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New Dual Monoclonal ELISA for Measuring Plasma Osteopontin as a Biomarker Associated with Survival in Prostate Cancer: Clinical Validation and Comparison of Multiple ELISAs

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BACKGROUND: A previously developed monoclonal/polyclonal ELISA (Mono/Poly) to detect plasma concentrations of osteopontin (OPN) was shown to provide prognostic information in breast, prostate, and other cancers. Here we describe the clinical validation of a new dual monoclonal (Dual Mono) assay. We compared both assays with 4 assays that recognize defined regions of OPN protein (dual polyclonal systems 5-1, 4-1, 4-3 and polyclonal-monoclonal system 1-3).

METHODS: OPN sequences recognized by the monoclonal antibodies that make up the Dual Mono ELISA were identified by Pepscan CLIPS™ analysis. Using the 6 ELISAs, we measured OPN in plasma from 66 patients with castration-resistant prostate cancer, and we assessed the ability of each assay to predict patient survival.

RESULTS: The assays varied in measured plasma OPN concentrations, with median values ranging from 112 to 1740 μg/L, and ability to predict patient survival. By Cox univariable regression of survival by tertiles of OPN, the Mono/Poly and Dual Mono ELISAs had the highest log-rank χ^2 values. After adjustment for risk factors independently associated with survival in our samples, OPN remained associated with survival only for the Mono/Poly and Dual Mono systems.

CONCLUSIONS: OPN plasma values varied significantly depending on the assay used. Only the Mono/Poly and Dual Mono systems were independently associated with survival in a population of men with castration-resistant prostate cancer. The availability of a clinically validated, dual monoclonal–based ELISA will provide consistent reagents for studies of OPN plasma concentrations in cancer and other pathologies.

Osteopontin (OPN)[8] is a secreted, integrin-binding phosphoprotein that has long been associated with cancer and other pathologies *(1–3)* and is an attractive candidate target for cancer therapy *(4, 5)*. Although many studies have suggested that OPN has potential clinical utility as a biomarker in multiple tumor types in both blood and tumor tissue, few studies have provided clinical outcome data on patients. Where clinical information has been provided, OPN concentrations have been associated with patient prognosis and survival, in studies of breast *(6, 7)*, prostate *(8, 9)*, and other *(10–12)* cancers. Recent work by Bramwell et al. *(7)* showed that serial monitoring for changes in plasma OPN concentrations over time in women with metastatic breast cancer provided information useful in patient management. Increased baseline plasma OPN concentrations were associated with poor survival, consistent with previous work *(6)*. More importantly, in multivariate analysis, an increase in plasma OPN of $>$250 μg/L at any time was the variable with the most prognostic value for poor survival (relative risk of death 3.261; 95% CI 1.716–6.198; $P = 0.0003$) in these patients *(7)*. Thus, although OPN is not a cancer-specific marker, there is ample and growing evidence that measurement of plasma OPN has potential clinical value in oncology.

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[8] Nonstandard abbreviations: OPN, osteopontin; CRPC, castration-resistant prostate cancer; mAb, monoclonal antibody; GST, glutathione *S*-transferase; pNPP, 4-nitrophenyl phosphate.

Currently, there is no standard ELISA for measuring OPN, a fact that hampers clinical development of OPN as a biomarker in cancer or other pathologies. Two recent studies have reported that OPN blood concentrations measured with different ELISAs give different absolute values *(13, 14)*. All ELISAs used in published studies employ polyclonal antibodies as assay components; the use of defined, monoclonal antibody reagents will facilitate the development of reproducible assays for clinical purposes.

Here we conducted a detailed analysis of plasma OPN as measured by multiple OPN ELISAs on a subset of plasma samples from castration-resistant prostate cancer (CRPC) (also known as hormone-refractory prostate cancer) patients from a previously reported study, for whom complete clinical outcome data were available *(8)*. We assessed the ability of each assay to predict patient survival and identified OPN sequences recognized by the antibodies that make up the Dual Mono ELISA by Pepscan CLIPS™ analysis. This report documents the first fully defined-epitope, monoclonal-based ELISA for OPN with demonstrated ability to provide clinical prognostic information in a cohort of cancer patients.

