Using a 6% risk of high-grade cancer as a cutoff, 428 of 1000 men could avoid biopsy, with 119 of 133 high-grade cancers detected and 14 of 133 missed.

#### 4.1.9 ConfirmMDx<sup>13</sup>

This is an invasive test. However NCCN includes it in the category we have defined because it can be done before a definitive diagnosis. GSTP1 is often methylated and thus under expressed. NCCN states:

*ConfirmMDx is a tissue-based, multiplex epigenetic assay that aims to improve the stratification of men being considered for repeat prostate biopsy. Hypermethylation of the promoter regions of GSTP1, APC, and RASSF1 are assessed in core biopsy tissue samples. The test, performed in one CLIA-certified laboratory, is not FDA approved.*

As they state on their web site<sup>14</sup>

*The use of epigenetic testing for prostate cancer detection using methylation specific PCR (MSP) and cancer-associated epigenetic biomarkers to improve upon histopathology has been well validated in both scientific and clinical studies. DNA methylation, the most common and useful measure of epigenetic abnormality testing, is responsible for the silencing of key tumor suppressor genes. DNA methylation biomarkers associated with prostate cancer have been extensively evaluated and more than 43 studies on the ConfirmMDx genes and technology have been published in peer reviewed, scientific and medical journals.*

*GSTP1 is the most intensely studied and widely reported epigenetic biomarker associated with prostate cancer diagnosis, encoding the glutathione S-transferase Pi 1 protein involved in detoxification, due to its high sensitivity and specificity. Complementing GSTP1, methylation of the APC and RASSF1 genes is frequently found in prostate cancer, and these markers have demonstrated a “field effect” aiding in the identification of biopsies with false-negative histopathological results.(1,2,17)*

We have considered methylations effects as significant.

## 4.2 4K TEST DETAILS

One of the more recent tests performed on a pre diagnostic basis is the 4K test. It uses several factors and has undergone multiple trials, As Bryant et al note from the more recent trial:

*In this study we demonstrate that a panel of four kallikrein markers— total PSA, free PSA, intact PSA, and hK2—can predict the result of prostate biopsy.*

<sup>13</sup> <http://mdxhealth.com/confirmmdx-prostate-cancer>

<sup>14</sup> <http://mdxhealth.com/confirmmdx-prostate-cancer>

*A statistical model based on the four markers improved this prediction above and beyond both PSA and age, as well as beyond a combination of total and free PSA. A decision analysis indicated that use of the statistical model to guide biopsy decisions would reduce the number of men receiving unnecessary biopsies, without substantially affecting the diagnosis of Gleason score 7 or higher (high-grade) cancers. Two initial studies on the Göteborg arm of the ERSPC demonstrated that the panel of markers improved prediction of biopsy outcome for both unscreened men and those with a previous PSA in the normal range.*

*Our results confirm these findings. The assays were subsequently modified, and a new statistical model was built based on a group of unscreened men in the Rotterdam arm of ERSPC. This was termed the “Rotterdam” model and was shown to improve the prediction of biopsy outcome over and above PSA in several independent validation cohorts, including unscreened men, men with prior screening, those with prior negative biopsy, and those subject to clinical work-up before biopsy.*

*The AUC results in these previous studies are very similar to those found here, including an AUC of 0.820 for high-grade cancer, representing an increment of 0.082 over age and PSA alone. For instance, in unscreened men in ERSPC, we reported an AUC of 0.825, 0.049 higher than the base model, and for previously screened men the AUC was 0.793, an increment of 0.094 beyond the base model.*

What is striking here is that large AUC number. This is exceedingly high and given the number of previous trials (see Benchikh et al, Vickers et al, Gupta et al for the various trials) this may be a quite useful metric.

#### 4.2.1 The Test

The 4K test is described somewhat in the papers by Bryant et al (2015) and Stattin et al (2015) and also in the 4K web site as indicated. Basically the following data is measured and entered:

1. Total PSA, tPSA
2. Free PSA, fPSA
3. Intact PSA, iPSA<sup>15</sup>
4. hk2 (or KLK2), hk2
5. Age, A
6. DRE, DRE
7. Prior Biopsy Status, PBS

Then a score is determined by a functional means as follows:

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<sup>15</sup> Note from the OPKO Brochure they state: *The iPSA test is a sandwich (noncompetitive) immunoassay that employs two distinct mouse monoclonal antibody products. The capture probe is a biotinylated, recombinant His6-Cys-tagged Fab fragment of the monoclonal antibody 5A10 with specificity for fPSA (of which iPSA is a component). The tracer is a Europium-labelled monoclonal antibody 4D4, with specificity to iPSA and complexed PSA (PSA-ACT). In combination, the reagents are specific for iPSA.*

$$f_{4K}(x) = f(tPSA, fPSA, iPSA, hk2, A, DER, PBS)$$

Just what that equation specifics are is considered proprietary. However the result is a number or percent that relates to a contemporaneous biopsy report. Namely  $f_{4K}$  equals:

$$f_{4K} = P[\text{Biopsy is Gleason 7 or higher and distant metastasis}] + n$$

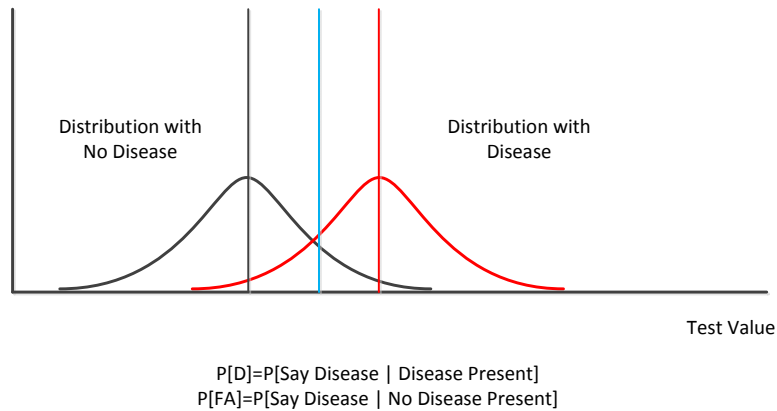
$n = \text{error}$

Namely the f number in a 4K measurement reflects the probability of there being an existing malignancy of considerable status and that the malignancy will lead to death in 20 years. The score goes from <1% to 95%.

