

A role for serum cytokines and cell adhesion molecules in the non-invasive diagnosis of colorectal cancer

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Abstract. Colorectal cancer (CRC) remains a major cause of cancer-related mortality. Consequently, new diagnostic and therapeutic approaches are being investigated including the serum levels of cytokines and other molecules, although the results are often inconclusive. Thus, the present study aimed to determine whether serum level of cytokines, cell adhesion molecules or matrix metalloproteinases (MMPs), alone or in combination, may contribute to the non-invasive diagnosis of CRC. The serum levels of nine cytokines [ILs; IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-17, IL-22 and IL-33, and interferon (IFN)- γ], two cell adhesion molecules (intercellular adhesion molecule-1 and P-selectin) and an MMP-7 were measured by ELISA in 33 patients with CRC and 35 healthy controls. Combined capacity of all molecules to detect the presence of CRC was assessed by logistic regression. Molecules and molecule combinations were tested for all stages and tumor grades. A significant increase was identified for IL-8 in patients compared with healthy controls; IL-10 was found to be significantly decreased. The biomarker potential of each significantly modified molecule was tested: IL-8 had a sensitivity of 0.865, a specificity of 0.600 and an area under the curve (AUC) of 0.777; for IL-10, sensitivity was 0.65, specificity was 0.69, with an AUC of 0.689. Logistic regression determined the best discriminative potential between patients

and control groups for the combination IL-4 + IL-6 + IL-8 + IFN- γ , with 0.97 sensitivity and 0.58 specificity. For the early stages of CRC, the combination IL-6 + IL-8 + IL-22 showed good performance. It was concluded that increased IL-8 had potential as single biomarker in CRC. Cytokine combinations are superior to single cytokine analysis in showing the presence of CRC.

Introduction

Colorectal cancer (CRC) is a disease with an increasing global incidence rate; it is currently the third most frequently diagnosed and the second leading cause of mortality among types of cancer worldwide (1). It has therefore spurred numerous efforts to improve treatment strategies, but also to develop new diagnostic approaches for an early and accurate detection. CRC is one of the types of cancer with a dominant inflammatory component and it frequently occurs against an inflammatory background (2). Cytokines, cell adhesion molecules and other molecules change their expression during the inflammatory process and this finally leads to alterations in their serum levels (3,4). At present, the diagnosis of CRC is based on non-invasive screening methods like guaiac-based fecal occult blood test (gFOBT) or the newer fecal DNA test, or on the more precise and accurate flexible sigmoidoscopy and colonoscopy. However, these methods are invasive, expensive or less sensitive in the diagnosis of CRC; consequently, alternative methods were tested for their diagnostic value in colorectal tumors (4,5).

One of these methods takes advantage of the increased serum levels of cytokines that accompanies inflammation in tumors (4). There have been numerous studies that have evaluated the potential diagnostic contribution of cytokines and other molecules to the diagnosis of CRC; the results were often inconsistent or inconclusive (4-7). Thus, the present study aimed to investigate whether serum levels of cytokines, cell adhesion molecules or matrix metalloproteinases (MMPs), alone or in combinations, could contribute to the non-invasive diagnostic of CRC.

The present study evaluated the serum level of nine cytokines (ILs; IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-17A, IL-22 and IL-33, and interferon (IFN)- γ), two cell adhesion molecules

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Abbreviations: AUC, area under the curve; CRC, colorectal cancer; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL, interleukin; MMP, matrix metalloproteinase; P-sel, P-selectin; ROC, receiver operating characteristic; Th, T helper

Key words: cytokine, cell adhesion, colorectal, cancer, diagnostic

[intercellular adhesion molecule-1 (ICAM-1) and P-selectin (P-sel)] and MMP-7. The cytokines were selected to represent the main immune networks in tumors, allowing not only their use as potential diagnostic tools, but also a global characterization of the immune response in colorectal cancer.

