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Small Integrin binding Ligand N-linked Glycoproteins, prostate-specific antigen and time to prostate cancer diagnosis

Alka Jain [a,b], Ying Ni [c], Daisy Zhang [c], Eleanor M. Simonsick [d], E. Jeffrey Metter [d,e], Kalu U. Ogbureke [f], Larry W. Fisher [g], Neal S. Fedarko [a,*]

[a] Department of Medicine, Johns Hopkins University, Baltimore, MD 21224, USA
[b] Staff Scientist, ThermoFisher Scientific, Middletown, VA 22645, USA
[c] Research Laboratory Core, Institute for Clinical and Translational Research, Johns Hopkins University, Baltimore, MD 21224, USA
[d] Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD 21225, USA
[e] Department of Neurology, University of Tennessee Health Science Center Memphis, TN 38163, USA
[f] Department of Diagnostic & Biomedical Sciences, School of Dentistry, University of Texas Health Science Center at Houston, Houston, TX 77054, USA
[g] Matrix Biochemistry Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

Background: Small Integrin Binding Ligand N-linked Glycoproteins (SIBLINGs[1]) were associated with cancer in cross-sectional studies. Whether SIBLINGs associate with preclinical disease is unknown. Methods: A retrospective longitudinal case control study was performed to determine the association of SIBLINGs and prostate-specific antigen (PSA) with preclinical disease. Paired serum samples from 109 cancer-free Baltimore Longitudinal Study on Aging participants were divided into those that were either most distal or proximal to diagnosis (cases) or censored (controls). Dentin sialophosphoprotein (DSPP), bone sialoprotein (BSP), osteopontin (OPN), and PSA were measured by immunoassay and dichotomized into low or high based on their respective cut-off values. Associations of time to diagnosis or death, modeled as disease-free survival (DFS) or overall survival (OS), were assessed using Kaplan Meier and Cox proportional hazard survival estimates on individual and aggregated biomarkers in distal or proximal sets separately. Models were adjusted for relevant covariates. A false discovery rate analysis assessed significance of hazard ratios (HRs) in sets. Results: Biomarkers/aggregates identified as true discoveries for DFS included DSPP + PSA, OPN + PSA, DSPP + BSP + PSA, DSPP + OPN + PSA, where unadjusted distal HRs ranged between 11 and 27 and after adjusting for age from 7 to 15, while proximal HRs ranged between 6 and 10 unadjusted and 5 to 12 after adjusting for age. For proximal OS, true discoveries included DSPP + BSP, DSPP + OPN, DSPP + BSP + OPN, and DSPP + OPN + PSA where unadjusted HRs ranged between 6 and 20 while age-adjusted HRs ranged between 5 and 12. Conclusions: These observations support SIBLINGs as biomarkers that associate with DFS and OS in prediagnosis samples.

Based on 2018 to 2022 data, at some point during their lifetime 12.8% of men will be diagnosed with prostate cancer and 2.4 % will die from the cancer [1]. Prostate cancer is a heterogeneous disease varying in presentation from localized indolent to a rapidly progressing lethal metastatic disease [2]. Disease progression and metastases are the cause of 90% of human cancer deaths [3]. An underlying assumption to prostate cancer clinical research has been that early detection and treatment will lead to improved quality of life and extended survival. An alternative approach to developing novel biomarkers of symptomatic cancer is to identify biomarkers of pre-symptomatic disease that can be used to assess risk status for disease development and subsequent progression. Identifying asymptomatic individuals at risk for disease diagnosis and progression would facilitate the targeting of surveillance and/or treatment to those who would benefit the most.

Members of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING[1]) gene family are intrinsically disordered, secreted glycoproteins that have multiple binding partners, including immunoregulatory proteins (complement factor H, inducible T-cell costimulatory ligand),

* Corresponding author.
E-mail address: nfedark1@jhmi.edu (N.S. Fedarko).

[1] Abbreviations: SIBLINGs, Small Integrin Binding Ligand N-linked Glycoproteins; DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; Baltimore Longitudinal Study of Aging (BLSA); DFS, disease-free survival; OS, overall survival; FDR, false discovery rate.

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Table 1
Characteristics, main predictors and outcomes for pre-diagnosis longitudinal samples.

Model Covariates	Longitudinal Samples Pre diagnosis		
	Distal (n = 109)	Proximal (n = 109)	P value
Age at visit, mean (SD), y	69 (10)	81 (8)	
median (range)	71 (41–86)	82 (53–95)	<0.0001
Race, % Caucasian	97	97	
BMI, mean (SD), kg/m^2	24.9 (2.4)	25.0 (3.0)	n.s.
18 < BMI < 25, number (%)	55 (50)	57 (52)	
25 > BMI < 30, number (%)	51 (47)	46 (42)	
BMI > 30, number (%)	3 (3)	6 (6)	
Systolic BP, mean (SD), mm Hg	138 (25)	145 (24)	≤0.005
Diastolic BP, mean (SD), mm Hg	82 (12)	78 (12)	n.s.
Hypertension, number (%)	75 (69)	84 (77)	
Predictors			
DSPP, mean (SD), ng/ml	175 (94)	223 (129)	
median (range)	162 (33–569)	209 (24–802)	<0.0001
BSP, mean (SD), ng/ml	101 (52.0)	142 (64.9)	
median (range)	91.9 (16.8–216)	153 (16.5–267)	<0.0001
OPN, mean (SD), ng/ml	15.8 (23.6)	30.7 (58.0)	
median (range)	3.08 (0.1–106)	113.9 (0.10–434)	<0.005
PSA, mean (SD), ng/ml	2.4 (2.0)	11.3 (38.5)	
median (range)	2.0 (0.2–10.8)	3.4 (0.4–352)	<0.0001
Outcomes			
PCA diagnosis %	81 (74 %)	81 (74 %)	
Deaths %	109 (100 %)	109 (100 %)	
Time to diagnosis, mean SD, y	15 ± 6	4 ± 4	
median (range)	16 (1–28)	3 (1–20)	<0.0001
Time to death, mean SD, y	18 ± 8	7 ± 5	
median (range)	20 (4–40)	6 (1–31)	<0.0001

Wilcoxon matched-pairs signed rank test.
Abbreviations: SD, standard deviation; y, years; BMI, body mass index; BP, blood pressure; DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate specific antigen. PCA, prostate cancer; n.s., not significant; y, years.
Hypertension defined as systolic BP ≥ 130 or diastolic BP ≥ 80 mm Hg.

receptors (integrins and CD44 variants) as well as specific proteases [4–6]. Normally restricted in expression to skeletal tissue and metabolically active ductal epithelial cells, SIBLINGs are also upregulated in certain cancers [5,7,8]. The association of SIBLING levels with established prostate cancer has been demonstrated using tissue arrays, immunohistochemistry, in situ hybridization of biopsies and ELISAs targeting individual SIBLINGs in serum [9–11]. The above observations were made in cross-sectional studies. The current study was undertaken to determine the association of time to prostate cancer diagnosis with serum SIBLING and PSA levels in longitudinal pre-diagnosis samples. A secondary outcome was to test for association between these biomarkers and overall survival.

Methods

Human subjects. Sets of serum samples were obtained under local approved protocols (Johns Hopkins Medicine Institutional Review Board). A longitudinal set of 218 pre-diagnosis serum samples from 109 men was obtained from the Baltimore Longitudinal Study of Aging (BLSA). The BLSA cohort study, run by the National Institute on Aging, has been previously used to assess the association of PSA velocity with prostate cancer aggressiveness [12]. Participants were disease-free on enrollment. Those under age 60 were assessed every 4 years, between ages 60–79 years were assessed every 2 years and aged 80 and older assessed annually. Assessments included comprehensive health, cognitive, and functional evaluations. Clinical measures associated with each BLSA sample include age, BMI, blood pressure, and PSA levels while for subjects who later developed prostate cancer, additional clinical measures included diagnosis date and disease stage. Ten samples had missing PSA values and clinical staging was incomplete for 25 subjects. All subjects in this set are currently deceased with known date of death. Hypertension was defined as systolic BP ≥ 130 or diastolic BP ≥ 80 mm Hg.

Immunoassays. Serum samples were stored at -80C until a controlled thaw and analysis by ELISAs. SIBLINGs possess an acidic pI, readily dissolve in aqueous solutions and they are intrinsically disordered. These physical properties and the buffering capacity of serum likely contribute to their ability to undergo at least 6 freeze/thaw cycles with little or no loss of signal. Analytical assays were performed without knowledge of the patients' clinical data or demographics. PSA levels for men were originally measured as part of the BLSA assessment using the Hybritech Tandem-R total PSA immunoradiometric assay (Beckman Coulter) and the clinically validated cut-off of 4.0 ng/ml was used for evaluation [13]. The ten samples with missing PSA values and 10 randomly selected samples were analyzed using the chemiluminescent Access Hybritech total PSA assay that uses the same monoclonal antibodies employed in the Hybritech Tandem total PSA assay and yields PSA concentrations based on calibration to the original Hybritech Tandem-R assay. The average coefficient of variance (%CV) between the original and rerun PSA values was 11 ± 5 %.

