Pro-inflammatory and anti-inflammatory cytokines as candidate markers in the diagnosis and assessment of Crohn's disease activity

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Abstract

Introduction: We evaluated the diagnostic characteristics of interleukin (IL)-12, interferon (IFN)-γ, IL-10 and IL-19 in the diagnosis and assessment of Crohn's disease (CD) activity.

Material and methods: We studied 49 CD patients assigned to the active (33 patients) and inactive (16 patients) disease subgroups and 31 healthy controls. Serum cytokines were measured using ELISA. Cytokine levels and their multiplication results were compared between the groups and their diagnostic characteristics were assessed.

Results: The levels of the studied cytokines except IL-10 and their multiplication results were significantly higher in CD patients than in controls ($p < 0.0001$–0.007) and in patients with active than inactive disease ($p < 0.0001$–0.023). In the diagnosis of CD, the [CRP] x [IL-19] and [IL-6] x [IFN-γ] results had a specificity of 0.96, positive predictive value (PPV) of 0.97 and positive likelihood ratio (LR+) of 15.0 and 15.5, respectively, while serum IL-19 and the [IL-6] x [IL-12] and IFN-γ] x [IL-12] results had a sensitivity of 0.9-0.96, negative PV (NPV) of 0.75–0.86 and negative LR (LR–) of 0.09–0.18. The area under the ROC curve (AUC) for these markers was 0.776–0.807. In the diagnosis of active CD, the [CRP] x [IL-10] and [CRP] x [IL-19] results had a specificity of 0.98, PPV of 0.96, LR+ of 33.4 and 30.5, and AUC of 0.896 and 0.895, respectively. Serum IFN-γ and the [CRP] x [IFN-γ], [CRP] x [IL-12] and [IFN-γ] x [IL-19] results had diagnostic sensitivity of 0.77–0.94, NPV of 0.86–0.93, LR– of 0.11–0.24 and AUC of 0.781–0.904.

Conclusions: Serum IFN-γ, IL-19 and some of the results of the studied cytokine levels' multiplication showed promising diagnostic performance in the diagnosis of CD and its active form, which requires further validation.

Key words: Crohn's Disease Activity Index, diagnostic characteristics, inflammation, interleukins.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory bowel diseases (IBD). CD is a transmural inflammation that can affect any part of the gastrointestinal tract, with a chronic relapsing clinical course,

risk of complications and deterioration of quality of patients' life [1, 2]. Although the pathogenesis of the disease is not fully explained, its important element is the dysfunction of the mucosal immune system caused by immunological and genetic factors, including mutations of the *NOD2* and *CARD15* genes, and changes in the intestinal microbiota [1, 2].

The inflammatory process involved in CD is characterized by unbalanced activity of Th1 and Th17 cells with increased production of many pro-inflammatory cytokines including interferon γ (IFN-γ), interleukin 17A (IL-17A), IL-17F, IL-23 and IL-12. Activity of regulatory T cells (Treg) is decreased with a reduction in the production of anti-inflammatory mediators [2, 3].

IFN-γ is one of the key pro-inflammatory cytokines involved in the pathogenesis of CD, exerting immunomodulatory and endothelial effects. It inhibits the activity of Th2 cells, leading to a reduction of the epithelial barrier function and disruption of the vascular barrier [3–5]. IL-12 induces the Th1 polarization and the secretion of IFN-γ, tumor necrosis factor α (TNF-α) and other mediators active in mucosal inflammation. Additionally, IL-12 works with IL-23 to promote the differentiation of Th17 cells [6].

IL-10 is a key anti-inflammatory cytokine influencing the production of pro-inflammatory mediators and other functions of many immunocompetent cells. Defects in IL-10 signaling have been reported to be associated with IBD, especially in children [7]. IL-19 has been found to inhibit the production of pro-inflammatory cytokines by macrophages, Th1 and Th2 cells and has been suggested to have an anti-inflammatory effect in colitis [8].

There is no gold standard in CD diagnosis. This disease is diagnosed on the basis of the clinical presentation, endoscopy with histology, imaging studies including computed tomography and magnetic resonance and laboratory tests [1, 9]. Clinical activity of the disease is assessed using the Crohn's Disease Activity Index (CDAI) and imaging studies. Laboratory tests performed for CD diagnosis include nonspecific inflammatory markers such as erythrocyte sedimentation rate (ESR), serum C-reactive protein (CRP), and fecal calprotectin [9], and antibodies to *Saccharomyces cerevisiae* to differentiate CD from UC.

Although the role of many cytokines in the pathogenesis and clinical course of CD is widely researched and well explained, they are not used for diagnostic purposes. Their possible use as markers in the diagnosis of CD must be preceded by an assessment of diagnostic performance. While there is ample evidence about the cytokine networks and serum level profiles in IBD, data on their diagnostic characteristics are scarce.

In our previous paper, we reported an evaluation of the diagnostic performance of serum CRP, IL-6, IL-17A, and IL-23 levels, and their multiplication results in the diagnosis of CD and the assessment of its activity. We found that IL-23 may be a promising candidate marker in the diagnosis of CD, and CRP may be useful in the assessment of disease activity [10]. Continuing this research in the current study, we looked at the diagnostic characteristics of selected pro- and anti-inflammatory cytokines, including IL-12, IFN-γ, IL-10 and IL-19, in the diagnosis and assessment of CD activity.

