

Development of a Novel Bladder Cancer Intervention Diagnostic Triage Assay Using Urinary Matrix Metalloproteinases (MMPs) as Clinical Biomarkers

Predictive

BIOSCIENCES

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Abstract

Background

With increasing cancer therapeutic efficacies and the development of better methodologies for early detection, the cancer survivor population has been growing steadily over the years. As this patient population grows, there is a clear need for the development of more cost effective clinical algorithms and assays that would result in more efficient management of cancer survivors.

We are developing a non-invasive diagnostic assay utilizing urinary Matrix Metalloproteinases (MMPs) as monitors of disease-free status and cancer recurrence in bladder cancer. In addition, we have incorporated a novel analytical algorithm called Clinical Intervention Determining Diagnostic (CIDD), which results in extremely high (~100%) Negative or Positive Predictive Value.

Methods

Urine samples were collected by clean catch or catheterization and stored in aliquots at -80°C. Samples from 84 bladder cancer patients and 448 cancer-free individuals were analyzed via ELISA and zymography to determine the levels of MMP-2, MMP-9 and MMP-9/NGAL.

Results

Initial studies using urinary levels of MMP-2, MMP-9 and MMP-9/NGAL to discriminate disease-free patients from those with bladder cancer resulted in approximately 80% (67/84) sensitivity and 71% (318/446) specificity, in the case of MMP-9, at a fixed protein level. However, applying our proprietary CIDD algorithm to MMP-9 levels correctly identified 42% of patients who were cystoscopy negative with a Negative Predictive Value of 98%.

Conclusions

CIDD deviates from current practice in the cancer biomarker field in that instead of providing a likelihood of having cancer, it allows the physician to rule out, with near 100% certainty, those patients who do not have cancer and who can be excluded from further intervention. In addition, CIDD could be applied to a symptomatic population, such as those presenting with hematuria, to rule out the possibility of cancer from a majority of patients.

Study Design

Our lab is developing the use of urinary matrix metalloproteinases to manage the care of cancer survivors by monitoring for recurrence in a non-invasive format and at greater frequency than currently possible. Based on previous studies which have shown that MMPs are found in the urine of cancer patients at greater frequency than in the urine of disease-free individuals, an initial proof of concept study was designed to compare urinary MMP-2, MMP-9 and MMP-9/NGAL levels in a set of 530 samples, including 84 samples obtained from bladder cancer patients. "Cancer-free" controls were obtained from patients who have had cancer, have been successfully treated and currently have "no evidence of disease (NED)", as well as from asymptomatic individuals (normals). Additional cancer-free controls were obtained from individuals who suffer from other urological conditions, such as kidney stones, bladder obstruction, incontinence, erectile dysfunction and urinary tract infections. Urine samples were assayed for MMP enzymatic activity by gelatin zymography and total MMP levels were quantitated via ELISA. To determine enzymatic activity, 30ul of unprocessed urine were mixed with 10ul of sample buffer, loaded and separated under non-reducing conditions in SDS-PAGE gels containing gelatin. Gels were incubated overnight in buffer containing calcium and then stained with coomassie blue. Enzymatic activity was visualized as a zone of clearance against a dark blue background where the gelatin was degraded by the MMP.

To determine total MMP levels, 50-100ul of neat urine were run on MMP-2, MMP-9 and MMP-9/NGAL specific ELISAs as per the manufacturer's instructions. Data analysis was accomplished via t-tests (assuming unequal variances) and receiver / operator characteristic (ROC) curves for all marker values. All studies were conducted in duplicate in a blinded fashion.