SIBLING levels were quantified using SIBLING ELISAs that had been used to determine biomarker cut-off values of 190 ng/ml for DSPP, 120 ng/ml for BSP and 11 ng/ml for OPN in 310 community-dwelling older adult males (see Supplemental Data: Methods). An underlying assumption to the current study's design was that this training set contains subjects who, at the time of sample collection, are likely to be preclinical and will be diagnosed in their lifetime. The 218 longitudinal samples are from older adult males who also have no symptoms of prostate cancer and contains some who will not develop prostate cancer and others who will be diagnosed with the disease at some future date. The analytical plan was to use SIBLING median values determined in the training group as the cut point for dichotomizing the biomarker data in the longitudinal study. The primary goal of this study is to address whether there is an association between SIBLING levels in preclinical samples and time to diagnosis. The association of dichotomized SIBLING levels and time to event (diagnosis, death) was assessed by Kaplan-Meier and Cox Proportional Hazard survival estimates.

Statistical analysis and scientific rigor. In modeling the relationship between biomarker and time to diagnosis as disease-free survival, censoring occurred for those subjects who were not diagnosed with prostate cancer. In modeling biomarker and time to death as overall survival, no censoring occurred as all subjects are deceased. Paired longitudinal serum samples from each BLSA participant were divided into those that were most distal or proximal to diagnosis (cases) or were censored (controls). No assumptions were made about the distribution of the data and non-parametric tests were employed. Comparisons between cases (those who eventually will be diagnosed with prostate cancer) and controls (those who will not be diagnosed with prostate cancer in their lifetime) used the Mann Whitney *U* test. Comparisons of measures between subjects' most proximal and distal samples were analyzed by Wilcoxon matched pairs signed rank test. Potential correlations between continuous measures (DSPP, BSP, OPN, PSA, age, BMI, time to events) were analyzed by Spearman correlation. Associations between dichotomized biomarkers and categorical measures (e.g., hypertension) were assessed by Goodman & Kruskal's lambda. The association of biomarkers with survival curves, median disease-free and overall survival times were modeled nonparametrically by Kaplan-Meier survival estimates. Biomarker levels were dichotomized into either low or high levels based on their respective cut-off values and separately analyzing samples grouped as most distal or proximal to diagnosis or censoring. Pairs of biomarkers were analyzed by stratifying samples into three groups: those with low levels of both biomarkers, high levels of one biomarker, and high levels of both biomarkers. Similarly, three biomarkers were stratified into four groups: those with all low biomarker levels, one high biomarker, two high biomarkers, and all

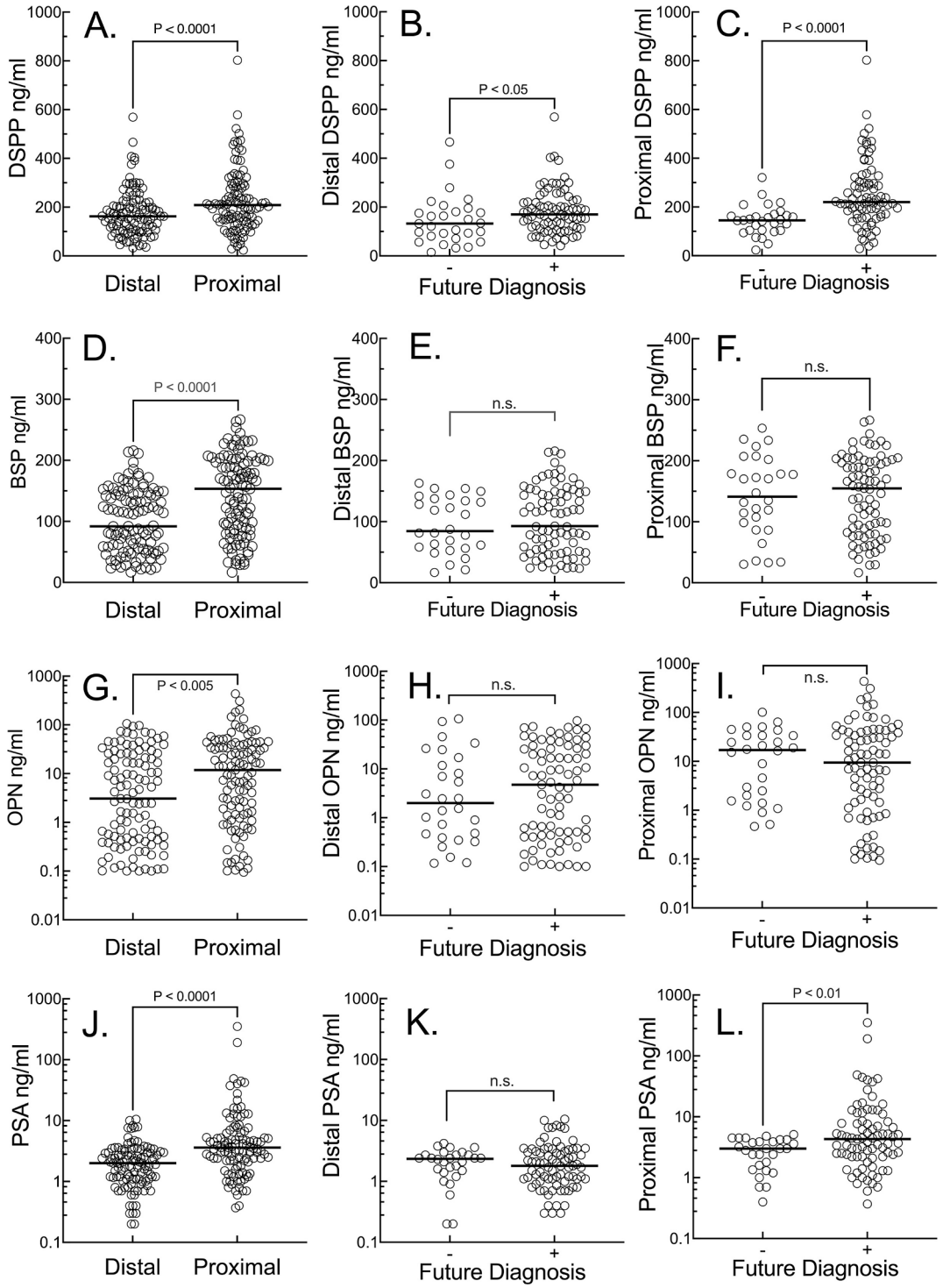

Fig. 1. SIBLING and PSA levels by time point and by future diagnosis. Longitudinal levels of SIBLINGs and PSA in serum from 109 donors were segregated into groups that were either most distal to or most proximal to time of diagnosis. Those groups were further stratified into subgroups that did not receive a diagnosis of prostate cancer (–) and those that did (+). Subgroups were compared for DSPP (**A, B, C**), BSP (**D, E F**), OPN, **(G, H, I)** and PSA (**J, K, L**). Comparisons were between most distal and proximal times to diagnosis/censoring (**A, D, G, J**); future diagnosis at the most distal times to diagnosis/censoring (**B, E, H, K**) and future diagnosis at the most proximal times to diagnosis/censoring (**C, F, I, L**). Abbreviations: DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; n.s., not significant.

three biomarkers high. A log-rank test was used to assess the p value for individual biomarkers, while a log-rank test for trend in the survival analysis was used to assess whether a linear trend was apparent between three or more survival curves. Hazard ratios were determined by Cox proportional hazard regression modeling using individual, pairs or trios of biomarkers dichotomized into low or high based on each biomarker's respective cut-off value and analyzing distal or proximal sets separately. There were four sets that were analyzed: distal disease-free survival, distal overall survival, proximal disease-free survival, and proximal overall survival. Comparisons of hazard ratios were performed within each set where there was a total of 14 different biomarker aggregates (4 individual biomarkers, 6 pairs and 4 trios of biomarkers). To assess

Table 2
Biomarker associations by Spearman correlation.