Now the ROC AUC for this test is 0.82.

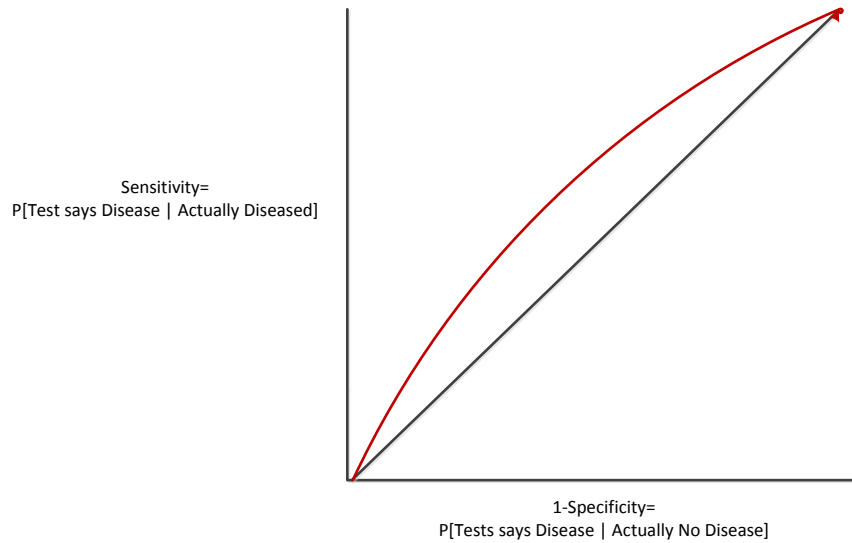
Let us examine this a bit from a statistical perspective.

First let us look at the PSA alone. It may look as follows.



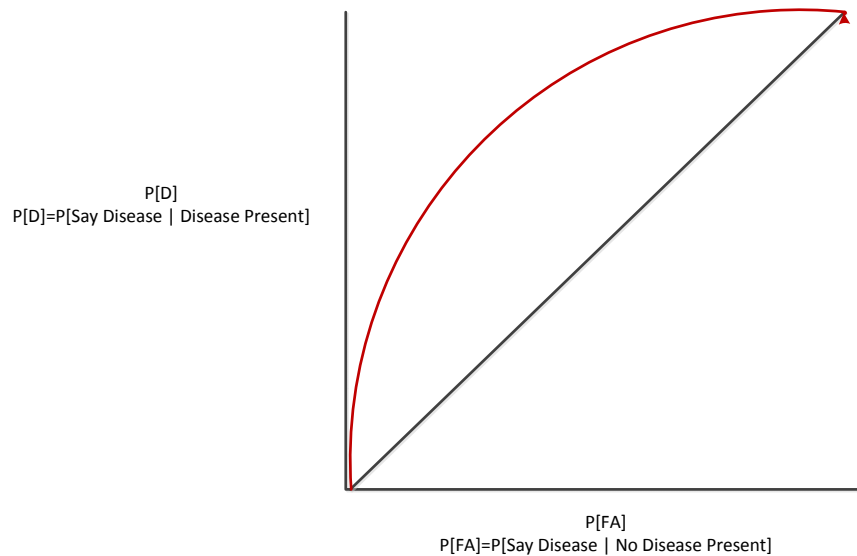
Here we have a PSA threshold of say 4.0. Now with this test we have many diseased states below 4.0 and many non-diseased states above. The red curve is the distribution of PSA give a PCa and the black is the PSA with no PCa. We could vary the blue decision line to try to change things but when we do we see poor performance. We could plot the Sensitivity vs the 1-Specificity of a PSA test as shown below:



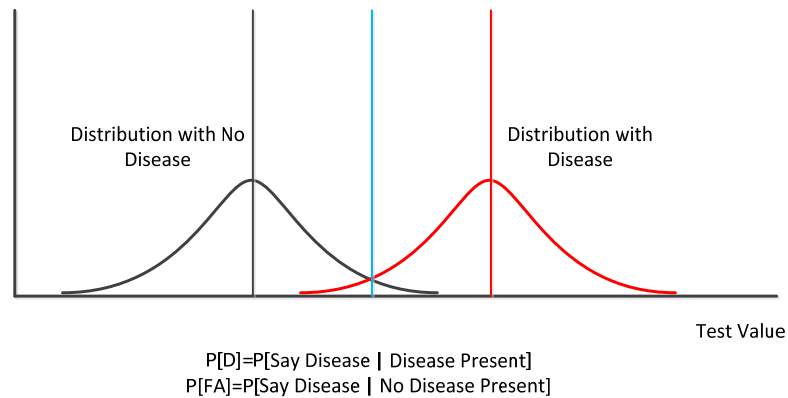


What this states is that as we try to get better detection we get poorer False Alarm rates. The red curve is almost linear and that means that if we want a 90% detection probability then we will suffer almost a 90% False Alarm rate. The measure of performance is the Area Under the Curve, AUC. The worst case AUC is a straight line, a coin toss if you will, and is 0.5.

Now with 4K one alleges gets a better discriminant. We depict that situation below:



Note that we now can get excellent Detection rates while suffering low False Alarm Rates. This means that the 4K tests look like the one below:



That is the distributions for disease and no disease are further apart and/or their variance or noise levels are lower.

This means that we have a much greater AUC and 4K argues for an 0.82 or higher AUC.