Urine Samples from Bladder Cancer Patients Contain Higher Levels of Enzymatically-active MMP-2, MMP-9 and MMP-9/NGAL than that from Cancer-free Individuals

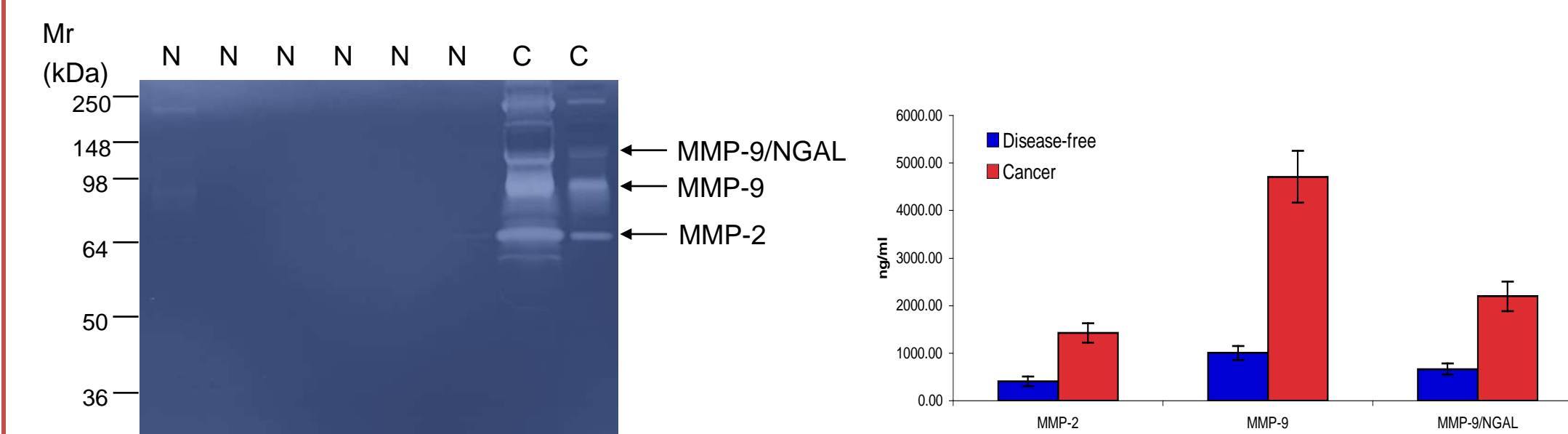
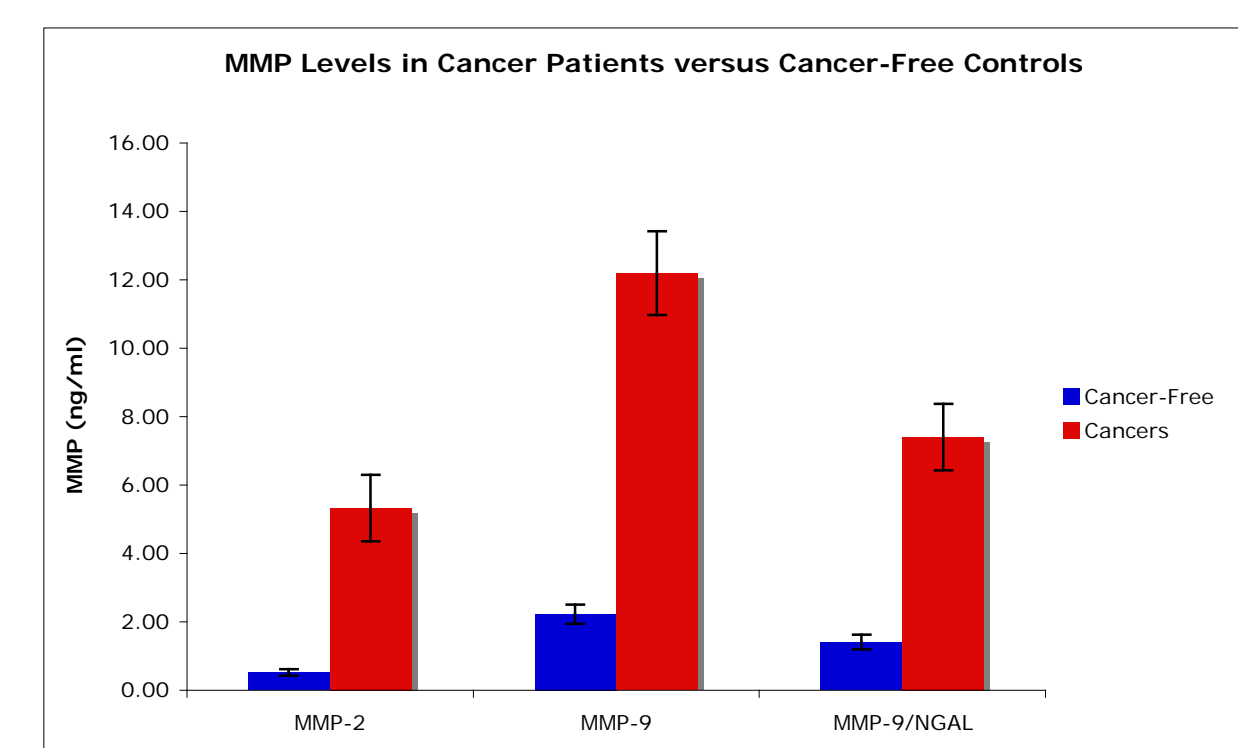