	DSPP			BSP			OPN			PSA		
Distal	r_s	95 % CI	P value	r_s	95 % CI	P value	r_s	95 % CI	P value	r_s	95 % CI	P value
Time to diagnosis	−0.28	(−0.45 to −0.10)	<0.005	0.04	(−0.16 to 0.23)	n.s.	−0.43	(−0.58 to −0.26)	<0.0001	−0.24	(−0.42 to −0.05)	<0.05
Time to death	−0.25	(−0.42 to −0.06)	<0.01	0.05	(−0.14 to 0.24)	n.s.	−0.44	(−0.58 to −0.26)	<0.0001	−0.30	(−0.46 to −0.11)	<0.005
Age	0.27	(0.08 to 0.44)	0.0005	0.02	(−0.18 to 0.21)	n.s.	0.40	(0.23 to 0.55)	<0.0001	0.42	(0.25 to 0.57)	<0.0001
BMI	0.10	(−0.10 to 0.28)	n.s.	0.02	(−0.18 to 0.21)	n.s.	0.01	(−0.19 to 0.19)	n.s.	−0.10	(−0.29 to 0.09)	n.s.
Hypertension	−0.02	(−0.21 to 0.17)	n.s.	0.20	(0.01 to 0.38)	<0.05	0.16	(−0.04 to 0.34)	n.s.	−0.03	(−0.23 to 0.16)	n.s.
Proximal												
Time to diagnosis	−0.34	(−0.50 to −0.16)	<0.0005	−0.27	(−0.44 to −0.08)	<0.005	0.10	(−0.09 to 0.29)	n.s.	−0.38	(−0.53 to −0.20)	<0.0001
Time to death	−0.20	(−0.38 to −0.01)	<0.05	−0.24	(−0.41 to −0.04)	<0.05	−0.36	(−0.52 to −0.18)	<0.0005	0.08	(−0.11 to 0.27)	n.s.
Age	0.25	(0.05 to 0.42	<0.01	0.07	(−0.12 to 0.26)	n.s.	0.34	(0.15 to 0.50)	<0.0005	0.05	(−0.15 to 0.24)	n.s.
BMI	−0.03	(−0.22 to 0.17)	n.s.	0.01	(−0.19 to 0.19)	n.s.	−0.01	(−0.20 to 0.18)	n.s.	−0.01	(−0.21 to 0.18)	n.s.
Hypertension	−0.07	(−0.26 to 0.12)	n.s.	−0.15	(−0.33 to 0.05)	n.s.	0.01	(−0.18 to 0.21)	n.s.	−0.08	(−0.27 to 0.11)	n.s.

Abbreviations: DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate specific antigen; r_s, Spearman correlation coefficient, 95% CI, 95% Confidence Interval; n.s., not significant.

hazard ratios within a set, a false discovery rate (FDR) analysis using the two-step method of Benjamini, Krieger and Yekutieli with a desired FDR Q of 1 % was employed [14].

Results

Longitudinal set characteristics

The characteristics, main predictors and outcomes associated with these samples obtained through the BLSA (n = 218) from subjects who were disease-free on enrollment and longitudinally followed are given in Table 1. These samples were from a broad age range (41–95 years). Almost all subjects were Caucasian and approximately 50 % had BMIs in the normal range while the percentage that were obese was less than 10 %. Systolic blood pressure was significantly different between the proximal and distal groups when compared by Wilcoxon matched pairs signed rank test. Hypertension was present in 69 % of subjects at the most distal time to diagnosis/censoring and the number increased to 77 % by the most proximal time to diagnosis. 74 % of subjects were eventually diagnosed with prostate cancer and 100 % are deceased. There was an average and median difference of at least 10 years between distal and proximal time to diagnosis and time to death for all subjects.

Biomarker distribution

Serum levels of DSPP, BSP, OPN and PSA exhibited a broad range of values and were significantly different between distal and proximal groups (Table 1, Fig. 1). The median value of DSPP at both the most distal and most proximal times to diagnosis/censoring was significantly higher in subjects who received a future diagnosis of prostate cancer when compared with those who remained disease-free (Fig. 1). BSP and OPN did not exhibit a difference in levels between subjects who received a future diagnosis of prostate cancer and those who remained disease-free at both the most distal and most proximal times to diagnosis/censoring. There was also no difference in median PSA levels between subjects who received a future diagnosis of prostate cancer and those who remained disease-free at the most distal times to diagnosis/censoring, while the difference was significant at the most proximal to diagnosis/censoring.

Biomarker associations

At the most distal time point, DSPP, OPN and PSA were negatively correlated with time to diagnosis, time to death, and positively correlated with age at visit, but not correlated with BMI or hypertension (Table 2). Distal BSP was correlated positively with hypertension. In the corresponding most proximal samples, DSPP associated negatively with time to diagnosis, time to death, and positively with age. Proximal BSP correlated negatively with time to diagnosis, time to death but not with age. Proximal OPN correlated negatively with time to death and positively with age. Proximal PSA was negatively correlated with only time to diagnosis (Table 2). Proximal levels of DSPP, BSP and OPN were not correlated with BMI or hypertension. DSPP and OPN were positively correlated at distal times to diagnosis/censoring (Spearman r = 0.25, 95 % CI 0.06 to 0.43, and $P < 0.01$). At proximal times to diagnosis/censoring, BSP and OPN exhibited a Spearman r of 0.20 with a 95 % CI of 0.007 to 0.38, $P < 0.05$. Proximal BSP and PSA exhibited a Spearman r of 0.19 with a 95 % CI of −0.0002 to 0.37), $P < 0.05$.

Biomarkers and time to events

Survival curves and time to diagnosis (disease-free survival) and time to death (overall survival) were profiled by Kaplan-Meier estimates of separately analyzed distal and proximal samples segregated into low versus high biomarker levels (Fig. 2). For the samples drawn most distal to diagnosis, high DSPP levels were associated with a 44 % decrease in median time to diagnosis and a 43 % decrease in time to death (Table 3). The probability of prostate cancer diagnosis (disease-free survival) and death (overall survival) was estimated by the Cox proportional hazard regression model. For the distal group, a subject with high DSPP had twice the probability of being diagnosed with prostate cancer by the next time point compared to subjects with low distal DSPP. A subject with high DSPP levels in the distal group had over twice the probability of having died by the next time point compared to a subject with low DSPP (Table 3). For the samples drawn most proximal to diagnosis, high DSPP levels were associated with an 80 % reduction in median disease-free survival and a 25 % reduction in median overall survival (Table 3). In the proximal group, a subject with high DSPP who had not been diagnosed with prostate cancer at a given time point had over 4 times the probability of being diagnosed by the next time point compared to a

