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Plasma Protein Profiling Reveal Osteoprotegerin as a Marker of Prognostic Impact for Colorectal Cancer

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Abstract

BACKGROUND: Due to difficulties in predicting recurrences in colorectal cancer stages II and III, reliable prognostic biomarkers could be a breakthrough for individualized treatment and follow-up. *OBJECTIVE:* To find potential prognostic protein biomarkers in colorectal cancer, using the proximity extension assays. *METHODS:* A panel of 92 oncology-related proteins was analyzed with proximity extension assays, in plasma from a cohort of 261 colorectal cancer patients with stage II-IV. The survival analyses were corrected for disease stage and age, and the recurrence analyses were corrected for disease stage. The significance threshold was adjusted for multiple comparisons. *RESULTS:* The plasma proteins expression levels had a greater prognostic relevance in disease stage III colorectal cancer than in disease stage II, and for overall survival than for time to recurrence. Osteoprotegerin was the only biomarker candidate in the protein panel that had a statistical significant association with overall survival ($P = .00029$). None of the proteins were statistically significantly associated with time to recurrence. *CONCLUSIONS:* Of the 92 analyzed plasma proteins, osteoprotegerin showed the strongest prognostic impact in patients with colorectal cancer, and therefore osteoprotegerin is a potential predictive marker, and it also could be a target for treatments.

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Background

Since the detection of carcinoembryonic antigen (CEA) in 1965 [1] a large number of biomarker candidates have been proposed to have a potential prognostic impact in colorectal cancer (CRC). However, CEA is still the only serologic marker recommended in surveillance for CRC by experts groups of American society of colon and rectal surgeons [2] and European society for medical oncology [3].

Due to the lack of sensitivity or specificity of the biomarker candidates and due to the polymorphism of the CRC and the tested cohorts none of the suggested biomarker candidates have shown superiority to CEA. The field is extensively expanding due to new analytic techniques such as next generation sequencing, which adds to the complexity of the information.

The present cohort has previously been used for several studies that have improved our understanding on both soluble and tissue prognostic biomarkers [4–14]. In this study, in search for prognostic biomarkers, the samples were assessed using the proximity extension assays (PEA) [15,16], and a protein panel consisting of 92 highly oncology-related protein biomarker candidates. In the multiplex PEA, each target protein is recognized by a pair of DNA-conjugated affinity binders such as poly- and monoclonal antibodies. Upon simultaneous target recognition the DNA arms on the antibodies are brought in proximity and hybridized to each other allowing an enzymatic DNA polymerization. The newly synthesized DNA

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molecule is then amplified using real-time qPCR. A combination of duel recognition and subsequent signal amplification results in detection of proteins with high specificity and sensitivity. The technology has now been widely used and is demonstrated to be suitable for multiplex and high throughput analyses of panels of proteins in large numbers of samples. The technology has, for instance, been used to identify novel biomarker candidates for small intestinal neuroendocrine tumor [17] to demonstrate the strong effect of genetic and lifestyle factors on protein biomarker levels [18] to identify circulating protein markers predicting of incident heart failure in the elderly [19] to reveal lower levels of several peripheral inflammatory protein biomarkers in women with antenatal depression [20]. The PEA has also been used to characterize exosomal proteome and to trace the exosomes to their originating cells and tissues [21].

The aim of this study was to investigate whether any of the selected biomarker candidates allow prediction of death or disease recurrence in patients with CRC.

Materials and Methods

Patient Samples

The study was prospective and the cohort included patients treated for CRC at the Department of Surgery, Central District Hospital, Västerås, County of Västmanland, Sweden, with a population of 260,000. The study period was between August 2000 and December 2003, and the inclusion criterion was a histologically verified adenocarcinoma of the rectum or colon. The total number of this patients cohort is 324, but for the present study samples from a subgroup of 270 patients were analyzed with disease stages II-IV, excluding disease stage I due to good prognosis with only one recurrence in that group.

Blood samples were collected into endotoxin-free tubes with EDTA one day prior to the planned resection of the CRC. For plasma preparation, the blood samples were centrifuged at 2,000×*g* for 10 min at room temperature, and plasma was transferred to a new tube and stored at −70°C until use. All assays were performed in a blinded manner.

Surveillance was according to national guidelines with computed tomography scan of thorax and abdomen after 1 and 3 years, and colonoscopy every 5 years up to 75 years of age for all patients. Patients with rectal cancer underwent rectoscopy or palpation of perineum every 6 months up to 3 years and then after 4 and 5 years from the operation. Additional radiological examinations outside the surveillance program were made if patients sought with symptoms suspecting recurrence of the CRC.

Information about disease stage, tumor differentiation grade, mucinous histology, death and cancer recurrence were collected from the histopathological, surgical and oncology records.

The latest update on the database was in May 2015 with new recurrences and the exact date of deaths recorded, which were available from the computerized hospital record system.

Protein detection

The PEA was performed using Olink Oncology I panel (Olink Proteomics, Uppsala, Sweden), according to the manufacturer's instructions and as described previously [15,21]. The list of the 92 oncology-related proteins included in the panel is summarized in Table 1. Briefly, 1 μl plasma sample was mixed with 3 μl incubation mix, containing a mixture of 92 probe pairs, in a 96-well plate. Each probe consisting of an antibody conjugated to a unique DNA oligonucleotide. The mixture was incubated at 4°C overnight, allowing recognition of target proteins by a pair of probes. Thereafter, 96 μl extension mix, containing PEA enzyme and PCR reagents, was added, the mixture was incubated for 5 min at room temperature before the DNA extension was initiated in a thermal cycler for 20 min at 50°C, followed by 17 cycles DNA amplification. A new mixture was prepared by adding 2.8 μl of the PCR products to 7.2 μl detection mix in a new 96-well plate from which 5 μl was transferred to a 96.96 Dynamic Array IFC (Fluidigm, South San Francisco, CA, USA) that was in advanced prepared and primed according to the manufacturer's instructions. The unique pair of primers for each protein was loaded in the other side of the array chip and the expression program was performed in a BioMark™ HD real-time PCR platform (Fluidigm, South San Francisco, CA, USA).