Materials and Methods

MATERIALS

Monoclonal antibodies (mAbs) mAb53 and mAb87B, raised by immunizing BALB/c mice with recombinant human OPN then generating and selecting hybridomas that produce OPN-specific antibodies, were purified as described *(15)*. Bacterially expressed recombinant glutathione *S*-transferase (GST)-tagged human OPN (GST-hOPN) was purified as described *(16)*. Rabbit polyclonal antihuman OPN antibodies were directed against bacterially expressed GST-hOPN *(17)*. Biotinylated polyclonal goat anti-rabbit immunoglobulins were obtained from Dako, alkaline phosphatase-conjugated streptavidin from Jackson ImmunoResearch Laboratories, and 4-nitrophenyl phosphate (pNPP) disodium salt hexahydrate tablets from Sigma.

HUMAN PLASMA SAMPLES AND STUDY POPULATION

Plasma samples were collected between January 1998 and September 2000 from 100 men with CRPC at the London Regional Cancer Centre as described *(8)*. Samples were collected after informed consent, under a protocol approved by the University of Western Ontario Ethics Review Board, and were stored at −80 °C. From this original collection, 66 plasma samples were used, based on availability of sufficient volume of sample to conduct the present study.

ELISAS

We showed previously that mAb53 does not recognize thrombin-cleaved OPN *(15)*. Because blood coagulation that occurs during preparation of serum results in cleavage of OPN by thrombin and 2 of the assay systems described here use mAb53 as capturing antibody, we used plasma (prepared with EDTA as anticoagulant) throughout the study. We used the Dual Mono ELISA kit (900–142; Assay Designs, Inc.), following instructions provided; human recombinant OPN is provided with this kit as a standard. We carried out the other ELISAs (Mono/Poly and systems 1-3, 4-1, 5-1, and 4-3) as described *(6, 17, 18)*, using as standard recombinant GST-hOPN (Mono/Poly) or human recombinant OPN, purified from CHO cells *(18)* (other systems). For each assay, triplicate readings of each sample were averaged.

PEPSCAN ANALYSIS

Synthetic peptides (300 pentadecamers) spanning the amino acid sequence of human OPN were synthesized on pins by Pepscan Systems *(19, 20)*. Each peptide was shifted by 1 amino acid with respect to the previous one and was screened for binding by treating with purified mAb87B or mAb53 followed, after washing, by alkaline phosphatase–labeled antibody to mouse IgG. Absorbance data for each peptide were obtained using pNPP substrate.

STATISTICAL ANALYSIS

We compared ELISA systems using ANOVA. The significance of multiple post hoc pairwise comparison tests was corrected by the Tukey approach. We evaluated agreement among the different ELISA systems for measuring OPN using the Bland–Altman approach, constructing both difference and ratio plots *(21)*. We estimated survival using the Kaplan–Meier technique and based on the date the OPN plasma sample was obtained. We categorized the OPN values into 3 groups at the tertile values and compared survival in the thirds using log-rank statistics. We evaluated the effect of the actual OPN values on survival using stepwise Cox regression, both unadjusted and adjusted for risk factors found to be independently associated with survival. Statistical analyses were performed using GraphPad Prism 4 (GraphPad Systems, Inc.) and SAS 9.1 (SAS Institute, Inc.), and plots were produced using GraphPad Prism. P values <0.05 were considered statistically significant.

Results

HUMAN OPN ELISA SYSTEMS

Fig. 1 diagrams the 6 human OPN ELISAs used, indicating the pairs of antihuman OPN antibodies used

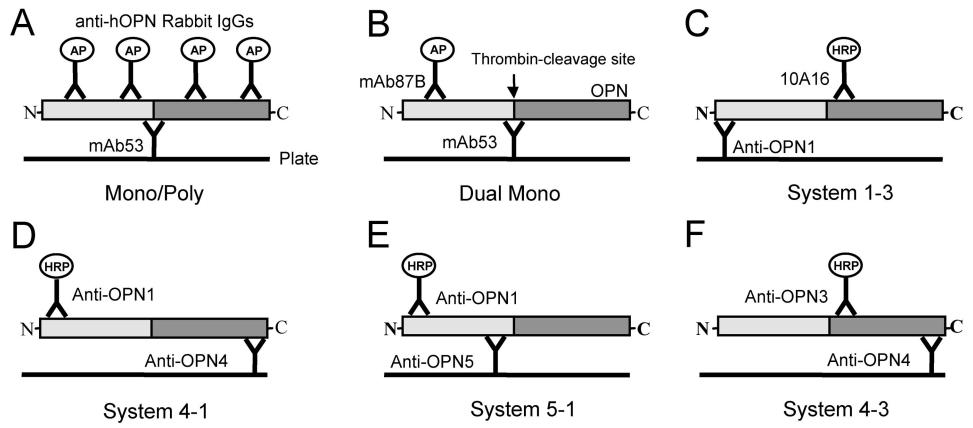

Fig. 1. Diagram of the OPN ELISA systems used in this study: Mono/Poly (A); Dual Mono (B); system 1-3 (C); system 4-1 (D); system 5-1 (E); and system 4-3 (F).