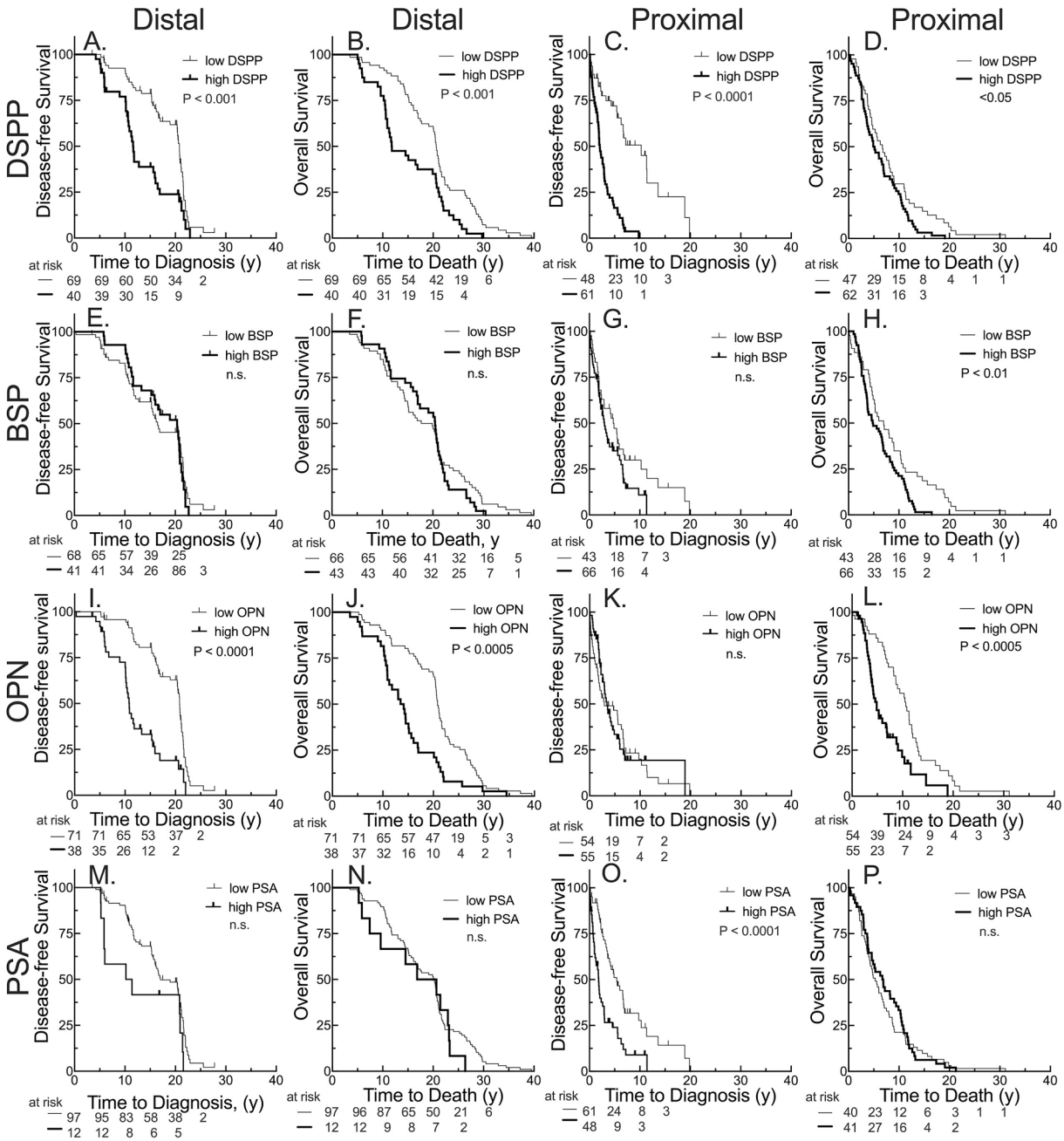

Fig. 2. Survival curves modeling time to diagnosis (disease-free survival) or time to death (overall survival). Longitudinal levels of SIBLINGs or PSA in serum from 109 donors were segregated as either most distal to or most proximal to time of diagnosis. Kaplan-Meier survival estimates were performed comparing high versus low predictor values at the most distal times to diagnosis/censoring (**A, B, E, F, I, J, M, N**) and most proximal times to diagnosis/censoring (**C, D, G, H, K, L, O, P**) for DSPP (**A, B, C, D**), BSP (**E, F, G, H**), OPN, (**I, J, K, L**) or PSA (**M, N, O, P**). The P value reported in each graph is from a Log-rank (Mantel-Cox) test. Abbreviations: DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; n.s., not significant. The number at risk are reported below each graph.

subject in the low DSPP proximal group. High DSPP levels in the proximal group associated with a 3-fold increase in the probability of dying by the next time point.

BSP levels were only modestly associated with time to event at the most proximal times to diagnosis/censoring, where a 37 % decrease in median disease-free and 30 % decrease in overall survival were observed. A subject with high proximal BSP levels had about twice the probability of dying by the next time point. High distal OPN levels were associated with a 2-fold increase in probability of diagnosis and death. There was no significant association between OPN and time to diagnosis at proximal times (Fig. 2, Table 3). However, high proximal OPN levels did associate with a decrease in disease-free survival and overall survival of 48 % and 34 %, respectively. A subject with a high proximal OPN levels had approximately twice the probability of dying by the next time point compared to a subject in the low OPN group. For the samples drawn most distal to diagnosis, survival curves were not different between low and high PSA levels for disease-free and overall survival (Fig. 2 and Table 3). For the samples drawn most proximal to diagnosis, high PSA levels were associated with a greater than 67 % reduction in median disease-free survival. A subject with elevated PSA levels in the proximal group who had not been diagnosed with prostate cancer at a certain time point had an over 2-fold probability of being diagnosed by

Table 3
Survival Estimates for individual biomarkers.

	Distal DFS	Distal OS	Proximal DFS	Proximal OS
DSPP				
Median survival, y				
low DSPP	20.8	20.6	10.3	6.71
high DSPP	11.6	11.8	2.06	5.01
HR, high DSPP	2.10 (1.33–3.28)	2.62 (1.63–4.18)	4.71 (2.77–8.40)	3.15 (1.90–5.45)
P value	≤0.001	<0.0001	<0.0001	<0.0001
BSP				
Median survival, y				
low BSP	16.5	19.9	4.72	6.97
high BSP	20.4	20.4	2.99	4.88
HR, high BSP	1.06 (0.66–1.65)	1.28 (0.80–2.02)	1.49 (0.94–2.42)	1.95 (1.20–3.27)
P value	n.s.	n.s.	n.s.	<0.01
OPN				
Median survival, y				
low OPN	20.8	20.8	2.89	10.6
high OPN	10.9	13.8	3.27	4.38
HR, high OPN	1.91 (1.22–3.99)	2.13 (1.36–3.35)	0.85 (0.55–1.33)	2.27 (1.41–3.69)
P value	<0.005	<0.001	n.s.	<0.001
PSA				
Median survival, y				
low PSA	17.0	20.1	5.41	5.28
high PSA	10.8	18.7	1.80	6.80
HR, high PSA	1.89 (0.94–3.46)	1.8 (0.89–3.33)	2.48 (1.58–3.90)	1.22 (0.78–1.90)
P value	n.s.	n.s.	<0.0001	n.s.

Abbreviations: DFS, disease-free survival; OS, overall survival; DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; n.s., not significant; y, years.
Hazard ratio determined by Cox proportional Hazard modeling of biomarkers dichotomized by their respective cut-off values. Reference is the group with both biomarkers low.

the next time point compared to a subject with low PSA.

SIBLINGs pairs and time to event

Serum levels of paired, dichotomized DSPP and BSP at most distal or proximal times to diagnosis/censoring were segregated into three groups: low levels of both DSPP and BSP, high levels of either DSPP or BSP, or high levels of both DSPP and BSP. Kaplan-Meier estimates of survival between the three groups was performed (Fig. 3, Table 4). A log-rank trend test revealed there was an association with survival. High distal DSPP and BSP associated with a 45 % reduction in median disease-free survival and a 2-fold increase in probability of a diagnosis event occurring (Table 3). A subject with high proximal DSPP and BSP, compared to the reference group, had an 80 % decrease in disease-free survival, a 39 % decrease in overall survival and a 5-fold increase in the probability of being diagnosed and dying by the next time point. With the pairing of BSP and OPN, there was no difference in median disease-free survival between subjects with high levels relative to subjects with low values (Table 4). Proximal overall survival was reduced 62 % and the probability of dying increased almost 5-fold in subjects with high BSP and OPN.

The pairing of DSPP with OPN yielded a log-rank trend test that was significant for disease-free and overall survival at both distal and proximal times to diagnosis/censoring (Fig. 3). Compared to subjects with low levels of both DSPP and OPN at the most distal time point, median disease-free was reduced 49 % and overall survival 45 % in subjects who had high distal DSPP and OPN (Table 3). Median disease-free survival was reduced 74 % and overall survival by 57 % in subjects with high proximal DSPP and OPN. A subject with high distal DSPP and OPN, compared to the reference group, had a 3- and 4-fold increase in probability of being diagnosed and dying, respectively, by the next time point. For proximal disease-free survival and overall survival, subjects with high DSPP and OPN had a 4-fold and 9-fold increase in hazard, respectively.

For DSPP paired with PSA, the trend across the three groups (both biomarkers low, one biomarker high, and both biomarkers high) and was significant except for overall survival at the most proximal time point (Fig. 4). Compared to the reference group (subjects with low distal levels of both DSPP and PSA), median disease-free survival was reduced 72 % in subjects who had both high distal DSPP and high PSA (Table 4). Distal median overall survival was reduced 65 % for high DSPP and PSA. Compared to the reference group, proximal median disease-free survival was reduced 86 % in subjects who had both high DSPP and PSA. Using the Cox proportional hazard estimator, a subject with high distal DSPP and PSA had an increase in probability of being diagnosed by the next time point of 20-fold and an increase in probability of dying of 5-fold, compared to the reference group. High proximal DSPP and PSA had a 11-fold increase in probability of being diagnosed by the next time point. Overall survival exhibited a 11-fold and 3-fold increase in probability of dying by the next time point for subjects with high distal and proximal DSPP and PSA, respectively.

When BSP and PSA were combined, the log-rank trend test and survival estimates were only significant at proximal times to diagnosis/censoring for disease-free survival (Fig. 4). A subject with high BSP and PSA had approximately a 68 % decrease in median disease-free and overall survival, and a 3-fold increase in probability of being diagnosed by the next time point, compared to subjects with low BSP and PSA. Combined OPN and PSA yielded a significant log-rank trend test for both distal and proximal disease-free survival and overall survival. For the group with high distal OPN and PSA, there was a 72 % decrease in median disease-free survival and a 51 % decrease in median overall survival. High proximal OPN and PSA were associated with a 61 % reduction in proximal median disease-free survival, and a 47 % decrease in median overall survival. High OPN and PSA at distal times to diagnosis/censoring was associated with an over 5-fold increase in probability of a prostate cancer diagnosis event occurring by the next time point. For distal overall survival, proximal overall and disease-free survival, a subject with high OPN and PSA had a 3-fold increase in probability of an event occurring by the next time point.

SIBLING and PSA trios and time to events

A composite of DSPP, BSP and OPN was made using dichotomized values and survival assessed for subjects with high levels of one, two or three of the biomarkers compared to the reference group – samples with low levels of all three biomarkers (Fig. 5). The log-rank trend across groups test was significant for disease-free and overall survival at both distal and proximal times to diagnosis/censoring. Subjects with high distal levels of DSPP, BSP and OPN had a 51 % decrease in disease-free survival and a 32 % decrease in overall survival (Table 4). A subject with all three biomarkers high had a 3-fold increase in probability of being diagnosed and 5-fold increase in risk of dying by the next time point, compared to the reference group. Subjects with high proximal DSPP, BSP and OPN had a 79 % decrease in median disease-free survival and a 73 % decrease in overall survival. High proximal DSPP, BSP and OPN was also associated with a 6-fold increase in probability of being diagnosed by the next time point and a 20-fold increase in hazard for overall survival. It should be noted that the confidence interval on this latter estimate is wide (see Table 5). The broad range likely reflects the limited number of subjects in the reference group (only 9 subjects had low levels of DSPP, BSP, and OPN).

