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Serum cytokine profile as a potential prognostic tool in colorectal cancer patients – one center study

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ABSTRACT

Aim: Comparison of 14 cytokines levels between a control group and prospectively enrolled CRC patients to confirm their significance in CRC development. We tested if a model based on 14 cytokines levels could predict prognosis in Caucasian CRC patients treated with 5-FU based chemotherapy.
Background: Novel prognostic tools in colorectal cancer (CRC) are necessary to optimize treatment, reduce toxicity and chemotherapy (CHT) costs.
Materials and Methods: We assessed prognostic significance of 14 cytokines: IL-1 beta, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12p70, IL-13, IL-17A in 75 prospectively enrolled CRC patients before initiation of palliative or adjuvant CHT and in 22 control subjects. Readings were taken using the Bio-Plex 200 System. Response to treatment was assessed after 6 months from initiation of CHT. The treated group was divided depending on the response into a progressors (death, progression of disease) and non-progressors group (stable disease, partial response, complete response).
Results: We found that increased concentration of IL-8 was a negative prognostic factor in the whole group and palliative subgroup, whereas increased level of IL-10, IL-7, and IL-12p70 was a negative predictor in the adjuvant group CHT.
Conclusions: We proposed a statistical model based on circulating cytokine levels, showing a good prognostic value in prediction of the response to CHT (AUC = 0.956). The model, including combined IL-2, IL-8, IL-10 and IL-13 levels, established in the whole treated group, should be validated in larger trials.

1. Introduction

Significant advances have been made in the colorectal cancer (CRC) therapy within the recent two decades. New therapies targeting the EGFR signaling pathway in a wild type RAS and VEGF regardless of the RAS status allowed for treatment individualization, better therapy outcomes with less toxicity for patients. Therapy based on most widely used TNM classification[1] could lead to overtreatment or undertreatment in some groups of patients.[2] Subjects with a known higher risk of progression could be proposed more aggressive therapy or more frequent follow up. However, accurate, non-invasive tools to assess patients' prognosis are still not available. Evidence of mutual connections between inflammation and carcinogenesis makes inflammatory cytokines exploratory targets as prognostic biomarkers.[3] Among other actions, these cytokines were found to modulate the immune response within tumor microenvironment.[4]

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Metaanalysis done in 2018 showed paucity and heterogeneity of studies performed so far assessing the prognostic value of cytokines in CRC.[2] Several cytokine-based prognostic models were proposed in CRC patients.[2] Some models showed promising prognostic utility; however, they need to be validated in different, possibly larger populations.

On the other hand, CRC incidence and prognosis vary significantly worldwide.[5,6] It might suggest that prognostic models could differ among populations.

In our study we compared 14 cytokines levels between a control group and prospectively enrolled CRC patients to confirm their significance in CRC development. We also tested if a model based on 14 cytokines levels could predict prognosis in Caucasian CRC patients treated with 5-FU based chemotherapy.

2. Material and Methods

2.1. Patients' enrollment and study design

Profile of 14 cytokines with a potential prognostic value in CRC patients was identified by the authors based on the literature review (IL-1 beta, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A). 85 consecutive inpatients, diagnosed with CRC (histologically confirmed adenocarcinomas), admitted in sequence for CHT to the Department of Internal Medicine and Oncology between May 2014 and October 2016, were screened for participation in this prospective study. Study design was accepted by the Ethics Committee (EC approval no: KNW/0022/KB1/155/14) and was conducted in accordance with the Declaration of Helsinki. All patients gave their informed consent to the investigation. Enrolled patients were Caucasians, residents of Poland. Exclusion criteria included targeted therapies, known genetic cause of CRC, acute and chronic infections, poor ECOG performance status (N = 10). Samples from 2 patients were not collected within the required timeframe; in addition, response data of one patient was not available (lost to follow up). Finally, data of 72 subjects, who had received at least one CHT cycle, were included in the analysis. Control group consisted of consecutive all-comer patients hospitalized in the Department of Endocrinology, admitted in sequence for diagnosis of hormonally inactive suprarenal incidentalomas or thyroid gland diseases (found free of disease).

To evaluate if selected cytokines could be used as prognostic markers, the whole group was divided into 2 subgroups of patients: progressors and non-progressors. Patients' clinical response was defined as positive (non-progressors' group) in the case of complete or partial response or stabilization of the disease. The negative response (progressors' group) was reported in the case of disease recurrence/progression or patient's death. Clinical response was assessed in accordance with clinical standards 6 months from the CHT initiation in all treated patients. Computed tomography, ultrasound and chest radiographs were utilized according to a standard approach to follow up on results of physical examination and lab results.

2.2. Assays

Fasting patients' blood samples were withdrawn from a cephalic vein before the CHT initiation and then processed for serum separation immediately. Blood samples were centrifuged at 300 g for 20 minutes and then kept in liquid nitrogen vapor until assessment. Serum levels of IL-1 beta, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12p70, IL-13, IL-17A were determined via multiplex Bio-Plex Pro™ kits (Bio-Rad®), in accordance with the manufacturer's instructions. Bead fluorescence readings were taken using the Bio-Plex 200 System high PMT (High RP1) setting and analyzed with Bio-Plex Manager version 6.1.0.727 (Bio-Rad Laboratories). The results are expressed in pg/mL if not stated otherwise.

2.3. Data analysis

Several authors found that a cytokine profile in metastatic CRC patients differs from those in less advanced stages.[7,14] Therefore, we performed a separate analysis for patients treated with palliative and adjuvant CHT. Palliative group consisted only of patients in CS IV (CS, *clinical* stage); adjuvant group included patients in CS II and III. Characteristics of all analyzed groups are shown in Tables 1 and 2.

