Two-stage classifiers that minimize PCA3 and the PSA proteolytic activity testing in the prediction of prostate cancer recurrence after radical prostatectomy

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Introduction: *Early biochemical recurrence after prostate cancer surgery is associated with higher risk of aggressive disease and cancer specific death. Many new tests are being developed that will predict the presence of indicators of aggressive disease like early biochemical recurrence. Since recurrence occurs in less than 10% of patients treated for prostate cancer, validation of such tests will require expensive testing on large patient groups. Moreover, clinical application of the validated test requires that each new patient be tested. In this report we introduce a two-stage classifier system that minimizes the number of patients that must be tested in both the validation and clinical application of any new test for recurrence.*

Materials and methods: *Expressed prostatic secretion specimens were prospectively collected from 450 patients prior to robot-assisted radical prostatectomy for prostate cancer. Patients were followed for 2.5 years for evidence of biochemical recurrence. Standard clinical parameters, the levels proteolytic activity of prostate specific antigen (PSA) and the levels of PCA3 RNA, PSA RNA and TMPRSS2:ERG fusion RNA were determined in each prospective patient specimen for subsequent correlation with biochemical recurrence.*

Results: *While levels of PCA3 and PSA proteolytic activity (PPA) in prostatic secretions provided an effective pre-surgical predictor of early biochemical recurrence in prostate cancer, application of the two-stage classifier shows that only 60% of the patients need these tests.*

Conclusion: *Two-stage classifiers can provide a parsimonious approach to both the validation and clinical application of biomarker-based tests. Adoption of the two-stage neutral zone classifier can reduce unnecessary testing in prostate cancer treatment.*

Key Words: PCA3, EPS, neutral zone classifier, two-stage classifier, prostate cancer, biomarkers, biochemical recurrence, urinary assay

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Introduction

Overtreatment remains a significant issue in prostate cancer management, and this has generated an ongoing search for new biomarkers that can predict cancer aggressiveness itself (e.g. NCCN risk group analysis), the likelihood of Gleason score (GS) upgrading, the presence of other adverse pathological factors or early biochemical recurrence. Reliable pretreatment

biomarkers of GS upgrading have not been found,[1] however, single marker results[2] and RNA profiling results (reviewed in[3]) have recently succeeded in providing measures of disease aggressiveness that can complement existing clinical tools.

In general, the comparative effectiveness of a given biomarker is determined from the area under the curve (AUC) generated in ROC analysis.[4,5] In applying this analysis to a patient population, a single cutoff point is chosen from the ROC curve of a multivariate classifier that includes baseline parameters (e.g. serum PSA, GS) and the biomarker (s) under consideration (see[2,6,7] for examples). Single cutoff points can be chosen in several ways, but each method splits patient populations into two groups: those with a probability of experiencing the event that is above the cutoff and those with a probability of experiencing the event that is below the cutoff. A disadvantage of this approach is that a significant false positive or false negative rate is often unavoidable. Moreover, this approach contributes to overtreatment and significantly affects the cost of administering tests for the biomarker, since each patient must be tested in order to use the classifier. In the short term, this has the unintended consequence of hindering the independent validation of new commercial biomarkers since the cost of testing large groups of patients with commercial tests cannot always be borne through ordinary research funding mechanisms.

Recently, we have proposed the use of the neutral zone classifier as an alternative approach to ROC analysis in medical research.[8] When used by itself, a neutral zone classifier has the advantage of segregating patient populations into three groups: those for whom the classifier predicts a relatively high probability of experiencing the event, those for whom it predicts a relatively low probability of experiencing the event, and those in a neutral zone for whom the classifier is ineffective at bringing clarity to the patient's risk. In this paper, using PSA proteolytic activity (PPA) as an illustrative biomarker of early biochemical recurrence, we demonstrate that serial application of a neutral zone classifier followed by a traditional Youden cutoff classifier[9] offers an additional advantage of reducing the number of patients that must be tested for the biomarker.

Materials and methods

Patient cohort

Between 2007 and 2013, expressed prostatic secretion (EPS) specimens were collected prior to surgery for prostate cancer from patients consented under an Internal Review Board approved protocol for the non-invasive-collection and biomarker analysis of EPS specimens. The EPS collection protocol, EPS processing and storage protocol have been described in detail.[10] Processed specimens were stored at -80°C until use. Supernatant fluid was used for PPA testing. PPA levels were determined on 450 specimens that have now been followed for biochemical recurrence for at least 2.5 years. Demographics are given in Table 1.