Materials and methods

Patients. The present study was a prospective, case-control study, in which 33 patients with colorectal malignant tumors and 35 age and sex-matched healthy controls were enrolled (Table I). The patients were treated in the Surgical Clinic of the Regional Institute of Gastroenterology and Hepatology (Cluj-Napoca, Romania), between March 2019 and March 2020.

The present study included patients with colorectal adenocarcinoma confirmed by biopsy and pathologic examination. The following subjects were excluded from the study: Patients and controls suffering from inflammatory diseases that may affect the serum levels of cytokines (such as collagen diseases, active rheumatoid arthritis or inflammatory bowel disease), active infections, active neoplasms in other locations; surgery, trauma or vascular events in the previous four months, severe organic deficiencies and patients with concomitant treatments that could alter the immune response (including chemotherapy or cortisol). The alcohol consumption and smoking status was comparable between the two groups.

The present study was approved by the Ethics Committee of the Iuliu Hațieganu University of Medicine and Pharmacy (Cluj-Napoca; approval no. 40/02.04.2018) and of the Regional Institute of Gastroenterology and Hepatology from Cluj-Napoca (approval no. 2769/1.03.2018). Written informed consent was obtained from each patient and healthy control.

ELISA analysis. A total of 10 ml serum was obtained from each patient and healthy control by venous puncture of the median cubital vein, followed by centrifugation for 10 min at 3,175 x g at room temperature. The serum was stored at -80°C before testing. ELISA kits (from Elabscience Biotechnology Inc.) with appropriate working ranges for the cytokines were used, including for IL-6 (cat. no. E-EL-H0102), IL-8 (cat. no. E-EL-H0048), IL-10 (cat. no. E-EL-H0103), IL-17A (cat. no. E-EL-H0105), IL-33 (cat. no. E-EL-H2402), IFN- γ (cat. no. E-EL-H0108), ICAM-1 (cat. no. E-EL-H2585) and P-sel (cat. no. E-EL-H0917). ELISA kits were also purchased from Biologend, Inc., including IL1 β (cat. no. BZ-437007) and IL-4 (cat. no. BZ-430307), and from R&D Systems Inc., including IL-22 (cat. no. RD-D2200) and MMP-7 (cat. no. RD-DMP700). Each cytokine was tested individually and not through multiplexed analysis, thus avoiding interactions between analytes in the testing process and also allowing to choose the range of each kit. All cytokines were analyzed in the present study, since they had values inside the assay working range and 100 μ l serum was used for each cytokine assay. Standard curves for molecules were generated, using the reference concentrations provided by the manufacturers. The results were read on a Biotek Synergy H1 Hybrid microplate reader (BioTek Instruments, Inc.). GraphPad Prism 6 (GraphPad Software, Inc.) was used for analysis.

Statistical analysis. The analysis of the statistical significance of differences in serum levels was performed either with the unpaired

t-test for normally-distributed data, or with the Mann-Whitney U test for non-normally distributed data. Differences in cytokine levels between stages or tumor grades were analyzed with either one-way analysis of variance (ANOVA) with Games-Howell post hoc test for data with normal distribution, or Kruskal-Wallis test with Dunn's post hoc test for other continuous data. Shapiro-Wilk test was used to test data normality.

To summarize the potential of all molecules to discriminate between patients with CRC and healthy individuals, logistic regression was performed; a stepwise approach was followed, excluding molecules with no significant influence on the dependent variable; different combinations of the remaining molecules were tested for their ability to discriminate between CRC and control samples. Logistic regression was used to check for confounding factors. Correlational analysis of Spearman coefficients was performed on all molecules; none of the molecules analyzed together in logistic regression had correlation coefficients above 0.7.