The CEA determination was on serum with a commercially available ELISA kit. The analysis is based on the principle of a Solid-Phase-Enzyme-Linked immunosorbent assay. According to the manufacturer's instructions, this assay has a detection limit of 1 ng/ml and the standard range is 5 to 75 ng/ml. (IBL; Immuno Biological Laboratories; http://www.ibl-hamburg.com).

The study was approved by the Regional Ethics Committee in Uppsala, Sweden (Dnr. 2000:001 and Dnr. 2009:345). Written study information was given to the patients, and all patients participating in the study gave a verbal consent. The verbal consent was approved by the ethical committee, and was documented in a questionnaire filled in by the patient or the researcher.

Statistical Analyses

Of the 92 measured proteins, the 78 proteins with less than 20% of the measured values below limit of detection (LOD) were included in the data analyses (Table 1).

Values for CEA measured using the ELISA kit were log-transformed before analysis. To avoid log of zero the transform $\log_2$ (CEA+ 1) was used.

The association between biomarkers and clinical parameters were measured univariately using Mann–Whitney test (gender, mucinous) or Spearman's correlation test (age, disease stage, tumor differentiation grade and CEA levels).

The association between levels of proteins and overall survival or time to recurrence was studied using Cox regression. For each protein a univariate Cox model is performed and summarized using the hazard ratio (HR) with 95% confidence interval and p-value. In addition, multivariate models with both protein level and clinical parameters as independent variable are computed and the association between survival/recurrence and protein level, adjusted for clinical parameters is assessed using the likelihood ratio test (p.lr). The clinical parameters included in the models are age and disease stage for the outcome overall survival and only disease stage for time to recurrence. Bonferroni's method for multiple testing correction was applied.

The recurrence or survival was illustrated with Kaplan–Meier curves, where the patients were divided into two groups with high or low protein levels using the median biomarker level as cut-off.

To investigate whether combination of more than one protein biomarker candidate did increase the prognostic significance, the most promising proteins were combined in a Cox regression model and a permutation test was adopted to check if the achieved association was stronger than expected by random.

Table 1. Proteins included in the Olink Oncology I panel sorted according to the short name and with the UniProt number given for identification