RNA preparation

RNA was prepared from the EPS sediment using the RNEasy mini kit (Qiagen, Germantown, MD, USA) as described by the manufacturer. Quantification of the amount of RNA obtained from each specimen

TABLE 1. **Patient demographics**

Patients with 2.5 yr follow up N = 450		
Factor	**Mean**	**Range**
Age	63.0 y	39 to 85y
Pre-Bx PSA	7.92 ng/mL	0.23 to 203 ng/mL
EPS volume	183.9 µL	0.5 to 3200 µL
Factor	N	%
Gleason sum		
4	1	0.2%
5	0	0.0%
6	169	37.6%
7	229	50.9%
8	27	6.0%
9	13	2.8%
10	1	0.2%
Ethnicity		
Hispanic	37	8.2%
Non-Hispanic	403	89.6%
Unknown	10	2.0%
Race		
African-American	21	4.6%
Asian	28	6.2%
Caucasian	378	84.0%
Native American	1	0.2%
Other	22	4.8%
T-stage		
T1a	1	0.2%
T1b	1	0.2%
T1c	354	78.7%
T2	2	0.44%
T2a	70	15.5%
T2b	14	3.1%
T2c	8	1.8%

was determined using a Nanodrop ND-1000 spectrophotometer (ThermoScientific, Waltham, MA, USA).

cDNA preparation

The SuperScript VILO cDNA Synthesis Kit (ThermoFischer Scientific, Claremont, CA, USA) was used to prepare cDNA as described by the manufacturer. In previous work[10] we noted the yield of PCR amplifiable product DNA was linear when 10 ng to 200 ng of input RNA was used for cDNA synthesis. Since RNA yield per specimen varied, a maximum of 200 ng of specimen RNA was used for cDNA synthesis and the resulting cDNA product was used for quantitative PCR amplification. The input RNA value was used as a normalization factor in evaluating subsequent RT-PCR results.

Quantitative RT-PCR

Quantitative RT-PCR assays for three prostate cancer biomarkers: PCA3 RNA, TMPRESS2:ERG fusion RNA and PSA mRNA were performed on each patient in the cohort. The details of each procedure have been described.[11]

PSA proteotytic activity assay (PPA assay)

The PSA proteolytic activity assay was initially developed in a collaboration between Northwestern University Feinberg School of Medicine and Ohmx corporation.[2] The PPA assay measures the activity of PSA (aPSA) rather than the amount of PSA protein.

The enzymatic assay was performed in a 96 well plate and using a Synergy 4 microplate reader (BioTek, San Diego, CA, USA). Because active PSA cleaves glutamine residues, a PSA specific peptide sequence ending in glutamine (Mor-HSSKLQ-AMC) was labeled with AMC (7-amino-4-methyl coumarin), a fluorescent dye. This peptide sequence was added to wells containing various known concentrations created by serial dilution of aPSA (2615 ng/mL to 26 ng/mL) in buffer A (50 mM Tris– HCl, 1.5 M NaCl, 2 mg/ml BSA, pH 7.5) to create a PSA standard curve. Active PSA (aPSA) was purchased from Scripps Laboratories; (San Diego, CA, USA). As the active enzyme recognizes the peptide substrate, the fluorescent dye that was released was quantified using an excitation wavelength of 380 nm and an emission wavelength of 450 nm. The reaction rate is linear for 45 minutes. The fluorescence values were collected every 2 minutes over 40 minutes at room temperature using top-read fluorescence mode to create a series of reaction rates. Plotting input PSA concentration as a function of observed PSA activity (aPSA) yields a standard curve used to calculate the PSA activity in an unknown sample. EPS supernatant specimens were diluted with buffer A, and 50 µL was loaded into each of three separate wells of a 96 well plate. The reaction was initiated by the addition of 50 µL of an 0.8 mM peptide (Mor-HSSKLQ-AMC) solution in buffer A.

Statistical methods

Traditional classifier

In a traditional classifier, a dichotomous outcome is linked to a decision statistic T where large values of T suggest membership in class 1 (C = 1) and small values of T suggest membership in class 0 (C = 0). Often the decision statistic T is the estimated probability of class 1, obtained from a fitted logistic regression model. In this paper, T is constructed from a logistic regression model using backward elimination of available biomarker measurements. The analyses are carried out using SAS/STAT software. Once significant variables were identified, a traditional classifier was constructed by choosing a single threshold from the ROC curve. The threshold is the value that maximizes the Youden index, defined as J = Sensitivity + Specificity -1. The procedure is illustrated in Figure 1a, where the desired threshold C´ is the value that maximizes the length of the vertical line between the minimum AUC for a test with no discriminatory value and the AUC values observed for the test under observation. The predicted membership class has the form:

$$\hat{C} = \begin{cases} 1 & , \text{if } T \geq C' \\ 0 & , \text{if } T < C' . \end{cases}$$

Neutral zone classifier

A neutral zone classifier $\hat{C}$ is constructed by choosing a threshold C_0 to control the false positive rate to be a α and a second threshold C_1 to control the false negative rate to be β. In our applications, both α and β were chosen to be as close to 0.05 as the granularity of the data permitted. The predicted membership class has the form:

$$\hat{C} = \begin{cases} 1 & , \text{if } T \geq C_1 \\ N & , \text{if } C_0 < T < C_1 \\ 0 & , \text{if } T \leq C_0 \quad , \end{cases}$$

where $\hat{C} = N$ assigns the neutral zone label, meaning the predictor values are too ambiguous to decide on class 0 or class 1. The value C_0 is chosen from the ROC curve as the threshold that corresponds to making the sensitivity equal to 1-β, and the value C_1 is the threshold that corresponds to making 1-specificity equal to α . The procedure is illustrated in Figure 1b.