For the trio of DSPP + BSP + PSA, log-rank trend test p values were significant at distal and proximal times to diagnosis (Fig. 5). A subject with high distal DSPP, BSP and PSA had a 62 % decrease in median

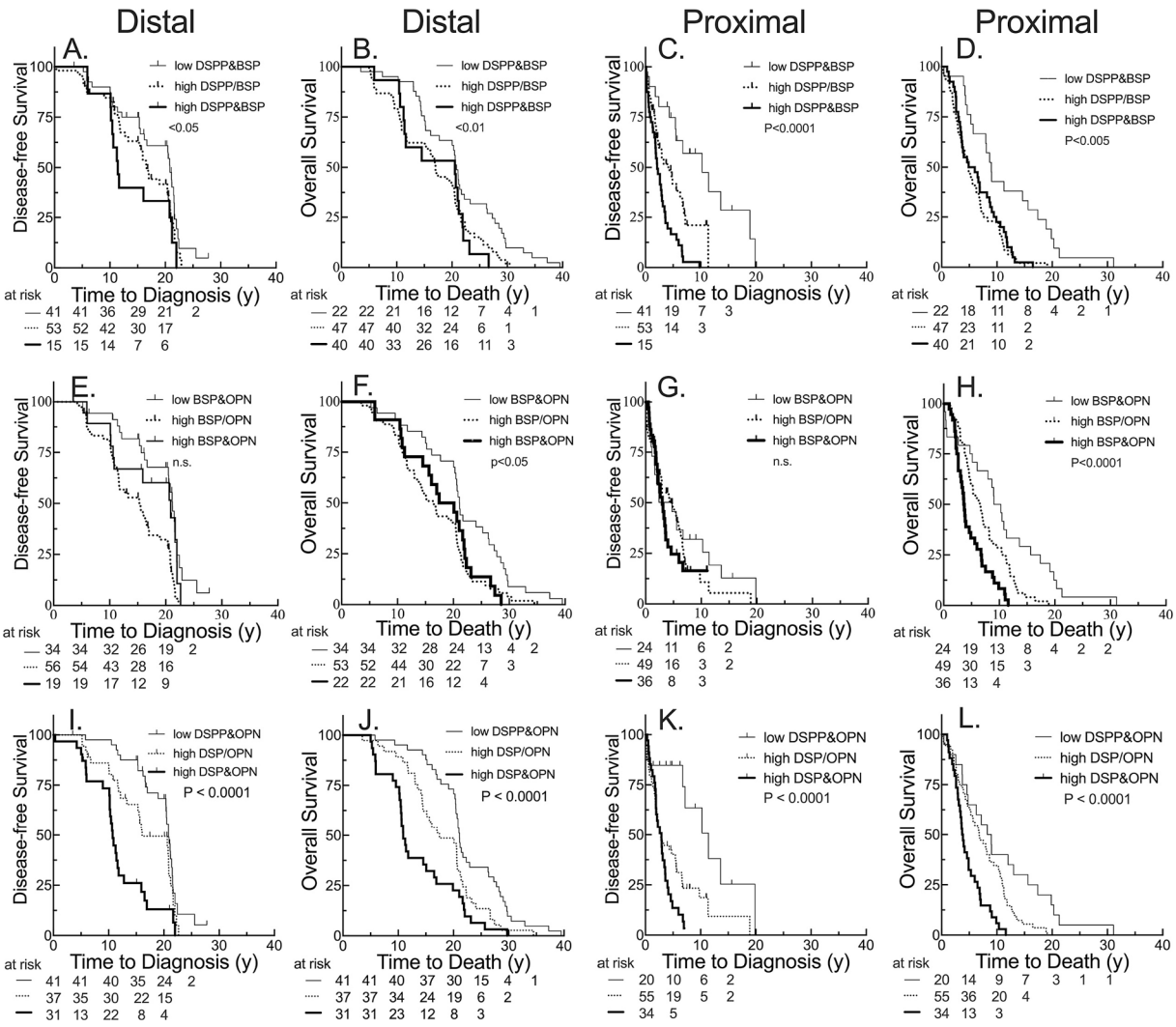

Fig. 3. Paired SIBLINGs and survival estimates. Kaplan-Meier survival estimates were performed between samples with low levels of SIBLINGs (low SIBLING-&SIBLING), high levels of one SIBLING (high SIBLING/SIBLING), or high levels of both SIBLINGs (high SIBLING&SIBLING). The pairing of DSPP with OPN (**A, B, C, D**), DSPP with BSP (**E, F, G, H**), and BSP with OPN (**I, J, K, L**) for disease-free survival (**A**, **C, E, G, I, K)** or overall survival (**B**, **D, F, H, J, L)** for the groups segregated as most distal to diagnosis (**A**, **B, E, F, I, J)** and most proximal to diagnosis **(C, D, G, H, K, L)**. The P value reported for each pairing/group is from a log-rank trend test for three or more survival curves. Abbreviations: DSPP dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin. The number at risk are reported below each graph.

Table 4
Survival Estimates for biomarker pairs.

	Distal DFS	Distal OS	Proximal DFS	Proximal OS
DSPP + BSP				
Median survival (y)				
low DSPP&BSP	20.8	20.8	10.3	9.00
high DSPP/BSP	16.6	17.0	4.14	4.84
high DSPP&BSP	11.4	20.5	2.09	5.44
HR, high DSPP&BSP	2.01 (1.00–3.84)	2.88 (1.39–5.71)	5.22 (2.61–11.5)	5.39 (2.66–12.0)
P value	<0.05	n.s.	<0.0001	<0.0001
BSP + OPN				
Median survival (y)				
low BSP&OPN	21.2	21.1	4.72	9.66
high BSP/OPN	15.3	16.5	4.32	6.49
high BSP&OPN	20.9	21.6	2.99	3.66
HR, high BSP&OPN	1.68 (0.86–3.24)	2.26 (1.14–4.44)	1.41 (0.76–2.69)	4.74 (2.35–10.0)
P value	n.s.	<0.05	n.s.	<0.0001
DSPP + OPN				
Median survival (y)				
low DSPP&OPN	20.8	21.1	10.3	8.83
high DSPP/OPN	16.06	16.5	2.89	6.49
high DSPP&OPN	10.7	11.5	2.62	3.78
HR, high DSPP&OPN	3.30 (1.85–5.83)	3.77 (2.10–6.75)	3.85 (1.95–8.05)	8.99 (4.24–20.7)
P value	<0.0001	<0.0001	<0.0005	<0.0001
DSPP + PSA				
Median survival, years				
low DSPP&PSA	20.6	27.3	11.4	5.69
high DSPP/PSA	12.8	16.6	3.60	5.99
high DSPP&PSA	5.86	9.60	1.61	6.09
HR, high DSPP&PSA	20.8 (6.39–58.2)	5.30 (1.79–12.7)	11.4 (5.52–25.4)	3.17 (1.66 to 6.40)
P value	<0.0001	<0.001	<0.0001	<0.001.
BSP + PSA				
Median survival (y)				
low BSP&PSA	16.7	19.9	5.41	6.97
high BSP/PSA	18.9	20.4	3.60	4.70
high BSP&PSA	20.9	21.4	1.75	6.80
HR, high BSP&PSA	1.36 (0.40–3.41)	1.44 (0.43–3.66)	3.24 (1.77–6.15)	2.01 (1.10–3.77)
P value	n.s.	n.s.	<0.0005	n.s.
OPN + PSA				
Median survival (y)				
low OPN&PSA	20.5	20.8	6.64	7.30
high OPN/PSA	15.3	15.8	3.27	6.72
high OPN&PSA	5.99	14.5	1.89	3.90
HR, high OPN&PSA	5.18 (1.74–12.5)	3.25 (1.09–7.88)	2.57 (1.30–5.10)	3.41 (1.70–6.88)
P value	<0.001	<0.05	<0.01	<0.005

Abbreviations: DFS, disease-free survival; OS, overall survival; DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; HR, hazard ratio; n.s., not significant.
Groups analyzed are: low levels of both biomarkers (e.g., low DSPP&BSP), high levels of one of the biomarkers (e.g., high DSPP/BSP), and high levels of both biomarkers (e.g., high DSPP&BSP).
Hazard ratio determined by Cox proportional Hazard modeling of biomarkers dichotomized by their respective cut-off values. Reference is the both biomarkers low group.

disease-free survival and a 11-fold increase in hazard. An 84 % reduction in median disease-free survival and 10-fold increase in probability of diagnosis was observed with all three biomarkers high at proximal times to diagnosis/censoring. There was a 4-fold increase in probability of dying by the next time point for subjects with high proximal DSPP, BSP and PSA (Table 5).