Long name	Short name	UniProt	Included in data analysis	Age	Gender	Disease stage	Differentiation grade	Mucinous histology	CEA
Adrenomedullin	AM	P35318	Yes	**3,49E-20**	0,92617	0,72735	0,02873	0,67277	0,00334
Amphiregulin	AR	P15514	Yes	**3,391E-06**	0,92226	0,11528	0,21812	0,21096	0,00248
B-cell activating factor	BAFF	Q9Y275	Yes	0,91083	0,06051	0,07610	0,05733	0,12110	0,53705
Betacellulin	BTC	P35070	No						
Ovarian cancer-related tumor marker CA 125	CA-125	Q8WXI7	No						
CA242 tumor marker	CA242	NA	No						
Carbonic anhydrase IX	CAIX	Q16790	Yes	0,00069	0,47516	0,97410	0,94444	0,02690	0,00458
Caspase-3	CASP-3	P42574	Yes	0,56527	0,18786	0,00128	0,05153	0,19824	0,00241
C-C motif chemokine 19	CCL19	Q99731	Yes	0,05049	0,34610	0,87958	0,08008	0,80637	0,06273
C-C motif chemokine 21	CCL21	O00585	Yes	0,28063	0,00266	0,14595	0,83141	0,98715	0,00850
C-C motif chemokine 24	CCL24	O00175	Yes	0,96578	0,55273	0,01597	0,76978	0,36390	0,02977
Tumor necrosis factor ligand superfamily member 8	CD30-L	P32971	Yes	0,26850	0,35114	0,97888	0,46516	0,52676	0,96319
CD40 ligand	CD40-L	P29965	Yes	0,83607	0,01342	0,00582	0,52538	0,11569	0,19351
Early activation antigen CD69	CD69	Q07108	Yes	0,00818	0,30036	0,00741	0,09338	0,00426	0,00250
Carcinoembryonic antigen	CEA	P06731	Yes	0,54055	0,77859	0,00012	0,48281	0,00109	**7,33E-102**
Macrophage colony-stimulating factor 1	CSF-1	P09603	Yes	0,00022	0,88912	0,57205	0,00816	0,13855	0,00108
Cystatin-B	CSTB	P04080	Yes	**7,886E-22**	0,88072	0,65541	0,16940	0,55341	0,00162
Cathepsin D	CTSD	P07339	Yes	0,00093	0,38429	0,07273	0,08670	0,09496	**7,252E-07**
C-X-C motif chemokine 10	CXCL10	P02778	Yes	**2,334E-10**	0,14283	0,78154	0,66320	0,83410	0,48876
C-X-C motif chemokine 11	CXCL11	O14625	Yes	0,00161	0,04333	0,00887	0,28002	0,08473	0,30461
C-X-C motif chemokine 13	CXCL13	O43927	Yes	**1,081E-09**	0,46852	0,91203	0,12193	0,03768	0,06175
C-X-C motif chemokine 5	CXCL5	P42830	Yes	0,70873	0,24760	0,37177	0,53957	0,79658	0,24615
C-X-C motif chemokine 9	CXCL9	Q07325	Yes	**8,542E-21**	0,56541	0,54938	0,08908	0,54116	0,16928
Epidermal growth factor	EGF	P01133	Yes	0,54590	0,27762	0,00190	0,67467	0,01591	0,19299
Epidermal growth factor receptor	EGFR	P00533	Yes	**1,406E-10**	0,99019	0,60245	0,53377	0,67444	0,10562
Extracellular matrix metalloproteinase inducer	EMMPRIN	P35613	Yes	0,00034	0,17781	0,66586	0,54466	0,67187	0,00862
Epithelial cell adhesion molecule	Ep-CAM	P16422	Yes	0,44307	0,93660	0,01392	0,53679	0,82782	0,30788
Erythropoietin	EPO	P01588	No						
Epiregulin	EPR	O14944	No						
Estrogen receptor	ER	P03372	No						
Receptor tyrosine-protein kinase erbB-2	ErbB2/HER2	P04626	Yes	0,03941	0,24925	0,37603	0,51445	0,50372	0,08963
Receptor tyrosine-protein kinase erbB-3	ErbB3/HER3	P21860	Yes	**8,384E-05**	0,00066	0,00069	0,80521	0,14831	0,21119
Receptor tyrosine-protein kinase erbB-4	ErbB4/HER4	Q15303	Yes	0,82819	0,83179	0,41870	0,95164	0,76564	0,33745
Fatty acid-binding protein, adipocyte	FABP4	P15090	Yes	**3,774E-09**	**1,788E-11**	0,28034	0,09066	0,95503	0,13437
Tumor necrosis factor receptor superfamily member 6	FAS	P25445	Yes	**3,84047E-14**	0,09023	0,01082	0,48624	0,25698	0,47818
Fas antigen ligand	FasL	P48023	Yes	0,55569	0,59287	0,84296	0,61888	0,98806	0,40235
Fms-related tyrosine kinase 3 ligand	Flt3L	P49771	Yes	0,24282	0,61635	0,77204	0,18778	0,26038	0,15515
Folate receptor alpha	FR-alpha	P15328	Yes	**1,014E-19**	0,43065	0,01288	0,20913	0,92390	0,26595
Follistatin	FS	P19883	Yes	**1,985E-05**	0,16060	0,98445	0,21999	0,92390	0,05947
Galectin-3	Gal-3	P17931	Yes	0,00776	0,01015	0,01083	0,31,996	0,06424	0,03338
Growth/differentiation factor 15	GDF-15	Q99988	Yes	**8,473E-10**	0,71091	0,03453	0,16352	0,83770	**7,016E-05**
Growth hormone	GH	P01241	Yes	0,01159	0,00861	0,48415	0,08031	0,02763	0,03192
Granulocyte-macrophage colony-stimulating factor	GM-CSF	P04141	No						
Granulocyte-macrophage colony-stimulating factor	HB-EGF	Q99075	Yes	0,50841	0,07316	0,04236	0,20270	0,56968	0,44013
Epididymal secretory protein E4	HE4	Q14508	Yes	**3,383E-31**	0,01152	0,10785	0,05111	0,88107	0,00674
Hepatocyte growth factor	HGF	P14210	Yes	0,00029	0,76283	0,06389	0,06963	0,42115	0,00022
Hepatocyte growth factor receptor	HGF receptor	P08581	Yes	0,50238	0,69615	0,88164	0,14970	0,90195	0,83913
Kallikrein-11	hK11	Q9UBX7	Yes	**9,316E-18**	0,00543	0,05690	0,12681	0,25784	0,01238
Interferon gamma	IFN-gamma	P01579	No						
Interferon gamma	IL-12	P29459/60	Yes	**7,216E-05**	0,13906	0,91534	0,20897	0,52228	0,35490
Interleukin-17 receptor B	IL-17RB	Q9NRM6	Yes	0,01113	0,03349	0,22225	0,37911	0,26720	0,49359
Interleukin-1 receptor antagonist protein	IL-1ra	P18510	Yes	0,04725	0,00019	0,01606	0,40775	0,33560	0,01576
Interleukin-2	IL-2	P60568	No						
Interleukin-2 receptor subunit alpha	IL-2RA	P01589	Yes	0,00038	0,09629	0,42806	0,30546	0,82064	0,07836
Interleukin-4	IL-4	P05112	No						
Interleukin-6	IL-6	P05231	Yes	**9,415E-05**	0,51447	0,05794	0,05714	0,02989	0,00011
Interleukin-6 receptor subunit alpha	IL-6RA	P08887	Yes	0,22498	0,54016	0,53,042	0,35189	0,25408	0,78588
Interleukin-7	IL-7	P13232	Yes	0,79912	0,51026	0,25951	0,59513	0,23885	0,82102
Interleukin-8	IL-8	P10145	Yes	0,00125	0,16531	0,04771	0,13796	0,21906	0,00182
Kallikrein-6	KLK6	Q92876	Yes	**4,571E-06**	0,01864	0,76124	0,62831	0,51184	0,17768
Latency-associated peptide transforming growth factor beta-1	LAP TGF-beta-1	P01137	Yes	**3,993E-05**	0,86392	0,02584	0,32598	0,97154	0,01379
Monocyte chemotactic protein 1	MCP-1	P13500	Yes	**0,000105**	0,31948	0,72352	0,77293	0,90291	0,10979
Melanoma-derived growth regulatory protein	MIA	Q16674	Yes	0,00041	0,03988	0,63645	0,45324	0,28398	0,54626
MHC class I polypeptide-related sequence A	MIC-A	Q29983	Yes	0,88835	0,27650	0,03513	0,51366	0,11723	0,01091
Midkine	MK	P21741	Yes	**2,672E-07**	0,35242	0,04709	0,14505	0,16343	0,00024
Matrix metalloproteinase-3	MMP-3	P08254	No						
Myeloperoxidase	MPO	P05164	Yes	0,01591	0,63493	0,09327	0,32617	0,02962	0,02671
Myeloid differentiation primary response protein MyD88	MYD88	Q99836	No						
Osteoprotegerin	OPG	O00300	Yes	**8,566E-23**	0,14780	0,45434	0,26413	0,59415	0,00043
Platelet-derived growth factor subunit B	PDGF subunit B	P01127	Yes	0,49582	0,25643	0,05685	0,50756	0,38431	0,62864
Platelet endothelial cell adhesion molecule	PECAM-1	P16284	Yes	0,04489	0,94508	0,27446	0,46361	0,58699	0,00061
Placenta growth factor	PlGF	P49763	Yes	**1,141E-18**	0,00259	0,84511	0,77597	0,78770	0,00105
Prolactin	PRL	P01236	Yes	0,40948	0,32592	0,04299	0,08377	0,17376	0,80941

TABLE 1 (*continued*)

