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Diagnostic Performance of a Novel Multiplex Immunoassay in Colorectal Cancer

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Abstract. *Background/Aim: We evaluated the diagnostic performance of a newly-launched magnetic bead-based multiplex immunoassay panel including cancer, apoptotic, immunological and angiogenesis biomarkers for differential diagnosis of colorectal cancer (CRC). Patients and Methods: Serum samples of 106 individuals comprising of 35 patients with CRC (23 colon cancer, 12 rectal cancer), 20 with respective benign colorectal diseases and 51 healthy controls were analyzed by the Milliplex™ MAP Human Circulating Cancer Biomarker Panel 1 run on the Bio-Plex™ 200 System. Results: IL-8, CEA, HGF, TNFα, CYFRA 21-1, OPN, TGFα, CA 19-9, CA 125, AFP and sFas showed significantly higher levels in cancer samples compared to healthy controls. It is noteworthy that comparing CRC and benign colorectal disease samples, many immunological and cell death markers were elevated as well. Exclusively, six markers were distinguished significantly between both groups: CEA showed the best performance in differential diagnosis reaching an AUC of 0.859 in ROC curve followed by CA 19-9, CYFRA 21-1, IL-8, CA 125 and OPN reaching AUCs between 0.696 and 0.744. Correlation with tumor stage was found for CEA, sFas and CYFRA 21-1. Finally marker scores were assembled showing that a combination of CEA and CA 19-9 had a higher AUC (0.893) compared to the biomarkers alone. Conclusion: Differential diagnosis of CRC can be improved by new biomarker classes and their combination assessed by novel multiplex immunoassay.*

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Colorectal cancer (CRC) is one of the most common cancers worldwide. With an estimated incidence of 447,000 new cases and 215,000 cancer related deaths in 2012, it is the second most common cancer in Europe (1). However, the treatability of CRC has been improved over the last decades resulting in a relative 5-year survival of 66.1% in 2005-2011 compared to only 49.8% in 1975-1977 (2). Especially the implementation of more efficient adjuvant chemotherapies in stage II-III CRC in the 1990s had a great impact by increasing the prognosis in these stages up to a mean 5-year survival of 66% (3). In the last decade, neoadjuvant and radiochemo-therapeutic regimes, as well as targeted therapies led to a further decrease of death rates of approximately 3% per year (4). But still only slightly more than 10% will survive a stage IV cancer more than 5 years (5, 6).

Screening programs for early diagnosis of CRC have been implemented in many western countries. In particular, colonoscopy and (immunochemical) fecal occult blood testing (FOBT and FIT) have demonstrated to decrease CRC-associated mortality (7-9). However, colonoscopy is an invasive procedure; therefore only a small percentage of individuals at-risk is willing to undergo this exam (10). FOBT is non-invasive, cost-efficient and well validated (LOE 1a) but it is limited by its low sensitivity for CRC (<50%) – especially for adenomas and early stage cancers – and its low specificity (11). The fecal immunochemical test (FIT) provides considerable advantages with its higher accuracy (7, 8). While screening programs have significantly reduced cancer mortality, still nearly half of CRCs are diagnosed at advanced stages III or IV (12, 13).

In contrast to screening in asymptomatic individuals, differential diagnosis approaches become relevant in patients presenting with specific cancer-related signs or symptoms. Markers should be sensitive, tumor- and organ-specific, distinguish malignant from benign lesions and correlate with tumor stage and prognosis (14). For both settings, blood-based biomarkers are promising diagnostic tools as they are easily obtained, robust, cost-efficient and mirror biochemical cancer characteristics. But still serum biomarkers are not recognized as relevant tools in cancer detection and definitive diagnosis of colorectal cancer is still done by imaging and histopathological assessment (14). Although discovered more than 50 years ago (15), carcinoembryogenic antigen (CEA) is the only marker recommended for supporting diagnosis and therapy monitoring by different societies like the National Academy of Clinical Biochemistry (NACB) (16), the European Group on Tumor Markers (EGTM) (17) and the American Society of Clinical Oncology (ASCO) (16).

Accuracy of cancer detection can be improved by assembling oncological and other biomarkers in patterns as already seen in CRC and other cancers (18-20). As development and progression of cancer disease is considered a complex process involving diverse cell death, proliferation and growth pathways, as well as an interaction with the tumor microenvironment and immunological reactions of the host, biomarkers reflecting these aspects are promising candidates for these panels (21-23).

A newly-launched Human Circulating Cancer Biomarker Multiplex Immunoassay enables parallel biomarker measurements and gives the possibility to depict this complex and dynamic interaction with the promise of being a faster, less volume wasting and more inexpensive tool (23). It comprises a widespread spectrum of established tumor markers such as CEA and cancer antigen 19-9 (CA 19-9), upcoming auspicious oncological markers as cytokeratin 19-fragments (CYFRA 21-1), soluble cell death markers sFas, its ligand sFasL and the tumor necrosis factor related apoptosis-inducing ligand (TRAIL), the angiogenetic marker vascular endothelial growth factor (VEGF) and immunological biomarkers like the growth proliferating cytokine stem cell factor (SCF), the interleukines 6 (IL-6) and 8 (IL-8), the macrophage migration inhibitory factor (MIF) and the tumor necrosis factor alpha (TNFα) (24-32).

