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Analysis of 32 Blood-Based Protein Biomarkers for their Potential to Diagnose Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is largely viewed as a preventable disease but the prevalence is increasing worldwide. Although many faecal and blood-based biomarkers have been proposed as potential diagnostic markers, none have been successful in large cohort studies. In this study, ELISA was used to evaluate 32 candidate protein biomarkers in a single cohort of CRC patients (n=95) and age/sex matched controls (n=50). Of these, 12 markers differed statistically between cases and controls. Receiver operating characteristic analysis identified IL8, Mac2BP, TIMP1, and OPN as the best performing markers for overall CRC diagnosis. However, further analysis determined that IL6, TGFB1, TIMP2 and IGF2 were most accurate at identifying early stage disease. We also assessed the correlation between markers and determined that the strongest correlations existed between VEGFA and TGFB1 (r=0.65, p<0.0001), M30 and M65 (r=0.59, p<0.001), and between TGFB1 and TIMP1 (r=0.55, p<0.0001). This analysis provides a consistent baseline for identifying a potential panel of diagnostic protein biomarkers in blood. Our results highlight protein biomarker combinations that reflect the disease process and which may provide the sensitivity and specificity required a reliable diagnosis of CRC.

Keywords: Colorectal cancer; Diagnosis; Blood-based; Protein biomarkers

Introduction

Worldwide, colorectal cancer (CRC) is the second most common cause of cancer-related death with an annual incidence over 1.2 million and an annual mortality over 600,000 [1]. The majority of cases are sporadic with 25-30% estimated to be due to hereditary factors [2,3]. For most sporadic CRC, an accumulation of somatic genetic and epigenetic mutations underlies the transformation from normal colonic mucosa to carcinoma and this transition is believed to occur over a long period of time, i.e., 10-15 years [4]. The high frequency of CRC, the long time frame for its development and the observation that most CRC arise from pre-malignant polyps make CRC an ideal target for population screening programs where detection and removal of premalignant (adenoma or polyp) or early stage malignant disease (Stage A) can potentially prevent the occurrence of CRC or at least significantly increase the likelihood of a complete cure. Due to the slow and multi-stage progression of this disease and the general absence of symptoms in the early stages, it is estimated that around 30-50% of patients have overt metastases at presentation [5].

Identification of non-invasive biomarkers for early detection of CRC, including detection of pre-malignant and clinically significant polyps and adenomas, is important for reducing both incidence and mortality. When diagnosed early, the 5 year the survival rate for CRC is 90-95% indicating a high curative rate. In comparison, when CRC is detected at later stages, the 5 year survival rate is significantly less (5-10%) [6]. Currently, the faecal occult blood test (FOBT), faecal immunochemical test (FIT), colonoscopy and sigmoidoscopy are the only clinically accepted diagnostic tests for CRC [7]. The FOBT and FIT are used to detect the presence of heme or blood in stool and whilst these tests have a relatively low cost, they are regarded as having poor sensitivity for early stage disease [8,9]. Because the presence of blood in stool is not specific for CRC, the FOBT and FIT also suffer from relatively high false positive rates. In contrast, while colonoscopies have high specificity for the disease, the procedure is highly invasive and expensive. Two of the most widely known serum protein biomarkers for gastrointestinal malignancies, including CRC, are the carbohydrate antigen CA19-9 and carcinoembryonic antigen (CEA) [10-12]. CEA, while useful for monitoring recurrence of CRC, exhibits specificity for cancer of 87%, but its sensitivity (35%) is too low to be useful for detection of CRC in an asymptomatic screening population [11]. Similarly, CA19-9 has limited utility as a diagnostic marker due to its lack of specificity for malignant disease [10].

Currently, but still in the research phase, are several promising DNA diagnostic biomarkers for CRC, in particular methylated septin 9 (mSEPT9) measured in plasma. A stool-based DNA test consisting of a panel of four methylated genes (BMP3, NDRG4, vimentin, TFPI2) and a mutant form of KRAS is also being developed [13,14]. Initial studies evaluating mSEPT9 indicated high detection rates for CRC [14-

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16], and this has been confirmed in recent multicentre trials [17-20]. While these reports indicate that the stool-based DNA test is superior to plasma mSEPT for CRC detection, data from larger, longer term studies in a screening populations are required to objectively compare the performance of these two tests with other screening modalities (FOBT and FIT) under population screening conditions.

Many reviews of CRC biomarkers have been published and many factors have been suggested for the lack of success of follow-up studies and lack of consistency of results between different biomarker studies. These factors include small cohort sizes, cohort composition, differences in sample handling and processing procedures, and over-representation of late stage disease patients which can bias biomarker sensitivity estimates [11,21-25]. Other factors that have hampered the use of biomarkers in the clinic include assay reproducibility, biomarker stability, and biomarker variability due to comorbidities and diurnal variation. In this study we have measured the concentration of 32 candidate CRC biomarkers in serum and plasma samples from a single cohort of CRC cases (n=95) and age/sex matched controls (n=50). These protein markers were selected based on in-house proteomic and gene expression experiments on colorectal cancer cell lines (in vitro data) and colorectal cancer tissue from patients. We assessed the usefulness of these markers for detecting CRC. Our analysis was designed to minimise the effect of sample collection, processing and storage and assay variability providing an accurate comparison of the performance of these blood-based protein biomarkers for CRC diagnosis.