Material and methods

The study included 49 Caucasian patients with CD, female/male 25/24, aged 36 ±14 years, and 31 healthy controls, also Caucasian, female/male 17/14, aged 52 ±16 years. The patients were diagnosed and treated in the Department of Gastroenterology and Hepatology of the University Hospital, Krakow, Poland. The diagnosis of CD was established by an experienced physician based on the clinical picture and colonoscopy with histology. CD patients were treated at the time of enrollment with glucocorticoids, 6-mercaptopurine, 5-aminosalicylate, infliximab and antibiotics in various combinations. CD patients with coexisting malignancy, endocrine disorders, diabetes, obesity, ischemic heart disease or systemic diseases were not enrolled in the study. In all CD patients the CDAI score was calculated, and they were assigned to the active (exacerbation, CDAI score ≥ 150, 33 patients) and inactive (CDAI score < 150, 16 patients) disease subgroups. The control group consisted of healthy subjects aged 20 to 61 years. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Signed informed consent was obtained from each subject prior to enrolling in the study. The Bioethical Commission of the Jagiellonian University, Krakow, Poland approved the study.

Laboratory tests

The serum was obtained from venous blood samples collected using S-Monovette tubes (Sarstedt, Germany). After clotting and centrifugation, the serum was aliquoted, and samples were stored at –70°C until assayed.

In our previous paper [10], we focused on the diagnostic performance of pro-inflammatory cytokines and CRP, while in this study we aimed to assess the diagnostic characteristics of pro- and anti-inflammatory cytokines involved in the pathogenesis and course of CD. Serum concentrations of IL-6 and CRP were determined using ELISA and immunonephelometry, respectively, as

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described elsewhere [10]. Serum levels of IL-12, IFN-γ, IL-10 and IL-19 were measured using the following ELISA reagents: Human IL12 (Interleukin 12) ELISA Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), reportable range 3.13–200 pg/ml; Human IFN-gamma ELISA, High Sensitivity (BioVendor, Brno, Czech Republic), reportable range 0.8–25.0 pg/ml; Human IL-10 High Sensitivity ELISA Kit (Diaclone SAS, Cedex, France), reportable range 1.0–50.0 pg/ml and Human IL19 (Interleukin 19) ELISA Kit (Elabscience Houston, USA), reportable range 31.25–2,000 pg/ml. All measurements were performed using the ELx808 spectrophotometer (BioTek R Instruments, Inc., Winooski, USA).

Multiplication results of [IL-6] x [IL-10], [IL-6] x [1L-19], [IL-6] x [IFN-γ], [CRP] x [IL-10], [CRP] x [1L-19], [CRP] x [IFN-γ], [IL-10] x [IL-19], [IL-10] x [IFN-γ], and [IL-19] x [IFN-γ], were calculated using standard concentration units, i.e. mg/l for CRP and pg/ml for interleukins. Serum levels of CRP and IL-6, analyzed elsewhere [10], were used only for these calculations.

Statistical analysis

All variables in the present study, except serum IL-10, showed in the Shapiro-Wilk test a non-Gaussian distribution, so the results are presented as medians and interquartile ranges (IQR, Q1–Q3). Medians were compared using the Kruskal-Wallis ANOVA test and the Dunn test. Spearman's rank coefficient (R) was used to assess the correlation. $P < 0.05$ was applied as the significance level.

Diagnostic characteristics in the diagnosis/exclusion of CD and active disease were assessed for serum IL-12, IFN-γ, IL-10, IL-19 levels, and their multiplication results. Relevant cut-off values were established based on the ROC (receiver operating characteristics) curve analysis, and diagnostic sensitivity and specificity, positive and negative predictive values (PPV, NPV), positive and negative likelihood ratios (LR+, LR-) and area under ROC curves (AUC) were calculated.

All statistical analyses were performed using the Statistica 13 software (TIBCO Software, Pao Alto CA, USA).

Results

Diagnosis/exclusion of Crohn's disease

Serum IL-12, IFN-γ and IL-19 levels were significantly higher in CD patients than in the control group ($p < 0.0001$–0.007). All multiplication results also differed significantly between CD patients and controls ($p < 0.0001$–0.017) (Table I).

In the diagnosis of CD, the [CRP] x [IL-19] and [IL-6] x [IFN-γ] results had diagnostic specificity of 0.96, PPV of 0.97 and LR+ of 15,0 and 15.5, respectively, while serum IL-19 and the [IL-6] x [IL-12] and IFN-γ] x [IL-12] results had diagnostic sensitivity of 0.9–0.96, NPV of 0.75–0.86 and LR– of 0.09–0.18. The AUC of these markers ranged

Table I. Comparison of median (mean for IL-10) concentration of studied cytokines in the serum and the multiplication results in patients with Crohn's disease (CD) and in the control group