Until now, there are no guidelines for multiplex assay configuration and implementation of these assays into clinical routine diagnostics failed due to technical and operational challenges (33). Many assays are for research-use-only (RUO) with not declared reagent stability that prevails high-quality *in vitro* diagnostic. Further problems are possible cross-reactivity and validation of these assays only in small clinical studies (34, 35).

As a first step of assay evaluation, we assessed the methodical quality and pre-analytical robustness of the cancer biomarker multiplex assay and found a good performance for most markers (36). On this steady bedrock we performed a clinical investigation testing the relevance of the included biomarkers for differential diagnosis in CRC and their correlation with clinical cancer characteristics.

Finally, we investigated marker combination scores including the best performing biomarkers to improve the sensitivity and specificity over those reached by CEA alone in clinical routine measurements.

Materials and Methods

Subjects and sample collection. Serum was collected from 106 individuals comprising of 35 patients with colorectal cancer (23 colon and 12 rectal cancer), 20 with respective benign colorectal diseases and 51 healthy controls. Among cancer patients, 21 had metastatic and 14 non-metastatic disease (one patient with TIS) (Table I).

Samples were taken at time of cancer disease before surgery or chemotherapy was performed. As the relevant control group, samples of patients suffering from benign colorectal diseases, *e.g.* chronic diseases like polyposis syndromes, non-hereditary polyps, inflammatory diseases like chronic or acute diverticulitis and colitis ulcerosa were included. In addition, serum of 15 male and 36 female healthy individuals was considered as control group. Pregnant women were excluded as well as persons with a cancer history. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (Nr. 319/12) and informed consent was obtained from study participants.

The patient sera were collected prospectively in the Department of Surgery and other Clinics of the Center of Integrated Oncology (CIO) Köln/Bonn at the University Hospital Bonn between 2010 and 2012. Transport, handling and storage of the samples were done in a standardized way in the Biofluid Biobank of University Hospital Bonn. In particular, blood was centrifuged at 3300g for 10 min, aliquoted in polypropylene tubes and stored at –80˚C until use. The history of every patient was thoroughly filed and information was stored pseudonymised.

Multiplex immunoassay. To determine biomarker concentrations, all samples were measured by use of the MILLIPLEX™ MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1, 96 Well Plate Assay, Cat. # HCCBP1MAG-58K (Merck Millipore, Billerica, MA) run on the Bio-Plex™ 200 system (Bio-Rad, Hercules, CA). Quality controls (QC1 and QC2), as well as a calibration curve based on 1:3 dilutions of the highest standard were used for quantification and as internal controls for intra- and inter-assay reproducibility. In addition, serum pools, produced as previously described, were used as external physiological controls (36). The detailed method and procedure are reported in Hermann *et al.* (36). Roughly summarized, it is a magnetic bead suspension multiplex assay methodologically based on flow cytometry.

In every bead, two fluorescent dyes are set, whose proportion to each other creates 100 distinguishable possibilities. On beads with identical characteristics a capture antibody is fixed covalently. The analyzed panel includes 24 biomarkers: the oncological biomarkers carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), total prostate-specific antigen (total-PSA), cancer antigen 15-3 (CA 15-3), cancer antigen 19-9 (CA 19-9), cancer antigen 125 (CA 125),

Table I. *Patient characteristics*.

Group	Total	Male	Female	Median age	(range) years
Healthy controls	51	15	36	39.4	(20.1-78.1)
Benign colorectal disease	20	8	12	54.7	(24.2-89.8)
Colon	18	8	10	56.3	(24.2-89.8)
Rectum	2	0	2	42.6	(39.4-46.0)
Colorectal cancer	35	22	13	69.4	(19.6-86.3)
Colon	23	13	10	68.0	(19.6-80.0)
Rectum	12	9	3	69.9	(46.0-86.3)
UICC stage					
0	1	1	0	74.2	
I	3	2	1	51.0	(47.8-61.5)
II	5	3	2	70.5	(57.7-73.7)
III	5	3	2	74.3	(68.1-82.0)
IV	21	13	8	73.5	(63.7-79.9)
All subjects	106				

cytokeratin 19-fragments (CYFRA 21-1), β-human chorionic gonadotropin (β-HCG), human epididymis protein 4 (HE4), osteopontin (OPN), prolactin, the cell death and angiogenesis markers soluble Fas (sFas), soluble Fas-ligand (sFasL), tumor necrosis factor related apoptosis-inducing ligand (TRAIL), vascular endothelial growth factor (VEGF) as well as the immunological markers interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNFα), transforming growth factor-α (TGFα), fibroblast growth factor-2 (FGF2), macrophage migration inhibitory factor (MIF), leptin, hepatocyte growth factor (HGF) and stem cell factor (SCF). Beads were pre-mixed and given into the wells containing diluted serum and reagents. After overnight incubation and fixation of the antigen (*e.g.* CEA) on the capture antibody linked to the magnetic microsphere, a biotinylated detection antibody was added. As reporter molecule streptavidin-phycoerythrin (PE) conjugate is introduced binding the biotinylated detection antibodies and completing the reaction. Finally two lasers interrogated the microspheres fast passing through the system. The first laser excites the discrete internal dye of the bead identifying the specific antibody and the other one the reporter molecule PE and thereby quantifying the result.