Then, to establish predictive significance of particular cytokines, we calculated receiver operator curves (ROC) for the whole group, palliative and adjuvant subgroups.

Next, we applied multivariate analysis to find the model which has the best predictive power regarding clinical response (progressors, non-progressors) for the whole group. All the patients' demographics, clinical and histopathological variables were tested for association with levels of immune mediators. These variables, together with the concentrations of IL-1 beta, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12p70, IL-13, IL-15, and IL-17A were tested as predictors of patients' favourable clinical response to CHT.

2.4. Statistical analysis

Continuous variables were presented as mean and standard deviation or median with the first and the third quartile when appropriate and compared using Student's t-test or Wilcoxon Rank-sum test, respectively. Nominal variables were described using counts and percentages and compared using χ^2 test or Fisher's exact test, when appropriate. Normality of data was tested using the Shapiro-Wilk test, homogeneity of variances was tested using the Levene test. All statistical tests were two-sided. In the case of more than two groups, comparisons were made using ANOVA or the Kruskal-Wallis test – selected based on the data normality and homogeneity of variance. Presented multiple comparisons were calculated using post-hoc tests appropriate for the performed analysis. Tukey's HSD test was used for post hoc analysis after ANOVA and the Steel-Dwass method after Kruskal-Wallis test.

For the construction of a prediction model, candidate cytokines were selected according to the following procedures. First, we nominated as the initial candidate, the cytokine that showed the smallest p-value in the simple logistic regression analysis, and excluded all cytokines whose correlation coefficients with the selected cytokine exceeded 0.7. Then, we identified another candidate cytokine with the next smallest p-value, and eliminated some of the remaining cytokines in the case where their correlation coefficients with the second selected cytokine were above 0.7. These procedures were repeated until candidate cytokines were finalized. By applying a backward elimination method to a logistic regression model with AIC as a target with all possible candidates of cytokines and fixed variables of age, clinical status and therapy, we constructed a prediction model for the response to treatment.

Results were expressed as odds ratios (OR) with corresponding 95% confidence intervals (95% CI; LCI – lower bound, UCI – upper bound). All results have been shown as mean (+/-SD) or median (Q1; Q3) for normally or not-normally distributed data, respectively.

Assuming 80% power and significance level of 0.05 at least 70 patients should be included to demonstrate 0.85 sensitivity and specificity compared to the null value of 0.65 in predicting a clinical response based on IL-8.

Data management and analysis activities were carried out in JMP® software, version 14.0.0 (SAS Institute Inc., 2018, Cary, NC,

Table 1
Characteristics of patients in the progressors' and non-progressors' groups.

Parameter	Non-progressors (NP) (n = 34)	Progressors (P) (n = 38)	Whole group (WG) (n = 72)	Test	*P*-value (NP vs. P)
Women	15 (44.1%)	21 (55.3%)	36 (50.0%)	P	0.35
Age (years) - mean ± SD	61.1 (±9.5)	66.4 (±9.2)	63.9 (±9.7)	T	0.02*
Radiotherapy	5 (14.7%)	9 (26.5%) N Missing: 4	14 (20.6%) N Missing: 4	P	0.23
Location:				P	0.42
- right colon	6 (17.6%)	8 (21.1%)	14 (19.4%)		
- left colon	8 (23.5%)	10 (26.3%)	18 (25.0%)		
- sigmoid colon	14 (41.2%)	9 (23.7%)	23 (31.9%)		
- rectum	6 (17.6%)	11 (28.9%)	17 (23.6%)		
CHT treatment:				P	0.18
Mayo Clinic regimen	10 (31.4%)	4 (10.5%)	14 (19.4%)		
FOLFIRI	13 (37.1%)	17 (44.7%)	30 (41.7%)		
FOLFOX4	7 (20.0%)	8 (21.1%)	15 (20.8%)		
LVFU2 (de Gramont regimen)	4 (11.4%)	9 (23.7%)	13 (18.1%)		
Clinical stage, CS:				P	0.02*
II	8 (23.5%)	3 (7.9%)	11 (15.3%)		
III	15 (44.1%)	11 (29.0%)	26 (36.1%)		
IV	11 (32.4%)	24 (63.2%)	35 (48.6%)		
Grading:				F	0.39
G1	3 (9.1%)	2 (5.4%)	5 (7.1%)		
G2	24 (72.7%)	23 (62.2%)	47 (67.2%)		
G3	6 (18.2%)	12 (32.4%)	18 (25.7%)		
	N Missing: 1	N Missing: 1	N Missing: 2		
response to treatment:				-	-
complete response	18 (52.9%)	0	18 (25.0%)		
partial response	10 (29.4%)	0	10 (13.9%)		
stable disease	6 (17.6%)	0	6 (8.3%)		
recurrence or progression death	0	34 (89.5%)	34 (47.2%)		
	0	4 (10.5%)	4 (5.6%)		

P – Pearson's χ^2 test; T – Student's T test; F – Fisher's exact test.
Chemotherapy schemes: Mayo Clinic regimen (5-FU; leucovorin), FOLFIRI (5-FU, leucovorin, irinotecan; FOLFOX4 (oxaliplatin; leucovorin; 5-FU); LVFU2 (de Gramont regimen: 5-FU; leucovorin).
* *P*-value < 0.05.

USA). A p-value less than 0.05 was considered to indicate statistical significance.