Variables	Control group, $n = 31$	CD, $n = 49$	P-value
IL-10 [pg/ml]	5.0 ±2.2	6.1 ±2.3	0.0357
IL-12 [pg/ml]	2.3 (1.5–4.5)	5.1 (2.8–8.3)	0.0027
IL-19 [pg/ml]	22.9 (17.0–39.5)	61.4 (35.7–134.0)	< 0.0001
IFN-γ [pg/ml]	2.9 (1.9–6.1)	6.6 (4.7–10.4)	0.0006
[CRP] x [IFN-γ]	2.8 (0.4–9.2)	37.6 (4.8–148.3)	< 0.0001
[CRP] x [IL-10]	4.4 (0.8–11.0)	26.2 (5.5–125.6)	0.0001
[CRP] x [IL-12]	2.0 (0.5–4.7)	18.0 (3.8–110.9)	< 0.0001
[CRP] x [IL-19]	26.3 (4.1–72.1)	465.6 (30.6–1555.0)	0.0007
[IL-6] x [IL-10]	6.9 (3.2–9.9)	20.2 (8.3–47.8)	< 0.0001
[IL-6] x [IL-12]	2.8 (1.9–6.8)	17.4 (4.6–51.8)	< 0.0001
[IL-6] x [IL-19]	36.4 (20.6–68.4)	283.3 (68.5–686.4)	0.0070
[IL-6] x [IFN-γ]	5.0 (2.2–9.5)	24.1 (7.0–68.6)	< 0.0001
[IFN-γ] x [IL-10]	12.9 (9.5–33.56)	41.3 (19.6–79.2)	0.0003
[IFN-γ] x [IL-12]	7.8 (3.8–16.2)	34.3 (12.4–76.7)	< 0.0001
[IFN-γ] x [IL-19]	104.9 (44.7–169.9)	421.2 (228.4–1030.5)	0.0006
[IL-10] x [IL-12]	11.0 (5.3–26.7)	31.8 (12.2–64.9)	0.0017
[IL-10] x [IL-19]	135.3 (57.4–319.6)	378.5(177.2–785.5)	< 0.0001
[IL-12] x [IL-19]	57.6 (29.3–294.6)	354.3 (120.3–795.5)	< 0.0001

Table II. Comparison of diagnostic characteristics of the studied cytokines and the multiplication results in the diagnosis of Crohn's disease

Variables	Cut-off value	AUC	Sensitivity	Specificity	PPV	NPV	LR+	LR–
IL-10 [pg/ml]	3.86	0.640	0.85	0.36	0.69	0.59	1.33	0.42
IL-12 [pg/ml]	1.76	0.708	0.90	0.43	0.73	0.71	1.58	0.23
IL-19 [pg/ml]	22.07	0.776	0.96	0.43	0.74	0.86	1.68	0.09
IFN-γ [pg/ml]	3.55	0.738	0.87	0.57	0.78	0.73	2.02	0.23
[CRP] x [IFN-γ]	12.34	0.793	0.69	0.89	0.92	0.62	6.20	0.35
[CRP] x [IL-10]	1.33	0.767	0.90	0.43	0.73	0.71	1.58	0.23
[CRP] x [IL-12]	4.23	0.797	0.75	0.75	0.84	0.64	3.00	0.33
[CRP] x [IL-19]	98.25	0.807	0.60	0.96	0.97	0.59	15.0	0.42
[IL-6] x [IL-10]	10.00	0.768	0.73	0.79	0.85	0.63	3.48	0.34
[IL-6] x [IL-12]	3.02	0.786	0.9	0.57	0.78	0.76	2.09	0.17
[IL-6] x [IL-19]	52.91	0.824	0.81	0.71	0.83	0.70	2.79	0.39
[IL-6] x [IFN-γ]	18.42	0.798	0.62	0.96	0.97	0.60	15.5	0.39
[IFN-γ] x [IL-10]	15.04	0.749	0.85	0.57	0.77	0.70	1.98	0.26
[IFN-γ] x [IL-12]	8.43	0.785	0.90	0.54	0.77	0.75	1.96	0.18
[IFN-γ] x [IL-19]	197.01	0.808	0.79	0.78	0.86	0.69	3.17	0.29
[IL-10] x [IL-12]	10.29	0.717	0.87	0.50	0.75	0.70	1.74	0.26
[IL-10] x [IL-19]	169.49	0.767	0.83	0.64	0.80	0.69	2.33	0.26
[IL-12] x [IL-19]	90.79	0.781	0.94	0.68	0.83	0.86	2.92	0.09

AUC – area under the ROC curve, PPV – positive predictive value, NPV – predictive value, LR+ – positive likelihood ratio [sensitivity/ (1–specificity)], LR– – negative likelihood ratio [(1–sensitivity)/specificity)].

Table III. Comparison of median (mean for IL-10) concentration of studied cytokines in the serum and the multiplication results in patients with inactive Crohn's disease (CD), active CD and in the control group