Statistical analysis. Biomarker levels between colorectal cancer patients and healthy controls were compared, as well as between colorectal cancer patients and patients with benign colorectal diseases. Log-transformation of biomarker levels was done for variance stabilization. For significance testing *t*-test was used or, when data were not following a normal distribution, Wilcoxon rank sum test. The significance level was adjusted to $p \leq 0.001$ following a Bonferroni correction for multiple testing. Then, areas under the curve (AUC) of receiver operating characteristic (ROC) curves and sensitivities of relevant biomarkers at 95% specificity versus the control group were calculated. The combination of significantly discriminating biomarkers was analyzed by a logistic regression approach.

Results

Markers that significantly discriminated between patients with colorectal cancer and healthy controls were IL-8, CEA, HGF, TNFα, CYFRA 21-1, OPN, TGFα, CA 19-9, CA 125, AFP and sFas (*p*-values ≤0.001, markers ordered according *p*-values). For all markers higher values were observed in cancer patients than in healthy controls. Other markers had a tendency to higher values in cancer patients as well, however the level of significance ranged between 0.05 and 0.001. Details are listed in Table II and an overview is given as a heat map in Figure 1 (Figure 1, Table II).

When comparing colorectal cancer and benign colorectal diseases the established biomarker CEA and additionally IL-8 were the only markers with significant differences ($p \leq 0.001$). Median concentration of CEA was 6.7 ng/ml (range: 0.4-150.0 ng/ml) in patients with cancerous lesions compared to 1.1 ng/ml (0.3-4.2 ng/ml) in those with benign colorectal diseases and 0.5 ng/ml (0.1-13.6 ng/ml) in healthy controls. Median level of IL-8 in cancer patients was 17.9 pg/ml (6.3-169.5 pg/ml) whereas in benign colorectal diseases a value of 10.1 pg/ml (5.0-28.5 pg/ml) and in healthy controls a value of 5.0 pg/ml (5.0-30.7 pg/ml) was observed. CA 19-9, CYFRA 21-1, CA 125, OPN and CA 15-3 reached significance levels between 0.05 and 0.001 for the discrimination between malignant and benign colorectal diseases (Figure 2, Table II).

Concerning the stage-dependency between non-metastasized and metastasized colorectal lesions, several markers had higher levels in advanced stages, CEA achieved the best performance ($p=0.0001$) followed by the cell death marker sFas ($p=0.0007$) and by CYFRA 21-1 ($p=0.0009$). Markers with lower significance levels ($0.001<p<0.05$) were CA 19-9, CA125, IL-8, prolactin and OPN (Table III).

Correlations between clinical relevant markers with correlation coefficients R>0.6 were observed for the comparisons of CEA/IL-8 (R=0.638), CEA/OPN (R=0.607), IL-8/TNFα (R=0.728), IL-8/TGFα (R=0.652), IL-8/HGF (R=0.621), IL-8/CYFRA 21-1 (R=0.621), OPN/sFas (R=0.691), OPN/HGF (R=0.681), TGFα/TNFα (R=0.683), TGFα/CYFRA 21-1 (R=0.616), and TGFα/HGF (R=0.604).