3. Results

The comparison of selected 14 cytokines levels in CRC patients before CHT to control group (CG) is presented in Table 3. IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-8 and IL-9 were significantly increased in CRC patients compared to controls. As the CRC group was heterogeneous, we compared the cytokine profile between adjuvant and palliative subgroups versus CG. Indeed, palliative patients showed different cytokine profile than adjuvant patients in comparison to controls.

Then, we compared cytokines profiles between non-progressors' and progressors' groups. In the progressors' group, IL-7 and IL-8 levels were significantly increased (Table 4).

Cytokines profile can vary on different stages of the disease in CRC patients.[7,8] To assess the relationship between a CRC stage and cytokine level in metastatic or non-metastatic CRC, we performed a separate response analysis in palliative (metastatic CRC patients) and adjuvant chemotherapy groups. Increased concentration of IL-8 in the palliative group was associated with a negative response to treatment. Results are shown in Table 5. Similar analysis performed in the adjuvant group showed that increased levels of IL-7, IL-10, IL-12p70 were associated with a negative response. Due to a low number of progressors in the adjuvant group, data are presented in the appendix (Table A.1).

4. Results of ROC analysis

To establish a potential prognostic significance of selected cytokines, we calculated ROC curves for each analyzed cytokine for palliative and adjuvant treatment subgroups. For the whole CRC group, the ROC analysis revealed acceptable prognostic value of IL-8 (sensitivity vs. specificity 0.823 vs. 0.632, AUC = 0.760 [95% CI: 0.647 – 0.873], cut-off = 20.28), as shown at Fig. 1.

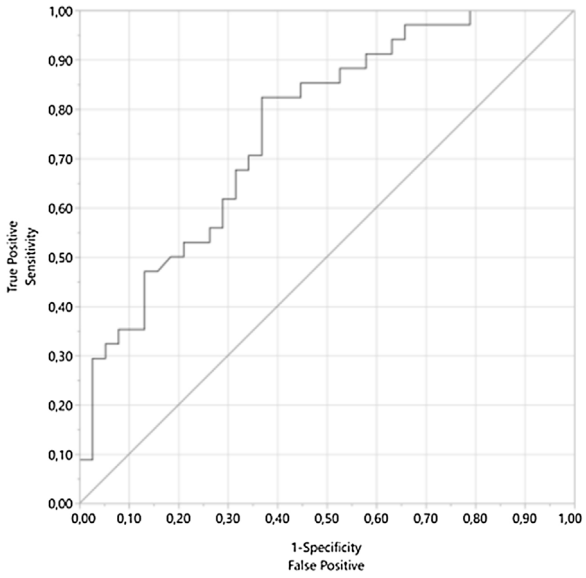

Fig. 1. Receiver operator curve of IL-8 for the whole CRC group.

The same relationship was found for IL-8 in the palliative group - ROC analysis revealed a good prognostic value of this cytokine (sensitivity vs. specificity respectively: 0.600 vs. 0.824, AUC = 0.770 [95% CI: 0.659 – 0.881], cut-off = 15.74), as presented in Fig. 2.

Table 2
Characteristics of patients in adjuvant, palliative and control groups.

Parameter	Adjuvant chemotherapy (A) (n = 23)	Palliative chemotherapy (P) (n = 49)	Control group (CG) (n = 22)	Test	*P*-value (A vs. P)
Women	9 (39.1%)	27 (55.1%)	15 (68.2%)	P	0.15
Age (years) - mean ± SD	60.6 ± 11.7	65.6 (±8.4)	63.5 (±13.4)	ANOVA	0.17
Radiotherapy	3 (13.6%) N Missing: 1	11 (23.9%) N Missing: 3	-	P	0.33
Surgery	23 (100%)	41 (83.7%)	-	F	0.049*
Location:				F	0.08
- right colon	1 (4.3%)	11 (22.4%)	-		
- left colon	6 (26.1%)	5 (10.2%)			
- sigmoid colon	12 (52.2%)	20 (40.8%)			
- rectum	4 (17.4%)	13 (26.5%)			
Chemotherapy:			-	-	-
Mayo Clinic regimen	11 (47.8%)	3 (6.1%)			
FOLFIRI	0	30 (61.2%)			
FOLFOX4	6 (26.1%)	9 (18.4%)			
LVFU2 (de Gramont regimen)	6 (26.1%)	7 (14.3%)			
Clinical stage, CS:			-	P	<0.001*
II	6 (26.1%)	5 (10.2%)			
III	16 (69.6%)	10 (20.4%)			
IV	1 (4.4%)	34 (69.4%)			
Grading:			-	F	0.58
G1	2 (9.1%)	3 (6.3%)			
G2	13 (59.1%)	34 (70.1%)			
G3	7 (31.2%)	11 (22.9%)			
	N Missing: 1	N Missing: 1			
Treatment response:			-	F	<0.001*
complete response	17 (73.9%)	1 (2.0%)			
partial response	1 (4.3%)	9 (18.4%) 5 (10.2%)			
stable disease	1 (4.3%)	30 (61.2%)			
recurrence or progression	4 (17.4%)	4 (8.2%)			
death	0				
Simplified response:			-	P	<0.001*
progressors	19 (82.6%)	15 (30.6%)			
non-progressors	4 (17.4%)	34 (69.4%)			

P – Pearson's χ^2 test; F – Fisher's exact test; ANOVA – Analysis of Variance.
Chemotherapy schemes: Mayo Clinic regimen (5-FU; leucovorin), FOLFIRI (5-FU, leucovorin, irinotecan; FOLFOX4 (oxaliplatin; leucovorin; 5-FU); LVFU2 (de Gramont regimen: 5-FU; leucovorin).
* *P*-value < 0.05.