Variables	Control group, n = 31	Inactive CD, n = 16	Active CD, n = 33	P-value
IL-10 [pg/ml]	5.0 ±2.2	5.3 ±2.3	6.6 ±2.2	0.0226
IL-12 [pg/ml]	2.3 (1.5–4.5)	3.4 (2.3–6.8)	5.1 (3.9–9.2)	0.0043
IL-19 [pg/ml]	22.9 (17.0–39.5)	50.2 (27.3–167.9)	73.0 (36.6–122.2)	0.0003
IFN-γ [pg/ml]	2.9 (1.9–6.1)	3.8 (2.6–7.0)	7.1 (5.3–11.1)	0.0001
[CRP] x [IFN-γ]	2.8 (0.4–9.2)	4.8 (0.7–12.6)	88.8 (27.5–322.4)	< 0.0001
[CRP] x [IL-10]	4.4 (0.8–11.0)	3.5 (1.7–17.3)	73.4 (20.4–174.5)	< 0.0001
[CRP] x [IL-12]	2.0 (0.5–4.7)	3.3 (0.9–6.6)	77.2 (15.5–204.7)	< 0.0001
[CRP] x [IL-19]	26.3 (4.1–72.1)	22.1 (12.3–309.0)	1256.0 (209.6–1865.7)	< 0.0001
[IL-6] x [IL-10]	6.9 (3.2–9.9)	10.0 (3.7–18.9)	31.9 (13.3–59.5)	< 0.0001
[IL-6] x [IL-12]	2.8 (1.9–6.8)	6.5 (3.0–15.1)	26.4 (11.0–97.9)	< 0.0001
[IL-6] x [IL-19]	36.4 (0.2–3.3)	73.0 (41.9–309.6)	353.0 (145.6–783.2)	< 0.0001
[IL-6] x [IFN-γ]	5.0 (2.2–9.5)	8.2 (2.5–18.4)	29.3 (22.3–117.7)	< 0.0001
[IFN-γ] x [IL-10]	12.9 (9.5–33.56)	17.7 (10.5–53.8)	44.2 (27.3–92.8)	0.0001
[IFN-γ] x [IL-12]	7.8 (3.8–16.2)	15.2 (8.6–44.5)	43.8 (22.8–89.2)	< 0.0001
[IFN-γ] x [IL-19]	104.9 (44.7–169.9)	344.3 (70.3–941.1)	584.6 (241.3–1105.2)	< 0.0001
[IL-10] x [IL-12]	11.0 (5.3–26.7)	15.5 (11.4–43.2)	35.4 (17.4–70.0)	0.0024
[IL-10] x [IL-19]	135.3 (57.4–319.6)	299.6 (122.4–721.5)	381.4 (186.0–862.6)	0.0003
[IL-12] x [IL-19]	57.6 (29.3–294.6)	255.0 (102.6–398.0)	370.6 (125.7–834.9)	0.0001

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from 0.776 to 0.807 (Table II). The diagnostic characteristics of the remaining cytokines and the multiplication results in the diagnosis/exclusion of CD were poor.

Diagnosis/exclusion of active Crohn's disease

The serum concentrations of the studied cytokines and all their multiplication results were significantly higher in CD patients with active than inactive disease and in patients with inactive disease than in the controls ($p < 0.0001$–0.023) (Table III). However, only serum IL-12 and IFN-γ significantly correlated ($p < 0.05$) with the CDAI scores (Figure 1).

In the diagnosis of active CD, the [CRP] x [IL-10] and [CRP] x [IL-19] results had a specificity of 0.98, PPV 0.96, LR+ 33.4 and 30.5, and AUC 0.896 and 0.895, respectively. Serum IFN-γ and the [CRP] x [IFN-γ], [CRP] x [IL-12] and [IFN-γ] x [IL-19] results had diagnostic sensitivity of 0.77–0.94, NPV 0.86–0.93, LR– 0.11–0.24 and AUC 0.781-0.904 (Table IV).

The remaining cytokines and multiplication results performed poorly in differentiating active and inactive CD.

Discussion

CD is an immune-mediated intestinal inflammation driven by activation of Th1 and Th17 cells with increased secretion of their cytokines like IL-12, IL-23, IL-17 and IFN-γ [1, 2], and disease activity is actually the degree of inflammation. Although there is a growing body of evidence of cytokine networks in IBD, the use of these mediators for diagnostic purposes is very limited. The 2016 European Crohn's and Colitis Organization (ECCO) Consensus recommends checking for chronic inflammatory response with serum CRP, ESR and fecal calprotectin in the diagnosis of CD [9]. Among them, only fecal calprotectin is specifically associated with intestinal inflammation.

There are many cytokines involved in the pathogenesis of CD, but data on their performance in the diagnosis of this disease are scarce. Therefore, the assumption of our study evaluating the diagnostic characteristics of IL-12, IFN-γ, IL-10 and IL-19 was the possibility of using inflammatory mediators involved in the CD pathogenesis as disease markers.