Figure 1 Gelatin zymography was used to assess the MMP enzymatic activity found in the urine of bladder cancer patients as compared to that from cancer-free individuals. MMP-2, MMP-9 and MMP-9/NGAL were detected at significantly higher frequency in the urine of cancer patients (C) compared to urine obtained from cancer-free controls (N). Results are shown graphically on the right. A representative zymography gel is shown on the left.

Higher Levels of MMP-2, MMP-9 and MMP-9/NGAL are Found in the Urine of Bladder Cancer Patients than in that from Cancer-Free Individuals



MMP-2					
	Cancer (n=84)	Cancer-Free Controls (n=446)	Healthy Controls (n=393)	Symptomatic Controls (n=133)	NED (n=12)
Mean	5.324	0.525	0.518	0.578	0.270
Median	1.388	0.243	0.239	0.274	0.168
p-value Cancer vs.		3.92E-04	3.94E-04	4.72E-04	1.32E-04

MMP-9					
	Cancer (n=84)	Cancer-Free Controls (n=446)	Healthy Controls (n=393)	Symptomatic Controls (n=133)	NED (n=12)
Mean	12.101	2.312	1.844	3.705	3.212
Median	7.563	0.207	0.212	0.164	0.077
p-value Cancer vs.		4.62E-14	1.77E-13	2.92E-09	0.00038

MMP-9/NGAL					
	Cancer (n=84)	Cancer-Free Controls (n=343)	Healthy Controls (n=276)	Symptomatic Controls (n=53)	NED (n=12)
Mean	2.403	1.518	0.971	4.461	1.077
Median	1.288	0.068	0.029	0.293	0.168
p-value Cancer vs.		7.38E-08	5.44E-04	0.00241	2.061E-05

Figure 2 Urine samples obtained from bladder cancer patients (n=84) and disease-free individuals (n=446) were analyzed by ELISA for absolute levels of MMP-2, MMP-9 and MMP-9/NGAL. Urine obtained from bladder cancer patients contained higher levels of MMPs as compared to controls. The graph above shows the mean protein values for the cancer and cancer-free controls. Mean and median levels of each marker are also shown in the table above. In all three cases, the mean marker concentration was significantly higher in the cancer cohort than in the cancer-free samples with p<0.0001.

Biomarker Performance

Standard Biomarker Performance as Determined by ELISA				
	Sensitivity [95% CI]	Specificity [95% CI]		
MMP-9	80% (67/84) [70%-88%]	71% (318/446) [65%-75%]		
MMP-2	62% (52/84) [51%-72%]	88% (393/446) [85%-91%]		
MMP-9/NGAL	73% (61/84) [62%-82%]	79% (271/341) [75%-84%]		

Standard Biomarker Performance as Determined by Zymography				
	Sensitivity [95% CI]	Specificity [95% CI]		
MMP-9	68% (55/81) [57%-78%]	78% (331/427) [73%-81%]		
MMP-2	56% (45/81) [44%-67%]	82% (352/427) [78%-86%]		
MMP-9/NGAL	58% (47/81) [47%-69%]	80% (342/427) [76%-84%]		

Table I Based on the ROC curves generated, two cutoff values per marker were used to calculate biomarker sensitivity and specificity. Results for each marker were calculated from both the ELISA and zymography data.