Receiver operating characteristics (ROC) curves for each molecule and for the logistic regressions were generated using XLstat software version 2021.3.1 (Addinsoft). All other statistical analyzes were performed with R software version 4.1.0 (8). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The biological background and the clinicopathological characteristics of the patients with CRC and those in the control group are summarized in Table I. The differences in the serum levels of molecules between patients and control groups are presented in Fig. 1A. A significant increase was observed for IL-8 serum levels in CRC compared with the control, whereas IL-10 was found significantly decreased. IL-1 β , IL-4, IL-6, IFN- γ , ICAM-1, MMP-7 and P-sel levels were increased, but not significantly. IL-17 and IL-33 were found slightly reduced in CRC patients, but with no statistical significance.

The modifications in the serum levels of molecules linked to the tumor TNM stage, WHO grade and location are presented in Fig. 1B-D. Significant increases with the tumor TNM stage were observed in IL-17 and IL-33 levels; IL-1 β also had significant modifications but exhibited a particular behavior, decreasing between stages I and II, and increasing in the later stages (Fig. 1B). IL-6, ICAM-1 and MMP-7 increased with stage, but without statistical significance. No stage-related modifications in the serum levels of IL-4, IL-8, IL-22, IFN- γ and P-sel were observed. The present study did not find any significant modification linked to the tumor WHO grade; however, some trends were observed. For example, IL-8, MMP-7 and ICAM-1 levels were increased in grade 1 compared with grade 2 tumors, whereas IL-6, IL-17 and P-sel levels were increased in grade 2 compared with grade 1 tumors (Fig. 1C). Only 2 patients had grade 3, so no significant comparisons were found.

Concerning the levels in the right and left colon (which included the left colon distally from the splenic flexure and the rectum) of the tumoral process, there were significant increases in the levels of IL-10 in the left locations; the adhesion molecules IL-8, ICAM-1 and P-sel as well as IL-33 were also increased on the left, but not significantly (Fig. 1D). IFN- γ was increased in the right colon tumors, without statistical significance. Finally,

Table I. Clinicopathological characteristics of patients with CRC and healthy control patients.

Clinicopathological characteristic	CRC patients, n=33	Healthy controls, n=35
Sex, n (%)		
Male	16 (48.5)	14 (40.0)
Female	17 (51.5)	21 (60.0)
Mean age, years	66.24	66.02
Tumor stage, n (%)		
I	7 (21.2)	
II	15 (45.5)	
III-IV	11 (33.3)	
Tumor WHO grade, n (%)		
1	10 (30.3)	
2	21 (63.6)	
3	2 (6.1)	
Tumor location, n (%)		
Right colon	10 (30.3)	
Left colon (including rectum)	23 (69.7)	
Tumor histology, n (%)		
Adenocarcinoma	33 (100.0)	
Comorbidity, n (%)		
Diabetes	6 (18.18)	7 (20.0)
Chronic cardiovascular disease (CAD, atrial fibrillation)	8 (24.24)	7 (20.0)
Cirrhosis	1 (3.03)	0 (0.0)
Ascites	1 (3.03)	0 (0.0)
Hypothyroidism	1 (3.03)	2 (5.71)
Chronic kidney disease	1 (3.03)	0 (0.0)
Adrenal adenoma	1 (3.03)	0 (0.0)
Obesity	1 (3.03)	1 (2.85)

CAD, coronary artery disease; CRC, colorectal cancer.

IL-1 β , IL-6, IL-17 and IL-22 and MMP-7 were not significantly modified in right compared with left tumor locations.

The biomarker potential of each significantly modified molecule was tested. The ROC curves are presented in Fig. 2. IL-8 had a sensitivity of 0.865, a specificity of 0.600 at a cutoff value of 20.741 pg/ml and an AUC of 0.777; for IL-10 sensitivity was 0.65, specificity was 0.69, with an AUC of 0.689.

Investigation of the differences between patients and controls linked to stage, tumor differentiation or locations showed significant increases in ICAM-1 for stage II in patients ($P=0.0127$; Fig. S1); for the rest of the molecules, there were no distinctive elements in different tumor stages compared with CRC in general. ICAM-1 showed some biomarker potential for stage II, with a sensitivity of 0.86, a specificity of 0.63 and an AUC of 0.724 (Fig. S1).