Materials and methods

Study Design

Patients were newly diagnosed cases of colorectal cancer (no previous history of disease) and blood was obtained prior to surgery (i.e., these are pre-surgical patients) via colorectal surgery preadmission clinics from a network of hospitals associated with the Victorian Cancer Biobank in Melbourne, Victoria, Australia, between 2005 and 2011. Patients with a previous history of CRC or who had already received chemo- and/or radio- therapy were excluded from this study. All research protocols used in this study was approved by the relevant Human Research Ethics Committees at Commonwealth Scientific Industrial Research Organisation, Adelaide, and the Royal Melbourne Hospital, Melbourne.

Serum and plasma samples from CRC patients (n=95) and healthy controls (n=50) were obtained and processed using methods previously described [26,27]. To minimise the effect of potential confounders, the normal and CRC cohorts were balanced for age, sex and disease stage. Briefly, blood was collected from each subject into serum separator tubes and EDTA plasma tubes. The blood was left at room temperature in the collection tube for 30 min and then centrifuged at (1,200 g, 10 min, room temperature). The supernatant was transferred to a new tube and centrifuged at (1,800 g, 10 min, room temperature). Aliquots of the resulting supernatant were frozen at -80°C until analysis. The time from sampling to freezing was 2 hrs.

Protein measurements in serum and plasma by ELISA

Serum and plasma were assayed using commercially available ELISA kits or reagents according to manufacturers' instructions unless otherwise specified. The following multiplex ELISA kits were sourced from R&D Systems (Minneapolis, MN, USA): multiplex kit for the chemokines ENA-78, MCP-1, and MIP-1β, the cytokine multiplex panel for analysis of TNF-α, IL6 and IL8, the multiplex panel for matrix metalloproteinases (MMP)-1, -3, -7, and -8. DuoSet ELISA kits for amphiregulin, DcR3, DKK3, and RegIV. ELISA kits for TGFB1, TIMP1, TIMP2, VEGFA and GRO-α were also purchased from R&D Systems (Minneapolis, MN, USA). ELISA kits for the following markers were also obtained: M30 and M65 (PEVIVA, Bromma, Sweden), IGFBP2 and IGF2 (DSL Inc., Webster, TX, USA), PKM2 (Schebo, Giessen, Germany), Mac-2BP (Bender MedSystems GmbH, Vienna, Austria), and OPN (Linco Research, St Charles, MO, USA). For the analysis of EpCAM, the DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) was used and chemiluminescent detection was performed using the Supersignal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

For CEACAM6, 96 well plates were coated with polyclonal rabbit anti-human carcinoembryonic antigen (DakoCytomation, Glostrup, Denmark) (2 μg/mL in carbonate buffer, pH 9.5). The standard curve (range 0.49 – 125 ng/mL) was prepared by serial dilution of the CEACAM6 recombinant protein (R&D Systems, Minneapolis, MN, USA). Biotinylated CEACAM6 monoclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA) was used for detection (1 μg/mL in PBS/1% BSA).

For SPONDIN-2, 96 well plates were coated with SPONDIN-2 monoclonal antibody (R&D Systems, Minneapolis, MN, USA) (4 μgmL carbonate buffer, pH 9.5). The standard curve (range 15.6 – 2000 ng/mL) was prepared by serial dilution of recombinant human SPONDIN-2 protein (Abnova, Taipei, Taiwan). Biotinylated anti-human SPONDIN-2 detection antibody (R&D Systems, Minneapolis, MN, USA) was prepared at a concentration of 800 ng/mL in PBS/3% BSA.

An in-house bead-based assay was used to measure P-cadherin. Monoclonal anti-human p-cadherin antibody (R&D Systems, Minneapolis, MN, USA) was coupled to carboxylated polystyrene beads (Luminex Corporation, Austin, TX, USA) according to the manufacturer's instructions. The standard curve (range 7.8 – 2000 ng/mL) was prepared by serial dilution of recombinant human p-cadherin protein (R&D Systems, Minneapolis, MN, USA). Streptavidin-R-phycoerythrin donkey anti-goat reporter (Thermo Fisher Scientific, Waltham, MA, USA) was used as a concentration of 0.4μg/mL (50 μL) for detection.

Two in-house quality control (QC) samples were included in each analysis. QC samples consisted of a pooled normal sample (n=41) and a pooled CRC patient sample (n=41). For commercially available ELISA kits, the intra-assay coefficients of variation (CV) were less than 10%, consistent with the manufacturers' specifications.