The combination of DSPP, OPN and PSA yielded a positive log-rank trend test at distal and proximal time to events (Fig. 5). A subject with high distal DSPP, OPN and PSA had a 72 % decrease in median disease-free survival and a 16-fold increase in hazard (Table 5). An 84 % reduction in median disease-free survival and 12-fold increase in probability of diagnosis was observed with all three biomarkers high at proximal times to diagnosis/censoring. Median overall survival was decreased 72 % and 56 % at distal and proximal times where there was a 4- and 10-fold increase in probability of dying by the next distal or proximal time point respectively, compared to subjects with low DSPP, OPN and PSA. A subject with high distal BSP, OPN and PSA had a 62 % decrease in median disease-free survival and 17-fold increase in hazard. An 68 % reduction in median disease-free survival and 3-fold increase in probability of diagnosis was observed with all three biomarkers high at proximal times to diagnosis/censoring. Median overall survival and hazard ratios were not significant at distal time points. There was a 57 % reduction in proximal median survival and a 5-fold increase in probability of dying by the next distal time point, compared to subjects with low BSP, OPN and PSA.

Survival model adjustments

Age is the most common risk factor for prostate cancer and age has a strong association with time to prostate cancer diagnosis and death [15]. Cox proportional hazard modeling with age as a covariate was carried out on individual biomarkers and pairs or trios of biomarkers to account for potential differences in age. Adjusting for age had a minimal effect on distal or proximal disease-free survival hazard ratios (Fig. 6). After adjusting for age, the only hazard ratios for distal overall survival that remained significant were with DSPP alone as the biomarker or with paired DSPP and OPN. When models of overall survival with distal levels of individual and grouped biomarkers were adjusted for age, only the paring of high DSPP and OPN remained significant. For both distal and proximal disease-free survival and proximal overall survival, biomarker aggregates that remained significant after adjusting for age include DSPP with OPN, DSPP with PSA, DSPP with BSP and PSA, and DSPP with OPN and PSA. BMI and hypertension have also been associated with prostate cancer risk [16,17]. Adjusting for BMI or hypertension in the Cox proportional hazard models did not change any of the hazard ratios significantly. Finally, because of the multiple comparisons made, an FDR analysis was carried out within each set (Fig. 6). Biomarkers/aggregates identified as true discoveries (marked by asterisks in the figure) changed little between distal and proximal disease-free survival. For disease-free survival at both distal and proximal time points, the biomarkers/aggregates identified in the age-adjusted models as discoveries were DSPP alone, PSA alone, BSP with OPN, DSPP with PSA, DSPP with OPN and PSA. The hazard ratios for distal overall survival did not qualify as true discoveries. For proximal overall survival, the biomarkers/aggregates identified as discoveries were DSPP alone, DSPP with BSP, BSP with OPN, DSPP with OPN, DSPP with OPN and PSA.

Discussion

Prostate cancer is the most common cancer in men and the second leading cause of cancer death in men [18]. The introduction of PSA into screening has been associated with an increase in the detection of early stage, localized cancers [6]. PSA is an organ-specific, but not cancer-

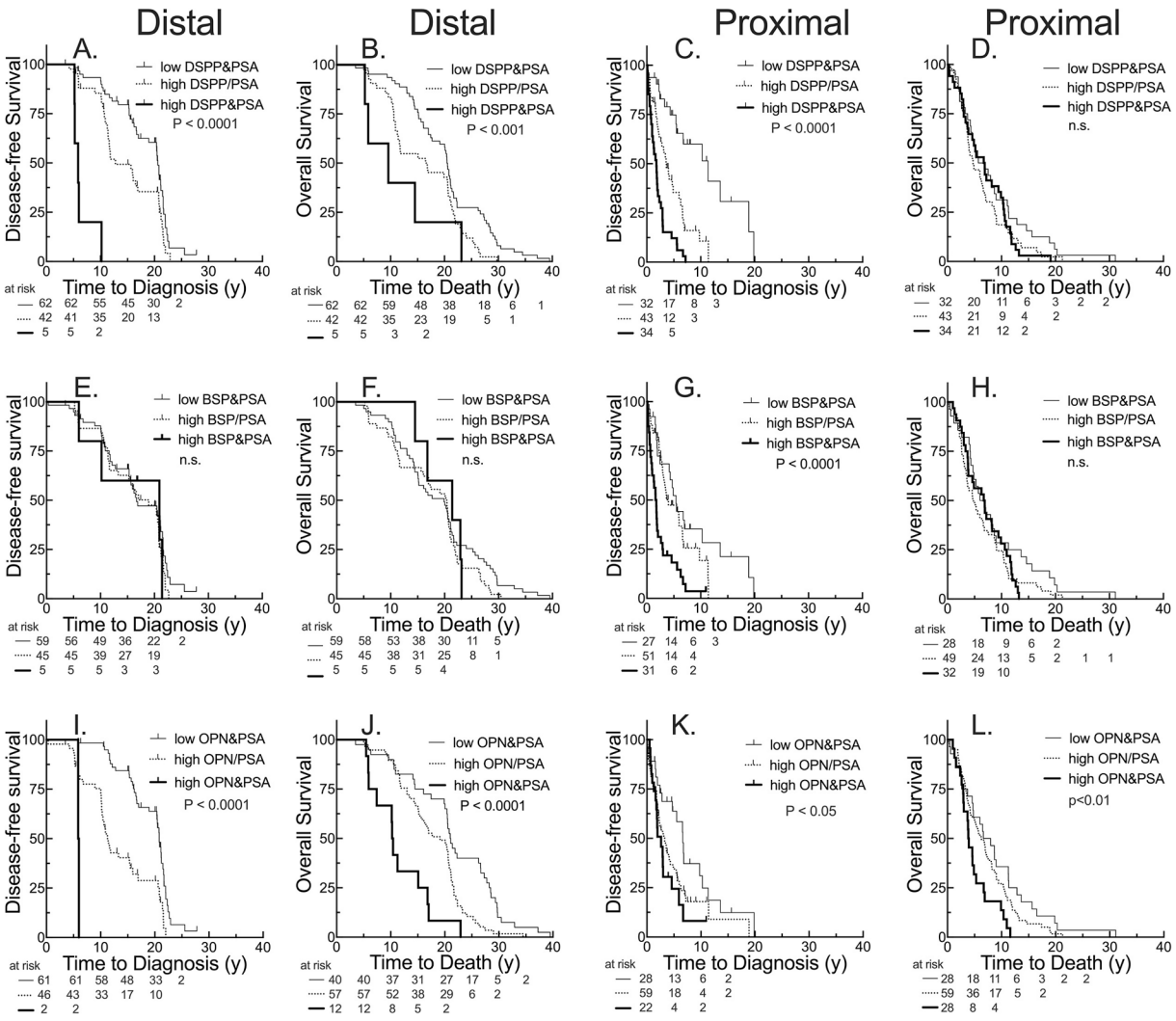

Fig. 4. Paired SIBLING and PSA levels and survival estimates. Kaplan-Meier survival estimates were performed using serum samples that were either most distal to or most proximal to time of diagnosis and comparing survival between samples with low levels of a SIBLING and PSA (low SIBLING&PSA), high levels of a SIBLING or PSA (high SIBLING/PSA), or high levels of both SIBLING and PSA (high SIBLING&PSA). Survival estimates were performed for DSPP with PSA (**A**, **B, C, D**), BSP with PSA (**E**, **F, G, H**), and OPN with PSA (**I**, **J, K, L**), modeling time to diagnosis (**A, C, E, G, I, K**) and time to death (**B, D, F, H, J, L**). The P value reported for each pairing is from a log-rank trend test for three or more survival curves. Abbreviations: DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen. The number at risk are reported below each graph.

specific, biomarker. Attempts to improve the detection of prostate cancer have included aggregated tests that incorporate urinary mRNA expression levels, multiplexed protein analysis, and clinical variables, blood biomarkers, genetic markers, and prostate examination [19,20]. While the use of multiple biomarkers in a combination with imaging modalities have also improved diagnostics and treatment prognosis [21,22], these advances do not address risk stratification at the level of neoplastic transformation or other early pre-symptomatic events in cancer development.

SIBLINGs are secreted and intrinsically disordered extracellular matrix proteins. They have multiple binding partners and can tether their respective ligands to the cell surface via interactions with integrins and CD44 variants [4,7,23–29]. Though initially isolated from mineralizing tissue, SIBLING expression by normal non-mineralizing tissue and upregulation in multiple cancer types were also observed [5,30]. The transcription factor RUNX2 has been implicated in regulating the expression of the SIBLINGs BSP [31,32], OPN [33], and DMP1 [34]. RUNX2 is expressed in multipotent mesenchymal cells, osteoblast-lineage cells, and chondrocytes [35]. RUNX2 is also expressed in the prostate where it has been associated with epithelial stem cells during development [36]. RUNX2 has been implicated in regulating the epithelial to mesenchymal transition, where epithelial cells progressively acquire characteristics of mesenchymal or fibroblasts-like cells [37,38] that contribute to prostate cancer invasiveness [39]. RUNX2 expression levels associated with increased Gleason score and metastasis of prostate tumors [40].