Correlation between molecules were tested prior to logistic regression analyses (Table SI). None of the molecules that had a correlation coefficient >0.7 were assessed together in logistic regression. For the combinations of molecules that were used (Fig. 3), IL4 + IL6 + IL8 + IFN- γ had a sensitivity of 0.97, a specificity of 0.58 and an AUC of 0.85; for IL4 + IL8 + IFN- γ ,

the sensitivity was 0.87, the specificity was 0.63 specificity and the AUC was 0.840; for IL4 + IL6 + IL8, there was 0.84 sensitivity and 0.63 specificity, with an AUC of 0.824; for IL6 + IL8, sensitivity was 0.84, specificity was 0.60 and AUC was 0.788; finally, for IL4 + IL8, there was a sensitivity of 0.84, a specificity of 0.66 and an AUC of 0.822.

The model with four cytokines had the best sensitivity (0.97) at the optimal cutoff point of 0.71. The equation of the model was: $Z = -7.77 + (3.297 \times \text{IL4}) - (0.03 \times \text{IL6}) + (0.086 \times \text{IL8}) - (0.103 \times \text{IFN}\gamma)$, where Z is the log(odds) for the positive diagnosis of CRC, -7.77 is the intercept of the y-axis, and 3.297, 0.03, 0.086 and 0.103 are the regression coefficients for each molecule. To test the potential of molecules for an early diagnosis in CRC, the present study performed logistic regression on stage I patients and controls. Table SII presents the logistic regressions and Fig. S2 presents the most significant of these. Some of the combinations tested for stage I had good discriminative potential, in particular, the combination IL6 + IL8 + IL22, with an AUC of 0.927, 0.85 sensitivity and 0.89 specificity (Fig. S2E). The same combination tested on all patients with CRC did not perform well (Fig. S2F).