Now we may have some issues with 4K. On the positive side it has gone through many tests. On the negative side:

1. It does not include temporal data. No velocity measurements are included. One may then ask if velocity is a key measure. As we have shown before, for example, HbA1c may affect PSA and perhaps that should be normalized.
2. It avoids prostate volume. As we have also shown before volume is a key metric, just by definition.
3. It ignores family history. That is strange since as is well known family history is a significant factor.
4. Also recall that if the test says 1% probability of disease then we have:

$$PD = P[\text{Say Disease}|\text{Disease}]$$

$$PFA = P[\text{Say Disease}|\text{No Disease}]$$

but

$$P[\text{Say Disease}|\text{Disease}]P[\text{Disease}] = P[\text{Disease}|\text{Say Disease}]P[\text{Say Disease}]$$

or

$$P[\text{Say Disease}|\text{Disease}] = \frac{P[\text{Disease}|\text{Say Disease}]P[\text{Say Disease}]}{P[\text{Disease}]}$$

or

$$P[\text{Disease}|\text{Say Disease}] = \frac{P[\text{Say Disease}|\text{Disease}]P[\text{Disease}]}{P[\text{Say Disease}]}$$

but

$$P[\text{Say Disease}] = P[\text{Say Disease}|\text{Disease}]P[\text{Disease}] + P[\text{Say Disease}|\text{No Disease}]P[\text{No Disease}]$$

or

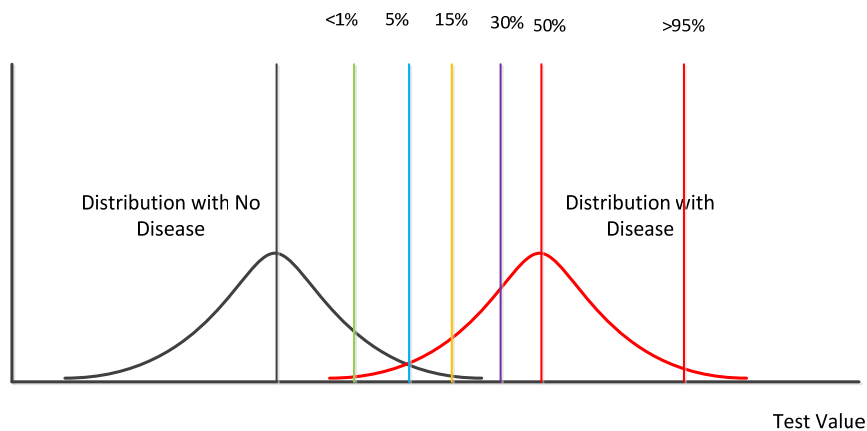
$$P[\text{Say Disease}] = PDp + PFA(1 - p)$$

where

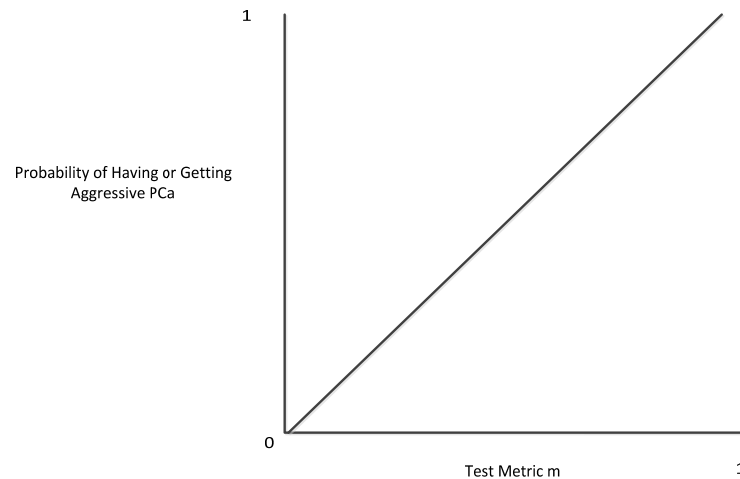
$$p = P[\text{Disease}]$$

Thus if PD is high and PFA is low, which occurs with a large AUC, then PD is almost p. Likewise PND, probability of no disease given disease, is likewise low if we say it is low.

Now the test creates measures as shown below. It scales along the horizontal axis in such a manner that it projects probabilities of having the disease as shown below:



The process first creates a test value. Then having some parametric measures of probabilities it creates measures of the probability of having the disease given your test metric. They then plot the results as shown below:



Thus a score of <1% means the patient has less than 1% chance of having PCa or even getting PCa. The overall performance of the test is quite impressive.

#### 4.2.2 Earlier Prognostic Data

As Stattin et al state additional insight about the same testing methodology wherein measuring PSA at 50 and 60 years of age can be prognostic of future results. They state:

*In this large representative cohort from Sweden, with >12 500 men followed for >15 yr and initially low rates of opportunistic PSA testing, PSA measured in cryopreserved blood collected at age 50 or 60 predicted metastasis at 15- to 20-yr follow-up.*

*In the subset of men with modestly elevated PSA, a prespecified model based on a panel of four KLK markers increased the predictive discrimination of metastasis. Risk stratification contributed by PSA was far greater than that reported for other risk factors such as race or family history. Among men with modestly elevated PSA at age 50 or 60, the four KLK panel yielded C-indexes from 0.82 to 0.88 for the prediction of documented distant metastasis. This can be compared with discrimination close to 0.60 for the Gail model that is used clinically to determine eligibility for breast cancer chemoprevention....*

*Our findings strongly support a risk-stratified approach to screening and biopsy. In men aged 50 yr, the 15-yr risk of metastasis among those in the top decile for PSA was 3.15%, sixfold higher than men with PSA below the median. The concentration of high-risk disease in this age group, with 48% of metastatic cases occurring in the men with PSA levels in the highest 10%, suggests that screening should focus on those men. In contrast, we were unable to identify a subgroup of men aged 40 yr at a substantially increased risk of distant metastasis within 15 yr, making it difficult to justify screening in this age group.*

*The identification of a small subset of men with elevated PSA at ages 50–60 yr with a substantially increased risk of developing metastatic disease many years later has important*

*implications for the development of novel preventive strategies. Use of the KLK markers as a reflex test may further refine stratification of risk.*

*The very low long-term risk for PCa and metastases in men with PSA <1 ng/ml was observed in earlier studies. Our findings are consistent with prior research demonstrating that men with a low PSA at age 60 have no mortality reduction from PSA screening but are at considerable risk of overdiagnosis. This supports the calls to limit screening in such men.....*

*We found that blood levels of PSA at ages 50 and 60 yr are prognostic of the long-term risk of metastatic PCa and that a panel of KLK markers is strongly predictive of distant metastasis documented many years later in men with a modestly elevated PSA. Our study has the following clinical implications.*

*First, widespread PSA testing at age 40 cannot be justified.*

*Second, screening can stop in men with PSA below the median (<1 ng/ml) at age 60 yr.*

*Third, for men in their fifties, screening could focus mainly on those in the top decile of PSA (>1.9 ng/ml) because close to half of the subsequent cases of distant metastasis are found in this group; men with lower PSAs should still be screened but less intensively.*

*Finally, four KLK markers measured in the blood can be used as a reflex test to aid biopsy decisions.*

The question one may ask here is that having long term PSA value and then using the 4K test may provide a highly reliable prognostic tool to ascertain the risk of high level of PCa. Stattin et al in their Table 2 show the risk profiles for men at 50 and at 60. Thus one possibly infer that the 50 and 60 year levels of say 0.6 and 1.2 respectively are high prognostic of low risk. Using Stattin et al numbers the relative risks would be 0.48 and 0.76 for a 20 year risk and 0.15 and 0.59 for a 15 year risk.