Tissue expression of SIBLINGs in established prostate cancer strongly correlated with the conventional histopathological prognostic indicators such as Gleason score, pathological staging and metastasis [41–45]. DSPP serum levels were elevated in early-stage prostate cancer and combining DSPP and PSA values increased sensitivity and specificity of detection of established prostate cancer [9]. DSPP and its proteolytically processed products – dentin sialoprotein and dentin phosphoprotein have been associated with mesenchymal and epithelial cell differentiation and in homeostatic regeneration [46–48]. DSPP expression and serum levels were elevated in the earliest stages of prostate cancer [9,43]. Suppressing DSPP expression in squamous cell carcinoma *in vitro* models was found to reduce the expression of genes involved in metastasis, angiogenesis, and epithelial to mesenchymal transition [49], downregulate cancer stem cell markers [50], and decrease cancer cell viability, migration and invasion while increasing apoptosis [51,52].

OPN has been proposed as a diagnostic biomarker for prostate cancer

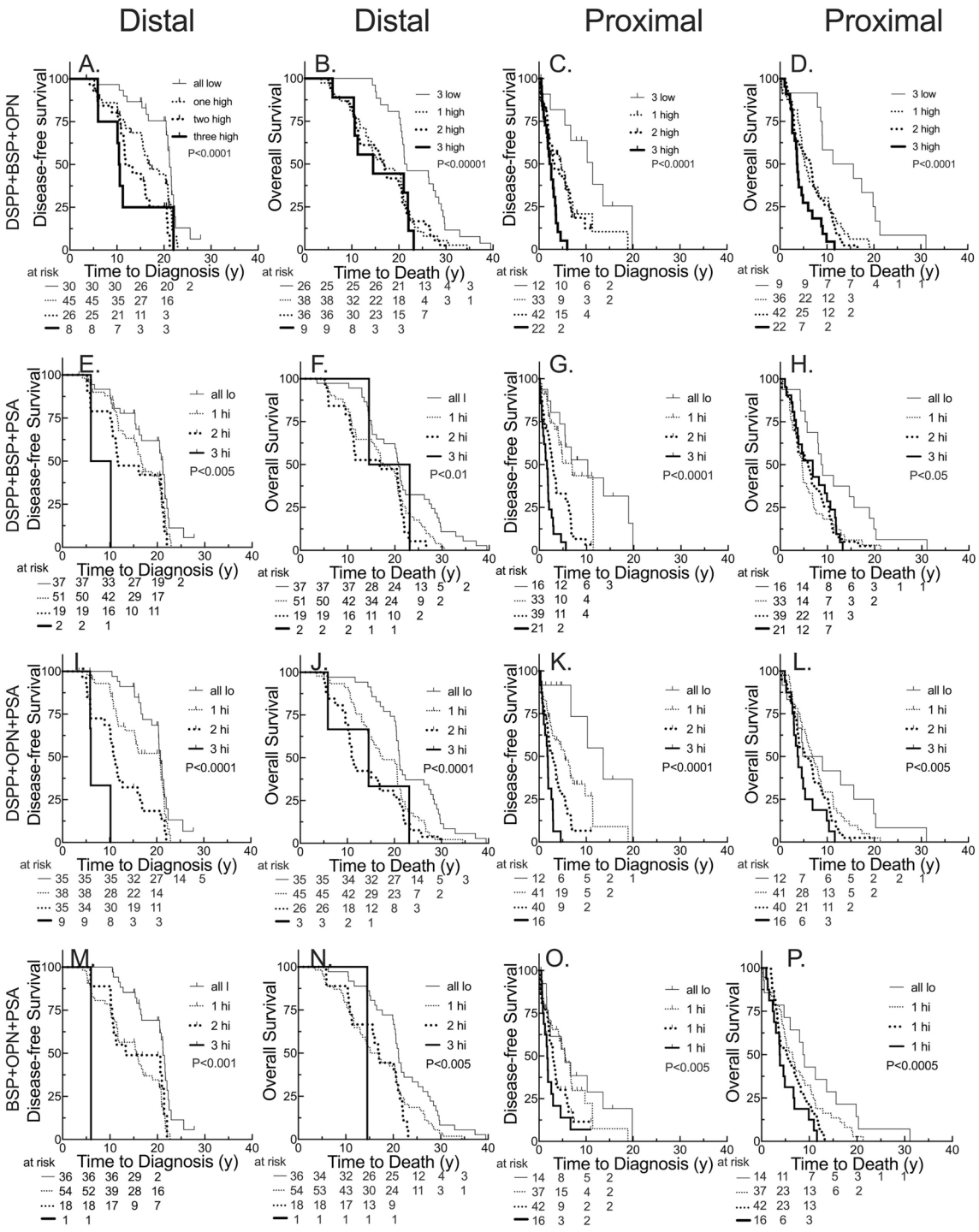

Fig. 5. Trios of SIBLINGs and PSA levels and survival estimates. Kaplan-Meier survival estimates were performed using serum samples that were either most distal to or most proximal to time of diagnosis and comparing survival between subjects with 1 biomarker high, 2 biomarkers high, or all 3 biomarkers high to subjects with low levels of all three biomarkers (reference). Survival estimates were performed for DSPP + BSP + OPN (**A, B, C, D**), DSPP + BSP + PSA (**E, F, G, H**), BSP + OPN + PSA (**I, J, K, L**), and DSPP + OPN + PSA, modeling time to diagnosis (**A, C, E, G, I, K, M, O**) and time to death (**B, D, F, H, J, L, N, P**). The P value reported for each pairing is from a log-rank trend test for three or more survival curves. Abbreviations: DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen. The number at risk are reported below each graph.

and a prognostic biomarker of response to treatment [45,53]. Underlying OPN biology are sentinel pathways involving neovascularization and angiogenesis, innate and adaptive immunity, hypoxia, stress response, and apoptosis [54–58]. BSP expression status in patients with moderately differentiated tumors or with pathologically confined tumors associated with higher risk of biochemical progression of prostate cancer [59]. High serum BSP levels were predictive of reduced postoperative and long-term survival [60]. Serum BSP and PSA doubling time were identified as independent predictors of prostate cancer metastasis to bone [44]. The above observations were focused on SIBLING expression or circulating levels and established cancer detection/prognosis.

The current study is the first to use longitudinal preclinical samples to assess SIBLING associations with time to events (diagnosis, censoring and death). In this study, individual biomarkers exhibited different

Table 5
Survival estimates of trios of biomarkers.

	Distal DFS	Distal OS	Proximal DFS	Proximal OS
DSPP + BSP + OPN				
Median survival, y				
All biomarkers low	21.2	21.4	11.4	13.5
1 biomarker high	17.0	16.9	4.72	5.28
2 biomarkers high	15.9	16.7	2.94	6.56
3 biomarkers high	10.4	14.5	2.44	3.66
HR, 3 biomarkers high	3.34 (1.34–7.69)	4.90 (1.91–11.8)	6.48 (2.65–17.8)	20.2 (6.98–74.5)
P value	<0.01	<0.0005	<0.0001	<0.0001
DSPP + BSP + PSA				
Median survival, y				
All biomarkers low	21.1	20.8	10.3	8.83
1 biomarker high	16.6	17.4	6.96	4.61
2 biomarkers high	11.7	16.8	2.91	5.99
3 biomarkers high	8.10	18.8	1.61	6.88
HR, 3 biomarkers high	10.8 (1.67–40.6)	2.84 (0.45–9.98)	9.95 (4.21–26.0)	3.92 (1.80–9.19)
P value	<0.005	n.s.	P < 0.0001	P < 0.001
DSPP + OPN + PSA				
Median survival, y				
All biomarkers low	20.8	21.0	11.4	8.68
1 biomarker high	20.8	20.1	5.41	6.56
2 biomarkers high	10.7	11.3	2.61	4.88
3 biomarkers high	5.86	5.86	1.86	3. 68
HR, 3 biomarkers high	16.1 (3.56–53.12)	4.42 (1.03–13.2)	12.1 (4.52–36.6)	9.67 (3.67–28.9)
P value	<0.0001	<0.05	<0.0001	<0.0001
BSP OPN + PSA				
Median survival, y				
All biomarkers low	21.2	21.2	5.50	8.83
1 biomarker high	15.3	15.2	5.56	6.49
2 biomarkers high	10.7	18.8	2.94	5.44
3 biomarkers high	5.86	18.8	1.75	3.82
HR, 3 biomarkers high	16.9 (2.54–66.2)	3.39 (0.53–12.2)	3.16 (1.36–7.64)	4.96 (2.07–12.4)
P value	<0.0005	n.s.	<0.01	<0.0005

Abbreviations: DFS, disease-free survival; OS, overall survival; DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; HR, hazard ratio; n.s., not significant.
Groups analyzed: low levels of all three biomarkers, high levels of one of the biomarkers, and high levels of two biomarkers, and high levels of all three biomarkers. Hazard ratio determined by Cox proportional Hazard modeling of biomarkers dichotomized by their respective cut-off values. Reference group is the all biomarkers low group.

associations with survival. High DSPP levels at the most distal and most proximal times to diagnosis/censoring were associated with a decreased time to prostate cancer diagnosis (reduction in median disease-free survival), whereas high BSP levels associated with decreased time to death (reduction in median overall survival) only at the most proximal times to diagnosis/censoring. High OPN levels at the most distal and proximal times to diagnosis/censoring were associated with decreased time to death, while high PSA levels only associated with decreased time to diagnosis at more proximal timepoints. For both pairs and trios of biomarkers, if DSPP was in the group, higher distal and proximal hazard ratios were observed. Proximal hazard ratios were higher when groups contained OPN, PSA or BSP. These observations are consistent with DSPP as a biomarker of early events in the development of cancer, while OPN, BSP and PSA are biomarkers of cancer progression.