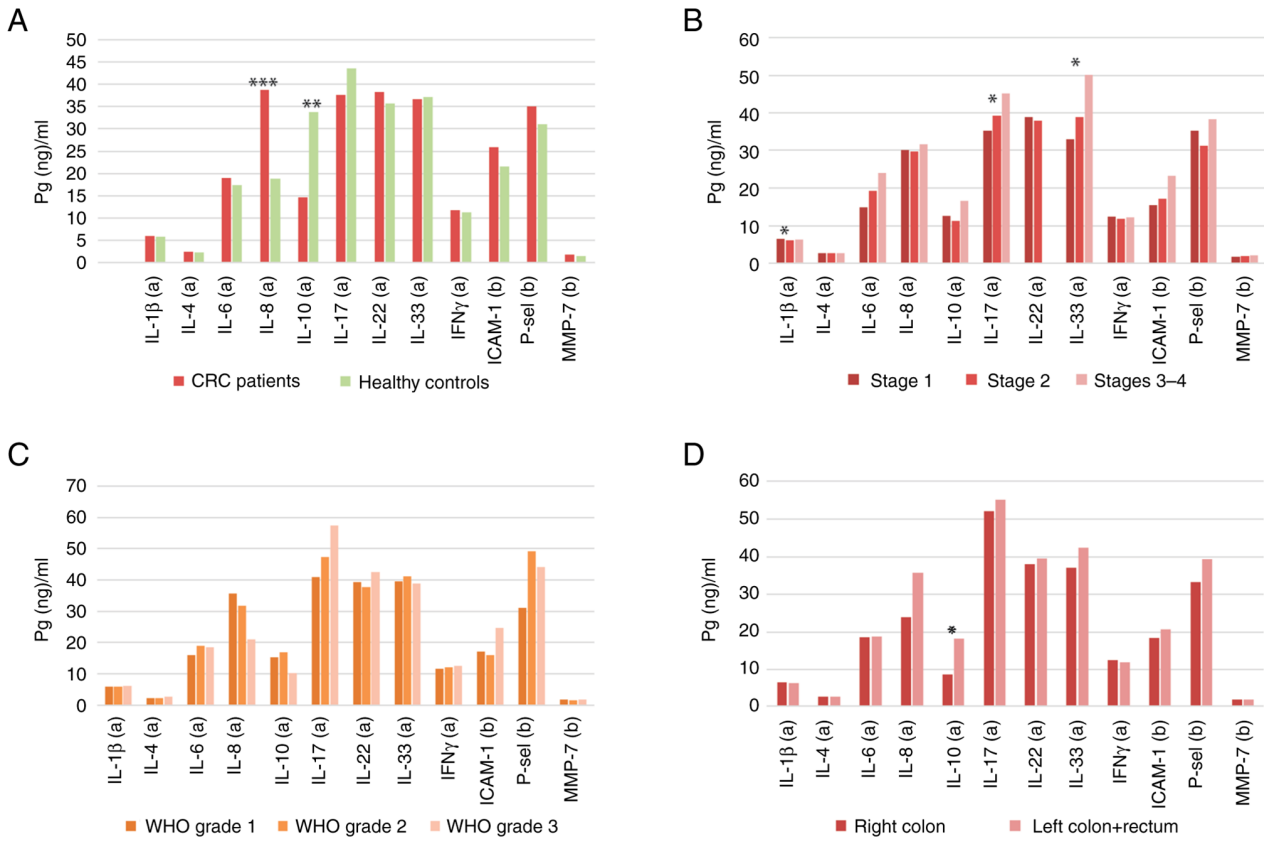


Figure 1. Serum levels of molecules in patients with CRC compared with healthy control patients and in different tumor stages, WHO grades or colon locations. (A) IL-8 exhibited a significant increase in patients with CRC compared with healthy controls, whereas IL-10 was found significantly decreased. (B) IL-1β, IL-17 and IL-33 had significant modifications with TNM stages. (C) No significant differences were found for the levels of molecules in different tumor WHO grades. (D) IL-10 significantly increased serum levels in left vs. right locations in the colon. *P<0.05; **P<0.01; ***P<0.001 vs. healthy controls or in different stages and locations; IFN, interferon; IL, interleukin; MMP7, matrix metalloproteinase 7; WHO, World Health Organization.