IL-12 is, along with other members of the IL-12 family (IL-2, IL-23, IL-27, IL-35), one of the
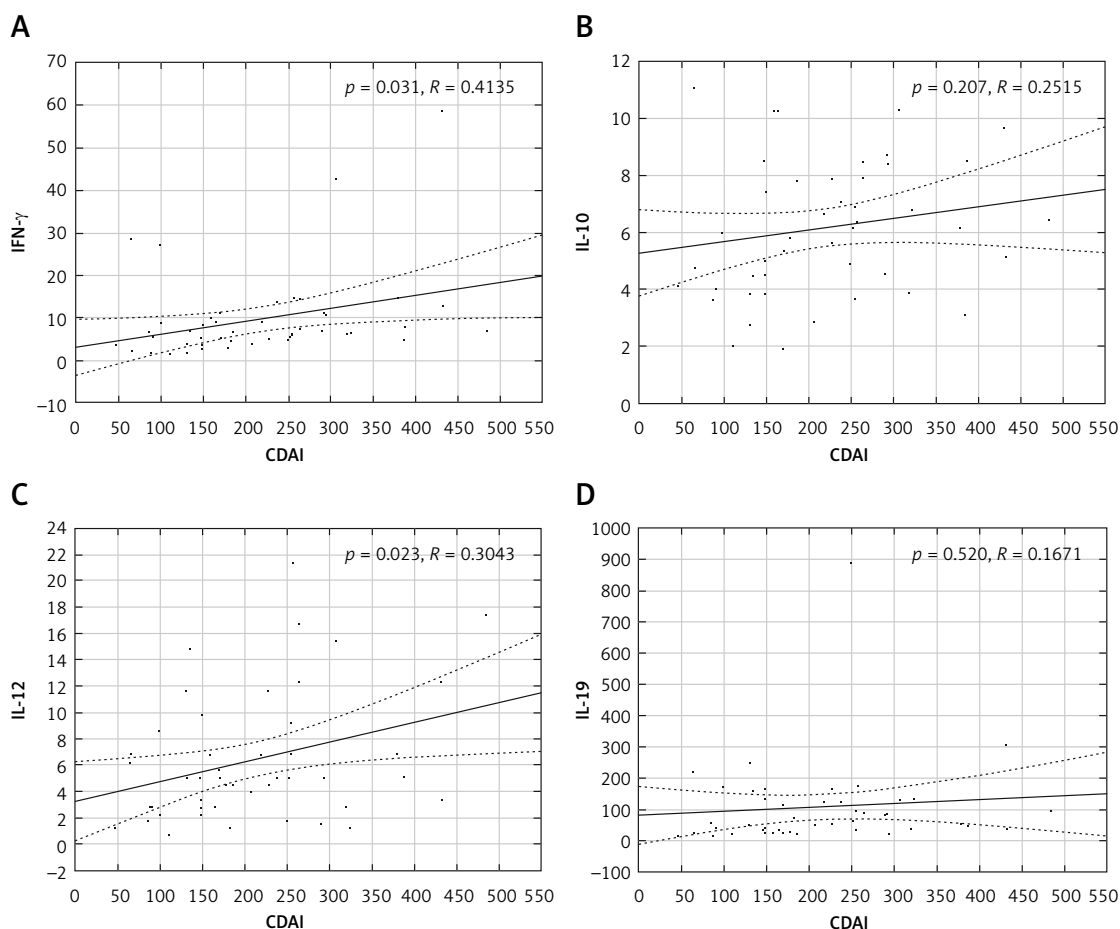

A

$p = 0.031, R = 0.4135$

B

$p = 0.207, R = 0.2515$

C

$p = 0.023, R = 0.3043$

D

$p = 0.520, R = 0.1671$

Figure 1. Spearman's correlation between serum levels of studied cytokines and CDAI scores

Table IV. Comparison of diagnostic characteristics of the studied cytokines and the multiplication results in the diagnosis of active Crohn's disease

Variables	Cut-off value	AUC	Sensitivity	Specificity	PPV	NPV	LR+	LR–
IL-10 [pg/ml]	5.64	0.685	0.71	0.64	0.57	0.76	2.00	0.45
IL-12 [pg/ml]	4.50	0.700	0.74	0.64	0.59	0.78	2.09	0.40
IL-19 [pg/ml]	52.23	0.705	0.68	0.69	0.60	0.76	2.18	0.44
IFN-γ [pg/ml]	4.54	0.784	0.94	0.60	0.62	0.93	2.34	0.11
[CRP] x [IFN-γ]	27.45	0.904	0.77	0.96	0.92	0.86	17.42	0.24
[CRP] x [IL-10]	31.96	0.896	0.74	0.98	0.96	0.85	33.4	0.26
[CRP] x [IL-12]	12.13	0.899	0.84	0.89	0.84	0.89	7.55	0.18
[CRP] x [IL-19]	638.80	0.895	0.68	0.98	0.96	0.82	30.48	0.33
[IL-6] x [IFN-γ]	21.91	0.852	0.81	0.91	0.86	0.87	9.07	0.21
[IL-6] x [IL-10]	21.53	0.816	0.64	0.87	0.77	0.78	4.92	0.41
[IL-6] x [IL-12]	21.76	0.817	0.61	0.89	0.79	0.77	5.51	0.44
[IL-6] x [IL-19]	251.94	0.817	0.74	0.84	0.77	0.83	4.62	0.31
[IFN-γ] x [IL-10]	22.07	0.790	0.87	0.64	0.63	0.88	2.45	0.20
[IFN-γ] x [IL-12]	22.80	0.787	0.74	0.78	0.71	0.83	3.48	0.29
[IFN-γ] x [IL-19]	197.01	0.781	0.90	0.64	0.64	0.91	2.54	0.15
[IL-10] x [IL-12]	24.96	0.713	0.74	0.69	0.62	0.80	2.39	0.38
[IL-10] x [IL-19]	746.00	0.723	0.36	0.89	0.69	0.67	3.19	0.73
[IL-12] x [IL-19]	108.90	0.736	0.90	0.56	0.58	0.89	2.03	0.17

AUC – area under the ROC curve; PPV – positive predictive value; NPV – predictive value; LR+ – positive likelihood ratio [sensitivity/(1–specificity)]; LR- – negative likelihood ratio [(1–sensitivity)/specificity)].

key mediators of the inflammatory response. IL-12 and IL-23, sharing the p40 subunit, cooperate in a common pathway with IL-23 dominance and play a pivotal role in the induction of mucosal inflammation in the pathogenesis of CD [6, 11, 12]. Increased expression in intestinal samples and higher serum levels of both IL-12 and IL-23 in CD patients have been reported [10, 13], but also no differences in serum IL-12 between CD patients and controls were found [14]. Previously, we found increased serum levels and very good diagnostic performance of serum IL-23 in the diagnosis of CD [10]. In the present study, serum IL-12 and its multiplication results were significantly higher in CD patients than in controls, and in CD patients with active disease (Tables I and II). Additionally, serum IL-12 correlated with the CDAI scores (Figure 1). However, serum IL-12 performed poorly in the diagnosis of CD, but when multiplied by serum levels of IL-6 and IFN-γ, these combined markers performed well in excluding CD (Table II). Serum IL-12 turned out to be useless in the diagnosis of active CD, and only the [CRP] x [IL-12] results performed better (Table IV). These differences in diagnostic characteristics between IL-23 [10] and IL-12 may reflect the predominance of IL-23 in their common pathway.