The limitations to the current study include a modest sample size of 109 subjects with 218 samples, limited diversity in the racial composition of longitudinal study group, missing staging data, and only two longitudinal time points for each subject were analyzed. The BSP assay exhibited the highest %CV values for controls and the widest 95 % confidence intervals. This, in combination with the limited number of samples may have affected detection of associations between BSP levels in preclinical samples and time to events (diagnosis, death). The translation of SIBLING measures to clinical utility has been hindered by their biology. SIBLINGs have splice variants, microheterogeneity in post-translational modifications, and cellular and extracellular sequestration that can complicate their quantitation [61–75]. For example, a specific OPN splice variant (OPNc) has been identified in breast cancer and, depending on the antibodies used in immunohistochemistry or immunoassays, the variant won't be detected [61,62]. The extent of phosphorylation and N-linked oligosaccharide modifications can also interfere with an antibody binding to its epitope.

The proteolytic processing of SIBLINGs also complicates their analysis. Very rapidly after its synthesis, DSPP is cleaved by bone morphogenetic proteins generating dentin sialoprotein (DSP) and dentin phosphoprotein containing fragments [76]. BSP may be processed by cathepsins and matrix metalloproteinases [77,78]. OPN cleavage by thrombin has been well characterized [79,80]. OPN and DMP1can be processed by matrix metalloproteinases (MMPs) [71,73,80,81]. Thus, ELISAs to measure DSPP, BSP and OPN may be detecting full length or fragments or both, depending on the detection antibody epitopes. Sandwich-based ELISAs can be engineered to be more specific than the competitive assay since the 'sandwich' involves two antibody recognition steps. In the current study, competitive ELISAs that measure the DSP portion of DSPP, total BSP (intact plus fragments) as well a sandwich-based ELISAs that measures intact OPN were used.

Observations from this current study are consistent with SIBLINGs meriting further study as serum biomarkers of events across the life history of prostate cancer development. Future studies of SIBLINGs and PSA with larger retrospective longitudinal repository study samples will help to define the furthest time before diagnosis where the biomarkers retain a predictive capacity. Such studies would also enable analysis of rates of changes in these biomarkers over time to be determined and the exploration of their predictive capability.

CRediT authorship contribution statement

Alka Jain: Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation. **Ying Ni:**

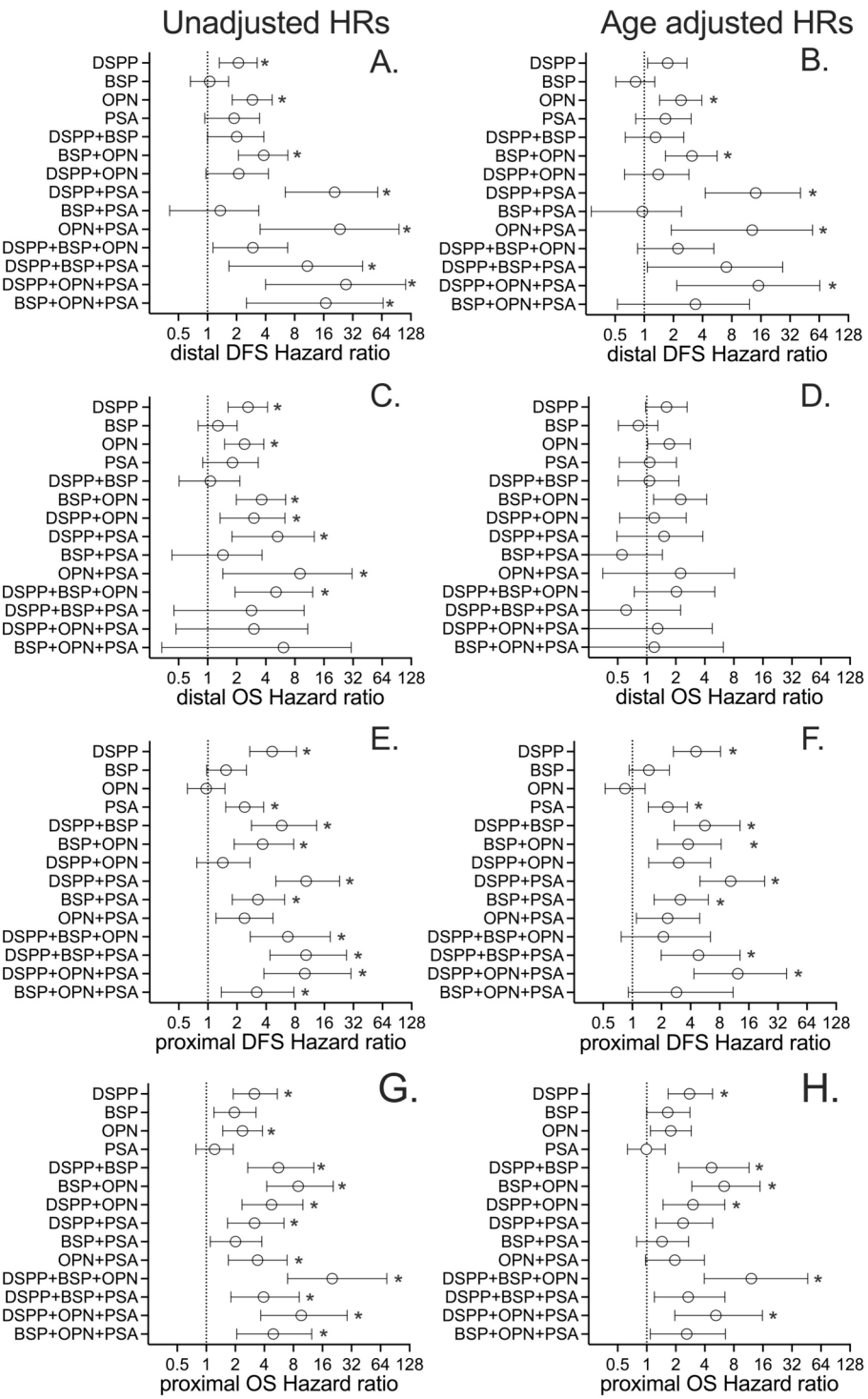

Fig. 6. Cox proportional hazard ratios. Proportional hazard modeling of unadjusted (**A, C, E, G**) and adjusted for age (**B, D, F, H**) hazard ratios for disease-free survival (**A**, **B, E, F)**, overall survival (**C**, **D, G, H)** for the groups segregated as most distal to diagnosis (**A**, **B, C, D)** and most proximal to diagnosis (**E, F, G, H)**. Asterisks indicate biomarkers that were identified as positive discoveries after applying the adaptive FDR method of Benjamini, Krieger and Yekutieli. Abbreviations: DSPP, dentin sialophosphoprotein; BSP, bone sialoprotein; OPN, osteopontin; PSA, prostate-specific antigen; DFS, disease-free survival; OS, overall survival.

Writing – review & editing, Resources, Methodology, Data curation. **Daisy Zhang:** Writing – review & editing, Methodology. **Eleanor M. Simonsick:** Writing – review & editing, Resources, Formal analysis, Conceptualization. **E. Jeffrey Metter:** Writing – review & editing, Resources, Formal analysis. **Kalu U. Ogbureke:** Writing – review & editing, Resources, Formal analysis, Conceptualization. **Larry W. Fisher:** Writing – review & editing, Resources, Conceptualization. **Neal S. Fedarko:** Writing – review & editing, Resources, Formal analysis, Conceptualization, Writing – original draft, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mbplus.2025.100171.

Data availability

The complete datasets for the data presented in the manuscript have been uploaded to the Mendeley Data repository and is available at doi: 10.17632/6x9z7t76hm.1.

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