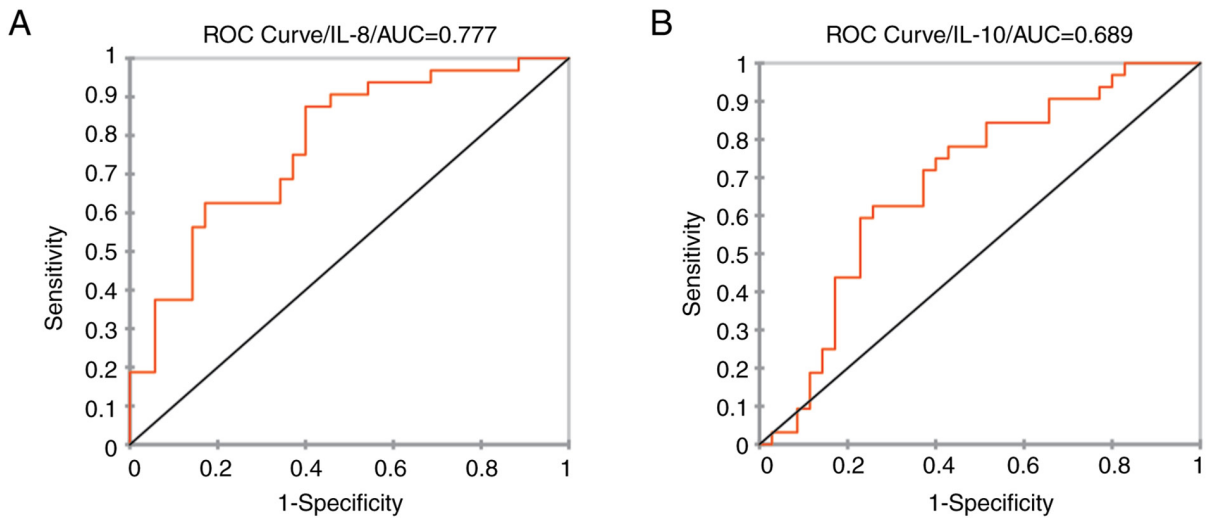


Figure 2. ROC curves for the significantly modified molecules. ROC curves for (A) IL-8, (B) IL-10. AUC, area under the curve; IL, interleukin; ROC, receiver operating characteristic.

Discussion

There are a number of studies which address the serum levels of cytokines and their possible diagnostic applications, many of which show increased levels of IL-8 in colorectal cancer patients (4-6). IL-1 exhibited no significant changes in a number

of studies (4-7). IL-6 level is found increased (9), including two meta-analyses (10,11), whereas another found no differences or even a decrease compared with healthy subjects (4). IL-4 and IFN γ were evaluated in two studies (4,5); one study showed significant differences (4), while the other found non-significant changes in these two cytokines (5). IL-17 was found

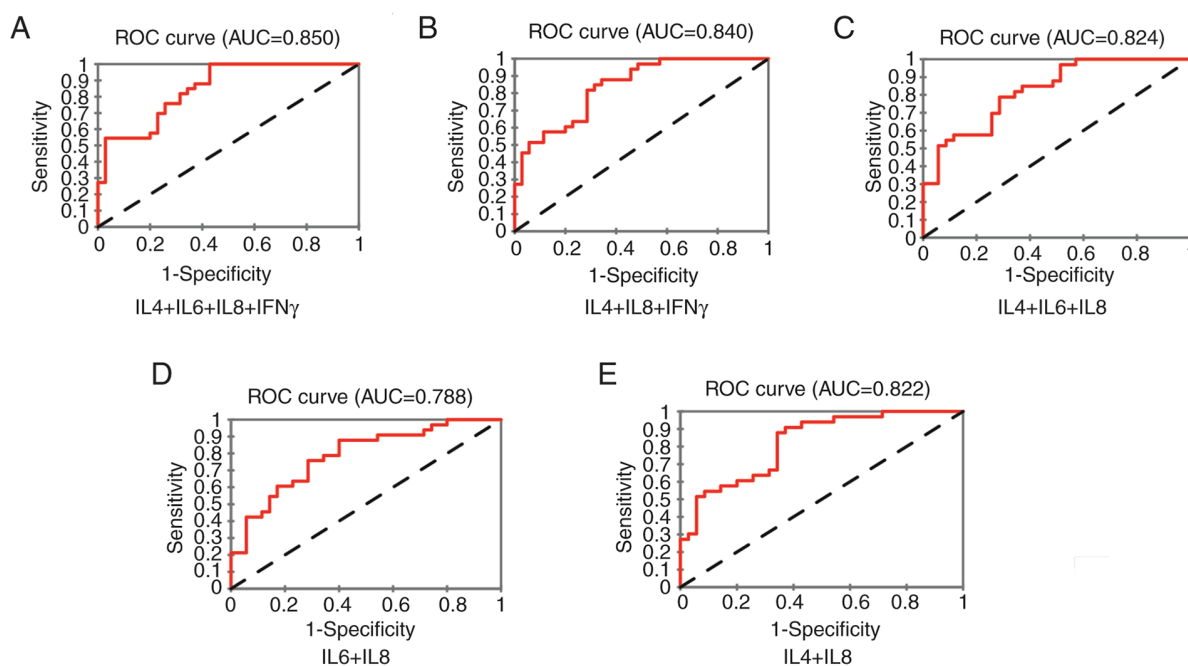


Figure 3. Logistic regression for the combination of molecules. ROC curves for logistic regression with different molecules combinations. (A) IL4 + IL6 + IL8 + IFN- γ , (B) IL4 + IL8 + IFN- γ , (C) IL4 + IL6 + IL8 (D) IL6 + IL8 and (E) IL4 + IL8. The combination IL4 + IL6 + IL8 + IFN- γ had the best performance, with 0.97 sensitivity, 0.58 specificity and AUC of 0.85. AUC, area under the curve; IFN, interferon; IL, interleukin; MMP7, matrix metalloproteinase 7; ROC, receiver operating characteristic.