## 5 POST PROGNOSTIC TESTS

Post Prognostic Tests are used after a diagnosis of PCa in removed tissues. They examine genetic expression profiles in an attempt to see how aggressive a cancer has been obtained.

There is always an interest in determining the prognostic value of tumors and hopefully staging treatment. There has been a recent flurry of interest in using cell cycle progression genes testing, a method of taking gene products from biopsy samples and then using them to ascertain the most likely progression of the tumor.

### 5.1 EXAMPLES

We examine two methodologies herein. One is CCP which is one methodology proposed to do this. The second is Oncotype DX which is similar in that it uses both a base set of genes and then a weighted set of other genes. The assumption in each appears to be that by doing a GWAS analysis and obtaining genes in some weighted fashion that one obtains a single number which is highly prognostic.

As Freedland et al noted:

*The cell cycle progression (CCP) score, a prognostic RNA signature based on the average expression level of 31 CCP genes, has been shown to predict biochemical recurrence (BCR) after prostatectomy and prostate cancer specific mortality in men undergoing observation. However, the value of the CCP score in men who received primary external beam radiation therapy (EBRT) is untested....*

*Of 141 patients, 19 (13%) had BCR. The median CCP score for patient samples was 0.12. In univariable analysis, CCP score significantly predicted BCR ( $p$ -value = 0.0017). The hazard ratio (HR) for BCR was 2.55 for a one-unit increase in CCP score (equivalent to a doubling of gene expression). In a multivariable analysis with Gleason score, PSA, percent positive cores, and androgen deprivation therapy, the HR for CCP remained significant ( $p$ -value = 0.034), indicating that CCP provides prognostic information that is not provided by standard clinical parameters. With 10-year censoring, the CCP score was associated with prostate cancer specific mortality ( $p$ -value = 0.013). There was no evidence for interaction between CCP and any clinical variable, including ethnicity.*

We take no position in this opinion paper regarding the efficacy of CCP or Oncotype DX as applied to PCa but we examine the original assertions in some detail. Conceptually it makes sense. It is as follows:

1. A handful of genes if over expressed, when combined with other metrics, can provide fairly accurate prognostic measures of PCa.

2. Selecting the genes can be accomplished in a variety of ways ranging from logical and clear pathway control genes such as PTEN to just a broad base sampling wherein the results have a statistically powerful predictive result.
3. Measuring the level of expression in some manner and from the measurements combine those in a reasonable fashion to determine a broad based metric.
4. Combining the gene expression metric with other variable to ascertain a stronger overall metric.

The CCP work to date has been focused somewhat on these objectives.

Let us now briefly update the work as detailed in the industry press. As indicated in a recent posting:<sup>16</sup>

*Cuzick and his colleagues initially measured the levels of expression of a total of 31 genes involved in CCP. They used these data to develop a predefined CCP “score” and then they set out to evaluate the value of the CCP score in predicting risk for progressive disease in the men who had undergone an RP or risk of prostate cancer-specific mortality in the men who had been diagnosed by a TURP and managed by watchful waiting. The findings of this study can be summarized as follows:*

*Among patients in the two RP cohorts*

1. *The CCP score could predict biochemical recurrence in univariate analysis (hazard ratio [HR] for a doubling in CCP = 1.89;  $p=5.6 \times 10^{-9}$ ).*
2. *The CCP score could predict biochemical recurrence in the final multivariate analysis (HR = 1.77;  $p=4.3 \times 10^{-6}$ ).*
3. *The CCP score and the PSA level were the most important and the most clinically significant variables in the best predictive model (the final multivariate analysis).*

*Among patients in the TURP cohort*

1. *The CCP score could predict time to death from prostate cancer in univariate analysis (HR = 2.92;  $p=6.1 \times 10^{-22}$ ).*
2. *The CCP score could predict time of death from prostate cancer in the final multivariate analysis (HR = 2.57;  $p=8.2 \times 10^{-11}$ ).*
3. *The CCP score was stronger than all other prognostic factors (although PSA levels added useful information).*

Thus there seems to be a strong belief in the use of CCP, especially when combined with other measures such as PSA.

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<sup>16</sup> <http://prostatecancerinfolink.net/2011/02/09/is-ccp-testing-really-the-prognostic-tool-we-need/>

The CCP test has been commercialized as Prolaris by Myriad. In a Medscape posting they state<sup>17</sup>:

*The Prolaris test, which measures the activity of cell cycle progression (CCP) genes in prostate cancer biopsy samples, was evaluated for its ability to predict either death from prostate cancer or biochemical recurrence in 5 company-sponsored studies, Dr. Cuzick reported.*

*It was tested at the time of disease diagnosis in 2 conservatively managed cohorts from the United Kingdom, after radical prostatectomy in 2 cohorts from the United States, and after external-beam radiation therapy.*

*In the studies, formalin-fixed prostate tissue from men with prostate adenocarcinoma was analyzed. A CCP score was calculated by measuring the average RNA expression of 31 CCP genes normalized by the average expression of 15 housekeeping genes as quantitated with reverse-transcriptase polymerase chain reaction, explained Dr. Cuzick.*

*A hazard ratio was then calculated for every unit change in CCP score for the risk for either biochemical recurrence or death from prostate cancer.*

*"A unit change is essentially a doubling in the expression of these cell cycle genes," he explained.*

*On multivariate analysis — variables ranged in the different studies but all included Gleason score and prostate-specific antigen (PSA) level — the predictive value of the CCP score for either outcome was "dominant" and "hugely significant" (hazard ratio, 2.6;  $P < 1010$ ), said Dr. Cuzick.*

*"PSA retained a fair amount of its predictive value, but the predictive value of the Gleason score "diminished" against the CCP score." he said. "Once you add the CCP score, there is little addition from the Gleason score, although there is some."*

*"Overall, the CCP score was a highly significant predictor of outcome in all of the studies," said Dr. Cuzick. "It was the dominant predictor in all but 1 of the studies in the multivariate analyses, and typically a unit change in the score was associated with a remarkably similar 2- to 3-fold increase in either death from prostate cancer or biochemical recurrence, indicating that this is a very robust predictor, and seems to work in a whole range of circumstances."*

Thus there is some belief that CCP when combined with other metrics has strong prognostic value.