Long name	Short name	UniProt	Included in data analysis	Age	Gender	Disease stage	Differentiation grade	Mucinous histology	CEA
Prostasin	PRSS8	Q16651	Yes	0,00048	**2,256E-07**	0,51364	0,92548	0,51110	0,00264
Prostate-specific antigen	PSA	P07288	No						
Regenerating islet-derived protein 4	REG-4	Q9BYZ8	Yes	**4,921E-09**	1,00000	0,62831	0,23509	**1,567E-05**	0,00011
Stem cell factor	SCF	P21583	Yes	0,09993	0,30924	0,17574	0,75212	0,26038	0,00033
E-selectin	SELE	P16581	Yes	0,00209	0,14824	0,03126	0,27168	0,15854	**1,115E-06**
Tissue factor	TF	P13726	Yes	**2,509E-14**	0,15361	0,03113	0,76205	0,73944	0,19597
Transforming growth factor alpha	TGF-alpha	P01135	Yes	**2,505E-09**	0,67218	0,25309	0,25537	0,71954	0,00038
Thrombopoietin	THPO	P40225	Yes	0,77874	0,03333	0,02902	0,30737	0,26182	0,93534
Angiopoietin-1 receptor	TIE2	Q02763	Yes	0,00138	0,88071	0,02018	0,80873	0,01405	0,00014
Tumor necrosis factor	TNF	P01375	No						
Tumor necrosis factor receptor 1	TNF-R1	P19438	Yes	**3,664E-16**	0,13451	0,91212	0,07303	0,28813	0,00135
Tumor necrosis factor receptor 2	TNF-R2	P20333	Yes	**2,425E-14**	0,11501	0,92249	0,05306	0,70327	0,00435
Tumor necrosis factor receptor superfamily member 4	TNFRSF4	P43489	Yes	**2,411E-10**	0,33579	0,30376	0,22775	0,91349	0,00018
Tumor necrosis factor ligand superfamily member 14	TNFSF14	O43557	Yes	0,18575	0,03336	0,37711	0,10951	0,02842	0,00022
Tartrate-resistant acid phosphatase type 5	TR-AP	P13686	Yes	0,32665	0,00092	0,00653	0,41962	0,19903	0,06662
Urokinase plasminogen activator surface receptor	U-PAR	Q03405	Yes	**1,330E-16**	0,34817	0,65382	0,03048	0,20760	**1,088E-05**
Vascular endothelial growth factor A	VEGF-A	P15692	Yes	**3,727E-09**	0,73671	0,34751	0,06310	0,48770	0,00288
Vascular endothelial growth factor D	VEGF-D	O43915	Yes	0,98837	0,17266	0,99152	0,43626	0,42716	0,98259
Vascular endothelial growth factor receptor 2	VEGFR-2	P35968	Yes	**9,449E-06**	0,13342	0,87055	0,73303	0,38426	0,66257

Association of proteins analyzed with PEA with clinical and histopathological parameters, in patients with diseases stage II-IV colorectal cancer, is demonstrated for successful analyses. $P < .000107$ are marked in bold text

Overall survival was measured from the date of surgery to the date of death from all causes. Time to recurrence was measured for disease stage II and III, from the date of surgery to the date of diagnosis of distant recurrence or to the date of death due to CRC, and censored at the date of death due to reasons other than CRC or at the last follow up. A second primary CRC/non-CRC was not regarded as a recurrence.

Results

Patient Characteristics

Of the 270 patients included in this study, samples from 9 patients were excluded due to low sample quality or technical reasons. The remaining 261 samples consisted of samples from 130 females and 131 males, with a median age of 70.5 (range 34–95) years. The cohort composed of 181 colonic and 80 rectal cancer patients. Disease stage II accounted for 127 cases, while 92 were stage III and 42 stage IV. The median follow-up time of surviving patients were 13 years (range 11.5–14.8) for which disease recurrences were observed for 18 patients with stage II (14%) and 39 patients with stage III (42%). Total of 173 patients were deceased (66%).

Protein detection

Samples were assessed for 92 proteins using the Multiplex Olink Oncology I panel (Table 1). There were no missing values for 68 proteins and 10 proteins had less than 20% missing values due to non-detectable levels of the proteins. These 78 proteins were used for further bio-statistical analyses, while the 14 proteins with higher missing value percentages were excluded from the analyses (Table 1).

Association between the protein biomarker candidates, the clinical parameters and CEA

In these comparisons the number of tests performed were 78*6 = 468, hence the significance threshold was set to $P = .05/468 = .000107$ according to Bonferroni's method.

Statistical significant association with age was observed for 32 proteins, while two proteins were associated with gender (Table 1). One protein was found to be associated with mucinous histology, and five proteins with CEA levels. No protein was found to be associated with disease stage or tumor differentiation grade (Table 1).

The CEA levels measured with PEA in this study had a strong correlation with the CEA value measured earlier using ELISA (Figure 1).

Association Between the Protein Levels and Overall Survival

With 78 proteins analyzed, the p-value threshold after multiple testing correction was set to 0.05/78 = 0.000641 (calculated based on Bonferroni's method). According to univariate Cox regression, 31 proteins were significantly associated with overall survival (Table 2). However, when the likelihood ratio *P*-value (p.lr) was calculated for overall survival, adjusting for age and disease stage, only one marker,

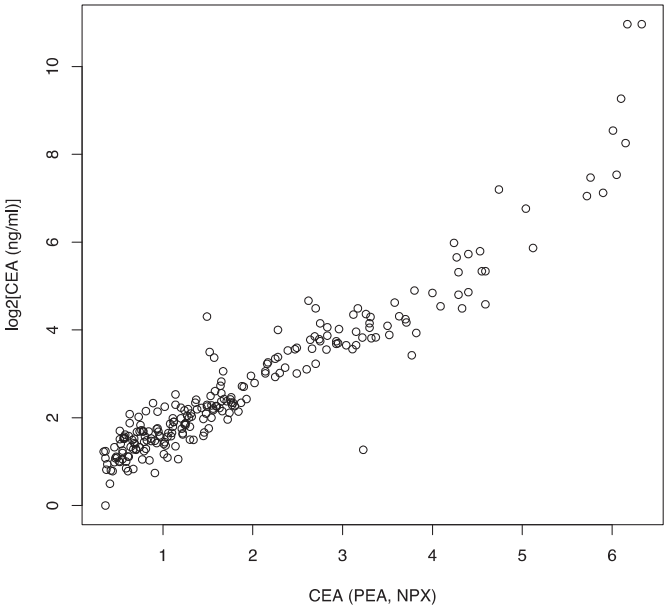

Figure 1. Comparison of carcinoembryonic antigen (CEA) measurements performed with conventional Solid-Phase-Enzyme--Linked immunosorbent assay (ELISA)(y-axis) compared to proximity extension assay (PEA) (x-axis).