IFN-γ is another key pro-inflammatory cytokine involved in the pathogenesis of CD. It activates macrophages and lymphocytes, and its additional role is disruption of the vascular barrier and reduction of the epithelial barrier function [2, 5, 15].

Higher serum IFN-γ levels in CD patients than in healthy subjects have been reported in many studies [16–19]. However, IFN-γ is not used as an inflammatory marker in the diagnosis of CD. Measurement of IFN-γ is only for differentiating between CD and intestinal tuberculosis (ITB). T-cell based IFN-γ release assays (IGRAs) provide good diagnostic performance for an accurate diagnosis of ITB [20]. Recently it was reported that serum IFN-γ in combination with IL-6 and fecal calprotectin constituted an index that 6 months after ileocolonic resection for CD had the AUC of 0.90 to predict an early recurrence [21].

In the present study, serum IFN-γ as well as its multiplication results were also significantly higher in CD patients than in controls, and in CD patients with active disease, correlating with CDAI scores (Table I and III, Figure 1). Not serum IFN-γ but the [IL-6] x [IFN-γ] results performed well in diagnosing CD, and [IFN-γ] x [IL-12] results performed well in excluding CD (Table II). Serum IFN-γ and its multiplication by CRP and IL-19 levels performed well in the exclusion of active CD. The AUC > 0.7 also indicated good diagnostic performance of these combined markers (Table IV) [22].

IL-10 as negative regulatory mediator is involved in reducing the effects of T lymphocytes in the intestinal mucosa [23–25]. It is suggested

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that Th1 and Th17 cells self-limit their inflammatory activity via IL-10 expression [26]. Mutations in IL-10 or its receptor genes have been reported to increase susceptibility to IBD [7, 27].

Elevated IL-10 mRNA expression in the intestinal mucosa was found in patients with both UC and CD [28, 29]. In patients with CD [30, 31] and with active disease [32] higher serum levels of IL-10 than in controls were found. Mitsuyama *et al.* found elevated serum IL-10 levels during recovery in IBD patients and suggested its use in monitoring disease status [33]. In other studies, serum IL-10 levels in patients with CD did not differ significantly from the controls [14, 34].

In the present study, serum anti-inflammatory IL-10 and its multiplication results were also significantly higher in CD patients than in controls and in CD patients with active disease (Tables I and III).

Only one combined marker, [CRP] x [IL-10], performed very well in the diagnosis of active CD with diagnostic specificity and PPV close to 1.0, LR+ above 30 and AUC > 0.8 (Table IV).

IL-19, belonging to the IL-10 superfamily, functions as an immunomodulatory cytokine [35, 36]. The role of IL-19 in IBD is not fully elucidated and data from *in vitro*, animal, and human studies are inconsistent. Fujimoto *et al.* described the possible anti-inflammatory effects of IL-19 in colitis [8]. Defects in IL-19 expression and an impaired response to this cytokine have been reported to possibly contribute to inflammation in active CD [36]. Fonseca-Camarillo *et al.* found elevated IL-19 and IL-24 gene expression and increased numbers of IL-19- and IL-24-producing cells in patients with active CD compared to healthy controls [37]. Increased IL-19 expression in biopsy specimens and elevated blood levels have been found in patients with active IBD [38].

We also found significantly higher serum IL-19 in CD patients compared to controls and in patients with active CD (Tables I and III). Serum IL-19 performed well in the exclusion of CD (Table II). On the other hand, the [CRP] x [IL-19] results performed well in the diagnosis of CD (Table II) and, with a different cut-off value, in the diagnosis of active CD with the same diagnostic characteristics as [CRP] x [IL-10] (Table IV).

In summary, serum levels of both pro- and anti-inflammatory cytokines studied were significantly higher in CD patients and in patients with active disease although all patients with CD were receiving anti-inflammatory therapy at the time of enrollment and blood collection. These findings may reflect the widely studied and reported cooccurrence of pro-and anti-inflammatory processes in IBD [39–41]. However, only the serum levels of pro-inflammatory cytokines, IFN-γ and IL-12, significantly correlated with CDAI scores, thus reflecting disease activity (Figure 1).

Among the studied cytokines, only IFN-γ and IL-19 showed promising diagnostic characteristics as single markers. Combined markers including [IL-6] x [IFN-γ], [IL-6] x [IL-12], and IFN-γ] x [IL-12] performed well in the diagnosis of CD, and [CRP] x [IL-10], [CRP] x [IFN-γ], [CRP] x [IL-12] and [IFN-γ] x [IL-19] performed well in the diagnosis of active disease. The [CRP] x [IL-19] results turned out to be useful in the diagnosis of both CD and its active form (Tables III and IV). Of note, three of the five combined markers that performed well in the diagnosis of active CD are the results of multiplying the levels of pro- and anti-inflammatory markers.