In this analysis we use CCP as both an end and a means to an end. CCP is one of many possible metrics to ascertain prognostic values. There is a wealth of them. We thus start with the selection of genes. The Appendix provides a description of all of them yet a more detailed pathway

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<sup>17</sup> <http://www.medscape.com/viewarticle/805351>



analysis is warranted but not included here not in the papers presented. We then examine classifiers for prognostic value. We first consider general issues and then apply them to the CCP approach. This is the area where we have the majority of our problems.

## 5.2 PCA METRICS: DO THEY WORK?

The recent report on such a cancer prognostic model such as Oncotype DX by Knezvic et al is a putatively prognostic method used in prostate cancer. Fundamentally what they do is examine cancer cells for the expression of various genes and examine three sets; baseline expressions, excess expressions and reduced expression. They use the baseline to set levels for excess and reduced. They then use the excess or reduced in a one dimension expression to determine a prognostic measure. This seems to be in contrast with work we reported on a few months ago<sup>18</sup>.

Like PSA measures, CA125, CEA, and the like, they try to reduce everything to a single number. We argue here that such an approach is problematic at best. Furthermore they fail totally to demonstrate any internal pathway influence. There is no predictive basis for their approach predicated upon the actual dynamics of the cell. It is purely correlative and there may be substantial confounders involved. This approach is an example of what we fell to be the poorer aspects of genomics applied to cancer prognostics.

In a recent study the authors develop a score called the GPS score which is based upon know malignant PCs cells and then argue that then score has significant prognostic value. The authors state:

*The Oncotype DX Prostate Cancer Assay has been clinically validated, demonstrating that the GPS, assessed in diagnostic biopsy tissue, can predict the likelihood of the presence of adverse pathology (high-grade and/or high-stage disease), and that it complements existing pre-treatment risk assessment tools such as PSA levels, Gleason Score, and clinical stage.*

*The assay is intended to help guide treatment decisions in early-stage prostate cancer, including the decision between immediate therapy and active surveillance. As evidence that the analytical assay was designed well for its intended use to test RNA from small biopsies, in a clinical validation study, valid GPS results were generated for more than 95% of samples requiring 1 mm and 30 microns of tumor tissue...*

They continue

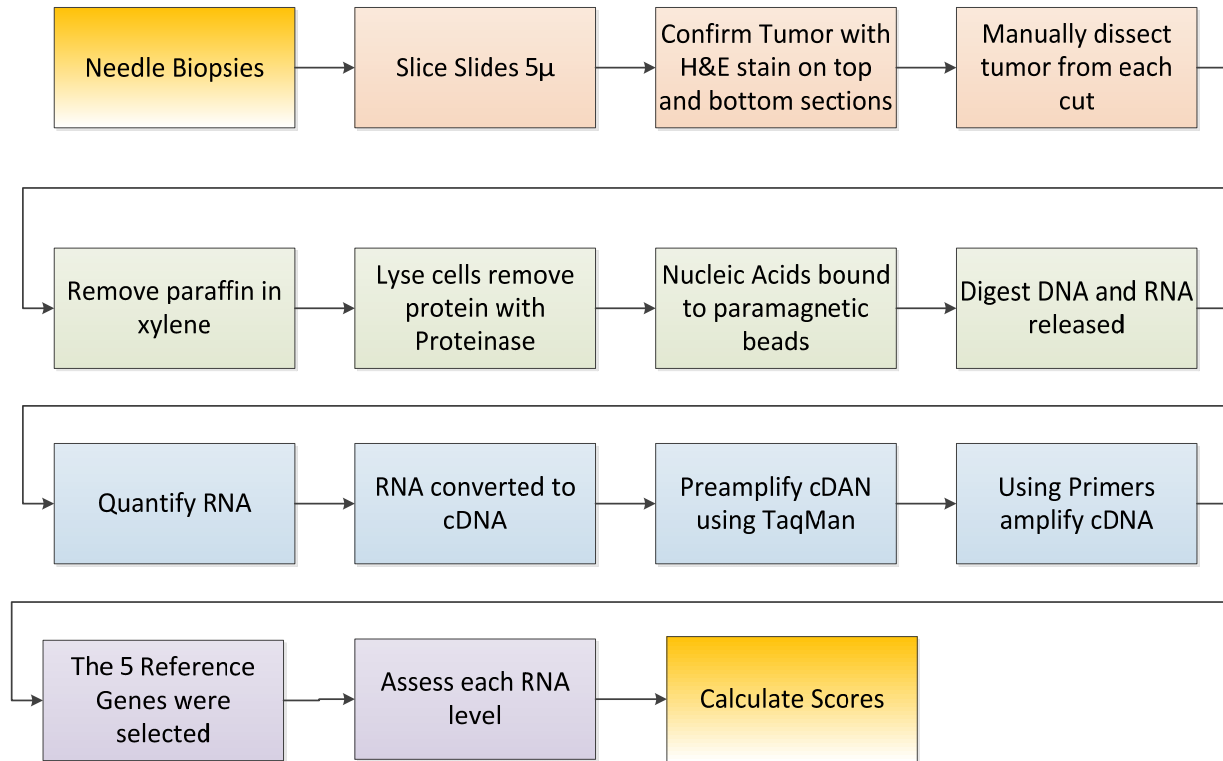
*Optimization of the Oncotype DX platform has enabled the development and analytical validation of the Oncotype DX Prostate Cancer Assay for use with prostate biopsy specimens. This RT-PCR assay has been clinically validated to predict the risk of high grade and/or non-organ confined disease at radical prostatectomy using biopsy samples containing as little as 1 mm of tumor tissue. The Oncotype DX Prostate Cancer Assay complements traditional clinical*

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<sup>18</sup> See <http://www.telmarc.com/Documents/White%20Papers/98%20CCP.pdf> Note that this study was based upon a different vendor with different genes.

and pathologic diagnostic features and will assist clinicians to discriminate patients with indolent prostate cancer from aggressive prostate cancer to help make the most appropriate treatment decisions.