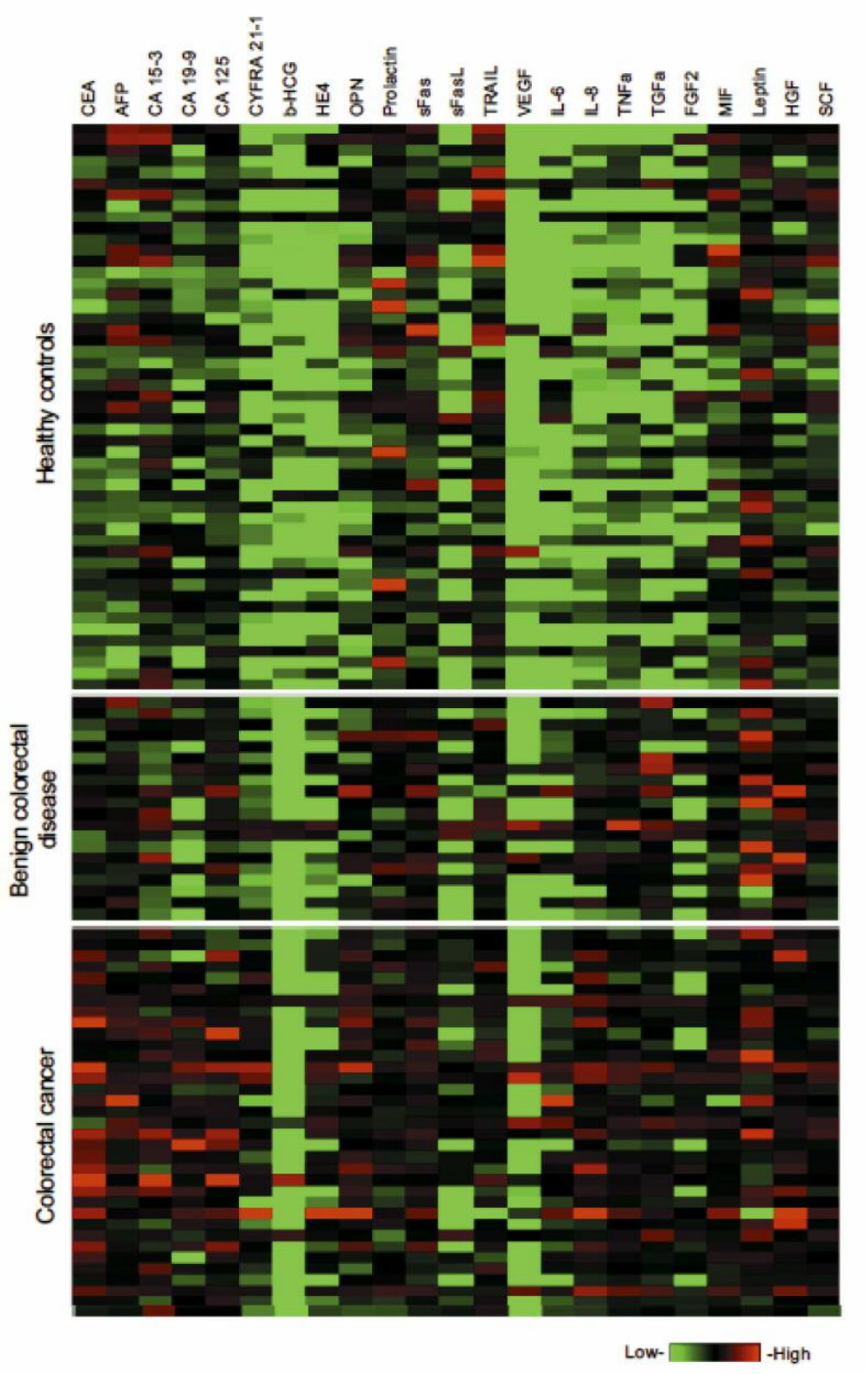

Figure 1. *Heat maps illustrate marker levels in all sera of healthy controls, patients with benign colorectal disease and colorectal cancer. Color changes from low to high levels range from bright green, dark green, black, dark red and bright red visualizing the marker level trends in the various groups.*