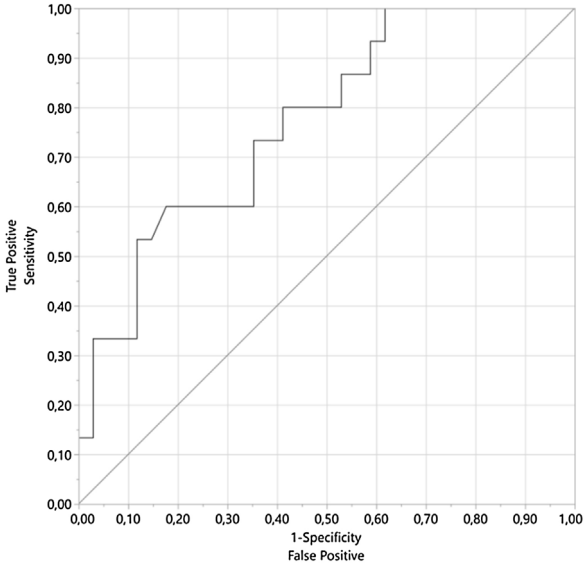

Fig. 2. Receiver operator curve of IL-8 in the palliative chemotherapy group.

In the adjuvant therapy group ROC analysis revealed a good prognostic value for several cytokines, such as: IL-1 beta (sensitivity vs specificity respectively: 1.000 vs.0.500, AUC = 0.710 [95% CI: 0.589 – 0.831], cut-off = 24.17), IL-1RA (0.789 vs. 0.750, AUC = 0.776 [95% CI: 0.666 – 0.886], cut-off = 392.07), IL-4 (0.895 vs. 0.500, AUC = 0.704 [95% CI: 0.583 – 0.825], cut-off = 12.42), IL-5 (0.684 vs. 0.750, AUC = 0.711 [95% CI: 0.589 – 0.831], cut-off = 41.05), IL-7 (0.842 vs. 1.000, AUC = 0.895 [95% CI: 0.817 – 0.973], cut-off = 9.43), IL-10 (0.789 vs. 1.000, AUC = 0.921 [95% CI: 0.853 – 0.989], cut-off = 18.95), IL12p70 (0.790 vs. 1.000, AUC = 0.882 [95% CI: 0.798 – 0.964], cut-off = 56.44), as shown at Fig. A.1 (appendix).

5. Multivariate model analysis

To establish a more accurate prognostic tool, we applied multivariate analysis to find the best correlation with clinical response for the whole group. We screened all assessed cytokines in order to include them in the multivariate response-prognostic model. From all analyzed cytokines in the simple models, IL-8 had the smallest p-value and no other cytokines were correlated with IL-8 for correlation >0.7. Then, the next cytokine with the smallest p-value was IL-7 and was not correlated with any remaining cytokines. Next was the IL-13 and it was correlated with IL-1beta, IL-4, IL-5, IL-6; hence, those were excluded from further analysis. Next was the IL-10, correlated with IL12p70, which was excluded. Next was the IL-17A which was not correlated with any of the remaining cytokines, and the same goes for IL-15. The last remaining cytokine was IL-1RA. All not excluded cytokines were included into further

Table 3
Clinical characteristics and serum cytokine profile in the whole, adjuvant, palliative and control groups.