The arrow in (B) indicates the thrombin cleavage site in OPN. Cleavage of the full-length OPN molecule results in an N-terminal and a C-terminal fragment, indicated by the 2 different gray scales. For each assay, the antibody used as capturing antibody is pictured below the OPN molecule, and the detecting antibody is pictured above it. Anti-OPN1, anti-OPN3, anti-OPN4, and anti-OPN5 are monospecific rabbit polyclonal antibodies affinity-purified against the immunizing peptides, and 10A16 is a mouse monoclonal antibody [Kon et al. *(18)*]. The peptides used to generate these antibodies are as reported [Kon et al. *(18)*, Kon et al. *(23)*]: anti-OPN1, 17-IPVKQADSGSSEEKQL; anti-OPN3/10A16, 170-KSKKFRRPDIQYPDATDE; anti-OPN4, 296-KHLKFRISHELDSASSEVN, anti-OPN5: 153-VDTYDGRGDSVVYGLRS. Numbers refer to the sequence of human OPNa, including the signal peptide. Sequences recognized by mAb87B and mAb53 were identified in this study (see online Supplemental Fig. 1). AP, alkaline phosphatase; HRP, horseradish peroxidase.

and the locations along the OPN protein backbone of the epitopes recognized by these antibodies.

The Mono/Poly assay (Fig. 1A) has been shown to provide prognostic information in prostate cancer *(8)*, as well as in metastatic breast cancer *(6, 7)*. This assay, as well as the Dual Mono assay (Fig. 1B), uses the monoclonal anti-hOPN antibody mAb53 as capturing antibody. On OPN cleavage by thrombin, the mAb53 recognition site is lost. Therefore, Mono/Poly and Dual Mono systems will not detect OPN fragments generated by thrombin-cleavage of full-length OPN. The same is expected for the systems 1-3 and 4-1, since the anti-OPN antibody pairs recognize epitopes on either side of the thrombin cleavage site. In contrast, systems 5-1 and 4-3 are able to recognize N-terminal or C-terminal fragments, respectively, generated upon thrombin cleavage. The antibody pairs used in the latter 4 systems (Fig. 1, C–F) are monospecific rabbit polyclonal antibodies affinity-purified against the immunizing peptides, except for system 1-3, which matches a rabbit polyclonal antibody with a mouse monoclonal antibody, 10A16 *(18)*. The detecting antibody for the Mono/Poly assay is a rabbit polyclonal antibody against full-length recombinant human OPN. Which epitopes on OPN are recognized by this polyclonal antibody is unknown, but there are likely to be several *(20)*. Importantly, the Dual Mono system uses a monoclonal antibody, mAb87B, as detecting antibody. Thus, the Dual Mono system is the first OPN ELISA to use 2 monoclonal antibodies, allowing for easier standardization of the assay. To gain a better understanding of the Dual Mono system, we first identified the OPN sequences to which monoclonal antibodies mAb53 and mAb87B bind.

IDENTIFICATION OF PEPTIDE SEQUENCES RECOGNIZED BY mAb53 AND mAb87B

mAb53 and mAb87B, specific to human OPN, were developed several years ago *(15)*. Here, we tested mAb53 and mAb87B for reactivity with 300 overlapping OPN peptides, each shifted by 1 amino acid and covering the entire sequence of human OPN, using a peptide microarray technology (Pepscan CLIPS analysis). The results, summarized in Supplemental Fig. 1 (which accompanies the online version of this article at www.clinchem.org/content/vol55/issue5), indicate that mAb87B shows maximum binding to a series of peptides that all have in common the amino acid sequence ^{75}PSKSNESH, located in the N-terminal half of the OPN molecule. For mAb53, maximum binding occurred to peptides with the common sequence ^{165}YGLRSK. These results are consistent with the loss

of mAb53 reactivity upon thrombin cleavage of OPN, as the thrombin cleavage site is contained within this sequence *(15, 22)*. Binding of mAb87B, at a position remote from the thrombin cleavage site, is unaffected by thrombin cleavage. Our results are also in line with observations by Kon et al. *(23)*, who measured the reactivity of mAb53 to synthetic peptides corresponding to sequences of human OPN, which indicated strong binding of mAb53 to a peptide with the sequence ^{162}SVVYGLRSKS, and strongly reduced binding to a ^{162}SVVYGLR peptide. mAb53 also failed to bind to a peptide with the sequence ^{153}VDTYDGRGDSVVYGLRS.