Table 2. Association of proteins levels with overall survival in patients with colorectal cancer (n = 261)

Long name	Short name	HR	l95	u95	p	p.lr
Adrenomedullin	AM	2.59	1.87	3.61	**1.491E-08**	0.0111
Amphiregulin	AR	1.49	1.28	1.73	**3.964E-07**	0.1812
B-cell activating factor	BAFF	1.32	0.92	1.90	0.12970	0.2739
Carbonic anhydrase IX	CAIX	1.19	0.98	1.43	0.07242	0.3842
Caspase-3	CASP-3	1.08	0.93	1.24	0.31013	0.9988
C-C motif chemokine 19	CCL19	1.18	1.00	1.40	0.05700	0.3188
C-C motif chemokine 21	CCL21	1.11	0.70	1.77	0.64948	0.4311
C-C motif chemokine 24	CCL24	1.12	0.94	1.33	0.20840	0.8201
Tumor necrosis factor ligand superfamily member 8	CD30-L	1.16	0.77	1.76	0.47228	0.6606
CD40 ligand	CD40-L	1.18	1.01	1.37	0.03420	0.1227
Early activation antigen CD69	CD69	1.23	1.03	1.47	0.02266	0.4955
Carcinoembryonic antigen	CEA	1.26	1.14	1.40	**1.16332E-05**	0.0082
Macrophage colony-stimulating factor 1	CSF-1	2.55	1.49	4.35	**0.00062**	0.0101
Cystatin-B	CSTB	1.86	1.51	2.29	**4.80892E-09**	0.1298
Cathepsin D	CTSD	1.42	1.19	1.70	**9.35197E-05**	0.1091
C-X-C motif chemokine 10	CXCL10	1.28	1.11	1.47	**0.00049**	0.3855
C-X-C motif chemokine 11	CXCL11	1.23	1.06	1.42	0.00777	0.6960
C-X-C motif chemokine 13	CXCL13	1.24	1.06	1.45	0.00594	0.5174
C-X-C motif chemokine 5	CXCL5	1.00	0.85	1.16	0.96249	0.7264
C-X-C motif chemokine 9	CXCL9	1.33	1.15	1.55	**0.00018**	0.9816
Epidermal growth factor	EGF	1.06	0.92	1.22	0.44618	0.8578
Epidermal growth factor receptor	EGFR	0.23	0.14	0.39	**2.84429E-08**	0.0028
Extracellular matrix metalloproteinase inducer	EMMPRIN	2.58	1.26	5.27	0.00920	0.2932
Epithelial cell adhesion molecule	Ep-CAM	1.08	0.94	1.25	0.26643	0.7436
Receptor tyrosine-protein kinase erbB-2	ErbB2/HER2	0.94	0.58	1.52	0.80336	0.5156
Receptor tyrosine-protein kinase erbB-3	ErbB3/HER3	0.73	0.39	1.37	0.32756	0.3980
Receptor tyrosine-protein kinase erbB-4	ErbB4/HER4	0.96	0.57	1.61	0.86894	0.7498
Fatty acid-binding protein. Adipocyte	FABP4	1.49	1.25	1.79	**9.48259E-06**	0.0288
Tumor necrosis factor receptor superfamily member 6	FAS	1.30	1.05	1.62	0.01768	0.3298
Fas antigen ligand	FasL	0.94	0.57	1.54	0.80033	0.6045
Fms-related tyrosine kinase 3 ligand	Flt3L	1.06	0.80	1.40	0.67798	0.9860
Folate receptor alpha	FR-alpha	2.52	1.60	3.95	**6.11404E-05**	0.2137
Follistatin	FS	1.80	1.26	2.58	0.00135	0.1898
Galectin-3	Gal-3	2.12	1.46	3.08	**7.24835E-05**	0.1979
Growth differentiation factor 15	GDF-15	1.42	1.26	1.60	**1.16265E-08**	0.0207
Growth hormone	GH	1.16	1.07	1.26	**0.00028**	0.0137
Granulocyte-macrophage colony-stimulating factor	HB-EGF	1.31	0.93	1.84	0.12002	0.3826
Epididymal secretory protein E4	HE4	2.77	2.05	3.75	**2.90167E-11**	0.0035
Hepatocyte growth factor	HGF	1.86	1.45	2.39	**1.07096E-06**	0.0021
Hepatocyte growth factor receptor	HGF receptor	0.82	0.22	3.11	0.77106	0.9033
Kallikrein-11	hK11	1.92	1.43	2.58	**1.59062E-05**	0.1055
Interferon gamma	IL-12	1.13	0.93	1.37	0.23144	0.4726
Interleukin-17 receptor B	IL-17RB	1.46	1.09	1.96	0.01153	0.0107
Interleukin-1 receptor antagonist protein	IL-1ra	1.39	1.11	1.72	0.00334	0.1613
Interleukin-2 receptor subunit alpha	IL-2RA	2.11	1.16	3.84	0.01463	0.3790
Interleukin-6	IL-6	1.29	1.16	1.44	**2.62106E-06**	0.0225
Interleukin-6 receptor subunit alpha	IL-6RA	0.95	0.65	1.38	0.77194	0.0977
Interleukin-7	IL-7	0.83	0.53	1.29	0.39981	0.2682
Interleukin-8	IL-8	1.14	1.03	1.28	0.01506	0.0803
Kallikrein-6	KLK6	1.90	1.29	2.79	0.00115	0.1824
Latency-associated peptide transforming growth factor beta-1	LAP TGF-beta-1	3.61	2.17	5.99	**6.90076E-07**	0.0052
Monocyte chemotactic protein 1	MCP-1	1.55	1.19	2.01	0.00097	0.0080
Melanoma-derived growth regulatory protein	MIA	1.22	0.81	1.84	0.33798	0.1350
MHC class I polypeptide-related sequence A	MIC-A	1.37	1.05	1.78	0.02055	0.1309
Midkine	MK	1.49	1.24	1.79	**1.75876E-05**	0.0171
Myeloperoxidase	MPO	1.47	1.06	2.03	0.01992	0.8639
Osteoprotegerin	OPG	3.33	2.38	4.66	**1.84908E-12**	**0.0003**
Platelet-derived growth factor subunit B	PDGF subunit B	1.07	0.91	1.25	0.41675	0.5598
Platelet endothelial cell adhesion molecule	PECAM-1	1.43	0.99	2.05	0.05674	0.3641
Placenta growth factor	PlGF	2.40	1.75	3.30	**6.24302E-08**	0.1707
Prolactin	PRL	1.10	0.91	1.32	0.33265	0.5899
Prostasin	PRSS8	1.97	1.38	2.82	**0.00020**	0.0271
Regenerating islet-derived protein 4	REG-4	1.51	1.22	1.86	**0.00014**	0.0129
Stem cell factor	SCF	0.88	0.69	1.13	0.31350	0.0831
E-selectin	SELE	1.08	0.90	1.31	0.40644	0.2589
Tissue factor	TF	2.36	1.60	3.49	**1.51693E-05**	0.0349
Transforming growth factor alpha	TGF-alpha	1.94	1.43	2.61	**1.58735E-05**	0.0417
Thrombopoietin	THPO	1.30	0.85	2.00	0.23054	0.5644
Angiopoietin-1 receptor	TIE2	1.13	0.65	1.98	0.65863	0.2310
Tumor necrosis factor receptor 1	TNF-R1	2.40	1.73	3.33	**1.68115E-07**	0.0153
Tumor necrosis factor receptor 2	TNF-R2	1.93	1.50	2.48	**3.01838E-07**	0.0263
Tumor necrosis factor receptor superfamily member 4	TNFRSF4	2.23	1.56	3.17	**9.16646E-06**	0.0970
Tumor necrosis factor ligand superfamily member 14	TNFSF14	1.20	0.94	1.52	0.14658	0.1851
Tartrate-resistant acid phosphatase type 5	TR-AP	1.36	0.94	1.99	0.10626	0.8858