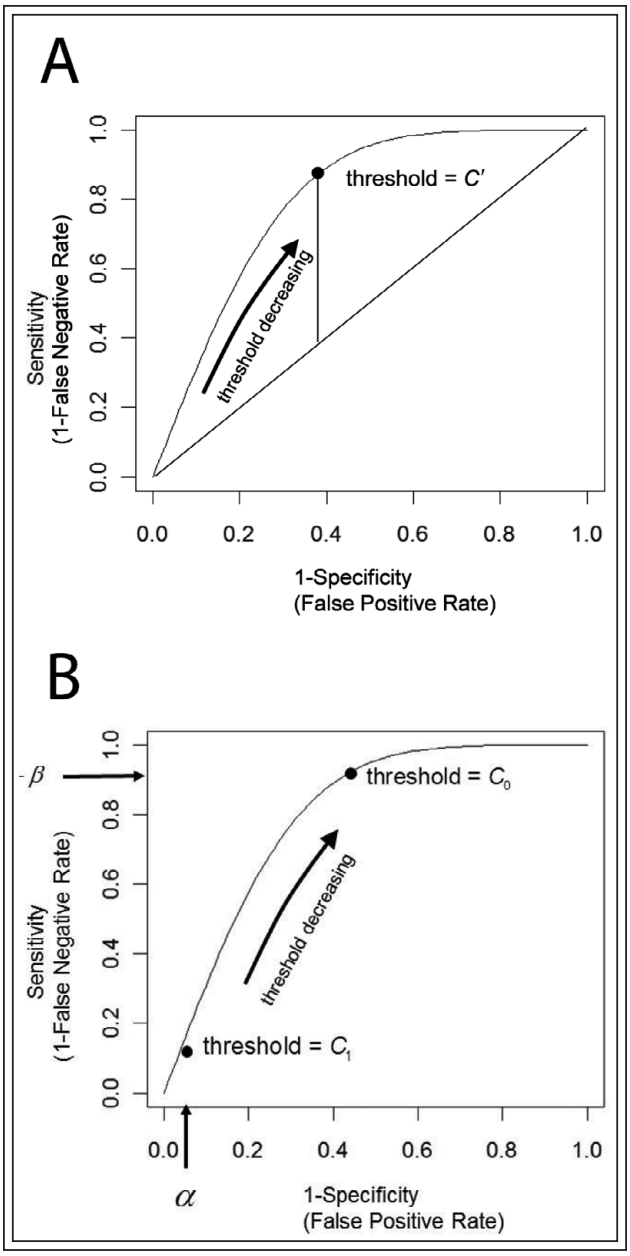

Figure 1. Schematic description of the thresholds used in the two classification methods. **a)** Finding the Youden threshold for a traditional classifier; **b)** Finding the two thresholds needed for a neutral zone classifier.

Serial application of a neutral zone classifier and a traditional classifier

A two-stage classifier with a definitive second stage combines a neutral zone classifier in a first stage with a traditional classifier in a second stage. In the first stage, the decision statistic T is developed through logistic regression analysis of all the patients, using backward variable selection on only those clinical variables that are routinely available. Thresholds C0 and C1 are selected from the resulting ROC curve to control the false positive rate and false negative rate to be as close to α and β, respectively, as is possible. The neutral zone classifier in the first stage creates a sub-population of patients that are classified as $\hat{C} = N$. Those patients classified as $\hat{C} = N$ in the first stage are used to construct the second stage of the two-stage classifier. The decision statistic T for the second stage is developed through logistic regression analysis using backward variable selection of both the clinical and laboratory variables. A single threshold C´ is selected from the resulting ROC curve that maximizes the Youden index. The second stage classifier is definitive in the sense that no patients in the sub-population are placed into a neutral zone. Consequently, the two-stage classifier with a definitive second stage ultimately predicts class membership for all patients.

Results

In previous work,[2] the levels of PPA in EPS were found to be inversely related to aggressiveness. In that study a group of 100 patients with available EPS specimens were studied. Aggressive prostate cancers were defined as those whose post surgical follow up showed distant or lymph node metastases, extracapsular extension, seminal vesicle invasion or prostate specific death. Non-aggressive prostate cancers were defined as those with postsurgical organ confined disease with GS of 6 or less and no evidence of biochemical recurrence within 2 to 5 years. Using a group of 450 patients for which EPS specimens were available, we extended those findings to NCCN risk groups, Figure 2. Using the NCCN guidelines for 2016, very low and low risk patients were segregated to group 1, intermediate risk patients to group 2 and high and very high risk patients to group 3.