Because a recombinant full-length OPN was used to generate these monoclonal antibodies, we cannot exclude that other sequences in OPN are part of a conformational epitope. However, our Pepscan analysis shows clearly that mAb53 and mAb87B have very strong binding interactions with OPN at residues 165–170 and 75–82, respectively.

PLASMA OPN VALUES FOR CRPC PATIENTS DIFFER ACCORDING TO THE ELISA ASSAY USED

We assayed plasma samples from 66 men with CRPC for OPN concentration using the 6 different ELISA systems. Fig. 2 shows that there was considerable variation in OPN concentrations measured with different ELISA systems, with median values between approximately 112 μg/L for the Mono/Poly assay to approximately 1740 μg/L for system 4-3. Median and mean OPN readings obtained with system 4-3 were considerably higher than with the other systems, which were more comparable to each other. System 4-3 also shows the greatest range of values (8–10 040 vs 4.4–451.3 μg/L for the Mono/Poly system and 56.1–471.6 μg/L for Dual Mono). When means were analyzed using 1-way repeated measures ANOVA, we obtained a P value <0.001. With the Tukey multiple comparison test, means were found to be not significantly different ($P > 0.05$), with 2 exceptions. The mean of system 4-3 was significantly different from all the other systems ($P < 0.001$), and the means of Mono/Poly and system 5-1 were significantly different from each other ($P < 0.05$). Mean and median values for all systems are presented in online Supplemental Table 1.

AGREEMENT AMONG OPN VALUES OBTAINED WITH DIFFERENT ELISA SYSTEMS

We next evaluated agreement among the ELISAs. Fig. 3A shows a pairwise correlation plot comparing OPN values for the Mono/Poly ELISA with those for the Dual Mono assay. The Pearson correlation coefficient (r) for this ELISA pair was 0.747 ($P < 0.001$) (Table 1).

Comparison of OPN plasma concentrations obtained by the Dual Mono system for the same 66 samples on 2 different days yielded a Pearson correlation coefficient of 0.991 ($P < 0.001$), whereas the Pearson correlation coefficient was 0.941 ($P < 0.001$) for OPN concentrations obtained by the Mono/Poly system on 2 different days (data not shown). A complete series of pairwise correlation plots comparing each system to each of the others is shown in online Supplemental Fig. 2; Pearson correlation coefficients for each ELISA pair are listed in Table 1. In summary, we found that the Mono/Poly system and the Dual Mono system both correlated well with each other ($r = 0.747$; $P < 0.001$) and with system 4-3 ($r = 0.849$; $P < 0.001$ and $r = 0.725$; $P < 0.001$, respectively) but less well with system 1-3 ($r = 0.399$; $P < 0.001$ and $r = 0.367$; $P = 0.002$, respectively), system 4-1 ($r = 0.609$; $P < 0.001$ and $r = 0.488$; $P < 0.001$, respectively), or system 5-1 ($r = 0.420$; $P = 0.007$ and $r = 0.318$; $P = 0.009$, respectively).

We used the Bland–Altman approach *(21)* to evaluate the agreement between concentrations ob-