TABLE 2 (*continued*)

Long name	Short name	HR	l95	u95	p	p.lr
Urokinase plasminogen activator surface receptor	U-PAR	4.66	2.90	7.49	**1.9837E-10**	0.0007
Vascular endothelial growth factor A	VEGF-A	2.46	1.76	3.45	**1.72093E-07**	0.0169
Vascular endothelial growth factor D	VEGF-D	1.30	0.89	1.92	0.17811	0.1677
Vascular endothelial growth factor receptor 2	VEGFR-2	0.48	0.26	0.90	0.02191	0.5169

Significant associations are marked in bold; the significance threshold is set to 0.05/78 = 0.000641 according to Bonferroni's methods for multiple testing correction.

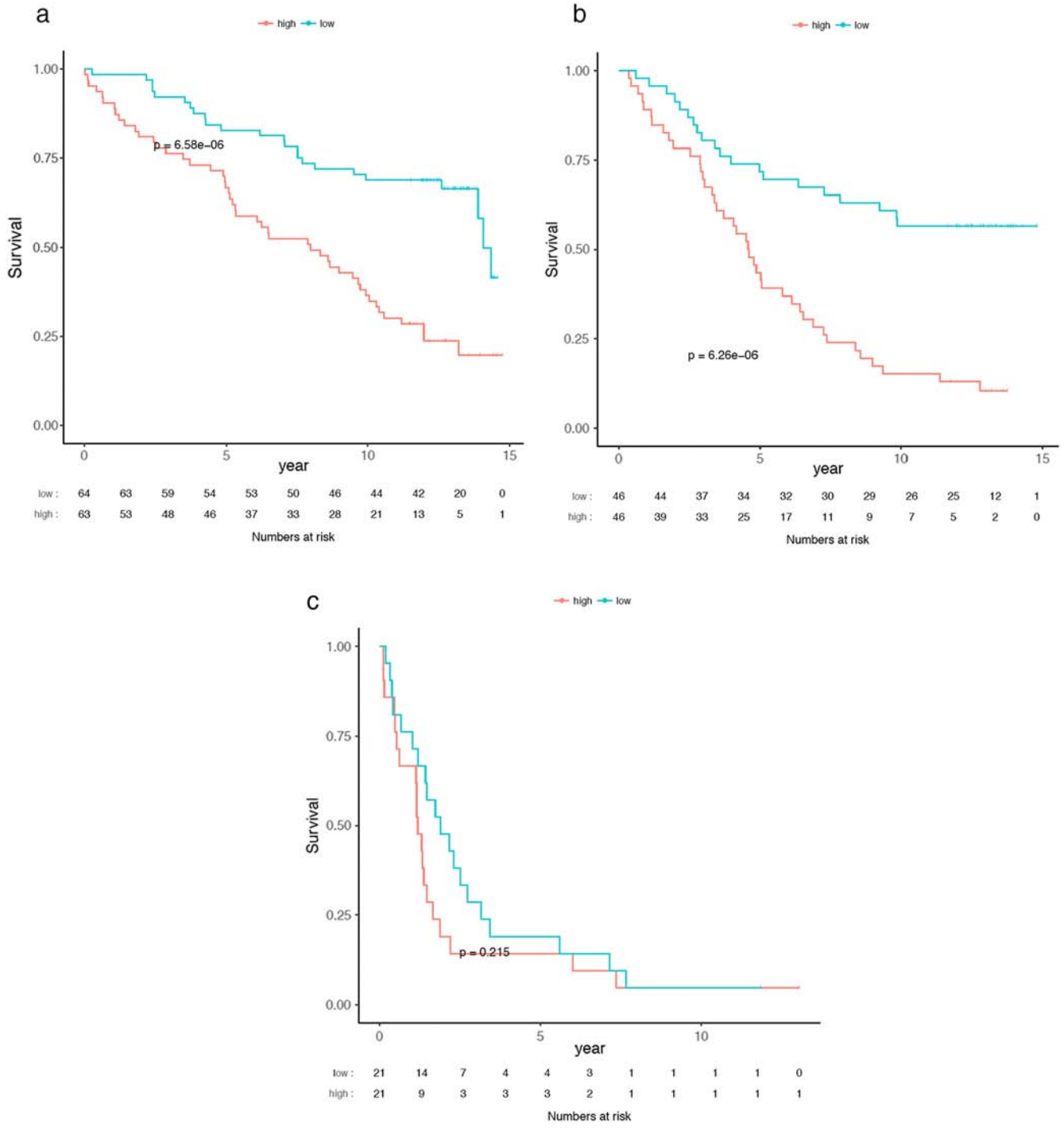

Figure 2. Kaplan Meier curves for osteoprotegerin representing overall survival in disease stages II-IV using the median value of the protein levels as the cut-off between the high and low groups. (A) Osteoprotegerin disease stage II. (B) Osteoprotegerin disease stage III. (C) Osteoprotegerin disease stage IV.