Data analysis

Multiplex panels and p-cadherin were analysed using the Luminex bead-based system (Qiagen, Hilden, Germany) and preliminary data was analysed using the Luminex IS2.3 software. For standard ELISAs, the absorbance was determined using the Wallac Victor3V Multilabel Counter microplate reader (Perkin Elmer, Waltham, MA, USA) set to 450 nm with wavelength correction at 570 nm. Preliminary data analysis for the standard ELISA assays was performed using Workout 2.0 software (DazDaq, Brighton, UK). The Prism software package (version 6, Graphpad Software Inc., San Diego, CA, USA) was used for statistical analysis. The non-parametric Mann-Whitney U test was used to determine the statistical difference between cancer and control patients. Receiver operator characteristic (ROC) curve analysis was performed to assess the diagnostic performance for each marker and to determine the sensitivity for each marker at 95% specificity. Spearman

correlation was used to determine correlations between markers. Statistical significance was defined as $p<0.05$.

Results

Study cohort

Table 1 summarises the characteristics of the cohort used in this study. The median age was 67 yrs (range 44-93 yrs) for CRC patients and 70 yrs (range 50-85 yrs) for the control group. The CRC patient group was further stratified according to Dukes' stage.

Characteristics	Control	CRC
N	50	95
Sex, N		
Female	25	50
Male	25	45
Median age, yrs (range)	70 (50-85)	67 (44-93)
Dukes' stage		
A		21
B		31
C		33
D		10

Table 1: Characteristics of the study cohort.

Biomarker analysis

Of the 32 proteins analysed, 23 protein markers were measured within the range of the ELISAs. Eight markers (MMP7, p-cadherin, RegIV, spondin-2, EpCam, GRO-alpha, amphiregulin, and DcR3) were undetectable in the serum or plasma of the majority of samples (CRC and control patients) and were excluded from further analysis. A summary of these 8 markers, including the standard curve range for each assay can be found in Supplementary Information 1. Of the 23 protein biomarkers that were detectable, 12 showed a significant difference ($p<0.05$) between the median values of the cancer and control patients (Figure 1 and Table 2). These 12 markers include Mac2BP, PKM2, IL8, IL6, IGFBP2, TGFB1, M65, IGF2, VEGFA, TIMP1 (measured in both serum and plasma), MMP1, and OPN. All of these proteins, with the exception of IGF2, were elevated in CRC patient samples in comparison to the control group. For IGF2, the