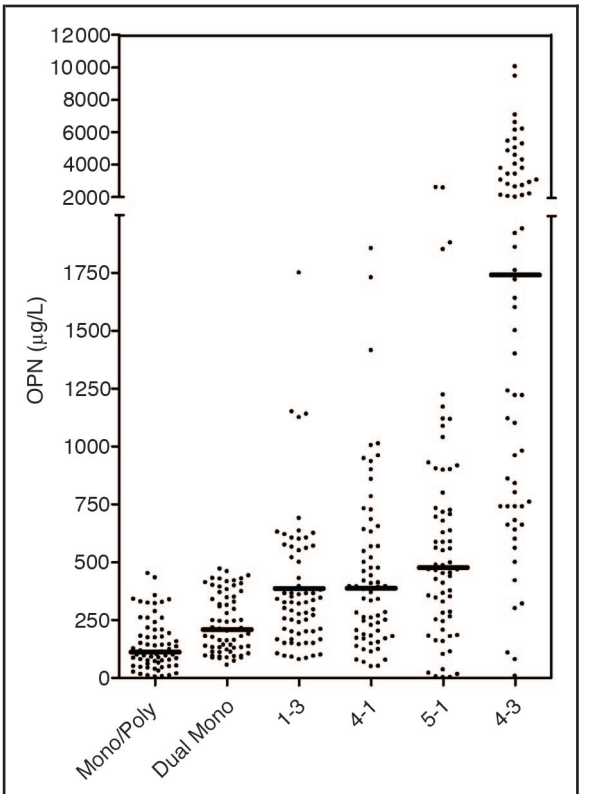

Fig. 2. Distribution of plasma OPN values from 66 patients with CRPC, measured using the 6 different ELISAs.

Each point represents the OPN concentration measured for 1 patient; line marks the median value for the indicated ELISA.

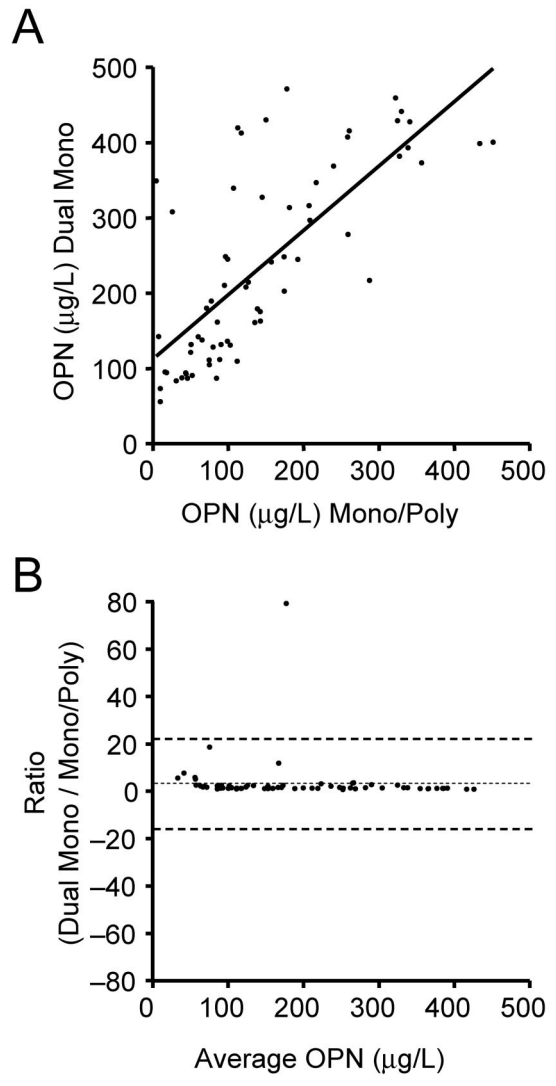

Fig. 3. Relationship between plasma OPN values determined by the Mono/Poly ELISA and the Dual Mono ELISA.

(A), Pairwise correlation between OPN plasma concentrations of 66 men with CRPC, obtained using the Mono/Poly ELISA and the Dual Mono ELISA. Pearson correlation coefficients (*r*) for these pairs of data sets are listed in Table 1. (B), Bland–Altman plot of the ratio between the OPN values of the Dual Mono system and the Mono/Poly system against the average of the 2 methods. The dotted line indicates the mean ratio (3.6) and the dashed lines indicate the 2 SD lines (95% limits of agreement) (−15.7 and 22.9, respectively).

tained with the Dual/Mono and Mono/Poly systems. When we plotted the difference of OPN concentrations between the 2 methods against their mean value, we observed a mean difference of 91.4 (95% limits of agreement −75.5–258.3; 95% CIs for these limits −111.9 to −39.2 and 222.0 to 294.6, respectively (online Supplemental Table 2). This indicates that despite high correlation, the agreement between the 2 systems is less good. When we plotted the ratio of the concentrations obtained with both methods (OPN Dual Mono/OPN Mono/Poly) vs the mean concentrations (Fig. 3B), we found a relatively constant ratio over the range of OPN concentrations tested, with a mean ratio of 3.6 (95% limits of agreement −15.7–22.9; 95% CIs −19.8 to −11.5 and 18.7 to 27.1, respectively). Sixty plasma samples showed ratios between 1 and 5. However, several samples had divergent ratios: 5 samples had Bland–Altman ratios between 6 and 12, 1 sample had a ratio of 19, and 1 sample had a ratio of 79, indicating poor agreement between the 2 methods for those patients. A complete list of results from the Bland–Altman approach (pairwise for all systems) is in online Supplemental Table 2.