Parameter	Whole group (WG) [n = 72]	Adjuvant (A) [n = 23]	Palliative (P) [n = 49]	Control group (CG) [n = 22]	Test	WG vs CG p value[@]	Test	A vs CG p value	P vs CG p value
BMI (kg/m^2)	25.7 (23.8; 27.8)	26.49 (24.31; 27.66)	25.49 (22.85; 28.12)	27.49 (24.70; 29.36)	U	0.13	SDM	0.64	0.25
Hemoglobin (g/dl)	12.3 (±1.6)	12.6 (±1.6)	12.2 (±1.7)	13.7 (±1.8)	T	<0.001*	HSD	0.06	0.001*
Erythrocytes (T/L)	4.4 (4.1; 4.7)	4.6 (4.3; 4.8)	4.3 (4.0; 4.7)	4.4 (4.0; 5.0)	U	0.44	SDM	0.98	0.48
White blood cells (G/L)	6.9 (5.4; 8.6)	6.7 (5.5; 7.8)	7.2 (5.3; 8.7)	6.6 (5.2; 8.7)	U	0.64	SDM	0.97	0.71
Platelets (G/L)	277 (196; 357)	280 (223; 305)	263 (185; 368)	237 (202; 291)	U	0.12	SDM	0.27	0.40
sGPT (U/l)	20.0 (15.0; 28.5)	18.0 (14.0; 25.0)	21.0 (15.5; 36.0)	18.0 (14.5; 23.5)	U	0.61	SDM	0.91	0.61
sGOT (U/l)	19.0 (14.0; 30.8)	15.0 (13.0; 20.0)	23.0 (16.5; 40.5)	22.0 (14.5; 25.5)	U	0.91	SDM	0.04*	0.46
IL-1 beta (pg/mL)	8.99 (6.99; 10.51)	9.07 (8.18; 10.69)	8.90 (6.76; 10.28)	5.56 (5.02; 7.21)	U	0.003*	SDM	0.003*	0.002*
IL- 1RA (pg/mL)	353.27 (281.08; 423.49)	352 (301.49; 424.42)	358.10 (267.82; 422.95)	265.24 (236.31; 381.13)	U	0.053	SDM	0.19	0.19
IL-2 (pg/mL)	2.19 (0.56; 3.98)	2.20 (0.56; 5.85)	2.02 (0.56; 3.96)	0.56 (0.56; 0.76)	U	0.014*	SDM	0.08	0.054
IL-4 (pg/mL)	8.86 (6.85; 10.97)	10.14 (8.18; 12.11)	8.80 (6.36; 10.06)	6.38 (5.75; 7.78)	U	0.005*	SDM	0.01*	0.047*
IL-5 (pg/mL)	34.86 (22.85; 41.16)	37.25 (26.10; 45.54)	34.58 (21.56; 38.61)	22.42 (21.08; 27.23)	U	0.016*	SDM	0.03*	0.12
IL-6 (pg/mL)	16.40 (11.15; 21.31)	14.05 (11.14; 23.70)	16.90 (11.04; 21.11)	9.50 (8.08; 11.88)	U	<0.001*	SDM	0.01*	0.01*
IL-7 (pg/mL)	9.13 (5.89; 11.43)	8.74 (6.26; 9.68)	9.30 (5.61; 12.56)	8.79 (6.73; 13.79)	U	0.82	SDM	0.87	0.99
IL-8 (pg/mL)	19.23 (14.68; 26.40)	17.71 (13.16; 20.02)	20.71 (15.57; 38.15)	13.12 (9.43; 16.25)	U	<0.001*	SDM	0.03*	<0.001*
IL-9 (pg/mL)	26.52 (20.58; 36.82)	24.27 (20.44; 48.57)	26.96 (20.67; 36.58)	20.13 (15.76; 28.31)	U	0.012*	SDM	0.11	0.04*
IL-10 (pg/mL)	14.61 (7.51; 30.49)	14.45 (7.84; 33.73)	14.77 (7.41; 24.76)	11.69 (8.20; 21.21)	U	0.73	SDM	0.81	0.99
IL-12p70 (pg/mL)	40.88 (18.49; 72.20)	49.58 (17.42; 87.60)	37.78 (18.55; 66.94)	34.02 (23.97; 53.65)	U	0.56	SDM	0.63	0.95
IL-13 (pg/mL)	9.01 (4.62; 14.43)	9.74 (4.49; 14.50)	8.44 (4.65; 14.67)	5.20 (3.73; 10.41)	U	0.07	SDM	0.40	0.16
IL-15 (pg/mL)	9.87 (9.87; 9.87)	9.87 (9.87; 9.87)	9.87 (9.87; 9.87)	9.87 (9.87; 9.87)	U	NA	SDM	NA	NA
IL17A (pg/mL)	117.03 (91.36; 138.11)	115.15 (89.90; 144.20)	120.40 (92.75; 136.76)	105.06 (94.09; 122.13)	U	0.34	SDM	0.57	0.74

SDM – nonparametric comparisons for all pairs using Steel-Dwass Method.
HSD – comparisons for all pairs using Tukey-Kramer Honest Significant Difference.
U – Mann Whitney U test (Wilcoxon rank-sum test).
T – Student's T test.
NA - P-value was not calculated because for majority of cases (>75%) values for IL-15 were equal to the limits of detection.
* *P*-value < 0.05.
@ Without adjustments for multiple comparisons.

Table 4
Concentrations of circulating markers in the non-progressors' and progressors' groups.

Parameter	Non-progressors(n = 34)	Progressors (n = 38)	Test	*P*
CEA (ng/mL)	2.62 (1.46; 6.88)	21.63 (5.19; 87.42)	U	<0.001*
CA19.9 (U/mL)	4.99 (2.00; 27.13)	13.60 (7.19; 86.47)	U	0.02*
IL-1 beta (pg/mL)	8.40 (4.30; 10.15)	9.32 (7.96; 10.68)	U	0.22
IL- 1RA (pg/mL)	349.47 (222.61; 451.31)	368.25 (296.50; 417.96)	U	0.55
IL-2 (pg/mL)	2.25 (0.56; 6.85)	1.96 (0.56; 3.95)	U	0.73
IL-4 (pg/mL)	8.84 (4.77; 11.16)	8.90 (7.81; 11.06)	U	0.47
IL-5 (pg/mL)	32.59 (16.83; 39.56)	35.84 (24.89; 42.39)	U	0.31
IL-6 (pg/mL)	14.26 (9.02; 20.60)	17.52 (11.98; 22.36)	U	0.12
IL-7 (pg/mL)	7.57 (5.52; 9.43)	9.70 (6.42; 14.75)	U	0.02*
IL-8 (pg/mL)	16.02 (11.67; 19.97)	21.63 (17.27; 41.27)	U	<0.001*
IL-9 (pg/mL)	25.86 (18.67; 35.07)	26.78 (21.99; 38.46)	U	0.45
IL-10 (pg/mL)	13.21 (6.52; 19.17)	16.28 (7.97; 34.79)	U	0.15
IL-12p70 (pg/mL)	37.79 (16.14; 55.04)	50.40 (22.07; 89.60)	U	0.11
IL-13 (pg/mL)	8.12 (4.39; 12.68)	10.09 (5.33; 16.43)	U	0.15
IL-15 (pg/mL)	9.87 (9.87; 9.87)	9.87 (9.87; 9.87)	U	NA
IL17A (pg/mL)	113.86 (63.55; 135.57)	122.10 (98.64; 139.58)	U	0.27

U – Mann Whitney U test (Wilcoxon rank-sum test).
NA - P-value was not calculated because for majority of cases (>75%) values for IL-15 were equal to the limits of detection.
All results have been shown as mean (±SD) or median (Q1; Q3) for normally on not-normally distributed data, respectively.
* *P*-value < 0.05.

Table 5
Concentrations of circulating markers in the palliative group depending on the response.