The approach is as follows as shown in the Figure below. Basically take the malignant cells and measure the expression of certain genes via their RNA using a baseline reference gene expression level.



Now the genes they have selected are categorized as follows. They have four categories related to PCa and one category for the purpose of setting a reference level.

<i>Stromal Gene</i>	<i>Cellular Organization Group</i>	<i>Androgen Group</i>	<i>Proliferation Group</i>	<i>Reference Genes</i>
BGN	FLNC	FAM13C	TPX2	ARF1
COL1A1	GSN	KLK2		ATP5E
SFRP4	TPM2	AZGP1		CLTC
	GSTM2	SRD5A2		GPSI
				PGK1

Now based upon the levels of expression of these genes against the gene reference level they have proposed a metric which they term the GPS metric which is a measure of prognostic value related to the aggressiveness of the cancer. The GPS metric is given by:

$$GPS = \begin{cases} 0 & \text{if } 13.4(GPS_u - 10.5) < 0 \\ 100 & \text{if } 13.4(GPS_u - 10.5) > 100 \\ 13.4(GPS_u - 10.5) & \text{otherwise} \end{cases}$$

The higher the GPS measure the arguably the greater the virulence of the cancer. The internal value above is given by:

$$GPS_u = 0.735(Stromal) - 0.368(Cellular) - 0.352(Androgen) + 0.095(Pr\ oliferation)$$

Finally the specific value calculations by class are given by:

$$Stromal = 0.527BGN + 0.457COL1A1 + 0.156SFRP4$$

$$Cellular = 0.163FLNC + 0.504GSN + 0.421TPM2 + 0.394GSTM2$$

$$Androgen = 0.634FAM13C + 1.079KLK2 + 0.642AZGP1 + 0.997SRD5A2^*$$

$$Proliferation = TPX2^*$$

where

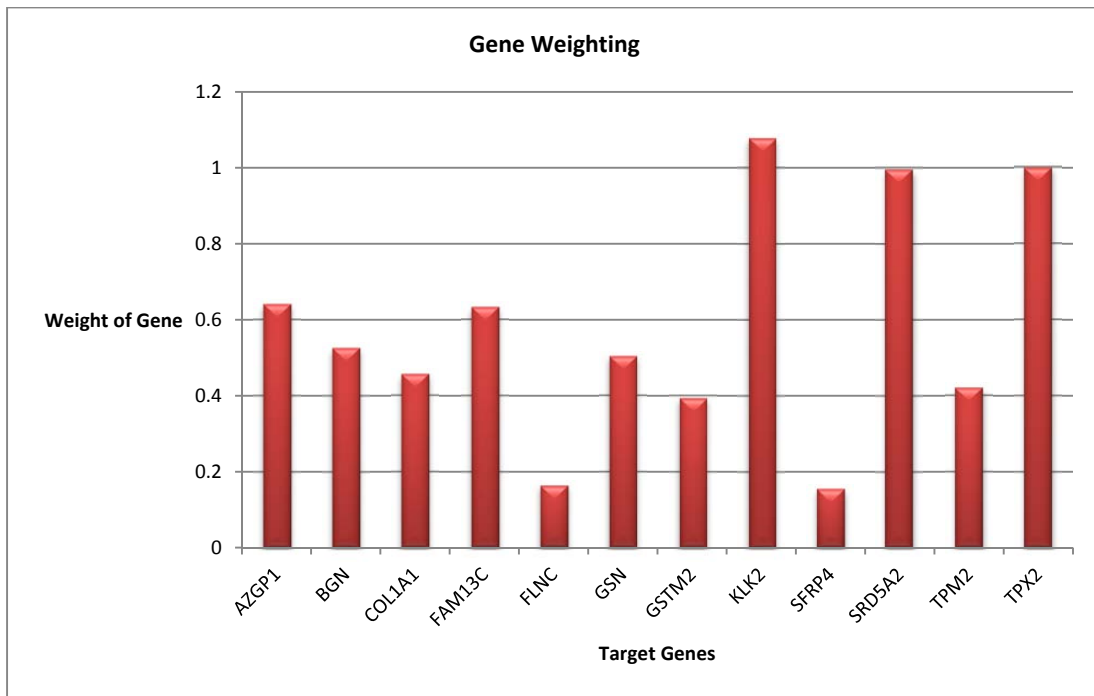
$$SRD5A2^* = \begin{cases} 5.5 & \text{if } SRD5A2 < 5.5 \\ SRD5A2 & \text{otherwise} \end{cases}$$

$$TPX2^* = \begin{cases} 5.0 & \text{if } TPX2 < 5.0 \\ TPX2 & \text{otherwise} \end{cases}$$

We summarize this below:

<i>Group</i>	<i>Genes</i>	<i>Weight</i>
<b><i>Stromal Gene</i></b>		
	BGN	0.527
	COL1A1	0.457
	SFRP4	0.156
<b><i>Cellular Organization Group</i></b>		
	FLNC	0.163
	GSN	0.504
	TPM2	0.421
	GSTM2	0.394
<b><i>Androgen Group</i></b>		
	FAM13C	0.634
	KLK2	1.079
	AZGP1	0.642
	SRD5A2	0.997
<b><i>Proliferation Group</i></b>		
	TPX2	1.00
<b><i>Reference Genes</i></b>		
	ARF1	
	ATP5E	
	CLTC	
	GPSI	
	PGK1	

We present graphically below the



From an earlier Press Release there was reported the results of a study stating<sup>19</sup>:

<sup>19</sup> <http://investor.genomichealth.com/releaseDetail.cfm?releaseID=762874>

*Results showed that the test, developed in collaboration with UCSF and Cleveland Clinic, strongly predicted disease aggressiveness ( $p=0.002$ ) offering information beyond currently available clinical factors, such as PSA and biopsy Gleason Score, to help physicians and their prostate cancer patients confidently choose the most appropriate treatment based on an individualized risk assessment.*

*Furthermore, this first-of-its-kind, multi-gene test has been validated to guide treatment decisions using the prostate needle biopsy sample taken before the prostate is removed -- thereby providing the opportunity for low risk patients to avoid invasive treatments such as radical prostatectomy or radiation.*