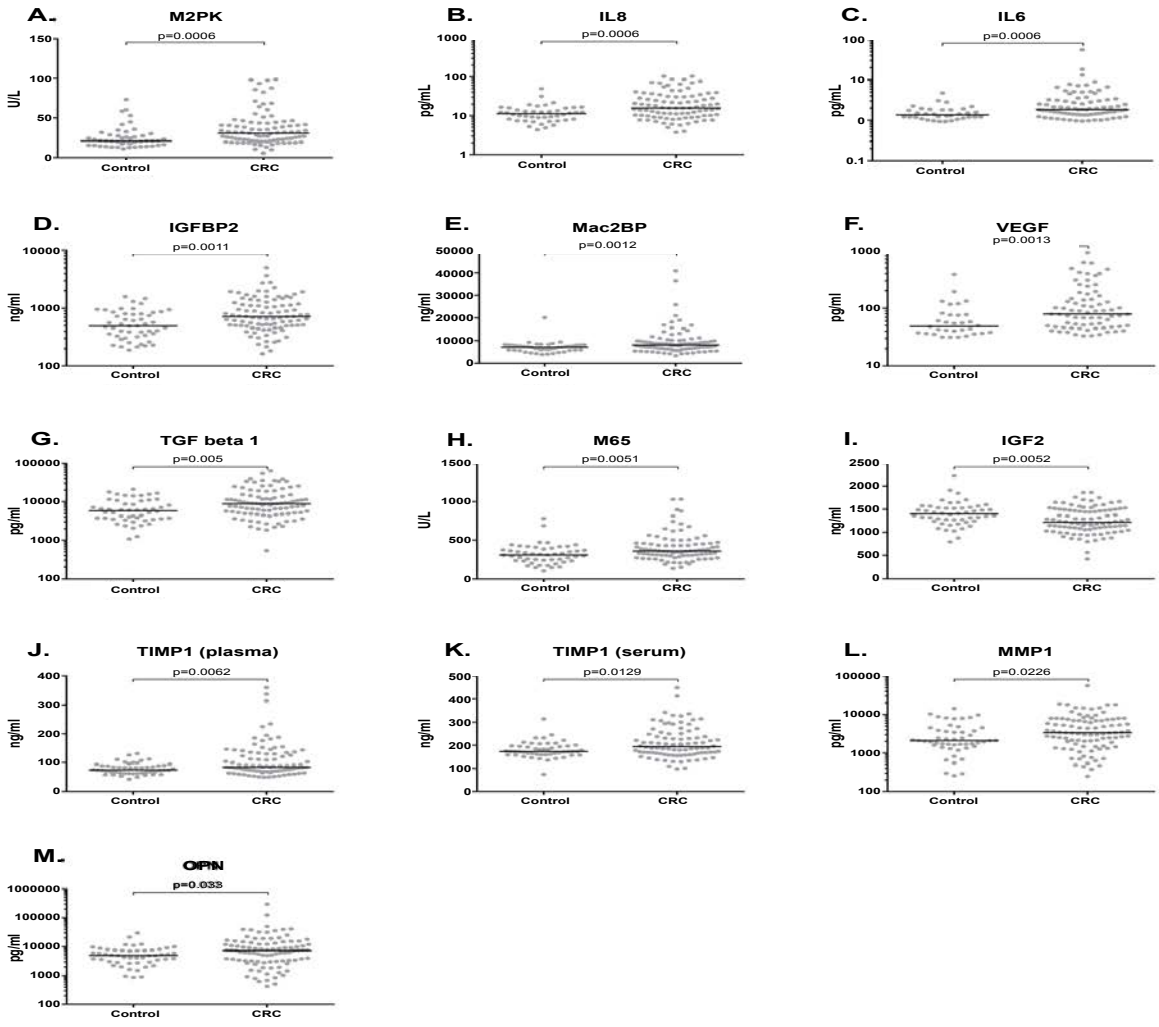

Figure 1: Scatter diagrams for the markers that are significantly different between the control and cancer patients ($p<0.05$) Scatter diagrams for the markers that are significantly different between the control and cancer patients ($p<0.05$).

			Control	Cancer	p value
Biomarker	**Units**	**Serum / plasma**	**median (range)**	**median (range)**	**cancer vs control**
				all cancers	
PKM2	U/ml	plasma	21.02 (11.26 - 73.3)	30.71 (5.81 - 98.80)	0.0006
IL8	pg/ml	serum	11.26 (4.36 - 49.89)	15.77 (3.71 - 103.5)	0.0006
IL6	pg/ml	serum	1.84 (0.95 - 55.80)	1.355 (0.93 - 4.740)	0.0006
IGFBP2	ng/ml	serum	490.5 (188.7 - 1583)	699.2 (163.3 - 3698)	0.0011
Mac2BP	ng/ml	serum	7126 (3918 - 20150)	8350 (4290 - 40870)	0.0012
VEGFA	pg/ml	plasma	48.69 (31.42 - 387)	78.96 (33.29 - 910.1)	0.0013
TGFB1	pg/ml	plasma	6034 (2070 - 21122)	8794 (1846 - 64230)	0.005
M65	U/L	serum	312.6 (143.3 - 783.2)	358.4 (132.8 - 1032)	0.0051
IGF2	ng/ml	serum	1399 (792.3 - 2230)	1221 (421.1 - 1864)	0.0052
TIMP1	ng/ml	plasma	74.89 (42.53 - 131.3)	84.31(49.9 - 359.6)	0.0062
TIMP1	ng/ml	serum	172.8 (74.3 - 314.4)	193.8 (98.6-449.4)	0.0129
MMP1	pg/ml	serum	2090 (258 - 14300)	3350 (249 - 56000)	0.0226
OPN	pg/ml	plasma	4980 (868 - 29600)	7410 (425 - 297000)	0.033
TNF-alpha	pg/ml	serum	7.55 (1.55 - 13.21)	7.85 (3.09 - 26.67)	0.1092
TIMP2	ng/ml	serum	82.5 (59.2 - 106.2)	77.6 (47.3 - 159.4)	0.1149
CEACAM6	ng/ml	plasma	1.62 (0.60 - 3.54)	1.63 (0.60 - 7.99)	0.1344
MIP-1beta	pg/ml	serum	77.00 (37.30 - 369.0)	66.95 (35.8 - 1510)	0.1355
Dkk3	pg/ml	plasma	58895 (37232 - 78500)	56473 (27726 - 75779)	0.3046
ENA-78	pg/ml	serum	1275 (302 - 5180)	1240 (264 - 4450)	0.4717
MMP3	pg/ml	serum	13250 (2280 - 37600)	11100 (2600 - 55500)	0.5033
TIMP2	ng/ml	plasma	90.71 (64.06 - 129.7)	90.86 (53.78 - 172.3)	0.6593
MCP1	pg/ml	serum	233.5 (80 - 526)	237.5 (20.3 - 795)	0.7915
MMP8	pg/ml	serum	8720 (1510 - 21600)	8190 (1490 - 50600)	0.9344
M30	U/L	serum	178.8 (85.7 - 500.1)	173.4 (87.17 - 901.6)	0.9791

Table 2: Summary of ELISA results for the biomarkers analysed in colorectal cancer and control patients ranked by p value.

median concentration was lower in CRC patients (1221 ng/mL, range 421.1-1864 ng/mL) when compared to the control group (1399 ng/mL, range 792.3-2230 ng/mL; p=0.005).

ROC analysis

ROC analysis was conducted to determine the ability of each marker to distinguish between the CRC and the control groups (Figure 2 and Table 3). At 95% specificity, IL8 was the best performing biomarker (sensitivity of 38%), followed by Mac2BP (sensitivity of 35%). At 95% specificity, TIMP1 measured in plasma and in serum at sensitivity of 33% and 28% respectively, followed by OPN (sensitivity of 31%) in plasma, and IL6 (sensitivity of 27%), M65 (sensitivity of 26%) and IGFBP2 (sensitivity of 25%) in serum. PKM2 and Dkk3 in plasma and MMP1, M30, ENA-78, MMP8, MIP-1beta, MMP3 and MCP1 in serum were the poorest performing biomarkers where the sensitivity for each at 95% specificity was less than 25%. In our previous study [26], we determined that CEA had a sensitivity of 14% at 95% specificity indicating that it is a poor biomarker for diagnosis of CRC, consistent with other reports.