Receiver-Operating Characteristic (ROC) Curves for MMP-2, MMP-9 and MMP-9/NGAL

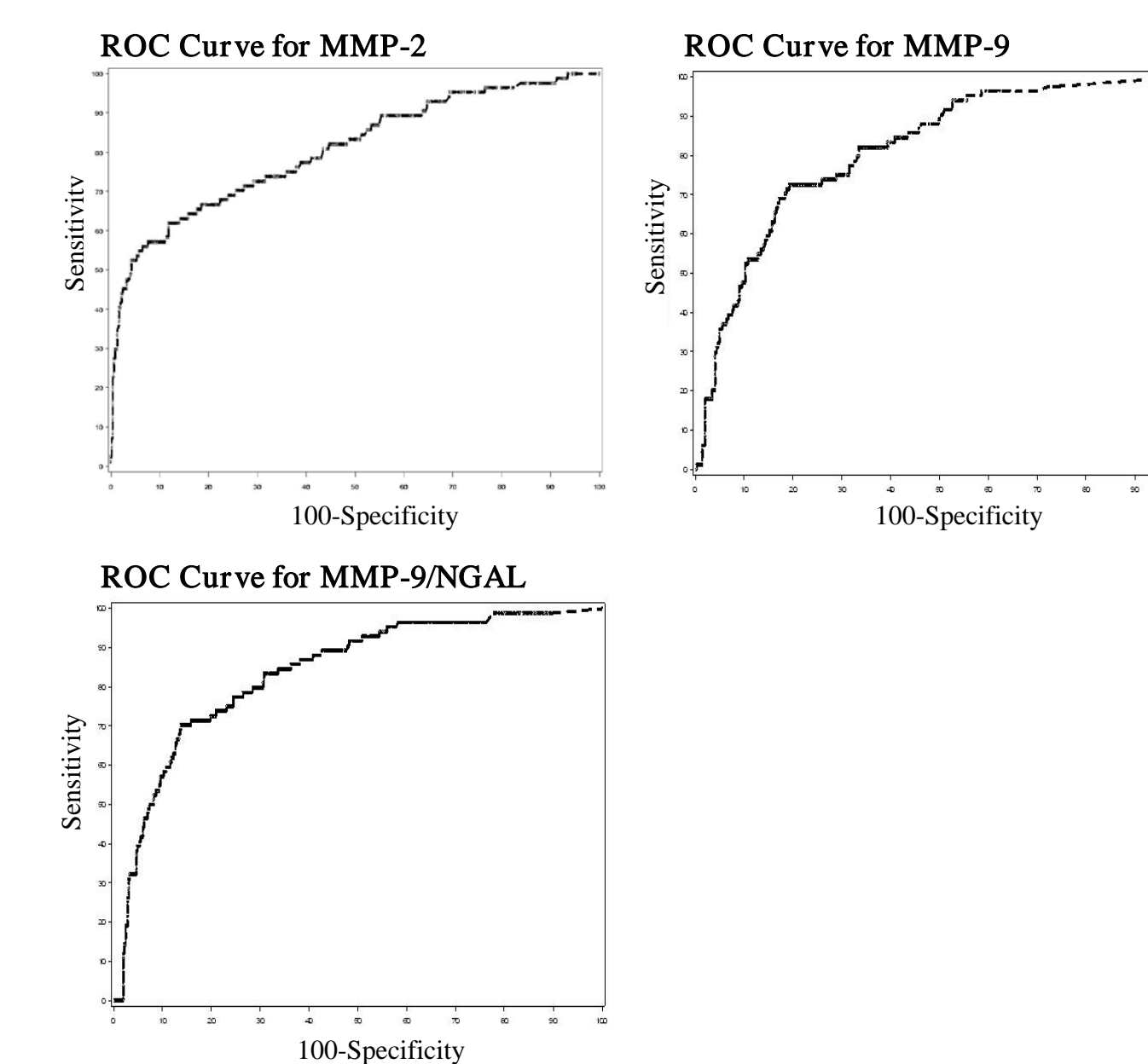


Figure 3 To measure the diagnostic accuracy of the markers, receiver-operating characteristic (ROC) curves were generated. The area under the curve for each marker was 80.6% for MMP-2, 83.9% for MMP-9 and 79.4% for MMP-9/NGAL.

Clinical Intervention Determining Diagnostic (CIDD)