*"The results of our study showed that the individual biological information from the Oncotype DX prostate cancer test tripled the number of patients who can more confidently consider active surveillance and avoid unnecessary treatment and its potential side effects. The test also identified a smaller number of patients who, despite seemingly low-risk clinical factors, had more aggressive disease and, would suggest that they consider immediate treatment," said Peter Carroll, M.D., MPH, professor and chair, Department of Urology, UCSF and principal investigator of this validation study.*

*"With these new study results, I believe we may be able to significantly increase the use of active surveillance, which has been limited to some extent by the absence of a validated genomic tool to more accurately distinguish low and high risk disease at the time of biopsy." Active surveillance is a treatment plan that employs careful and consistent monitoring of the cancer in a man's prostate without removing it. Under active surveillance, patients have regular check-ups and periodic PSA blood tests, clinical exams and potential biopsies to closely monitor for signs of prostate cancer progression.*

*The Oncotype DX prostate cancer test measures the level of expression of 17 genes across four biological pathways to predict prostate cancer aggressiveness. The test results are reported as a Genomic Prostate Score (GPS) that ranges from 0 to 100 and is combined with other clinical factors to further clarify a man's risk prior to treatment intervention.*

Now there are many significant issues in this analysis.

1. The weights are arguably chosen to maximize the risk of missing an aggressive PCa. However I have not yet seen adequate clinical evidence to that effect.
2. Prior proposed genes and the ones included herein are shown below, one from the study currently in discussion and the other from a prior study of a Myriad genetic profile:

<i>Target Genes Oncotype DX</i>	<i>Housekeeping Genes Oncotype DX</i>	<i>Target Gene Myriad</i>	<i>Housekeeping Gene Myriad</i>
<b>AZGP1</b>	<b>ARF1</b>	<b>ASF1B</b>	<b>CLTC</b>
<b>BGN</b>	<b>ATP5E</b>	<b>ASPM</b>	<b>MMADHC</b>

<i>Target Genes Oncotype DX</i>	<i>Housekeeping Genes Oncotype DX</i>	<i>Target Gene Myriad</i>	<i>Housekeeping Gene Myriad</i>
COL1A1	CLTC	BIRC5	MRFAP1
FAM13C	GPSI	BUB1B	PPP2CA
FLNC	PGK1	C18orf24	PSMA1
GSN		CDC2	PSMC1
GSTM2		CDC20	RPL13A;LOC728658
KLK2		CDCA3	RPL37
SFRP4		CDCA8	RPL38
SRD5A2		CDKN3	RPL4
TPM2		CENPF	RPL8
TPX2		CENPM	RPS29
		CEP55	SLC25A3
		DLGAP5	TXNL1
		DTL	UBA52
		FOXM1	
		KIAA0101	
		KIF11	
		KIF20A	
		MCM10	
		NUSAP1	
		ORC6L	
		PBK	
		PLK1	
		PRC1	
		PTTG1	
		RAD51	
		RAD54L	
		RRM2	
		TK1	
		TOP2A	

It should be obvious that these two tests are dramatically different. Yet they claim similar results. The question is; what genetic expression has gone astray? Why, for example, do we see such a massive disparity? Frankly, other than CLTC we see no other commonality. What causes these disparate expressions? The answers are left hanging. At least with PSA we have some clear cause and effect. Here, at best, we have some correlative values.

With such disparate sets of genes one wonders why and how these tests can be compared if at all. Or, are these results just suggestive and are neither causative nor resulting from the lesions.

3. In the current test under discussion the cells used for extraction are arguably from the prostate biopsy. The Myriad appear to be more wide spread.

4. Are these tests worth anything? Furthermore, groups are offering tests to assess risks based upon genetic profiles. As stated<sup>20</sup>:

*Myriad also rolled out new tests. In September, the company launched its myRisk Hereditary Cancer™ test, a 25-gene panel covering eight major cancers (breast, colorectal, endometrial, gastric, melanoma, ovarian, pancreatic, and prostate) at an average selling price of \$3,700. In October the company introduced myPlan Lung Cancer, which carries a \$3,400 list price; followed in November by myPath Melanoma, which has an average selling price of \$1,500. By 2015, Myriad has said, it expects to discontinue several current tests, including the BRACAnalysis test at the center of the Supreme Court case.*

Just because some genetic profile may have some correlative relationship the genetic profile is not causative. Tests like these can be costly and of yet to be fully justified clinical value. Take a melanoma, if one has a suspect pigmented lesion then a simple excision and competent path study should suffice. That is an order of magnitude less than the genetic profile. In fact if one were to do a profile it should be of the melanocytes and not of the cells in general.

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<sup>20</sup> <http://www.genengnews.com/insight-and-intelligence/the-10-biggest-events-of-2013/77899986/>

## 6 DISCUSSIONS

The challenge of Pre Detected PCa tests is truly a compelling task. We know that PCa can become a highly heterogeneous genetically challenging cancer and that in its early stage if detected can be treated with low morbidity and mortality. However, even with multi core biopsies, 24 and above cores, one may miss the lesions even aided by MRI and ultrasound. Thus having non-invasive tests would be a significant step forward.

PSA is notoriously problematic. It is smoke to PCa as the first indicator of a potential problem. As Pashyan et al state:

*These data show that personalised screening with eligibility for screening based on an absolute risk that is dependent on age and polygenic risk and equivalent to the risk threshold for eligibility based on age alone could reduce the number of people eligible for screening while detecting the majority of the cancers identified through a programme based on age alone. Alternatively, screening the same number of individuals in a personalised screening programme could potentially detect a greater number of cases than a screening programme based on age alone.*

There are more and more personalized screenings. In this report we have attempted to bring some of them to light.

### 6.1 CTCs AND PCA

Circulating Tumor Cells, CTCs, are now capable of being extracted efficiently from patients. From Haber's Lab at Dana Farber his team has published a paper in Science by Yu et al describing the results. The results are worth examining.