Table 3. Association of protein levels with time to recurrence in patients with colorectal cancer

	HR	l95	u95	p	p.lr
Disease stage II and III					
EGFR	0.43	0.18	1.03	0.059	0.020
GDF-15	1.35	1.08	1.69	0.008	0.021
MIC-A	1.57	0.96	2.54	0.070	0.037
CXCL10	0.77	0.56	1.04	0.091	0.039
IL-6	1.24	1.02	1.50	0.031	0.047
SCF	0.72	0.47	1.09	0.120	0.050
FABP4	1.41	1.03	1.91	0.030	0.090
HGF receptor	0.15	0.01	1.49	0.104	0.105
Disease stage II					
HGF receptor	0.01	0.00	0.27	0.009	0.009
EGFR	0.29	0.05	1.59	0.155	0.156
GDF-15	0.87	0.52	1.46	0.606	0.599
MIC-A	1.10	0.45	2.70	0.828	0.827
CXCL10	0.75	0.40	1.39	0.359	0.346
IL-6	1.20	0.84	1.73	0.312	0.333
SCF	0.84	0.40	1.77	0.643	0.649
FABP4	0.85	0.45	1.60	0.612	0.609
Disease stage III					
GDF-15	1.44	1.13	1.83	0.003	0.005
FABP4	1.49	1.05	2.10	0.024	0.028
MIC-A	1.84	1.06	3.19	0.030	0.024
SCF	0.57	0.34	0.96	0.036	0.047
EGFR	0.42	0.16	1.10	0.076	0.079
CXCL10	0.75	0.54	1.04	0.082	0.073
IL-6	1.22	0.97	1.54	0.083	0.085
HGF receptor	0.95	0.05	18.60	0.972	0.972

HR: hazard ratio, l95: lower 95% confidence interval, p95: upper 95% confidence interval, p.lr: p-value calculated from the likelihood ratio.

osteoprotegerin, met the significance threshold (p.lr = .00029; Table 2). See also separate Kaplan–Meier survival analysis on osteoprotegerin for disease stages II, III and IV (Figure 2, *A–C*).

Seven other proteins could be found to have a trend for association with overall survival defined as p.lr < .01 (Table 2).

Combinations of biomarkers according to the description written in material and methods did not result in improved overall survival prediction (data not shown).

Association Between the Protein Levels and Time to Recurrence

No statistical significant associations were observed between the levels of the 78 proteins analyzed and the time to recurrence with univariate Cox regression, when the significance threshold was set to 0.05/78 = 0.000641 (Bonferroni). However, eight proteins with p.lr < .05 in both disease stage II and III, or separately in disease stage II or III, revealed a trend in the association with time to recurrence (Table 3).

Hepatocyte growth factor receptor was the only protein with p.lr < .05, in disease stage II, with low protein levels associated with higher risk of recurrence (Figure 3*A*), however in both disease stages as well as in disease stage III only, no trend with disease recurrence could be seen.

In disease stage III only growth differentiation factor 15 (GDF-15) had p.lr < .01 (Figure 3*B*), and fatty acid-binding protein, adipocyte (FABP4) (Figure 3*C*), MHC class I polypeptide-related sequence A (MIC-A) and stem cell factor (SCF) had a p.lr < .05.

Although not statistically significant in the Cox regression analysis, C-X-C motif chemokine

(CXCL10) had an interesting Kaplan–Meier curve with many early recurrences seen in patients with low CXCL10 levels in disease stage III (Figure 3*D*).

Combinations of biomarkers according to the description in Materials and Methods did not improve the predictive value of the biomarkers in regard of time to recurrence (data not shown).

Discussion

The present study reveals that there are several soluble protein biomarker candidates of interest in the prediction of survival and disease recurrences in patients with CRC. However, only one protein, osteoprotegerin, did show a statistical significant association with survival.

Overall the biomarker candidates had a stronger non-significant association with overall survival than time to recurrence, which could be due to the fact that there are more endpoints to calculate on when overall survival is used, as the number of deaths exceeds the number of recurrences in this patient cohort. Another explanation could be that the protein expression levels may reflect other conditions leading to death not caused by the CRC or age [22].

The same observation, with stronger non-significant association of biomarker candidates with prognosis in disease stage III compared with disease stage II, was observed. Also here, an explanation could be that there are more recurrences in disease stage III than in disease stage II, generating more endpoints in disease stage III. A more likely explanation is that disease stage III patients do already have more disseminated disease, generating higher levels of these proteins from the tumor itself, or due to the response of the immune system.

Osteoprotegerin was the only protein with a significant association with overall survival after correction for age and disease stage. This association was found to be strongest in disease stage III. However, it could not be associated with disease recurrence. As the name indicates, osteoprotegerin is a protein with a role in bone homeostasis; it is also named as tumor necrosis factor receptor superfamily member 11B (TNFRSF11B). The difference between osteoprotegerin and the other members of the TNF receptor family is its lack of a trans membrane domain, resulting in osteoprotegerin acting as a decoy receptor, neutralizing TNF-related apoptosis inducing ligand function by binding to it [23]. The protein inhibits apoptosis by binding to cell death receptors 4 and 5 [23,24] and is in CRC cells regulated by β-catenin [25]. Expression of osteoprotegerin has been demonstrated to be involved in distant metastases in previous studies [23,26,27]. Using immunohistochemical analysis of tumor tissues, it has been demonstrated that overexpression of osteoprotegerin is associated with recurrence of CRC [23]. The protein has also been studied as a potential target for treatment of CRC with antibodies that antagonize osteoprotegerin, thus increasing tumor cell sensitivity to TNF-related apoptosis inducing ligand, and has successfully been used in animal models to treat tumor-induced bone disease [23,24]. One study has revealed that high serum levels of osteoprotegerin in patients with stage IV CRC was associated with poor prognosis [28] and one clinical trial has reported the use of osteoprotegerin construct in the treatment of cancer patients [29]. Increase in osteprotegerin during neoadjuvant therapy for advanced rectal cancer has on the other hand been associated with better progression free survival [30].