increased in some studies (4,12), but the majority reported no difference or even decrease in CRC patients (13-15). Two studies (10,12), one of them a meta-analysis (10), showed a high level of IL-22 in CRC patients. IL-10 is generally found increased in CRC patients (13,16); however, Abtahi *et al* (17) show decreased levels compared with healthy patients, whereas Yamaguchi *et al* (4) found no difference.

The adhesion molecules ICAM-1 and P-sel are generally increased in CRC patients (18,19). The same is true of MMP-7, which correlates with the tumor stage (20). Some studies report low levels of ICAM-1 or P-sel as the disease progresses (21,22). Concerning the studies with multiple cytokines, Yamaguchi *et al* (4) show a profile with moderate increase in proinflammatory cytokines, IL-8, IL-12, IL-17A, TNF- α and IFN- γ , as well as increases in IL-4, IL-9 and some proinflammatory chemokines (such as CXCL-10 and CCL-3 and 4). Kantola *et al* (5) found a profile with significant increases of IL-6, IL-7 and IL-8, as well as non-significant increases in IL-12, IFN γ and CXCL-10. Pengjun *et al* (23) found IL-8, TNF- α and MMP-7 as potential serum biomarkers, while IFN γ , IL-6 and IL-10 were not significant in discriminating between CRC and normal serum.

The potential value as biomarkers for diagnosis and prognosis was tested for the following molecules: IL-4, IL-6, IL-8 and IL-10, P-sel and MMP-7 (4-6,9,16,18,24), as well as for multi-cytokine profiles (4,5,23), highlighting the potential of all these molecules for being such biomarkers. Other biomarker molecules for CRC were found to be IL-7, IL-9, CCL-11 and CXCL-10 (4,5). However, there was an inconsistency in the aforementioned studies concerning cytokine levels, some showing increased and others showing decreased levels for the same molecule. This inconsistency was also observed concerning the biomarker potential of

these cytokines, neither molecule being found as universal biomarker for CRC (5,23).

In this context, the present study selected molecules representing the main immune networks that are present in colorectal tumors: IL-1 β , IL-6, IL-8 and IL-33 for the inflammatory network, IFN- γ for the T helper (Th)1 and IFN- γ -secreting network, IL-4 for the Th2 network, IL-17A and IL-22 for the Th17 and Th22 networks, and IL-10 for the suppressive network. Two adhesion molecules, ICAM-1 and P-sel, were also tested, as was MMP-7, which is produced as a consequence of the tumor development process. In addition to its diagnostic utility, such a profile may allow a global characterization of the immune response in colorectal tumors. The profile that was obtained suggested a moderate increase in the inflammatory compartment (mainly IL-1 β and IL-8) and in the Th1 and Th2 networks, no difference in the Th17 response and a reduction in the suppressive network, reflected in the significantly low levels of IL-10 level. However, the increases are not notable, some of them being even not significant, the most likely causes being the low immunogenicity of colorectal cancer and the generally weak response that the organism mounts against tumors.

Not all the significantly modified molecules showed biomarker potential. The present study highlighted that increased IL-8 had the capacity to discriminate between CRC and normal serum (Fig. 2).