The ability of each marker to discriminate disease stage from the control group was also evaluated (Table 4). Of the 32 markers evaluated, only IL6, TIMP2 (measured in serum), IGF2 and TGFB1 appear to identify patients with early stage disease (i.e., stage A disease, $p<0.05$). Although M65 was able to identify stage A disease with the highest sensitivity (38%) at 95% specificity, the area under the ROC curve was not statistically significant (p=0.057). Only TGFB1 and IL6 were able to identify patients with either Stage A or B disease ($p<0.05$). Although IL8 was the best performing biomarker for diagnosing CRC overall (Table 3), it was most successful at identifying stage C and D disease where its sensitivity was 53% ($p<0.0001$) and 75% (p=0.004), respectively. IL6 and IGF2 were the most successful markers for identifying stage A disease (sensitivities of 30% and 29%, respectively, $p<0.05$) however their sensitivity detecting for stage B disease dropped to 24% ($p<0.05$) and 19% (p=0.382), respectively.

Correlation between markers

Since the markers measured in this study represent different biological aspects of CRC (eg, inflammation, angiogenesis, metastasis, growth factor production, apoptosis), the Spearman correlation was used to determine if any relationship existed between any of the markers. Although correlations between many biomarker pairs were found to be significant, the majority of the correlations were weak ($r<0.3$), including correlations found between the 12 significant biomarkers (Supplementary information 2). Table 5 lists the marker pairs with Spearman $r>0.3$. As expected, plasma concentrations of TIMP1 and TIMP2 correlated strongly with their respective measurements in serum (r=0.63, $p<0.0001$ and r=0.74, $p<0.0001$, respectively).

The strongest correlations were observed between VEGFA and TGFB1 (r=0.65, $p<0.0001$), M30 and M65 (r=0.59, $p<0.0001$), and between TGFB1 and TIMP1 measured in plasma (r=0.55, $p<0.0001$). For the inflammatory markers and chemokines, correlations were weak between IL6 and IL8 (r=0.274, p=0.002), between IL8 and MCP1 (r=0.218, p=0.012) and ENA-78 correlated weakly with both MCP1 (0.272, p=0.001) and MIP1B (r=0.197, p=0.024).

Discussion

We have evaluated 32 protein biomarkers for their utility as diagnostic markers of CRC. Although there is an abundance of literature evaluating potential biomarkers for CRC, it is difficult to compare the performance of individual biomarkers due to the differences in cohort sizes and compositions. Differences in sample handling, storage