Early biochemical recurrence after surgery or radiation is widely accepted as an indication of disease progression[12] that also identifies aggressive disease with increased risk of prostate specific mortality.[13-15] Pre-treatment parameters like serum PSA level, GS and primary Gleason pattern provide strong indicators of early biochemical recurrence. Men with high risk disease defined as those with primary Gleason pattern 5 in a single biopsy core or primary Gleason pattern 4 in four or more biopsy cores are at highest risk for early biochemical recurrence.[16] Moreover, among patients with early biochemical recurrence, those with primary pattern 4 or greater and extraprostatic extension post surgery are at highest risk for prostate specific mortality.[17]

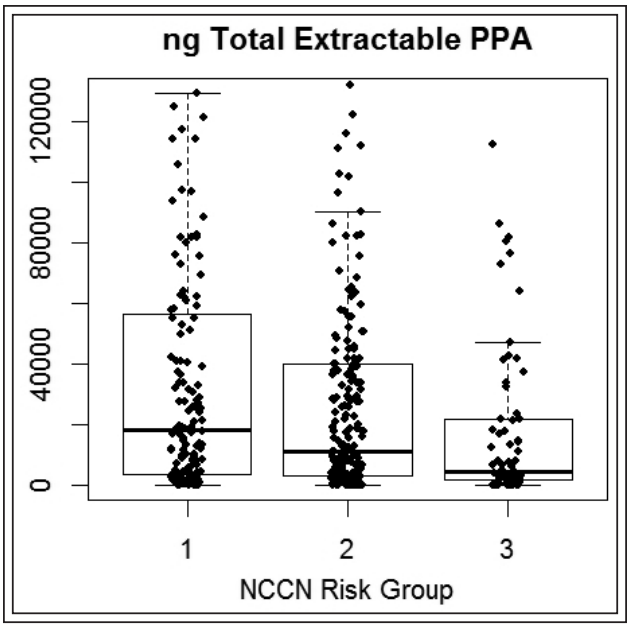

Figure 2. Inverse relation between total extractable PPA and NCCN risk.
As noted in materials and methods, PPA is given in ng proteolytically active prostate specific antigen/mL PBS extraction fluid. Total ng extractable PPA is the amount extracted from the EPS specimen obtained from each patient. Group 1 includes all prostate cancer patients classified by 2016 NCCN guidelines as low and very low risk. Group 2 includes all prostate cancer patients classified by 2016 NCCN guidelines as intermediate risk. Group 3 includes all prostate cancer patients classified by 2016 NCCN guidelines as high or very high risk.

Given the correlation of PPA with both NCCN risk shown here and an independent measure of prostate cancer aggressiveness,[2] we tested its ability to serve as a predictor of biochemical recurrence by including it in a list of 10 biomarkers comprising PPA and its normalized variants, TMPRSS2:ERG mRNA, PSA mRNA, PCA3 RNA, EPS specimen volume and total recovered specimen RNA. To provide a benchmark context for a neutral zone classifier, we first show results for the traditional Youden cutoff[9] classifier. Backward variable selection for a logistic regression model using SAS/STAT software determined that PCA3 RNA normalized to the RNA quantity used for cDNA production, and the total ng of extractable PPA were the only two laboratory variables that contributed significantly to the prediction of recurrence. Results obtained from constructing a traditional Youden cutoff[9] classifier for segregating patients according to their probability for recurrence are shown in Figure 3.

It can be seen in Figure 3 that the classifier has a false positive and false negative rate of 11.4% and 28.6%, respectively. While the 95.6% negative predictive value of the test suggests that it would be useful, this approach requires testing all of the 450 patients. The cost of the Progensa PCA3 test performed at a certified lab is about $760 per patient. Although the price of the commercial PPA test has not yet been determined, it would probably be intended to compete with tests like the GPS score from Oncotype[6] and/or the CCP score from Polaris.[18] These tests are priced near $3000/patient. If we assume that the cost of the single variable PPA test would be approximately $1000, the overall cost of testing this group would be about $792,000 beyond the standard clinical tests generally employed. The most significant effect of an expenditure of this type is to preclude verification of commercial biomarker validation by independent research groups since the cost of reimbursing the cost of commercial tests cannot always be borne by ordinary research funding mechanisms.

To test the idea that serial use of a neutral zone classifier followed by a traditional classifier might reduce both the need for the test and its associated costs, we introduce a two-stage classification procedure. In the first stage, a neutral zone classifier using only routinely available clinical variables (biopsy GS, serum PSA and age) is used to control the false positive and false negative rates to acceptable minima.[8] The neutral zone classifier only makes confident predictions for a subset of patients and places the remaining patients in a neutral zone for further analysis in the second stage. Patients in the neutral zone were subsequently tested for the PCA3 and PPA biomarkers, and then analyzed with a second stage traditional Youden cutoff[9] classifier, Figure 4. Although the neutral zone approach reduces the number of patients for whom PCA3 and PPA testing would be required by nearly 40%, the estimated cost of performing both tests in constructing the model would still be over $482,000. While the two tests interact in logistic regression, we asked whether or not the efficacy of the predictions made by the test would be affected by omitting one of the two assays in the second stage of the neutral zone approach. When the PPA test was omitted, PCA3 was eliminated in model selection process as a significant laboratory variable. On the other hand, when PCA3 was omitted, PPA remained as a significant lab variable, Figure 5.