They state in an earlier version on breast cancer:

*Circulating tumor cells (CTCs) are present at low concentrations in the peripheral blood of patients with solid tumors. It has been proposed that the isolation, ex vivo culture, and characterization of CTCs may provide an opportunity to noninvasively monitor the changing patterns of drug susceptibility in individual patients as their tumors acquire new mutations. In a proof-of-concept study, we established CTC cultures from six patients with estrogen receptor-positive breast cancer. Three of five CTC lines tested were tumorigenic in mice.*

*Genome sequencing of the CTC lines revealed preexisting mutations in the PIK3CA gene and newly acquired mutations in the estrogen receptor gene (ESR1), PIK3CA gene, and fibroblast growth factor receptor gene (FGFR2), among others. Drug sensitivity testing of CTC lines with multiple mutations revealed potential new therapeutic targets. With optimization of CTC culture conditions, this strategy may help identify the best therapies for individual cancer patients over the course of their disease.*



We have examined the pathways above extensively in the past and WNT is a well-known target. I hear Haber talk this past week at NYAS and his talk was quite informative. I was especially impressed by the means used to extract CTCs that alone is worth a look.

My general concern however is several fold:

1. CTCs can come from anywhere. As we have discussed before the work of Gundem et al demonstrated a complex proliferation of genetic profiles in AR PCa. Thus one may be able to gain some prognostic information but not localization.
2. There is always the issue of stem cells. Again what cells may get extravasated is not the same as what cells are proliferating.
3. Cell communication via exosomes is a concern as is the ECM issues of localized growth.

This is an extraordinary useful tool and definitely worth following. The current Science work by Miyamoto et al on PCa states:

*Prostate cancer is initially responsive to androgen deprivation, but the effectiveness of androgen receptor (AR) inhibitors in recurrent disease is variable. Biopsy of bone metastases is challenging; hence, sampling circulating tumor cells (CTCs) may reveal drug-resistance mechanisms. We established single-cell RNA-sequencing (RNA-Seq) profiles of 77 intact CTCs isolated from 13 patients (mean six CTCs per patient), by using microfluidic enrichment.*

*Single CTCs from each individual display considerable heterogeneity, including expression of AR gene mutations and splicing variants. Retrospective analysis of CTCs from patients progressing under treatment with an AR inhibitor, compared with untreated cases, indicates activation of noncanonical Wnt signaling ( $P = 0.0064$ ). Ectopic expression of Wnt5a in prostate cancer cells attenuates the antiproliferative effect of AR inhibition, whereas its suppression in drug-resistant cells restores partial sensitivity, a correlation also evident in an established mouse model. Thus, single-cell analysis of prostate CTCs reveals heterogeneity in signaling pathways that could contribute to treatment failure.*

The last sentence is the most powerful and disturbingly consistent observation. PCa is just "too sneaky". It does not follow simple lines of development. It is not a BRAF V600 melanoma, it is not a Vogelstein colon cancer progression.

Frankly, it is for this reason alone that the USPTF recommendations on PSA testing are cruel and unusual. It is clear that PCa is a highly complex cancer and one that lends itself to multiple parallel paths resulting in some significantly high mortality rates. The very thought of taking the only tool and refusing to use it may, in my opinion, almost border on the criminal.

## 6.2 SPECIFICS

Not all tests are equal. However there are some key specifics.

1. The Pre Tests do allow for clinically established reasons to reduce the need for biopsies. The low AUC of PSA alone is mitigated by the substantially higher AUCs of the newer tests.
2. The newer Pre Tests do not measure specific genetic profiles. They do measure KLK gene expressions and these may be adequate.
3. The majority of tests fail to include temporal data. Namely temporal data often reflects a change and change often reflects a malignancy. Change may also reflect other changes as well as we have shown with increasing HbA1c.
4. Family history is useful but can be problematic since we do not fully understand the inheritance process.
5. Blanket rejection of PSA is unreasonable and unjustified. If there were but a PSA test and the second resort was biopsy then one can possible consider a modification but not rejection. Yet with the proliferation of adjuncts as described herein, then the PSA lends itself to ongoing utility in establishing a need to perform other non-invasive testing.
6. Post Testing is problematic. The problem is that one can possibly determine the aggressiveness of a PCa yet have no way to address it. Thus one wonders what benefit some of them provide.

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## 8 WHITE PAPERS

The following are related White Papers we have written in the recent past which may facilitate the material contained herein. <http://www.telmarc.com/White%20Papers/default.html>

- No 127 STAT3 and PCa: of Mice and Men, August 2015.
- No 126 Prostate Cancer Metastases: Some Simple Cases, (July 2015)
- No. 125 CRISPR and Cancer: Revised (April 2014)
- No. 124 CRISPR Cas9: A Genomic Tool (April 2015)
- No. 123 Metformin, Statins and PCa (February 2015)
- No. 121 Sirt1, Exosomes and Prostate Cancer (January 2015)
- No. 120 CNVs and Prostate Cancer (December 2014)
- No. 119 SNPs and Prostate Cancer (October 2014)
- No. 118 Vitamin D and Prostate Cancer (October 2014)
- No. 117 SPDEF, ETS Transcription Factors and PCa (October 2014)
- No. 116 Methylation, Prostate Cancer, Prognostics (August 2014)
- No. 112 Prostate Cancer: miR-34, p53, MET and Methylation (May 2014)
- No. 111 CRISPR and Cancer (April 2014)
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- No. 104 Prostate Cancer and Blood Borne Markers (December 2013)
- No. 103 Prostate Cancer Indolence (December 2013)
- No. 102 MDS and Methylation (August 2013)
- No. 101 Exosomes and Cancer (August 2013)
- No. 100 lncRNA and Prostate Cancer (July 2013)
- No. 99 SNPs and Cancer Prognostics (July 2013)
- No. 98 CCP and Prostate Cancer (July 2013)
- No. 95 MER Tyrosine Kinase Receptors and Inhibition (June 2013)
- No. 93 Cancer Cell Dynamics Methylation and Cancer (April 2013)
- No. 91 Methylation and Cancer (March 2013)
- No. 88 Extracellular Matrix vs. Intracellular Pathways

No. 87 Prostate Cancer Prognostic Markers

No. 86 Cancer Models for Understanding, Prediction, and Control

No. 85 Prostate Cancer Stem Cells

No. 84 Epistemology of Cancer Genomics

No. 83 Prostatic Intraepithelial Neoplasia (

No 82 Prostate Cancer: Metastatic Pathway Identification (February 2011)

No 80 PSA Evaluation Methodologies (December 2010)

No 79 The PSA Controversy (November 2010)