These findings suggest considering the use of combined inflammatory markers in these settings, as reported by Cerrillo *et al.* and Kiernan *et al.* [21, 31]. Billiet *et al.* found that the ratio of TNF-α/CRP may predict non-response to treatment with infliximab [42]. The search for markers for CD diagnostics continues and goes beyond inflammatory cytokines. Recently, Wędrychowicz *et al.* found that plasma antimicrobial peptides (elafin, cathelicidin, α-defensins) are associated with the phenotype and location of CD lesions in children [43]. Overall, the analysis of the immune response cannot be overestimated in clinical practice, as demonstrated in the LATE-COVID-Kids Study of the pediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 (PIMS-TS) [44].

Completing the CDAI is time-consuming, requires prior complete blood count (CBC) checks, and sometimes requires additional consultations, e.g. by an ophthalmologist. Part of the patient's response is subjective, which should also be considered a limitation. Regardless of this, we used CDAI as a reference in the assessment of the diagnostic characteristics of the cytokines studied to check the agreement between these tools. For CD monitoring purposes also endoscopy including small-bowel capsule endoscopy and cross-sectional imaging studies utilizing ultrasound, computed tomography, and magnetic resonance are in use [45]. However, these diagnostic tools are resource dependent, not widely available, and have some limitations.

In patients with CD, laboratory tests such as CBC are routinely performed at control visits, so more tests may be ordered. We found that serum IFN-γ, IL-19 and results of multiplication of studied cytokine levels as combined markers can effectively preclude or confirm CD and its active form. Markers with such diagnostic characteristics could be considered a complementary tool and later possibly an alternative to CDAI in the assessment of CD activity.

The important point is that currently all cytokine assays used in our study are approved for research use only. Possible adoption of them as

candidate and later recommended markers would require appropriate analytical standardization in accordance with the regulations in laboratory diagnostics. On the other hand, serum CRP and IL-6 measurements are routinely available. It should also be taken into account that the use of combined markers would complicate the diagnostic process and increase its costs.

Due to the small size of the studied groups, our results should be considered preliminary, requiring verification.

In conclusion, this is our latest study evaluating the possibility of extending the panel of inflammatory markers used in CD diagnosis and assessment of its activity. It could make the clinical CD workup faster and more convenient for the patient, easier and cheaper. Our results suggest the need for further evaluation of IFN-γ, IL-19 and selected combined markers, including correlation with the CDAI scores and results of imaging studies.

Funding

No external funding.

Ethical approval

Approval number: 122.6120.239.2016.

Conflict of interest

The authors declare no conflict of interest.

References

1. Feuerstein JD, Cheifetz AS. Crohn disease: epidemiology, diagnosis, and management. Mayo Clin Proc 2017; 92: 1088-103.
2. Zhang Y, Li Y. Inflammatory bowel disease: pathogenesis. World J Gastroenterol 2014; 20: 91-9.
3. Sanchez-Munoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. World J Gastroenterol 2008; 14: 4280-8.
4. Cuia D, Huanga, G, Yanga D, et al. Efficacy and safety of interferon-gamma-targeted therapy in Crohn's disease: a systematic review and meta-analysis of randomized controlled trials. Clin Res Hepatol Gastroenterol 2013; 37: 507-13.
5. Langer V, Vivi E, Regensburger D, et al. IFN-γ drives inflammatory bowel disease pathogenesis through VEcadherin-directed vascular barrier disruption. J Clin Invest 2019; 129: 4691-707.
6. Aggeletopoulou I, Assimakopoulos SF, Konstantakis C, et al. Interleukin 12/interleukin 23 pathway: biological basis and therapeutic effect in patients with Crohn's disease. World J Gastroenterol 2018; 24: 4093-103.
7. Krawiec P, Pawłowska-Kamieniak A, Pac-Kożuchowska E. Interleukin 10 and interleukin 10 receptor in paediatric inflammatory bowel disease: from bench to bedside lesson. J Inflam 2021; 18: 13.
8. Fujimoto Y, Aono K, Azuma YT. The clarified role of interleukin-19 in the inflammatory bowel disease and hypersensitivity: insights from animal models and humans. J Vet Med Sci 2019; 81: 1067-73.
9. Gomollón F, Dignass A, Annese V, et al. on behalf of ECCO. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. J Crohn's Colitis 2017; 11: 3-25.
10. Slowinska-Solnica K, Pawlica-Gosiewska D, Gawlik K, et al. Serum inflammatory markers in the diagnosis and assessment of Crohn's disease activity. Arch Med Sci 2021; 17: 252-7.
11. Peluso I, Pallone F, Monteleone G. Interleukin-12 and Th1 immune response in Crohn's disease: pathogenetic relevance and therapeutic implication. World J Gastroenterol 2006; 12: 5606-10.
12. Arnold Greving CN, Towne JE. A role for IL-12 in IBD after all? Immunity 2019; 51: 209-11.
13. Parronchi P, Romagnani P, Annunziato F, et al. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. Am J Pathol 1997; 150: 823-32.
14. Martinez-Fierro ML, Garza-Veloz I, Rocha-Pizaña MR, et al. Serum cytokine, chemokine, and growth factor profiles and their modulation in inflammatory bowel disease. Medicine 2019; 98: 38: e17208.
15. Strober W, Fuss IJ. Pro-inflammatory cytokines in the pathogenesis of IBD. Gastroenterology 2011; 140: 1756-67.
16. Singh UP, Singh NP, Murphy EA, et al. Chemokine and cytokine levels in inflammatory bowel disease patients. Cytokine 2016; 77: 44-9.
17. Korolkova OY, Myers JM, Pellom ST, et al. Characterization of serum cytokine profile in predominantly colonic inflammatory bowel disease to delineate ulcerative and Crohn's colitides. Gastroenterology 2015; 8: 29-44.
18. Vasilyeva E, Abdulkhakov S, Cherepnev G, et al. Serum cytokine profiles in children with Crohn's disease. Med Inflam 2016; 2016: 7420127.
19. Chen P, Zhou G, Lin J, et al. Serum biomarkers for inflammatory bowel disease. Front Med 2020; 7: 123.
20. Chen W, Fan JH, Luo W, et al. Effectiveness of interferon-gamma release assays for differentiating intestinal tuberculosis from Crohn's disease: a meta-analysis. World J Gastroenterol 2013; 19: 8133-40.
21. Cerrillo E, Moret I, Iborra M, et al. A nomogram combining fecal calprotectin levels and plasma cytokine profiles for individual prediction of postoperative Crohn's disease recurrence. Inflamm Bowel Dis 2019; 25: 1681-91.
22. Šimundić AM. Measures of diagnostic accuracy: basic definitions. eJIFCC 2008; 19: 203-11.
23. Shouval DS, Ouahed J, Biswas A, et al. Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. Adv Immunol 2014; 122: 177-210.
24. Castillo P, Kolls JK. IL-10: a paradigm for counterregulatory cytokines. J Immunol 2016; 197: 1529-30.
25. Fu SH, Chien MW, Hsu CY, et al. Interplay between cytokine circuitry and transcriptional regulation shaping helper T cell pathogenicity and plasticity in inflammatory bowel disease. Int J Mol Sci 2020; 21: 3379.
26. Cope A, Le Friec G, Cardone J, et al. The Th1 life cycle: molecular control of IFN-gamma to IL-10 switching. Trends Immunol 2011; 32: 278-86.
27. Glocker EO, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 2009; 361: 2033-45.
28. Schreiber S, Heinig T, Thiele G, et al. Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. Gastroenterology 1995; 108: 10.