From a practical point of view, application of the serial classification model shown in Figure 4 to a new patient is straightforward. First, using standardized versions of the patient's PSA, GS and age values,

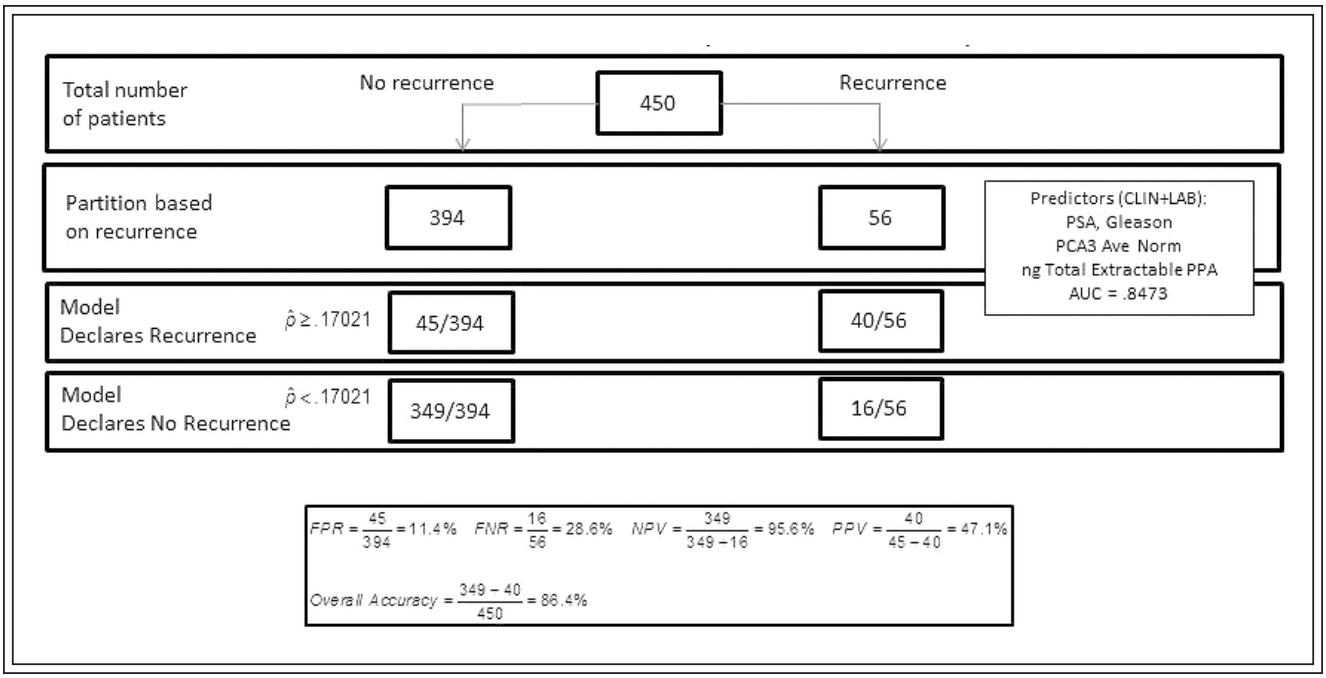

Figure 3. Traditional Youden cutoff classifier.
This classifier partitions the patients into two groups based on the Youden cutoff of $\hat{p} = 0.17021$, with a true positive rate of 71.4%, a true negative rate of 88.6%, a false negative rate of 28.6%, and a false positive rate of 11.4%. The estimated overall accuracy of the classifier is 86.4%. Five-fold cross validation estimates of the false negative rate, false positive rate and overall accuracy are 32.1%, 11.9%, and 85.6%, respectively. The fitted probability of recurrence, $\hat{p}$, is calculated as

$$\hat{p} = \frac{\exp(-2.80 + 1.02 \times PSA_s + .596 \times GS_s + .318 \times PCA3_s - 2.094 \times PPA_s)}{1 + \exp(-2.80 + 1.02 \times PSA_s + .596 \times GS_s + .318 \times PCA3_s - 2.094 \times PPA_s)}$$

where

$$PSA_s = (PSA - 7.92)/11.44$$
$$GS_s = (GS - 6.76)/.748$$
$$PCA3_s = (PCA3 - 86.66)/254.77$$
$$PPA_s = (PPA - 35{,}466.66)/57{,}427.77$$

are standardized versions of serum PSA, GS, PCA3 Ave Norm, and ng total extractable PPA.
The strengths of the classifier is its negative predictive value of 95.6% and its overall accuracy of 86.4%. Predictors (CLIN +LAB): GS: Biopsy Gleason Score, PSA: Serum PSA, PCA3: PCA3 RNA normalized to the amount of RNA use in cDNA production and ng extractable PSA Proteolytic Activity.
PPV = positive predictive value; NPV = negative predictive value; FNR = false negative rate; FPR = false positive rate.

compute $\hat{p}_1$, Figure 4. Classify the patient as likely to have recurrence if $\hat{p}_1 \geq .29167$ and unlikely to have recurrence if $\hat{p}_1 \leq .04839$. If $.04839 < \hat{p}_1 < .29167$, then obtain the two laboratory variables PCA3 and PPA. Using standardized versions of the patient's PSA, PCA3 and PPA values, compute $\hat{p}_2$ from the probability formula for the second stage traditional classifier, Figure 4. Classify the patient as likely to have recurrence if $\hat{p}_2 \geq .20211$, and unlikely to have recurrence if $\hat{p}_2 < .20211$. If the serial classification procedure in Figure 5 were to be used, the only change is to calculate $\hat{p}_2$ from the revised formula in Figure 5, where only PPA is called for.