	Non-progressors(n = 15)	Progressors (n = 34)	Test	*P*
CEA (ng/mL)	5.40 (1.94; 83.25)	28.49 (6.12; 116.21)	U	0.19
CA19.9 (U/mL)	12.17 (5.65; 120.17)	19.40 (7.21; 103.20)	U	0.84
IL-1 beta (pg/mL)	8.25 (2.21; 10.00)	9.32 (7.55; 10.38)	U	0.22
IL- 1RA (pg/mL)	358.10 (194.95; 567.55)	355.05 (286.71; 408.89)	U	0.80
IL-2 (pg/mL)	3.03 (0.56; 7.41)	1.35 (0.56; 3.63)	U	0.38
IL-4 (pg/mL)	7.35 (±3.10)	8.8 (±2.7)	T	0.10
IL-5 (pg/mL)	29.96 (7.83; 36.98)	35.63 (23.46; 39.39)	U	0.19
IL-6 (pg/mL)	15.15 (5.53; 20.49)	17.52 (11.79; 21.61)	U	0.19
IL-7 (pg/mL)	7.92 (±4.20)	10.5 (±6.3)	T	0.16
IL-8 (pg/mL)	15.41 (10.56; 21.02)	22.16 (17.27; 45.01)	U	0.003*
IL-9 (pg/mL)	30.50 (15.08; 37.06)	26.78 (21.51; 36.57)	U	0.95
IL-10 (pg/mL)	13.62 (4.50; 19.84)	15.39 (7.69; 29.67)	U	0.41
IL-12p70 (pg/mL)	37.63 (15.51; 52.01)	39.37 (21.32; 79.12)	U	0.22
IL-13 (pg/mL)	7.83 (4.57; 11.98)	10.09 (5.06; 15.34)	U	0.23
IL-15 (pg/mL)	9.87 (9.87; 9.87)	9.87 (9.87; 9.87)	U	NA
IL17A (pg/mL)	109.01 (30.07; 134.99)	122.10 (97.66; 138.65)	U	0.28

U – Mann Whitney U test (Wilcoxon rank-sum test).
T – Student's T test.
NA - P-value was not calculated because for majority of cases (>75%) values for IL-15 were equal to the limits of detection.
All results have been shown as mean (±SD) or median (Q1; Q3) for normally on not-normally distributed data, respectively.
* *P*-value < 0.05.

Table 6
Statistical characteristics of parameters used in the prognostic model for the whole treated group, presented in Fig. 3.

Parameter	per	Odds Ratio	Lower 95%	Upper 95%	*P*-value
IL-8	1 pg/mL increase	0.900	0.781	0.968	<0.001*
IL-13	1 pg/mL increase	0.776	0.602	0.916	0.002*
IL-10	1 pg/mL increase	0.923	0.825	0.994	0.008*
IL-2	1 pg/mL increase	2.841	1.566	7.652	<0.001*
Age	1 year increase	0.864	0.730	0.968	0.009*
Clinical stage, CS	II$	1	-	-	0.23
	III	0.127	0.004	4.445	
	IV	1.376	0.105	17.254	
Chemotherapy	palliative$	1	-	-	<0.001*
	adjuvant	155.689	4.158	5829.884	

* *P*-value < 0.05.
$ reference group.

multiple regression analysis. We found that in the whole CRC group IL-2, IL-8, IL-10 and IL-13 incorporated in the multivariate model showed the best prognostic value of patient's clinical response (Fig. 3, Table 6).

The multivariate model showed a good prognostic value (sensitivity vs. specificity respectively: 0.911 vs. 0.895 AUC = 0.956 [95% CI: 0.905 – 1.000].

6. Discussion

In our study we confirmed the presence of significant cytokine profile differences between CRC patients and the control group. Levels of IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9 were significantly increased in the CRC group. These results are in accordance with a recently published study of Yamaguchi et al.[9] which showed

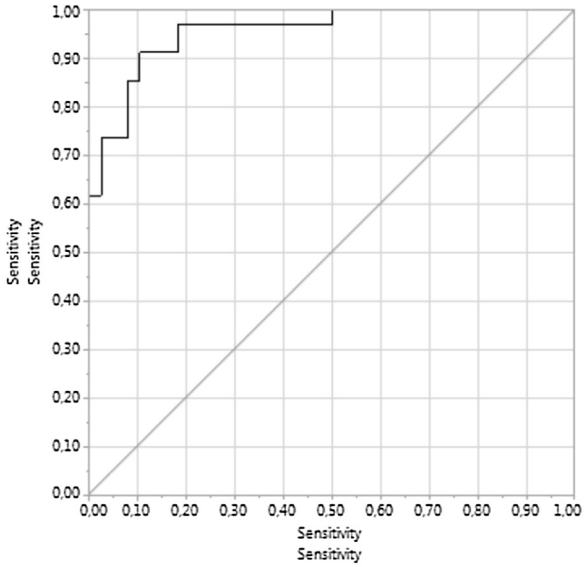

Fig. 3. Prognostic model of positive response for the whole treated group, including combined IL-2, IL-8, IL-10 and IL-13 concentrations.

increased levels of IL-4, IL-8, IL-9 and IL-17A in the whole group of CRC patients. Other authors observed increased serum levels of IL-1RA, IL-4, IL-6, IL-7, IL-8, CCL2 and PDGF-BB in CRC patients with distant metastasis.[7]