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29. Niessner M, Volk BA. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). Clin Exp Immunol 1995; 101: 428-35.

30. Meng D, Liang L, Guo X. Serum interleukin-10 level in patients with inflammatory bowel disease: a meta-analysis. Eur J Inflam 2019; 17: 1-7.

31. Kiernan MG, Calvin Coffey J, Sahebally SM, et al. Systemic molecular mediators of inflammation differentiate between Crohn's disease and ulcerative colitis, implicating threshold levels of IL-10 and relative ratios of pro-inflammatory cytokines in therapy. J Crohns Colitis 2020; 14: 118-29.

32. Kucharzik T, Stoll R, Lügering N, et al. Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). Clin Exp Immunol 1995; 100: 452-6.

33. Mitsuyama K, Tomiyasu N, Takaki K, et al. Interleukin-10 in the pathophysiology of inflammatory bowel disease: increased serum concentrations during the recovery phase. Med Inflam 2006; 2006: 26875.

34. Nielsen OH, Køppen T, Rüdiger N, et al. Involvement of interleukin-4 and -10 in inflammatory bowel disease. Dig Dis Sci 1996; 41: 1786-93.

35. Fonseca-Camarillo G, Yamamoto-Furusho JK. Interleukins involved in inflammatory bowel disease as new therapeutic targets. Cur Immunol Rev 2013; 9: 86-92.

36. Cantó E, Garcia Planella E, Zamora-Atenza C, et al. Interleukin-19 impairment in active Crohn's disease patients. PLoS One 2014; 9: e93910.

37. Fonseca-Camarillo G, Furuzawa-Carballeda J, Granados J, et al. Expression of interleukin (IL)-19 and IL-24 in inflammatory bowel disease patients: a cross-sectional study. Clin Exp Immunol 2014; 177: 64-75.

38. Steinert A, Linas I, Kaya B, et al. The stimulation of macrophages with TLR ligands supports increased IL-19 expression in inflammatory bowel disease patients and in colitis models. J Immunol 2017, 199: 2570-84.

39. Chen ML, Sundrud MS. Cytokine networks and T cell subsets in inflammatory bowel diseases. Inflamm Bowel Dis 2016; 2: 1157-67.

40. Friedrich M, Pohin M, Powrie F. Cytokine networks in the pathophysiology of inflammatory bowel disease. Immunity 2019; 50: 992-1006.

41. Lee SH, Kwon J, Cho ML. Immunological pathogenesis of inflammatory bowel disease. Intest Res 2018; 16: 2642.

42. Billiet T, Cleynen I, Ballet V, et al. Evolution of cytokines and inflammatory biomarkers during infliximab induction therapy and the impact of inflammatory burden on primary response in patients with Crohn's disease. Scand J Gastroenterol 2017; 52: 1086-92.

43. Wędrychowicz A, Tomasik P, Kowalska-Duplaga K, et al. Plasma elafin, cathelicidin, and α-defensins are increased in paediatric inflammatory Crohn's disease and reflect disease location. Arch Med Sci 2021; 17: 1114-7.

44. Jatczak-Pawlik I, Lewek J, Czkwonianc E, et al. LATE-COVID-Kids Study. Biochemical and cardiovascular predictors of PIMS-TS risk in children after COVID-19 recovery: preliminary results of the LATE-COVID-Kids Study. Arch Med Sci 2022; 18: 545-52.

45. Benitez JM, Meuwis MA, Reenaers C, et al. Role of endoscopy, cross-sectional imaging and biomarkers in Crohn's disease monitoring. Gut 2013; 62: 1806-16.