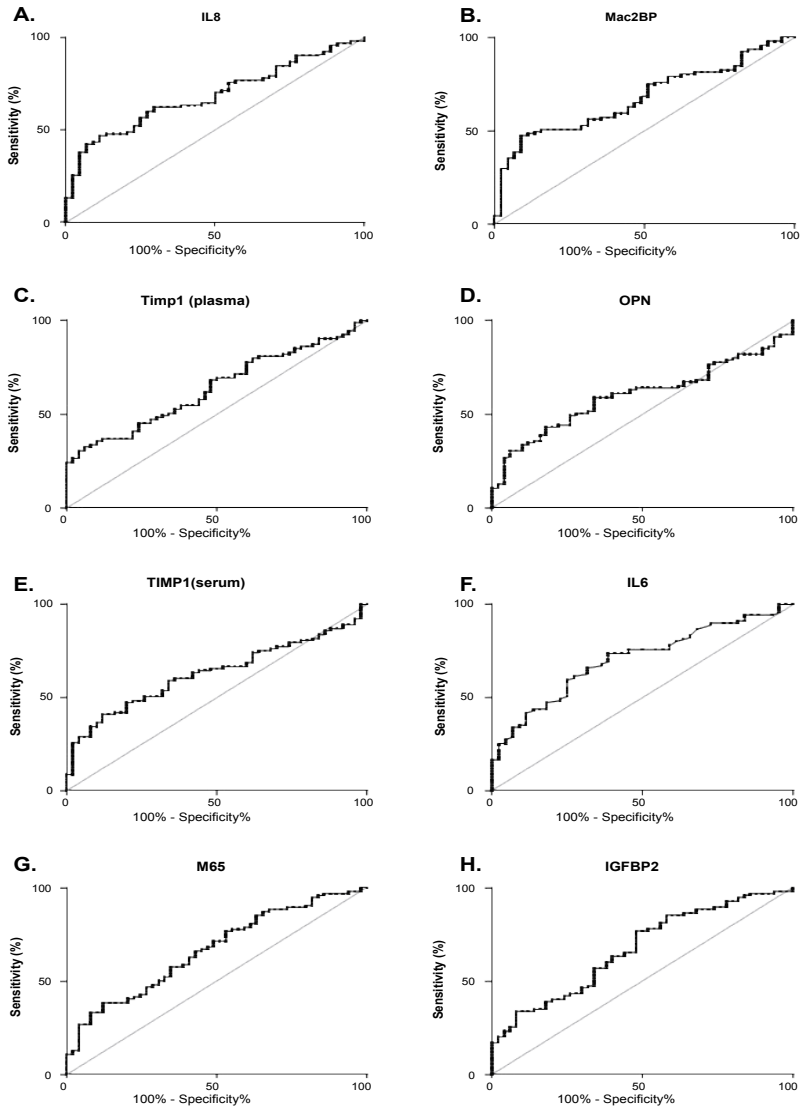

Figure 2: Receiver operator characteristic curves for the eight markers with the highest sensitivity at 95% specificity.

conditions and processing for reported studies also make comparisons difficult. This report is one of the few studies evaluating a large number of proteins (> 20 proteins) in the same patient cohort [28,29], and furthermore, our cohort was balanced for age, sex and disease stage. Of the 32 biomarkers investigated, 12 were found to be significantly different between the control and CRC patient group. Of these, IL8, Mac2BP, OPN and TIMP1 (measured in both serum and plasma) were the best performing biomarkers for diagnosing CRC (sensitivities of 38%, 35%, 31%, 33% and 28%, respectively, at 95% specificity). A fixed specificity of 95% was chosen to minimise the number of false positive cases as we consider this to be an important aspect of a diagnostic assay and so that we can compare any single biomarker to the performance of the FIT assay used in screening programs.

Our data shows that the serum and/or plasma levels of proteins involved in similar pathophysiological processes did not necessarily correlate strongly. For example, proteins such as the interleukins and chemokines that are involved in the inflammatory and immune process only weakly correlated with each other, and a similar result was observed for the MMPs and TIMPs which are involved with tissue remodelling. Although the lack of correlation between markers with similar biological function is surprising, Bunger et al recently published that in a panel of 12 cytokines measured in the sera of 100 CRC patients and controls, only IL8 discriminated between controls and cancer patients and only poor to moderate correlation was found between the cytokines measured [30].

Strong correlations were observed between VEGFA and TGFB1 ($r=0.65$, $p<0.0001$), TGFB1 and TIMP1 ($r=0.55$, $p<0.0001$), and between VEGFA and TIMP1 ($r=0.47$, $p<0.0001$). Although cytokines have been reported to induce the expression of these markers, only relatively weak correlations were found between TIMP1 and IL8 and IL6 ($r=0.36$ – 0.44) and between IL6 and VEGFA ($r=0.24$). In a study by Biasi et al [31], no significant difference was found between VEGFA measured in the circulation of CRC patients and controls and a weak negative trend with disease stage was observed with TGFB1 with statistical significance occurring at stage C disease only. The authors did not

	AUC (95% CI)	p value	Sensitivity (%) at 95% specificity	Cut off value (95% specificity)
IL8	0.68 (0.59 - 0.77)	0.0006	38	>21.86
Mac2BP	0.67 (0.58 - 0.76)	0.0009	35	>9304
TIMP1 (plasma)	0.64 (0.55 - 0.73)	0.0061	33	>110.5
OPN	0.61 (0.52 - 0.70)	0.0329	31	>11800
TIMP1 (serum)	0.63 (0.54 - 0.72)	0.0133	28	>232.0
IL6	0.70 (0.61 - 0.80)	0.0002	27	>2.895
M65	0.66 (0.56 - 0.75)	0.0016	26	>472.4
IGFBP2	0.67 (0.57 - 0.76)	0.0008	25	>1225
TIMP2 (serum)	0.58 (0.48 - 0.67)	0.1144	25	<64.67
TIMP2 (plasma)	0.52 (0.43 - 0.62)	0.6578	24	<74.65
IGF2	0.64 (0.55 - 0.73)	0.0040	23	<1040
VEGFA	0.67 (0.58 - 0.77)	0.0005	23	>132.5
CEACAM6	0.58 (0.48 - 0.67)	0.1340	22	>2.558
TGFB1	0.65 (0.56 - 0.74)	0.0027	22	>16195
TNF-alpha	0.58 (0.49 - 0.69)	0.1088	20	>11.51
PKM2	0.70 (0.60 - 0.79)	0.0001	19	>60.39
Dkk3	0.50 (0.41 - 0.60)	0.9502	18	<42184
MMP1	0.62 (0.52 - 0.71)	0.0226	15	>9130
M30	0.51 (0.41 - 0.61)	0.8417	13	>374.2
MMP8	0.51 (0.41 - 0.61)	0.7808	12	>18650
ENA-78	0.54 (0.44 - 0.64)	0.4705	10	<488.0
MIP-1beta	0.56 (0.46 - 0.66)	0.2245	9	<26.35
MMP3	0.53 (0.44 - 0.63)	0.5744	7	<5460
MCP1	0.51 (0.41 - 0.61)	0.7899	6	>416.0
AUC: Area under the receiver operator characteristic curve				

Table 3: Sensitivity of biomarkers at 95% specificity.

report a correlation for these two markers. In contrast, our study found that the circulating level of these proteins strongly correlated, and were statistically higher in the CRC cohort in comparison to the control group. Furthermore, both markers demonstrated the highest sensitivity for predicting stage B disease. VEGFA, TGFB1 and TIMP1 reportedly encourages metastatic spread by influencing different aspects of the tumor-stromal environment including promotion of angiogenesis, by stimulating cell migration and invasion, or by promoting epithelial to mesenchymal transition [32,33].