Discussion

Two central conclusions can be drawn from the results. First, as a biomarker that is inversely correlated with NCCN risk group, Figure 2, PPA was found to be an effective pre-surgical biomarker for the prediction of early biochemical recurrence in patients who will undergo radical prostatectomy, Figures 3, 4 and 5. Second, for ROC based analysis of the data, the two-stage classifier with a traditional second stage provides an alternative to the traditional single-stage approach that significantly reduces the overall cost of both model building and

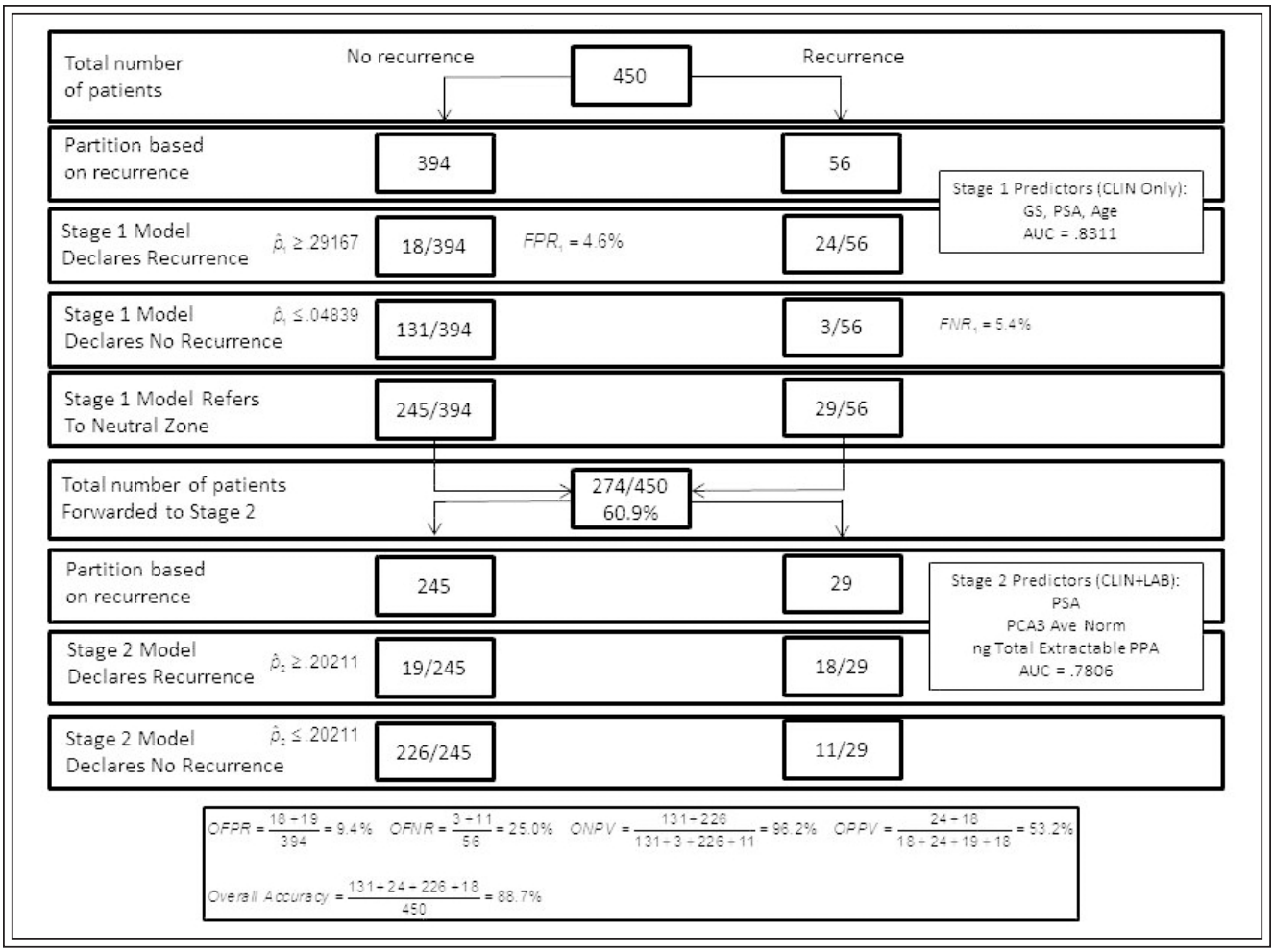

Figure 4. Serial application of a neutral zone classifier and a traditional classifier.
In this approach the standard clinical information on each patient is first used in a neutral zone classifier that controls both false positive and false negative rates at ≤ 5.4% by classifying only the patients with probabilities of recurrence greater than or equal to a threshold of = 0.29167 or less than or equal to a threshold of $\hat{p}_1 = 0.04839$. The value of $\hat{p}_1$ is the fitted probability of recurrence based on just using the clinical variables, and is given by the formula $\hat{p}_1 = \frac{\exp(-2.44 + .924 \times PSA_s + .621 \times GS_s + .332 \times Age_s)}{1 + \exp(-2.44 + .924 \times PSA_s + .621 \times GS_s + .332 \times Age_s)}$
where PSAs and GSs are as previously defined, and where $Age_s = (Age - 63.00)/8.04$. The 274 patients in the neutral zone who cannot be given a confident answer regarding recurrence within 2.5 yrs are then tested with the PCA3 and PPA tests and the results are reanalyzed with a traditional Youden cutoff ($\hat{p}_2 = 0.20211$) classifier.[9] The value of $\hat{p}_2$ is computed for patients that were placed into the neutral zone, and is the fitted probability of recurrence based on including PPA as a predictor given by the formula $\hat{p}_2 = \frac{\exp(-3.028 + 1.931 \times PSA_s + .463 \times PCA3_s - 3.227 \times PPA_s)}{1 + \exp(-3.028 + 1.931 \times PSA_s + .463 \times PCA3_s - 3.227 \times PPA_s)}$.

The application of the two-stage classifier results in an overall true positive rate of 75.0%, an overall true negative rate of 90.6%, an overall false negative rate of 25.0%, and an overall false positive rate of 9.4%. The estimated overall accuracy of the classifier is 88.7%. Five-fold cross validation estimates of the false negative rate, false positive rate and overall accuracy are 39.3%, 9.6%, and 86.7%, respectively. Moreover, this approach retains a high overall negative predictive value at 96.2%and a high overall accuracy of 88.7%. CLIN only: GS: Biopsy GS, PSA: Serum PSA and Age. CLIN +LAB: PSA: Serum PSA, PCA3: PCA3 RNA normalized to input RNA used for cDNA production and PPA: Total ng Extractable PSA Proteolytic Activity.
OPPV = overall positive predictive value; ONPV = overall negative predictive value; OFNR = overall false negative rate; OFPR = overall false positive rate.