It is more likely that the protein biomarkers are related to the tumor disease itself if a prognostic effect is seen in time to recurrence than overall survival only [31]. Using correction for multiple comparisons none of the biomarker candidates did meet the *P*-value threshold set by the Bonferroni method. However there are some proteins worth of mention showing a trend of association with recurrence.

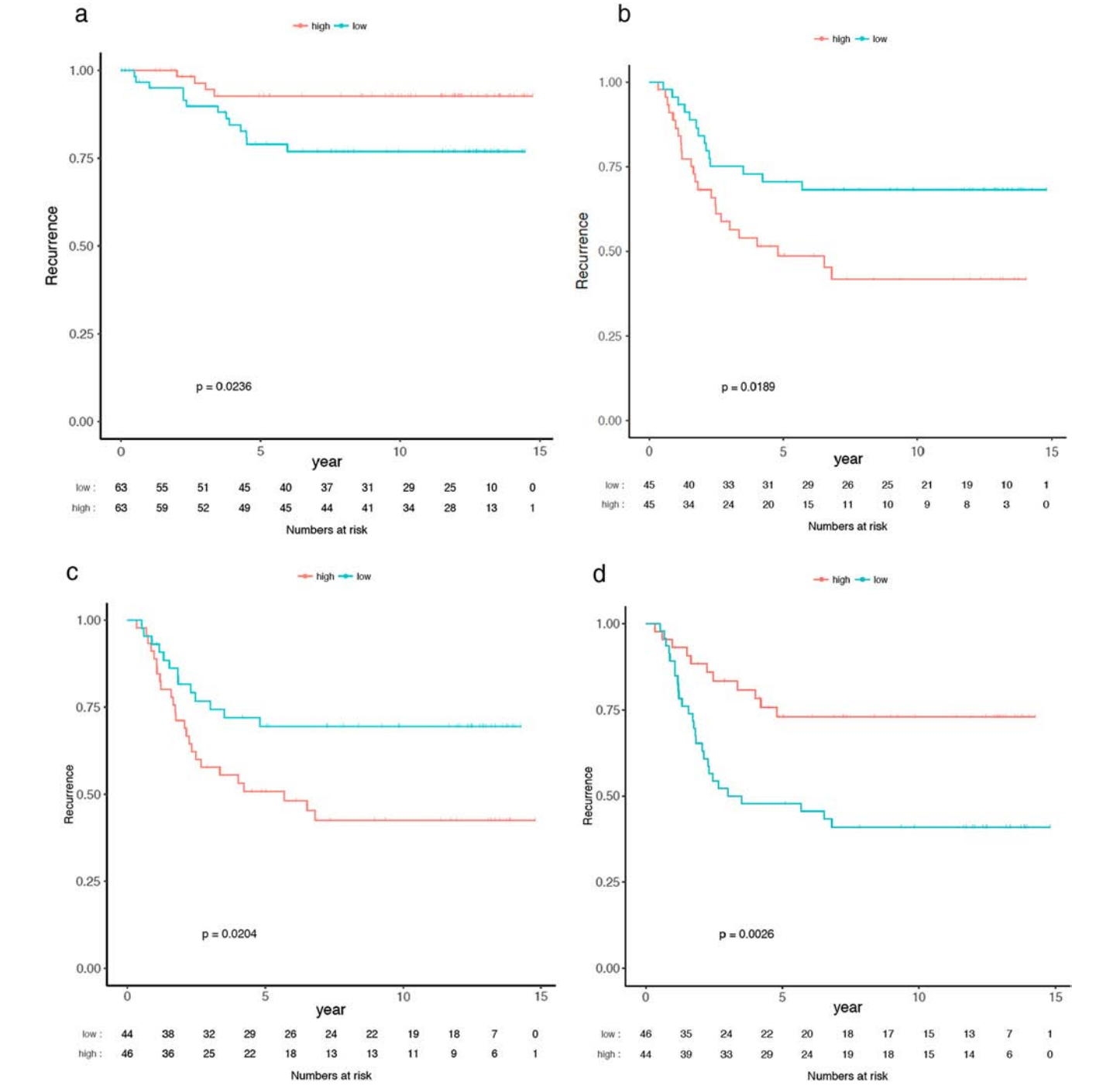

Figure 3. Kaplan Meier curves representing time to recurrence for hepatocyte growth factor receptor (HGF receptor) disease stages II (3a), growth differentiation factor 15 (GDF-15) disease stages III (3b) and fatty acid-binding protein, adipocyte (FABP4) disease stages III (3c) and C-X-C motif chemokine (CXCL10) III disease stages (3d).

GDF-15 is a biomarker studied previously on the present cohort using immunohistochemistry on the primary tumor, which revealed that moderate to high staining intensity was related to higher risk for recurrences compared with none or low staining intensity [14]. In the present study GDF-15 was related to recurrence in disease stages II and III when analyzed together and in disease stage III when analyzed separately, but not in disease stage II.

In current study, low levels of hepatocyte growth factor receptor, also known as c-MET revealed a trend of higher risk of recurrence in disease stage II, but not in disease stage III or disease stage II and III analyzed together. Increased expression of c-MET measured by immunohistochemistry on the primary tumor is associated with worse prognosis in CRC [32]. C-MET inhibitors are now used in clinical trial as a therapeutic agent against several cancer types including CRC [33].

Another observation from the data presented in this study was that a significant association with age was observed for 32 protein biomarkers. This is confirmed by other recent studies, using PEA, demonstrating altered levels of proteins correlated to the age of the

individuals [34,35]. Age should therefore be included as a variable in multivariate analysis during survival calculations based on biomarker levels in blood, as was done in the present study. Storage time, storage temperature and sample handling are other factors that may affect the levels of protein abundance [34,36]. The protein measurements were made more than 10 years after the biobanking of the plasma in the present cohort, and it was sampled in a period of 3 years so it is possible that the storage time can affect the protein abundance between the individuals in this cohort.

In this study, Bonferroni's method for corrections of multiple testing was used, but in order to not miss potential biomarkers of interest; it was motivated to discuss some of the markers revealing only a trend when it came to the prognostic associations. However, as with all biomarker studies, the results have to be verified by independent cohorts to assure the true value of the findings herein.

Conclusions

Of the 92 analyzed plasma proteins, osteoprotegerin demonstrated the strongest prognostic impact in patients with colorectal cancer, suggesting osteprotegerin as a potential predictive marker and also a plausible target for treatments.

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