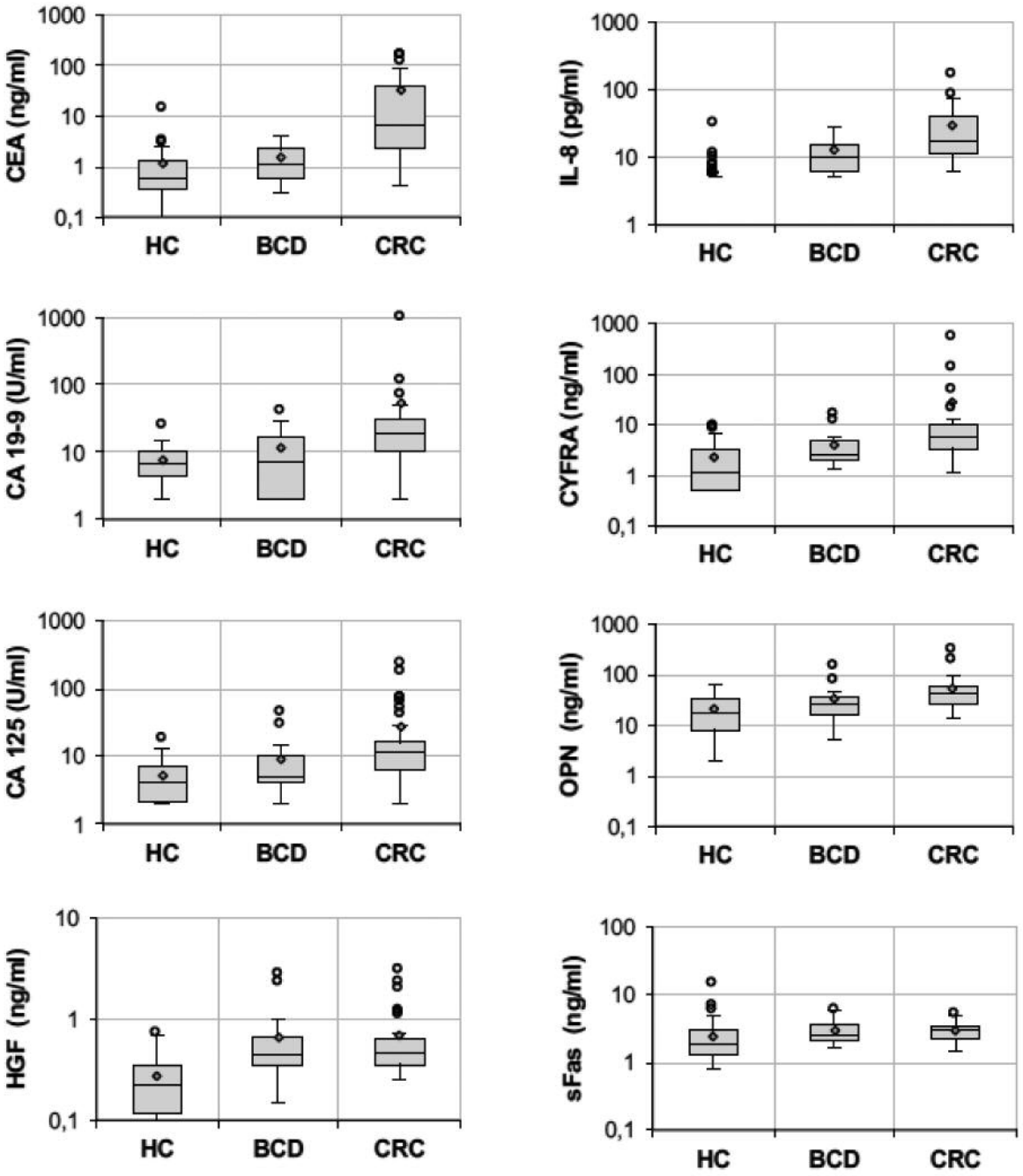

Figure 2. *Box plots show marker distribution in various diagnostic groups. Box plots for biomarkers CEA, IL-8, CA 19-9, CYFRA 21-1, CA 125, OPN, HGF and sFas indicate medians, means, interquartile ranges, whiskers and outliers for the groups of healthy individuals, patients with benign and malignant colorectal disease.*

The power of discrimination between groups is best described by ROC curves and sensitivities at a defined specificity. For the comparison between patients with colorectal cancer and healthy controls, IL-8 showed the best performance with an AUC of 0.976 in ROC curves and a sensitivity of 85.7% at 95% specificity followed by CEA with an AUC of 0.915 and a sensitivity of 68.8% at 95% specificity. TNFα had an AUC of 0.892 and a sensitivity of 80.0% while CA 19-9 had an AUC of 0.841 and a sensitivity of 60.0% at 95% specificity.

Table II. *Biomarker concentration for each tested marker and subgroup as well as discriminative power in the intergroup comparison.*