ASSOCIATION OF OPN VALUES WITH OVERALL SURVIVAL

The Mono/Poly system has previously been shown to discriminate between CRPC patients with poor vs good prognosis *(8)*. A main goal of the present work was to determine if any or all of the other assays provide similar, clinically relevant prognostic information. For each ELISA system, patients were grouped into tertiles according to their plasma OPN concentrations (high, intermediate, and low) as indicated in the inserts of each Kaplan–Meier plot (Fig. 4). Our results show that the OPN ELISA systems vary in their association with overall survival in this group of patients. Fig. 4 indicates that for all 6 ELISA systems, patients below the lowest tertile for OPN plasma concentrations had the best prognosis [Mono/Poly, median survival >24 months (Fig. 4A), Dual Mono, >24 months (Fig. 4B); system 5-1, 17 months (Fig. 4E); 4-1, 19 months (Fig. 4D); 4-3, 17 months (Fig. 4F); 1-3 16 months (Fig. 4C)]. For systems 1-3 and 4-3 (Fig. 4D and 4F, respectively), however, estimated survival in the lower and middle third largely overlapped. Conversely, patients in the highest third had the worst prognosis (median survival 6–8 months). The Dual Mono and the Mono/Poly assays allowed the greatest distinction between the 3 tertile-derived groups, with median survival times for high, intermediate, and low OPN concentrations at >24, 15, and 7 months, respectively, for the Dual Mono (Fig. 4B) and >24, 16, and 6 months for Mono/Poly (Fig. 4A). On the other hand, system 1-3 (Fig. 4F) showed the least distinction between the 3 tertile groups, with median survival times of 16, 15, and 8 months.

We evaluated the association of OPN concentration and survival using the log-rank statistic across ter-

Table 1. Pearson correlation coefficients (*r*) and *P* values among OPN measurements using different ELISA systems.

	Dual Mono	1-3	4-1	5-1	4-3
Mono/Poly	$r = 0.747$; $P < 0.001$	$r = 0.399$; $P < 0.001$	$r = 0.609$; $P < 0.001$	$r = 0.420$; $P = 0.007$	$r = 0.849$; $P < 0.001$
Dual Mono		$r = 0.367$; $P = 0.002$	$r = 0.488$; $P < 0.001$	$r = 0.318$; $P = 0.009$	$r = 0.725$; $P < 0.001$
1-3			$r = 0.623$; $P < 0.001$	$r = 0.508$; $P < 0.001$	$r = 0.407$; $P < 0.001$
4-1				$r = 0.760$; $P < 0.001$	$r = 0.581$; $P < 0.001$
5-1					$r = 0.353$; $P = 0.004$

tiles, with χ^2 values reported in Table 2. Of the 6 ELISAs, the Mono/Poly and Dual Mono assays showed the strongest association with survival ($\chi^2 = 19.256$ and 17.056, respectively). Mono/Poly and Dual Mono also showed the strongest association with survival when OPN concentrations were treated as a continuous variable in Cox regression analysis ($\chi^2 = 24.023$ and 23.976, respectively). System 1-3 had the weakest association with survival, with the association being nonsignificant ($P = 0.134$) when thirds were compared using the log-rank test ($\chi^2 = 4.021$) and significant ($P = 0.032$) when OPN concentration was treated as a continuous variable ($\chi^2 = 4.582$). Systems 4-3, 4-1, and 5-1 all showed association with survival that was not quite as strong as the mAb53-based assays (Mono/Poly and Dual Mono). System 1-3 does not appear to reliably discriminate patient survival in this group of patients. We examined the effect of known risk factors on survival in our sample, and found several that were associated with overall survival (online Supplemental Table 3). Only hemoglobin and alkaline phosphatase activities were found to be independently associated with survival. When adjusted for these 2 variables, the effects of the Mono/Poly ($\chi^2 = 5.124$, $P = 0.024$) and Dual Mono ($\chi^2 = 6.476$, $P = 0.011$) systems were significant, but effects of the other ELISAs were not (online Supplemental Table 4).