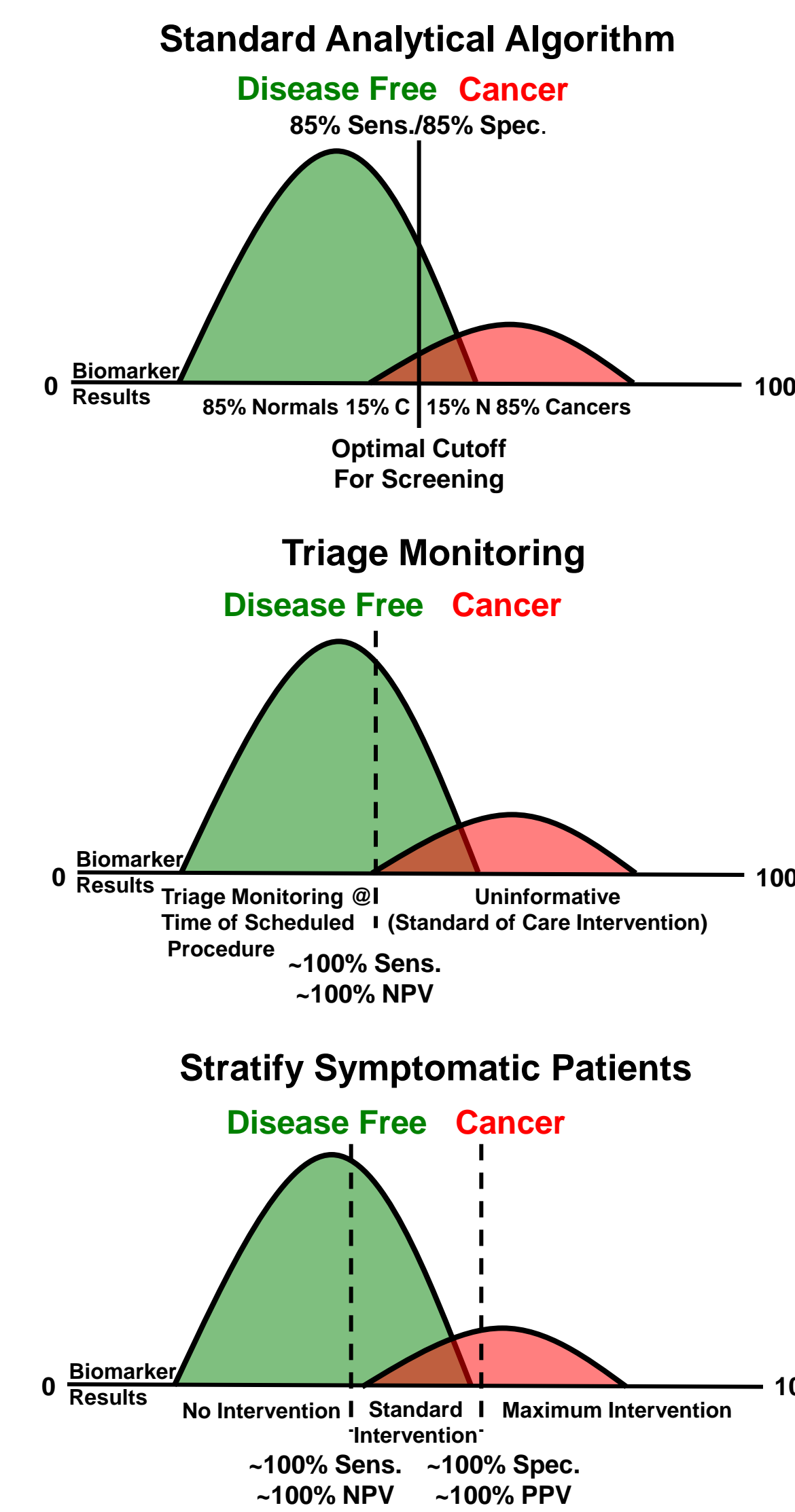


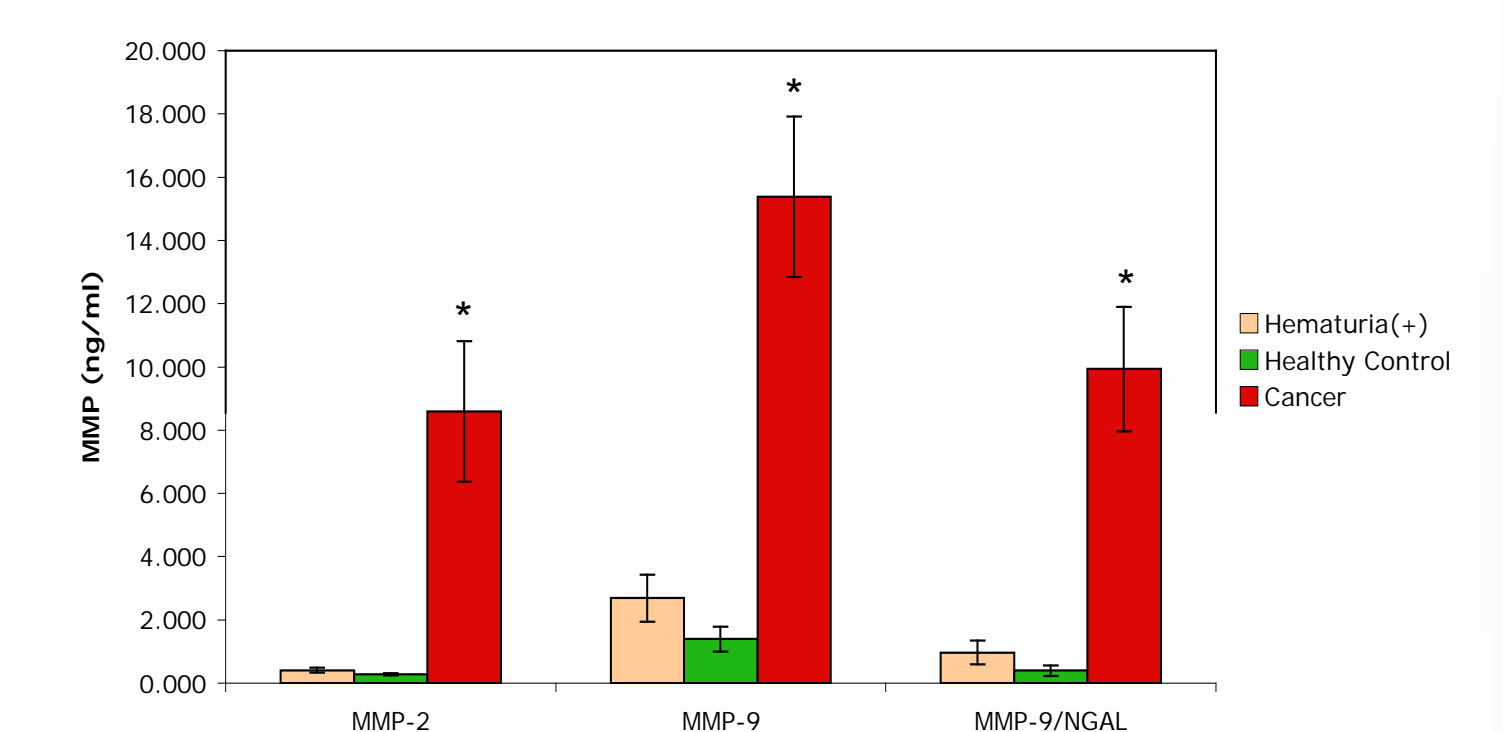
Figure 4 Figure 4 graphically displays the concept underlying CIDD. In this example, patient urine sample levels of MMPs are measured and then are compared to one or more threshold levels of the same biomarker to stratify the patients. Patients are assigned to one of three groups based upon the comparison of the patient's biomarker level and the threshold values. The first group consists of patients who have biomarker levels that are below a threshold level that is unambiguously-associated with non-recurrence of a disease with which the biomarker is associated. Under Triage Monitoring, patients in this group could forego additional tests. The second group consists of patients who have biomarker levels that are unambiguously-associated with recurrence of the disease, and should have increased monitoring relative to the standard of care. The final group of patients have biomarker levels that are between the two thresholds and therefore are subject to standard screening assay.