Biomarker	Unit	Healthy controls		Benign colorectal diseases		Colorectal cancer		HC *vs.* CRC	BCD *vs.* CRC
Oncological biomarkers									
CEA	ng/ml	0.5	(0.1-13.6)	1.1	(0.3-4.2)	6.7	(0.4-150.0)	**$p<0.001$**	**$p<0.001$**
AFP	ng/ml	1.1	(0.5-9.7)	2.5	(0.9-8.1)	2.6	(1.0-22.5)	**$p<0.001$**	ns
CA 15-3	U/ml	11.1	(2.0-36.6)	8.3	(3.7-35.1)	13.6	(4.8-69.0)	$p<0.05$	$p<0.05$
CA 19-9	U/ml	6.9	(2-24.1)	7.0	(2.0-38.9)	19.4	(2.0-976.6)	**$p<0.001$**	$p<0.05$
CA 125	U/ml	4.0	(2.0-18.5)	5.0	(2.0-43.0)	11.4	(2-227.8)	**$p<0.001$**	$p<0.05$
b-HCG	mU/ml	0.2	(0.2-2.0)	0.2	(0.2-2.1)	0.2	(0.2-5.3)	ns	ns
CYFRA 21-1	ng/ml	1.1	(0.5-9.5)	2.4	(1.3-16.5)	5.6	(1.2-566.5)	**$p<0.001$**	$p<0.05$
HE4	ng/ml	2.0	(2.0-8.0)	2.0	(2.0-11.0)	2.6	(2.0-70.1)	$p<0.05$	ns
OPN	ng/ml	17.3	(2.0-63.0)	26.7	(5.3-147.0)	44.0	(13.1-306.5)	**$p<0.001$**	$p<0.05$
Prolactin	ng/ml	8.4	(1.2-246.6)	10.5	(3.3-37.8)	9.0	(3.4-27.1)	ns	ns
Apoptosis markers									
sFas	ng/ml	1.8	(0.8-15.3)	2.5	(1.7-6.0)	3.0	(1.4-5.4)	$p<0.001$	ns
sFasL	pg/ml	50.0	(50.0-178.8)	50.0	(50.0-111.2)	50.0	(50.0-88.7)	ns	ns
TRAIL	pg/ml	86.5	(35.8-360.9)	90.0	(39.5-161.9)	100.6	(25.1-172.4)	ns	ns
Angiogenesis marker									
VEGF	pg/ml	50.0	(50.0-743.0)	50.0	(50.0-743.6)	50.0	(50.0-926.1)	ns	ns
Immunological markers									
IL-6	pg/ml	2.0	(2.0-10.2)	2.0	(2.0-15.0)	2.9	(2.0-61.9)	$p<0.05$	ns
IL-8	pg/ml	5.0	(5.0-30.7)	10.1	(5.0-28.5)	17.9	(6.3-169.5)	**$p<0.001$**	**$p<0.001$**
TNFa	pg/ml	5.0	(5.0-18.0)	8.7	(5.0-85.1)	10.2	(5.0-26.2)	**$p<0.001$**	ns
TGFa	pg/ml	10.0	(10.0-52.6)	18.7	(10.0-122.4)	18.3	(10.0-64.1)	**$p<0.001$**	ns
FGF2	pg/ml	50.0	(50.0-260.9)	85.3	(50.0-225.6)	89.9	(50.0-235.9)	$p<0.05$	ns
MIF	ng/ml	0.4	(0.1-13.3)	0.6	(0.2-1.0)	0.7	(0.1-3.5)	$p<0.05$	ns
Leptin	ng/ml	10.4	(2.9-48.8)	14.2	(0.5-80.9)	11.6	(0.5-76.0)	ns	ns
HGF	ng/ml	0.2	(0.1-0.7)	0.4	(0.2-2.7)	0.5	(0.3-3.1)	**$p<0.001$**	ns
SCF	pg/ml	42.1	(20.0-209.6)	56.5	(33.7-135.4)	67.4	(31.5-152.1)	$p<0.05$	ns

Concentrations are given as median and range of the observed concentration levels, discriminative power as *p*-value of the respective intergroup comparison. Significant differences ($p<0.001$) are highlighted in bold. Nonetheless, markers showing values between 0.05 and 0.001 are also given. HC: Healthy control, BCD: benign colorectal disease, CRC: colorectal cancer, ns: not significant.

Most relevant for differential diagnosis is the comparison between colorectal cancer and benign colorectal diseases. Here, CEA showed the best performance with an AUC of 0.859 and a sensitivity of 65.7% at 95% specificity followed by IL-8 with an AUC of 0.744 and a sensitivity of 31.4% as well as by CA 19-9 with an AUC of 0.740 and a sensitivity of 37.1% at 95% specificity. While combination of CEA and IL-8 did not increase AUC (0.861), the combination of CEA and CA 19-9 led to an improved AUC of 0.893. Sensitivity of the marker combinations at 95% specificity were 65.7%, respectively (Figure 3).

Discussion

Many efforts in diagnostic oncology aim at improving the early diagnosis of cancer and accurate differentiation between benign and malignant lesions in order to offer the most appropriate therapy options. In colorectal cancer, performance of (immunochemical) fecal occult blood test (FOBT or FIT) and colonoscopy are well established in national screening programs as they have shown to improve colorectal cancer-related mortality (7-9). While blood-based markers are considered promising diagnostic tools, they have only rarely been recommended for cancer detection and to a greater extent for disease monitoring during and after therapy in international guidelines (16, 17). Although the established marker CEA was discovered more than 50 years ago (15), studies are still pending to investigate the defined use of this marker in clinical practice. Beyond oncological biomarkers, new approaches also address other markers of cell death, proliferation, interaction with the tumor microenvironment and immunological host reactions to form more meaningful marker patterns (18-23).

Recently, a multiplex immunochemical magnetic bead assay was launched as research-use-only (RUO) device that enabled the parallel assessment of 24 biomarkers involved in different pathophysiological fields of cancer development, thereby saving sample volume, analysis time, costs and