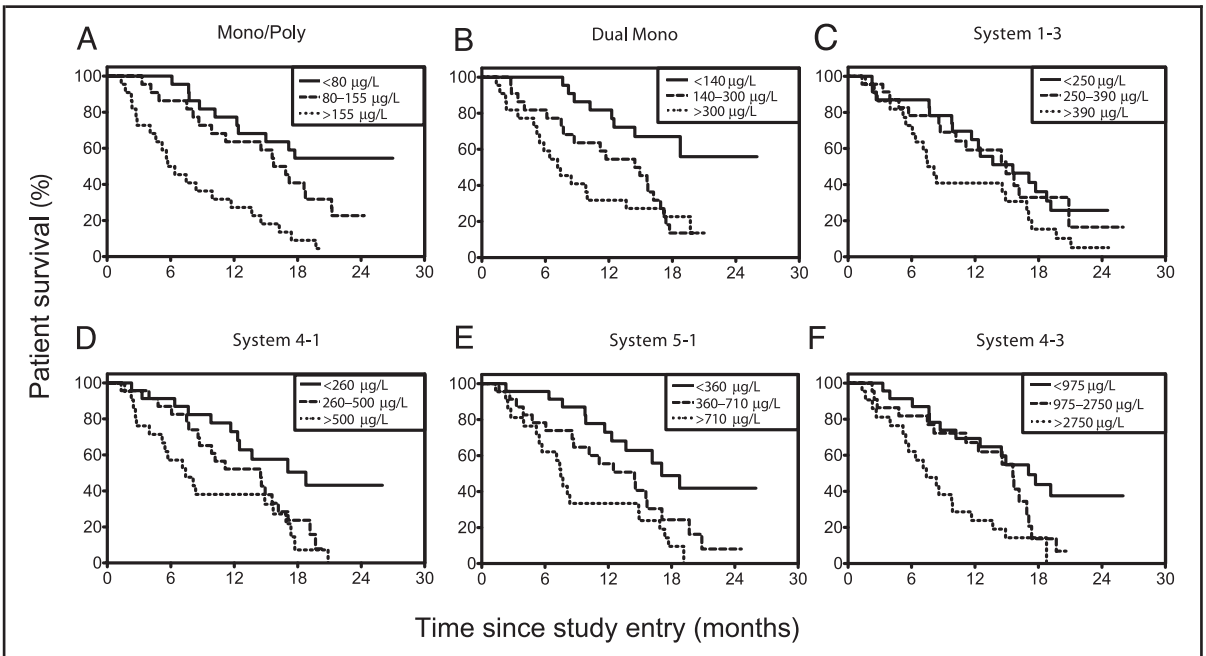

Fig. 4. Association of plasma OPN concentrations with patient survival in men with CRPC.

Kaplan-Meier survival curves determined for the Mono/Poly assay (A), Dual Mono assay (B), system 1-3 (C); system 4-1 (D); system 5-1 (E); and system 4-3 (F). OPN values from 66 patients were divided into 3 equal tertiles (high, intermediate, and low OPN) as indicated for each curve.

Table 2. Association of OPN concentration and CRPC patient survival.

	Log-rank test based on thirds		Cox univariable regression (untransformed)	
System	χ^2 (*df* = 2)	*P*	χ^2 (*df* = 1)	*P*
Mono/Poly	19.256	<0.001	24.023	<0.001
Dual Mono	17.036	<0.001	23.976	<0.001
1-3	4.021	0.134	4.582	0.032
4-1	9.214	0.010	15.384	<0.001
5-1	10.649	0.005	4.876	0.027
4-3	11.671	0.003	19.188	<0.001