These results could be interpreted from the immunosuppressive tumor microenvironment perspective. Several authors proposed a shift towards Th2 secreted immunosuppressive cytokines in the CRC development,[10,11] which could contribute to an impaired systemic cytotoxic response.[12] Immunosuppressive cytokines are secreted mainly by lymphocytes Th2: IL-4, IL-5, IL-6, IL-9, IL-10, IL-13,[13] whereas Th1 lymphocytes synthesize primarily anti-tumor cytokines: IL-2, IL-15 and IFN-gamma. In CRC patients, decreased levels of circulating Th1 cytokines (IL-2, IL-15, IFN-gamma) were found, whereas cytokines produced by Th2 lymphocytes remained within normal concentrations or their levels were increased.[4,14,24]

In our study a movement towards Th2 cytokine levels in the whole group was not shown; increased levels of both Th2 (IL-4, IL-5, IL-6, IL-9) and Th1 (IL-2) cytokines were observed in comparison to the control group. Looking at the palliative group, an increased IL-4, IL-6, IL-9 levels were noted. In the adjuvant group we found increased concentrations of IL-4, IL-5, IL-6 vs. the control group. Thus, our results confirm the presence of alterations in the levels of Th1 and Th2 cytokines in the whole CRC patients group and also in different stages of colon cancer.

Analysis of the progressors vs. non progressors group in the whole CRC group showed significantly increased IL-7 and IL-8 levels. Both interleukins are important factors in the colon cancer development. IL-8 is known to promote angiogenesis and metastatic processes[15] in CRC patients. IL-8 was also confirmed to be a negative prognostic factor for CRC.[16,17] IL-8 promotes tumor growth, metastasis, angiogenesis and resistance to chemotherapy in the cell culture model of colon cancer.[18] Some authors showed that an increased level of IL-8 could also be a marker of a more aggressive disease in the negative response group.[19] IL-8 is expressed in significantly higher levels in the tumor tissue, compared to benign adenomas and the colon inflammatory diseases.[20] Significance of increased IL-7 level in the CRC patients is not well established. IL-7 is required for T-cell development as well as the survival and homeostasis of mature T-cells.[21] Recombinant IL-7 could be effective in improving the immune function in patients with lymphopenias.[22] The study of Krzystek-Korpacka et al. assessing the IL-7 level in 110 patients with colon cancer showed a significantly higher level of IL-7 in plasma of CRC patients and benign adenomas, in comparison to a control group.[23] In our study we managed to confirm this relationship only for progressors vs. non progressor group. Higher level of IL-7 was found in patients with lymph nodes and distant metastasis and also in patients with right sided-colon tumors.[23] Moreover, TNF-alfa, IL-10 and PDGF-BB levels were found to be independent predictors of increased plasma IL-7 level.[23] Other authors found that the presence of metastasis was associated with a significant increase in IL-4, IL-6, IL-7, IL-8, MCP-1, and PDGF-BB.[7,24] Results of our study are in accordance with these papers, confirming the association of IL-7 with the negative prognosis.

In the palliative group, progressors showed a significantly higher level of IL-8 versus non progressors. In the adjuvant group, the progressors' group recorded increased levels of IL-7, IL-10, IL-12p70 (Table A.1). IL-10 is involved in immunosuppressive microenvironment within the tumor.[25] Several cells in the intestine have been shown to produce IL-10, including T cells, monocytes, macrophages, and epithelial cells.[26,27] Also, CRC cells can secrete IL-10 directly or after CEA or IL-6 stimulation.[28,29,30] High preoperative serum levels of IL-10 correlate with poor progression free survival and overall survival of CRC patients.[31] It was shown that IL-10 serum levels increase over time during progression in CRC patients.[4,14,32] It has been found that an elevated IL-10 level is associated with a negative prognosis.[33] Some authors noted that an increased plasma level of IL-10 could be the result of immunosuppressive activity of the tumor.[14] Our data could indicate that IL-10 together with IL-7 would play a role in the negative response in the adjuvant therapy group; however, a small size of the progressors in this group (n = 4) limits the value of these conclusions.

IL-12p70 stimulates production of IFN-gamma,[34] limiting metastasis and tumor development. Increased concentration of IL12p70 in the progressors' group could be a sign of anti-tumor immunological response. Further studies are necessary to analyze this occurrence.

Usage of a cytokine panel instead of single markers could contribute to more accurate assessment of patient immunological state and prognosis.[7,24] When assessing multiple cytokines levels, it might be possible to reveal more specific sets of cytokines for different cancers and disease stages. On the other hand, balance should be found between the number of markers and prognostic accuracy to enable easy and cost-effective clinical application. Therefore, models involving a lower number cytokines, with satisfactory prognostic specificity, could be advantageous.

In the current paper, we tried to examine if the model based on serum concentration of 14 cytokines in CRC patients could show a prognostic value in the Polish population. The best predictive model adjusted for age, type of chemotherapy (palliative vs. adjuvant) and clinical stage included combined levels of IL-2, IL-8 IL-10 and IL-13. In our model, three cytokines exhibit pro-tumor or immunosuppressive activity: IL-8, IL-10 and IL-13[32,35,36] and one, IL-2, shows mainly anti-tumor action.[37] In our model, an increased level of IL-2 and decreased levels of IL-8, IL-10, IL-13 in the non-progressors' group was observed. This cytokine profile could reflect systemic immune response in these patients.