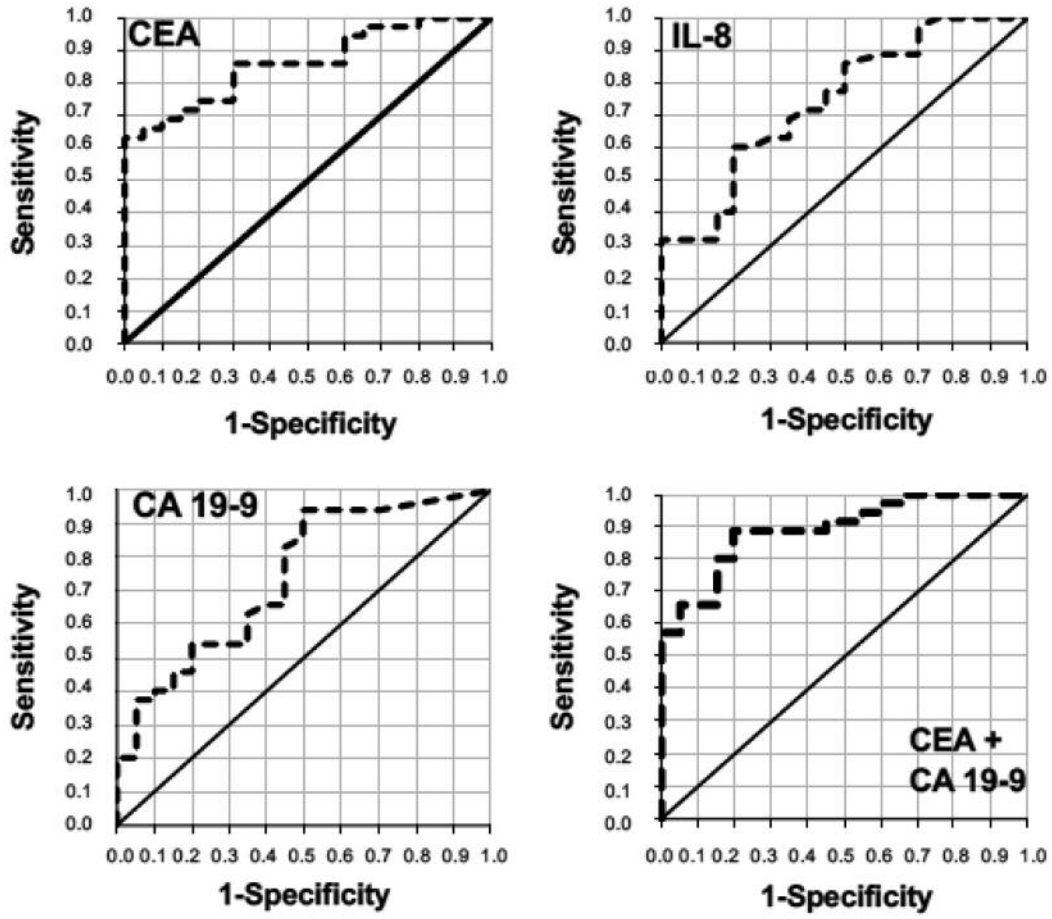

Figure 3. *Receiver operating characteristic (ROC) curves indicate best discriminating markers for comparison of patients with colorectal cancer and benign colorectal diseases. Single markers with best discrimination between benign and malignant colorectal diseases were CEA (area under the curve=AUC 0.859), IL-8 (AUC 0.74) and CA 19-9 (AUC 0.74). Combination of CEA and CA 19-9 improved diagnostic AUC to 0.893.*

Table III. *Discriminative power between non-metastatic (M0) and metastatic (M1) colorectal cancers.*

	Tumor markers									
	CEA	AFP	CA 15-3	CA 19-9	CA 125	CYFRA 21-1	b-HCG	HE4	OPN	Prolactin
M0 *vs*. M1	**0.0001**	0.7931	0.9306	***0.0161***	***0.0074***	**0.0009**	0.9815	0.4028	***0.0231***	***0.0067***
	Apoptosis markers			Angiogenesis marker						
	sFas	sFasL	TRAIL		VEGF					
M0 *vs*. M1	**0.0007**	0.4483	0.4227		0.3162					
	Immunological markers									
	IL-6	IL-8	TNFa	TGFa	FGF2	MIF	Leptin	HGF	SCF	
M0 *vs*. M1	0.3961	***0.0084***	0.2107	0.1820	0.7058	0.4586	0.8047	0.2158	0.1866	

p-Values indicate the discriminating power of measured parameters between the subgroups of non-metastatic (M0) and metastatic (M1) colorectal cancers. Significant differences ($p<0.001$) are highlighted in bold. Nonetheless, markers showing values between 0.05 and 0.001 are also highlighted (bold and italics).

providing a comprehensive picture of biochemical processes. In a previous study, the methodical and pre-analytical accuracy of included markers was demonstrated which is an essential basis for further clinical testing (36).

In the present clinical assessment, significantly higher levels, not only of markers associated with colorectal cancer such as CEA and CA 19-9, but also of other oncological biomarkers like CA 125, AFP, CYFRA 21-1 and HGF, as well as of cell death and immunological markers sFas, TNFα, TGFα, OPN, and IL-8 were observed when compared to healthy controls. For apoptotic markers sFas and MIF, this issue has already been discussed (37). These results are quite remarkable as the pattern of these markers may help in the early detection of malignant lesions. However, most levels of TNFα, TGFα, IL-8, ß-HCG, HE4, sFasL, VEGF, IL-6, and FGF-2 were close to the level of quantification; as imprecision was higher in this low value range, clinical results of these markers have to be interpreted with care.