A significant and strong correlation was found between M30 and M65 (r=0.59, p<0.001). M30 and M65 represent caspase cleaved cytokeratin 18 and total cytokeratin 18, respectively. Whereas M30 is reported to be a marker of tumour cell apoptosis, M65 is reported to be a marker of apoptosis and necrosis [34]. Our data supports current literature which indicates circulating levels of M30 and M65 as markers of tumour burden and may be useful as diagnostic markers for epithelial cancers [35-38]. These markers have also been evaluated in pre-clinical models of cancer to assess drug and/or treatment efficacy [39-43].

The analysis that we report here is one of few studies that provide a consistent baseline for identifying a potential panel of diagnostic markers for CRC. Based on our analysis of 32 protein biomarkers in the same patient cohort, no single biomarker adequately discriminated between controls and CRC patients to be useful in a diagnostic or screening application. Further experiments are required to determine if identified protein biomarker combinations that reflect the disease process provide the sensitivity and specificity required for CRC diagnosis. Our study also highlights that a panel of markers

	Stage A			Stage B			Stage C			Stage D		
	AUC (95% CI)	p value	Sensitivity (%) at 95% specificity	AUC (95% CI)	p value	Sensitivity (%) at 95% specificity	AUC (95% CI)	p value	Sensitivity (%) at 95% specificity	AUC (95% CI)	p value	Sensitivity (%) at 95% specificity
IL8	0.50 (0.34 - 0.67)	0.970	10	0.67 (0.55 - 0.80)	0.013	24	0.77 (0.65 - 0.88)	<0.0001	53	0.82 (0.60 - 1.05)	0.004	75
Mac2BP	0.64 (0.48 - 0.80)	0.073	29	0.66 (0.53 - 0.79)	0.022	38	0.71 (0.59 - 0.84)	0.001	39	0.68 (0.47 - 0.89)	0.112	25
TIMP1 (plasma)	0.62 (0.47 - 0.77)	0.115	23	0.66 (0.53 - 0.78)	0.018	36	0.63 (0.49 - 0.77)	0.047	39	0.65 (0.48 - 0.82)	0.132	20
OPN	0.51 (0.34 - 0.68)	0.900	24	0.73 (0.61 - 0.84)	0.001	42	0.52 (0.38 - 0.66)	0.761	16	0.73 (0.50 - 0.95)	0.023	60
TIMP1 (serum)	0.54 (0.37 - 0.70)	0.631	25	0.68 (0.56 - 0.81)	0.006	28	0.70 (0.58 - 0.83)	0.002	37	0.53 (0.30 - 0.76)	0.751	30
IL6	0.65 (0.50 - 0.81)	0.047	30	0.71 (0.59 - 0.83)	0.003	24	0.74 (0.63 - 0.86)	0.0003	30	0.62 (0.39 - 0.85)	0.281	13
M65	0.64 (0.50 - 0.79)	0.057	38	0.54 (0.42 - 0.68)	0.468	10	0.75 (0.65 - 0.86)	0.0002	25	0.77 (0.57 - 0.96)	0.008	50
IGFBP2	0.63 (0.48 - 0.78)	0.087	29	0.71 (0.60 - 0.82)	0.001	23	0.59 (0.47 - 0.72)	0.160	21	0.88 (0.78 - 0.97)	0.0002	40
TIMP2 (serum)	0.65 (0.49 - 0.81)	0.047	28	0.53 (0.37 - 0.68)	0.692	28	0.55 (0.41 - 0.68)	0.462	15	0.69 (0.48 - 0.91)	0.054	40
TIMP2 (plasma)	0.55 (0.40 - 0.72)	0.442	24	0.54 (0.04 - 0.68)	0.590	29	0.53 (0.40 - 0.67)	0.609	12	0.59 (0.37 - 0.81)	0.372	30
IGF2	0.66 (0.50 - 0.82)	0.035	29	0.56 (0.42 - 0.69)	0.382	19	0.71 (0.59 - 0.82)	0.001	27	0.68 (0.49 - 0.87)	0.074	10
VEGFA	0.58 (0.43 - 0.74)	0.262	14	0.76 (0.64 - 0.88)	<0.0001	40	0.67 (0.55 - 0.79)	0.010	13	0.64 (0.45 - 0.84)	0.159	20
CEACAM6	0.50 (0.35 - 0.65)	0.970	14	0.52 (0.39 - 0.65)	0.766	20	0.59 (0.47 - 0.72)	0.149	19	0.84 (0.70 - 0.97)	0.001	50
TGFB1	0.66 (0.52 - 0.80)	0.029	19	0.72 (0.60 - 0.83)	0.001	32	0.63 (0.51 - 0.76)	0.047	16	0.51 (0.29 - 0.07)	0.940	18
TNF-alpha	0.52 (0.37 - 0.67)	0.778	10	0.65 (0.52 - 0.78)	0.032	21	0.54 (0.41 - 0.68)	0.537	21	0.69 (0.48 - 0.91)	0.082	38
PKM2	0.65 (0.50 - 0.79)	0.051	19	0.67 (0.55 - 0.79)	0.010	16	0.72 (0.60 - 0.83)	0.001	15	0.79 (0.63 - 0.94)	0.004	40
Dkk3	0.51 (0.36 - 0.67)	0.821	0	0.52 (0.38 - 0.65)	0.789	10	0.52 (0.38 - 0.65)	0.820	21	0.57 (0.34 - 0.80)	0.475	30
MMP1	0.56 (0.40 - 0.73)	0.399	14	0.62 (0.49 - 0.75)	0.065	16	0.64 (0.52 - 0.77)	0.029	15	0.61 (0.42 - 0.81)	0.258	10
M30	0.54 (0.38 - 0.70)	0.600	14	0.62 (0.49 - 0.74)	0.080	19	0.63 (0.51 - 0.75)	0.041	12	0.60 (0.39 - 0.81)	0.321	30
MMP8	0.54 (0.38 - 0.69)	0.642	6	0.54 (0.41 - 0.67)	0.544	17	0.54 (0.41 - 0.66)	0.577	12	0.70 (0.49 - 0.91)	0.056	22
ENA-78	0.60 (0.46 - 0.75)	0.172	14	0.59 (0.37 - 0.64)	0.956	7	0.50 (0.37 - 0.63)	1.000	6	0.62 (0.43 - 0.80)	0.250	20
MIP-1beta	0.59 (0.44 - 0.73)	0.260	5	0.52 (0.39 - 0.65)	0.769	7	0.57 (0.45 - 0.70)	0.268	9	0.60 (0.40 - 0.80)	0.307	20
MMP3	0.51 (0.34 - 0.67)	0.950	14	0.55 (0.41 - 0.68)	0.466	10	0.53 (0.40 - 0.66)	0.696	12	0.69 (0.55 - 0.84)	0.054	10
MCP1	0.57 (0.43 - 0.72)	0.329	14	0.52 (0.39 - 0.64)	0.815	3	0.58 (0.46 - 0.71)	0.201	12	0.55 (0.34 - 0.77)	0.592	20