Triage of Bladder Cancer Survivors using CIDD

	CIDD
MMP-9	98% NPV [95-100%] with 42% exclusion [37-47%]
MMP-2	96% NPV [92-98%] with 45% exclusion [40-50%]
MMP-9/NGAL	97% NPV [93-99%] with 41% exclusion [36-47%]

Table II The table above shows the results of application of CIDD to the ELISA results presented here. In the case of MMP-9, at 98% negative predictive value, 42% of disease-free individuals would be excluded from follow-up tests, with the remaining patient population receiving existing standard of care monitoring.

Triage of Hematuria Positive Patients using CIDD



*Statistically Different from Hematuria(+)/Cysto(-) and Healthy Controls

	n	Cutoff	Sens.	Spec.	NPV	POE
MMP-2	22 Cancers (Hema+/Cysto+) 50 Hema+/Cysto- 100 Healthy Controls	0.284ng/ml	100% (22/22) [85-100%]	62% (31/50) [47-75%]	100% (31/31) [89-100%]	62% (31/50) [47-75%]
MMP-9	22 Cancers (Hema+/Cysto+) 50 Hema+/Cysto- 100 Healthy Controls	0.143ng/ml	100% (22/22) [85-100%]	44% (22/50) [30-59%]	100% (22/22) [85-100%]	44% (22/50) [30-59%]
MMP-9/NGAL	22 Cancers (Hema+/Cysto+) 50 Hema+/Cysto- 100 Healthy Controls	0.058ng/ml	100% (22/22) [85-100%]	54% (27/50) [39-68%]	100% (27/27) [87-100%]	54% (27/50) [39-68%]

Figure 5 Bladder cancer patients often initially present with blood in the urine. However, only a small fraction of people with blood in the urine are positive for bladder cancer. To determine whether MMP levels could be used in conjunction with CIDD to distinguish between individuals who unequivocally do not have cancer and those that should follow standard of care, we analyzed a set of 172 urine samples for MMP levels. In all cases, mean MMP values were significantly higher in the cancer group than in either the hematuria+/cystoscopy- or normal control group. The Table above shows the performance of each marker when CIDD is applied. For all three markers, with 100% NPV, greater than 40% of true negatives would be excluded from further tests.

Summary

- Initial studies comparing samples obtained from bladder cancer patients and control samples demonstrated that MMP-2, MMP-9 and MMP-9/NGAL are found at significantly higher levels and at greater frequency in the urine of bladder cancer patients, as determined both by ELISA and zymography.
- Sensitivity and specificity, as determined by ELISA were 62% and 88% for MMP-2, 80% and 71% for MMP-9, and 73% and 79% for MMP-9/NGAL.
- Application of CIDD to the data established new cutoffs to be set for each marker, where at near 100% negative predictive value (NPV) this algorithm enables physicians to exclude a large portion of patients from unnecessary follow-up tests.
- In addition, MMP levels and CIDD could be used to triage symptomatic patients who present with hematuria into those who do not need further tests and those who should continue to receive the standard of care.