Discussion

Although OPN (over-)expression is not cancer specific, there is growing evidence that OPN is a valuable prognostic blood marker in oncology that correlates with disease progression and patient survival. Using our previously developed Mono/Poly OPN ELISA, we have shown that plasma OPN concentrations in women with metastatic breast cancer were increased relative to healthy female volunteers or patients who had completed primary treatment and were free of disease *(6)*. Increases in OPN plasma concentrations, in serial samples from a cohort of 158 women undergoing treatment for newly diagnosed metastatic breast cancer, predicted shorter survival *(7)*. Likewise, we showed that OPN plasma concentrations are increased in men with CRPC and that higher plasma concentrations were associated with shorter survival *(8)*. Although these studies are promising, an important clinical goal is to have a fully defined (monoclonal antibodies rather than polyclonal antibodies), clinically validated (able to provide prognostic information) OPN ELISA. Of the human OPN ELISA systems currently available commercially, including those from R&D Systems, Immuno-Biological Laboratories Co., Inc. (system 1-3 in our study), and the Dual Mono System (Assay Designs, Inc.), only the latter uses 2 robust monoclonal antibodies to human OPN. Here we found a strong correlation between OPN values read using the mAb53-based Mono/Poly system and the Dual Mono system. We also carried out pairwise comparisons by using the Bland–Altman approach. For the Dual Mono and Mono/Poly systems, the ratio of OPN values against their mean did not differ much over the range of OPN concentrations. A few samples had a much higher than average ratio, due to a relatively high OPN concentration in the Dual Mono system and/or a relatively low concentration in the Mono/Poly system for those samples. Most importantly, however, both systems appeared to have equivalent prognostic value in this cohort of CRPC patients.

Comparison of the OPN values for the Mono/Poly assay obtained during the current study with those published *(6)*, using the same Mono/Poly assay on the same subset (n = 66) of patients, showed that we now obtain somewhat lower OPN values. This could be due to aging of the samples, which were kept at −80 °C but went through a number of freeze–thaw cycles, or to the use of a different preparation of GST-hOPN standard for the assay. Nevertheless, there was good correlation between the previous and current OPN values for the Mono/Poly assay, with a Pearson correlation coefficient of 0.807 ($P < 0.001$).

Differences in OPN concentrations obtained with the 6 different OPN ELISA systems may result in part from the use of different calibration standards in these assays (see Materials and Methods). In addition, proteolytic processing and posttranslational modification of OPN may cause variation between assays. Human full-length OPN consists of 314 amino acids, including an N-terminal 16-residue signal peptide, which is cleaved upon secretion. In solution, OPN is believed to adopt a flexible conformation with limited secondary structure, including 3 α-helices and 2 β-strands *(24)*. Proteolytic fragmentation of OPN, by thrombin (peptide bond 168–169) or matrix metalloproteinases (peptide bonds 166–167, 201–202, and 210–211) *(25, 26)*, can generate OPN fragments having potential physiological and pathological importance. The Mono/Poly and Dual Mono systems and systems 1-3 and 4-1 recognize only OPN that is not thrombin cleaved. In contrast, systems 5-1 and 4-3 can detect N- or C-terminal fragments of OPN, respectively. Because OPN concentrations obtained with system 4-3 are in most cases much higher than those obtained with the other systems, it is attractive to speculate that a C-terminal OPN fragment(s) is present in these samples. A cluster of C-terminal OPN fragments has been identified in urine from patients with early-stage ovarian cancer, but not from healthy female volunteers *(27)*. Future work may show whether different OPN ELISA systems are optimal for specific cancer types.

Human OPN can be posttranslationally modified at up to 36 phosphorylation, 6 *O*-glycosylation, and 2 *N*-glycosylation sites, and variability at many of these sites has been reported *(28, 29)*. One sulfated tyrosine has been identified in urinary OPN *(28)*. Interestingly, the mAb87B recognition site (^{75}PSKSNESH) was shown to be subject to phosphorylation, at residues Ser-76, Ser-78, and Ser-81, in human OPN purified from urine and milk *(28, 29)* and murine OPN from *ras*-transformed fibroblasts and osteoblasts *(30)*. This region of OPN also contains a potential

N-glycosylation site at Asn-79 *(31)*. Such modifications may affect binding of the mAb87B antibody, which was raised against unphosphorylated, bacterially expressed GST-hOPN. Sequences recognized by anti-hOPN1, anti-hOPN3, and anti-hOPN4, but not those recognized by anti-hOPN5 and mAb53, may also contain modifications *(28, 29)* that may affect antibody binding and thus ELISA readings. Much remains to be learned about the significance of OPN posttranslational modifications, in either normal physiology or cancer, and whether these differ between individuals or are health related (e.g., tumor-expressed OPN or stress-associated OPN may be differentially phosphorylated).

In conclusion, we have clinically validated a new OPN ELISA system, which uses a pair of defined monoclonal antibodies. The overall performance of this assay appears comparable to our previously described Mono/Poly system, although for some patients the results may vary depending on which system is used. Both assays only detect OPN that has not been cleaved by thrombin. The use of monoclonal antibodies by the Dual Mono assay will allow for better standardization, which will be of prime importance in clinical settings and for comparing data from different research studies.

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