Table 4: Biomarker sensitivity at 95% specificity according to disease stage.

Marker 1	Marker 2	Spearman R	P value
TIMP2 SERUM	TIMP2 PLASMA	0.740	<0.0001
VEGFA	TGFB1	0.649	<0.0001
TIMP1 SERUM	TIMP1 PLASMA	0.626	<0.0001
M65	M30	0.587	<0.0001
TGFB1	TIMP1 PLASMA	0.552	<0.0001
VEGFA	TIMP1 PLASMA	0.470	<0.0001
IL8	TIMP1 SERUM	0.440	<0.0001
OPN	IGFBP2	0.418	<0.0001
IL8	TIMP1 PLASMA	0.406	<0.0001
TIMP1 PLASMA	MAC2BP	0.404	<0.0001
TGFB1	DKK3	0.401	<0.0001
TIMP2 PLASMA	TIMP1 PLASMA	0.393	<0.0001
M65	TIMP1 PLASMA	0.383	<0.0001
IL8	M65	0.382	<0.0001
TIMP1 PLASMA	IGFBP2	0.380	<0.0001
IL6	IGF2	-0.374	<0.0001
IL6	PKM2	0.373	<0.0001
VEGFA	MAC2BP	0.363	0.0002
IL6	TIMP1 SERUM	0.360	<0.0001
DKK3	TIMP1 PLASMA	0.356	<0.0001
IL8	MAC2BP	0.348	<0.0001
VEGFA	TIMP1 SERUM	0.347	0.0003
M65	IGFBP2	0.342	<0.0001
IL6	TIMP1 PLASMA	0.342	<0.0001
CEACAM6	TIMP1 SERUM	0.337	<0.0001
M30	IGF2	-0.334	<0.0001
TIMP1 SERUM	MAC2BP	0.325	0.0001
TGFB1	MAC2BP	0.313	0.0002
CEACAM6	TIMP1 PLASMA	0.309	0.0002
PKM2	TIMP1 SERUM	0.306	0.0003
TGFB1	TIMP1 SERUM	0.303	0.0003
TIMP1 SERUM	IGFBP2	0.300	0.0003

Table 5: Correlation between markers.

representative of different biological processes in the carcinogenesis pathway, including inflammation, the immune response, or apoptosis may be most optimal. Simultaneous measurement of these potential biomarker combinations in a large and well-defined cohort is required to evaluate their true diagnostic ability.

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