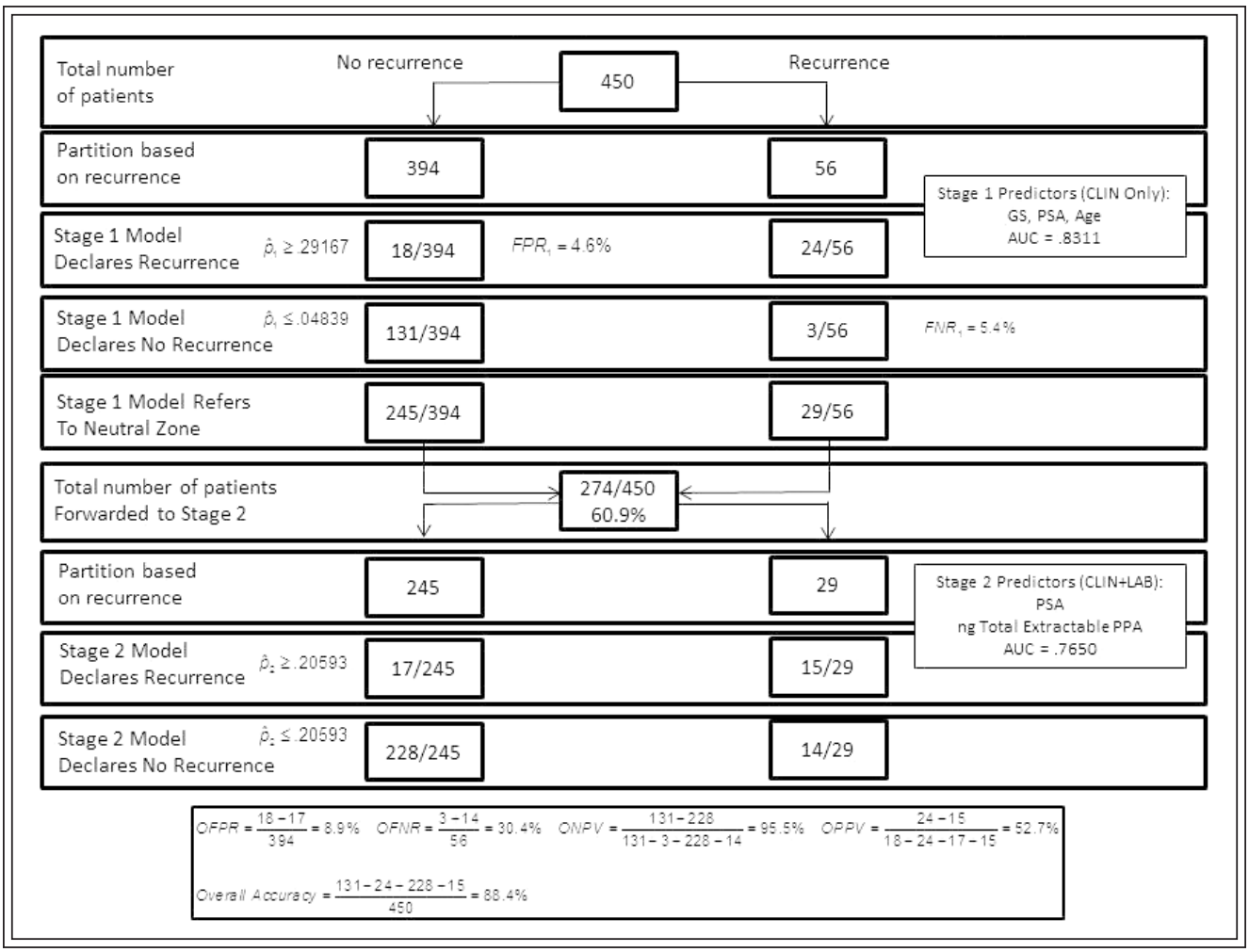

Figure 5. Serial application of a neutral zone classifier and a traditional classifier removing PCA3 variable.
In this classifier, the first stage neutral zone classifier is identical to that shown in Figure 4. In the second stage, where a traditional Youden cutoff classifier[9] is used, PCA3 is dropped from the set of predictors in an effort to reduce the cost of follow up for patients placed into the neutral zone in the first stage.
The revised formula for $\hat{p}_2$ is $\hat{p}_2 = \frac{\exp(-2.668 + 1.929 \times PSA_s - 1.987 \times PPA_s)}{1 + \exp(-2.668 + 1.929 \times PSA_s - 1.987 \times PPA_s)}$.

This two-stage classifier results in an overall true positive rate of 69.6%, an overall true negative rate of 91.1%, an overall false negative rate of 30.4%, and an overall false positive rate of 8.9%. The overall negative predictive value and overall accuracy remain relatively high at 95.5% and 88.4%, respectively.
CLIN only: GS: Biopsy Gleason Score, PSA: Serum PSA and Age. Clinical +LAB: PSA: Serum PSA and PPA: Total ng Extractable PSA Proteolytic Activity.
OPPV = overall positive predictive value; ONPV = overall negative predictive value; OFNR = overall false negative rate; OFPR = overall false positive rate.

the cost of applying the model in new patient testing, without sacrificing accuracy. The two-stage approach, Figures 4 and 5, permits expensive testing to be confined to a much-reduced subset of the patient cohort by confining the need for the test to a smaller subset of the patient population compared to the traditional approach in which all patients would be required to take the tests. The traditional approach called 389/450 patients correctly for an accuracy of 86.4%, while the sequential neutral zone approach called 399/450 patients correctly for an accuracy of 88.7%. Consequently, the combined improvement in avoiding unnecessary testing and

comparable accuracy make the two-stage approach superior to the traditional approach when evaluating this biomarker for the prediction of biochemical recurrence. In effect the neutral zone first stage in the two-stage classifier codifies some aspects of clinical intuition where additional tests are required only in those cases where available testing has produced ambiguous results. An additional advantage of the two-stage classifier as applied here is that even patients for whom standard clinical tests suggest extremely aggressive disease (e.g. GS 9, serum PSA > 10) can often be assured that available treatment options will permit their disease to remain under control in the sense that it will not undergo biochemical recurrence or progression for an extended period of time.