Many immunologic and cell death markers were found in benign colorectal diseases too, as already seen in breast cancer (38). In consequence, there is no discrimination between benign and malignant colorectal diseases which would be most relevant for differential diagnosis. Only CEA, IL-8 and partly CA 19-9 demonstrated discriminatory potential achieving AUCs of 0.859, 0.744 and 0.740 in ROC curves and sensitivities of 65.7%, 31.4% and 37.1% at 95% specificity. The combination of CEA and CA 19-9 resulted in an improved AUC of 0.893. Additionally, CYFRA 21-1, CA 125, OPN and CA 15-3 showed a promising trend for discrimination.

The high accuracy of CEA for detection of colorectal cancer is in line with earlier studies supporting the clinical validity of the multiplex immunoassay (39). In accordance with reports from other studies, CA 19-9 is less sensitive than CEA for colorectal cancer detection. However, it has considerable prognostic impact, particularly in advanced cancer stages (16, 40, 41). The high diagnostic sensitivity of IL-8 is a new finding that is backed by some preliminary earlier reports. Bălăşoiu *et al.* described a significant increase of IL-8 levels in the supernatant of tumor cells as well as in serum of cancer patients with higher values in advanced tumor stages (42). In addition, elevated IL-8 levels in patients with liver metastases were found to be associated with worse disease-free and overall survival (43). This is in concordance with stage dependency observed in our cohort.

Although present only at low levels in patient sera, CYFRA 21-1 showed some discriminative power which is in line with earlier studies (44). This may be explained by the fact that cytokeratins are not only general tumor markers for epithelial cancers but also biomarkers of cell death (22) that are released in cases of enhanced cellular turnover. As part of a biomarker panel with CEA, seprase, OPN, ferritin and anti-p53, sensitivity for early colorectal cancer detection was improved to 69.6% at 95% specificity and 58.7% at 98% specificity as compared to 43.9 % at 95% specificity for CEA alone (19). As a limitation of the present study, CYFRA 21-1 was found to be a critical parameter in the initial methodical analyses (36) and has to be interpreted with caution. While CA 125 is often used as tumor marker in ovarian cancer, it is elevated in the sera of patients with adenocarcinoma of other sites (14) as well as in patients with peritoneal or pleural effusions, with liver dysfunctions or renal failure (45). Similarly, elevated CA 15-3 levels cannot only be observed in serum of breast cancer but also in patients with adenocarcinoma of other sites up to a certain extent, explaining a potential utility of being included in biomarker panels for detection of colorectal cancer (14, 46, 47). OPN is described to promote invasiveness as well as progression of colorectal cancer (48, 49) and has shown some diagnostic potential (50, 51). Furthermore, it has already been included in a multiple biomarker panel leading to promising results (19). Like cytokine markers, TNFα, TGFα, as well as sFas were not only elevated in colorectal cancer, but also in benign colorectal diseases. In addition, sFas showed a clear stage dependency in colorectal cancer. This is in line with other studies that found higher sFas levels in patients with advanced tumors, poor differentiation, tumor invasion (52) and in metastatic colorectal cancer (53).

According the recommendations of the European Group on Tumor Markers (EGTM) (54) serum samples from all diagnostically-relevant groups, *i.e.* patients with colorectal cancer, healthy controls and patients with benign colorectal diseases were included in the present study. Blood samples were collected following predefined standards of transport, handling and storage in the Biofluid Biobank Bonn. Prior to testing on its relevance for colorectal cancer detection, the multiplex immunoassay was rigorously checked on its methodical performance and on conceivably influencing pre-analytical factors (36). Measurements were performed by trained staff and quality controls were run with every test: Artificial materials and physiological serum pools were used to control interassay precision; benign, malignant and healthy samples were randomly distributed on the same plates to avoid uncontrolled variances. Statistical evaluation was done independently from analytical processes and results were corrected for multiple testing according Bonferroni. As the study was planned as a pilot study using a new multiplex immunoassay, the number of patients was limited in all groups and will have to be validated by further studies.

Although the assay is only available as research use only (RUO) assay it enables the parallel assessment of various biomarker classes relevant to cancer development and progression. Many oncological, cell death and immunological markers showed promising potential for the discrimination between colorectal cancer patients and healthy controls. However, the relevant differentiation of patients with

malignant and benign colorectal diseases was only achieved by the already established biomarker CEA and CA 19-9 as well as by the newly-identified IL-8.

Conclusion

The newly-available multiplex cancer biomarker panel provides great potential for research approaches when a multitude of biomarkers mirroring different pathophysiological processes of cancer development has to be tested on its clinical utility. Compared to single ELISA tests, multiplexing enables a faster, more cost-efficient and less volume consuming assessment of a considerable number of analytes at the same time. In our setting, the combination of CEA and CA 19-9 showed the best performance in colorectal cancer diagnostic and IL-8 was identified as further valuable marker. Thus, this pilot study presents a robust basis for further validation studies.

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