All four cytokines included in the prognostic model (IL-8, IL-10, IL-13 and IL-2) play an important role in the inhibition of cellular immunity and immunosuppressive response in the tumor microenvironment. IL-8 is considered to be a pro-tumor and angiogenetic factor in the tumor growth.[38] IL-10 is a cytokine with confirmed immunosuppressive action.[31] There has been no unequivocal confirmation of pro-tumor or anti-tumor IL-13 action so far. This immunosuppressive, anti-inflammatory cytokine is secreted mainly by Th2 helper lymphocytes but also natural killer T cells, mast cells and other cells.[39] It was shown that IL-4 and IL-13 may stimulate survival of colon cancer cells.[40] On the other

hand, in the retrospective study assessing IL-13 plasma level before surgery in 241 CRC patients, a significantly lower concentration of IL-13 was shown in subjects with lymph nodes metastasis, lymph vessels invasion and distant metastasis, as well as poor survival prognosis.[41]

The only interleukin with anti-cancer properties seen in the model is IL-2, mainly produced by Th1 lymphocytes. IL-2 induces proliferation and activation of cytotoxic T lymphocytes, and NK cells, and could be an indicator of systemic anti-tumor activity.[42,43] Recombinant forms of IL-2 are used in the therapy of malignant melanoma and renal cancer,[44] other IL-2 derivative has been accepted by FDA for cutaneous T-cell lymphoma.[45]

Several authors have already assessed the inflammatory prognostic value of circulating cytokines in CRC. Findings were summarized in a recently published meta-analysis.[2] The studies included in metaanalysis varied in terms of patient population, number and selection of cytokines. The authors concluded that further research was required to establish and validate prognostic score based on multiple cytokine levels.[2] Although inflammatory parameters included in these analyses were heterogonous, they also included several cytokines assessed in our study: IL-1 beta, IL-2, IL-4, IL-6, IL-7, IL-8 and IL-10.

Chang et al.[46] in 2016 assessed IL-1 beta, IL-6, TNF-alpha and CRP levels in 164 CRC patients with CRP < 5 mg/L from Taiwan before treatment and showed that such assessments may help identify patients at risk of early cancer progression. In another paper, Chen et al. in 2015[47] developed a cytokine-based prognostic classifier based on 17 cytokines which were affecting the overall survival (FGF-2, TGF-alpha, Flt-3 L, GM-CSF, INFa2, GRO, IL-10, MCP-3, MDC, sIL-2Ra, IL-2, IL-7, IL-8, MCP-1, MIP-1b and VEGF). The authors claim that cytokine based classifier might serve as a novel screening tool for identifying metastatic CRC patients with a high risk of short overall survival. However, these conclusions are based on Asian CRC patients and it is not known if the results could be applied to the Caucasian population.

Vayrynen et al.[48] looked into the levels of circulating 13 cytokines: IL-1RA, IL-4, IL-6, IL-7, IL-8, IL-9, IL-12, IFN-gamma, CXL10, CCL2, CCL4, CCL11 and PDGF-BB as well as pathology biopsies analysis. Their work revealed several potentially clinically relevant prognostic markers, including CD31T cells, CD831 dendritic cells, serum CCL4, and serum IFN-gamma.

On the other hand, Di Caro et al.[49] found that CXCL8, VEGF and Pentraxin-3 were associated with an increased risk of disease recurrence independently of TNM staging in 69 all stages CRC patients. Shizamaki et al.[50] assessed a pre-operative high level of serum IL-6 and the blood granulocytes/lymphocytes (G/L) ratio; these two parameters appeared to be significant predictive factors for cancer progression and poor prognosis.

These papers show that inflammatory markers based prognostic tools have a promising clinical potential in different CRC populations. Different populations are characterized by various CRC incidence and prognosis.[5] We contributed to the research performed so far adding data regarding the Polish population. This would be of importance, as Poland belongs to a group of countries with a high and increasing incidence and mortality due to CRC.[51]

To our knowledge, this is the first model assessing the prognostic value of four mentioned interleukins in Polish CRC patients receiving 5-FU based therapy. Models based on circulating cytokines levels may be considered as an attractive, additional tool in the clinical decision-making process for CRC patients. Larger studies are necessary to validate their clinical utility.

The present study followed the REMARK guidelines for the assessment of prognostic tumor markers[52]; however, we admit several limitations of the study. Assessed CRC patients' population was heterogeneous, including patients before and after surgery, before and after radiotherapy; included patients were in different disease stages. The follow up period was relatively short (6 months).

Some of these limitations could be addressed. Nagtegaal at al.[53] reported that, although preoperative RT/CRT potentially affected immune cell densities and serum cytokine levels, their correlation data were parallel in patients who did not receive RT/CRT. According to the authors, this indicated that preoperative RT/CRT was not a significant confounding factor. Other authors indicated[54] that sex, tumor, site, and N-stage had little association with the serum cytokine fluctuations reported. The authors conclude that the presence of a systemic inflammatory response was associated with IL-1RA, IL-6, IL-7, IL-8, IL-9, IL-12, IFN-gamma, IP-10, MCP-1, MIP-1B, and PDGF-BB levels. The results suggest that tumor factors have less effect than the activation of the systemic inflammatory response for serum cytokine concentrations. This finding was in line with the report that the majority of patients do not resolve their systemic inflammatory response, following a potentially curative surgery.[54,55] The heterogeneity of study populations diminishes the generalizability of our findings. However, the results could contribute to assessing the mechanisms of the systemic response and prognosis in CRC patients.

In conclusion – stratifying patients, who are likely to rapidly progress, is crucial in modern oncology treatment. Cytokine-based models seem to show a promising clinical value. We proposed a circulating cytokines based model, showing the prognostic value in foreseeing response to the 5-FU based therapy in Caucasian CRC patients. The model, including combined IL-2, IL-8, IL-10 and IL-13 levels in the CRC group, could provide rationale for larger scale clinical studies validating presented results.

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Competing interests

None declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.rpor.2020.08.004.

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