The two-stage classifier in Figure 4 correctly identifies 357 of the 394 (90.6%) patients that did not have a recurrence within 2.5 yrs after surgery, and correctly identifies 42 of the 56 patients (75%) that did have a recurrence. In contrast to the traditional method, this approach requires testing only 274 of the 450 patients. Given a combined price for the PCA3 and PPA test of $1760.00/patient, the overall cost of testing this group for model development would be reduced from $792,000 to by about $309,760 to $482,240, thereby facilitating the independent verification of commercial biomarker results. Moreover, omitting the PCA3 test did not degrade the accuracy of the test and would result in an additional reduction in cost of model development of $208,240, so that the model development for the accurate prediction of cancer control as indicated by the absence of recurrence within 2.5 years, could be completed for $274,000 versus $792,000.

The PPA test has certain apparent advantages over existing predictors of risk. For example, unlike RNA profiling tests, the assay itself can be successfully carried out on each patient without regard to the availability of cancer foci or the quantity or quality of RNA in the specimen. Moreover, the prediction of cancer control allows high risk patients to be included in the tested group. Within the group for which 2.5 year follow up was available, 44 patients were classified as high or very high risk patients by NCCN criteria. Twenty-five of these patients were given ADT or EBRT without an indication of biochemical recurrence and did not experience biochemical recurrence or become castrate resistant within 2.5 years. With regard to the validation of PPA as a recurrence biomarker, the limitations of the study are that it was developed on a patient group that was predominantly Caucasians from a single institution practicing robot-assisted radical prostatectomy.

The value of the method suggested here is that it comprises a general approach to the parsimonious application of laboratory tests that extends the applicability of ROC methodology. □

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