Combinations of molecules were tested by logistic regression; all showed discriminative potential for CRC, providing possible diagnostic approaches for the clinician (Fig. 3). Concerning the potential of these molecules, alone or in combinations, to detect the early stages of the tumoral process, the combination IL-6 + IL-8 + IL-22 showed a good discriminative potential for this stage (Fig. S2E). The same combination, tested in all CRC patients, did not have notable performances

(Fig. S2F). However, this result should be considered cautiously, given the small number of subjects in stage I that were tested in the present study. The results should be interpreted in the context of the variability of the results in IL testing in CRC, highlighted in the aforementioned studies. Within this variability, certain patterns can be observed. For example, from the few studies with multi-cytokine analysis aforementioned, including the present study, the main patterns that emerge are those with increased IFN- γ , IL-12 and CXCL-10, along with a moderate increase in Th2 and Th17 cytokines (4), and patterns with non-significant increases of these cytokines (5,23). Inflammation was found increased by all studies, through its representative molecules (IL-6, IL-8, ICAM-1 and P-sel) (4,5,18,19,23). The most constantly increased cytokines that all studies, including the present study, have found significantly increased is IL-8, followed by IL-6.

This behavior of ILs and adhesion molecules in CRC may be explained by the heterogeneity of colorectal cancer in histological structure and molecular mechanisms, which leads to different immune infiltration and, by consequence, to different cytokine production patterns (25).

The heterogeneity of the immune infiltration in tumors and its consequence, the variability of the seric cytokine profiles, makes it difficult to find reliable biomarkers in colorectal cancer; a good strategy may be to choose cytokine combinations that cover all possible patterns, such as IL6 + IL8 + IL4 + IFN- γ , or IL-6 + IL-8 for the patterns with only inflammation. An alternative is to use molecules that have been found constantly elevated in CRC, such as IL-8 and IL-6 or, in other studies, IL-7 and IL-9 (4,5).

The results of the present study were generally in line with other studies on this topic (4,5,18,19,23); however, owing to the small sample size, these results should be validated on larger population samples before translation into the clinic. An area that the study did not cover is represented by cytokine level increases in precancerous lesions, such as polyps, familial polyps or adenomas; some of the molecules studied, such as IL-4 and IL-17, increased in expression along the adenoma-carcinoma sequence (26). Such an approach would be useful as a non-invasive method of differentiating between malignant and non-malignant lesions.

Compared with the gFOBT test, which has a sensitivity of 31% and a specificity of 87% (27), the combinations of ILs used in the present study have sensitivities that range between 84 and 97% and specificities ranging between 58 and 66%. Cytokine testing is superior in terms of sensitivity but less specific compared with the gFOBT test; cytokine testing is also more expensive. However, since a cancer has to be diagnosed, high sensitivities are preferable; the combined use of cytokine testing and gFOBT test would provide a combination of high sensitivity and specificity.

A significant challenge is that serum cytokines levels also increase in inflammatory diseases. However, these increases are much more prominent in inflammation than in cancer, which could help to differentiate between the two (28-30); the pattern of these increases could also be helpful, as it has been shown that there is a complex pattern of these increases in CRC (4,5,23), whereas the immune response in inflammatory diseases is not as complex, being usually Th1, Th2 or Th17-driven.

Using the right strategy, cytokines may have a role in the diagnosis of colorectal neoplasias, along with their emerging role as a prerequisite for future personalized immuno-therapies in cancer (31). It is a non-invasive and inexpensive method, which proved to be accurate in terms of results, and may be considered to have its place in the diagnostic strategies in colorectal cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

OF conceived the study and wrote the manuscript; IBN, LB and OZ made substantial contributions to analysis and interpretation of data; FZ made substantial contributions to acquisition of data; VC made important contributions to the conception and design of the work, being also involved in drafting the manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript. LB and OZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study obtained the approvals of the Ethics Committee of the Iuliu Hatieganu Medicine and Pharmacy University (Cluj-Napoca, Romania; approval no. 40/02.04.2018) and of the Regional Gastroenterology and Hepatology Institute (Cluj-Napoca, Romania; approval no 2769/1.03.2018) and the written informed consent from each patient and healthy control.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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