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Research Article

Comparative Evaluation of Four Methods for Fecal Calprotectin Testing: One-Step Card Test, A Point-of-care Lateral Flow Assay, A Standard and an Automated ELISA

Tsvetelina Velikova[1], Rayna Shentova[2], Zoya Spassova[3], Kalina Tumangelova-Yuzeir[4], Ekaterina Krasimirova[4], Ekaterina Ivanova-Todorova[4], Dobroslav Kyurkchiev[4], Christo Zhelev[2], Iskra Altankova[1]

[1]Clinical immunology, University Hospital Lozenetz, Sofia, Bulgaria

[2]Department of Gastroenterology and Hepatology, University Pediatric Hospital "Prof. Ivan Mitev", Sofia, Bulgaria

[3]Clinic of Gastroenterology, University Hospital "St. Ivan Rilski" – Medical University, Sofia, Bulgaria

[4]Laboratory of Clinical Immunology, University Hospital "St. Ivan Rilski", Sofia, Bulgaria0

Abstract

Objective: We aimed to compare four methods for fecal calprotectin (FC) measurement in view of technical/performance characteristics, diagnostic sensitivity and specificity, positive and negative predictive value, coincidence and correlations between tests, and clinical usefulness, in both adults and children.

Patients and methods: We examined the stool samples of 41 patients with inflammatory bowel disease (IBD) (25 adults and 16 children), and 12 healthy persons by One-step card test, a point-of-care assay Quantum Blue and two types of ELISA (Ridascreen and Alegria).

Results: The Quantum Blue system possessed the highest diagnostic sensitivity to determine IBD patients, followed by the two ELISAs. All tests exerted 100% specificity and 100% PPV, except the Ridascreen ELISA method - 92% and 94-97%, respectively. The best NPV belonged to the Quantum Blue system, especially for the CD patients, the other three tests showed similar results. The coincidence of the results varied between 85.36-97.56% among tests with the maximum coincidence for both ELISA methods and moderate to strong correlations between tests. We documented a significant relationship between the FC level and the duration of the disease, presence of complications, the type of treatment, serum iron and platelets counts, depending on the chosen method.

Conclusion: Our results demonstrated that all four methods exhibited comparable results, making them acceptable and convenient for clinical use for both adults and children. For clinical practice, we may suggest One-step card test for screening, followed by testing with Quantum Blue (at the clinical site) or ELISA method (at the immunological laboratory).

Keywords: Fecal calprotectin; Intestinal inflammation; Point-of-care testing; Pediatric fecal calprotectin; Enzyme immunoassay; One-step card test

Introduction

Although the histological examination of biopsied mucosa remains the "gold standard" for the characterization of intestinal inflammation, much more convenient for routine practice is the determination of leukocyte products associated with inflammation in feces such as calprotectin, lactoferrin, S100A12, lysozyme, leukocyte esterase, even cytokines. Described last century as a heterodimeric calcium-binding protein with bacteriostatic and fungistatic effect, fecal calprotectin (FC) is now increasingly prevalent in gastroenterological practice because of its bowel specificity [1]. The determination of FC reflects activation of neutrophils and their influx into the intestinal lumen during inflammation indirectly [1]. As the FC is released into the intestinal lumen, it is absorbed by fecal material passing through without being affected by any medications and enzymatic degradation [2]. FC is increased when intestinal inflammation is present and has been shown to have high diagnostic accuracy for discriminating organic inflammation (like in Inflammatory Bowel disease–IBD) from functional disorders (as in Irritable bowel syndrome–IBS) [3]. Indeed, elevated FC was observed in patients with IBD, but also colorectal cancer, diverticulous disease, celiac disease, cystic fibrosis, rheumatoid arthritis, bacterial infection, necrotizing enterocolitis, premature neonates, NSAIDs usage [1,3-5], etc. FC is handy in the detection of intestinal inflammation, especially when the affected area of the gastrointestinal tract is not accessible for

*Corresponding author: Tsvetelina Velikova, Clinical immunology, University Hospital "Lozenetz", Kozyak 1 Str., Sofia 1407, Bulgaria. E-mail: tsvelikova@medfac.mu-sofia.bg

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endoscopic examination. Furthermore, FC correlates well with endoscopic and histological findings in IBD. Thus, it could be screening method that can reduce the number of unnecessary invasive procedures in adult patients by 76% and in children by 35% [6]. Previous studies documented that FC is an equally useful marker for both adults and children [3,5]. Still, more studies for FC usefulness in clinical gastroenterology practice are needed.

FC is a non-invasive and very reliable marker for clinical practice due to its stability in feces out of the body (up to 7 days of sampling at room temperature or months when frozen) [2]. Several methods have been developed to test FC - qualitative and quantitative tests. Standard immunoenzyme assays are validated for measuring FC, but they have some disadvantages – they are time-consuming and labor intensive. Recently, a point-of-care (POC) desk-top devise to measure FC in a fast and efficient manner has been developed [7]. Simpler, quicker and more easy tests for qualitative or semi-quantitative analysis of FC also exist [8,9]. All commercially available methods for FC testing differ in many aspects, and there is a lack of enough studies comparing them.

The purpose of our study was to compare four methods for FC measurement – One-step card test, a point-of-care lateral flow assay Quantum Blue, a standard and an automated ELISA in view of technical/performance characteristics, diagnostic sensitivity and specificity, positive and negative predictive value, coincidence and correlations between tests, and clinical usefulness of the tests. We aimed to establish a useful clinical algorithm for intestinal inflammation in a study group comprises of both adults and children with IBD on the basis of the obtained results.

Patients and Methods

Subjects of the study

Fifty-three persons were included in the study: 41 patients with IBD-20 (48.8%) with ulcerative colitis-UC, and 21 (51.2%) with Crohn`s disease–CD, as well as 12 age and sex-matched healthy persons. From the patients, 16 were men (39%), and 25–women (61%). The overwhelming proportion of patients was at the state of activity–34/41 (82.9%).

Twenty-five of the IBD patients were adults, recruited at the Clinic of gastroenterology at University Hospital St. Ivan Rilski, Sofia, at mean age 41 ± 16 (18-71) years. The diagnosis of all patients was based on the standard criteria of ECCO Consensus for CD (2010) and UC (2012), which include a set of anamnestic, clinical, laboratory and instrumental studies.

Sixteen of the IBD patients were children, recruited at the Department of Gastroenterology and Hepatology, University Pediatric Hospital "Prof. Ivan Mitev", Sofia, at mean age 11 ± 4 (0-17) years. The diagnosis of the pediatric patients was made according to the Porto criteria for the diagnosis of IBD in children and adolescents.

The exclusion criteria for both adult and children IBD patients were the following but not limited to: acute diarrhea, proved infectious diarrhea, NSAIDs usage, melena, epistaxis, menorrhagia, other systemic severe or psychiatric illness.

All subjects of the study were informed about the purpose of the experiment, and a written informed consent was obtained from all participants. If the patient was under 18 years old, the informed consent was signed by their parents/legal guardians. The study design was approved by the Ethic Committee of the Medical University of Sofia, and the research was performed according to the local hospitals` ethic considerations.

Preparation of stool samples

Participants were provided with a tube equipped with a spatula to collect fecal material. Every sample was refrigerated at -70°C immediately after transportation to the laboratory. Before being tested, samples were entirely thawed at room temperature.

Methods for fecal calprotectin testing

FC in stool samples was evaluated by four methods:

1. Qualitative chromatographic immunoassay – One-step card test (CerTest Calprotectin, CerTest Biotec, Spain).
2. Quantitative lateral flow sandwich immunoassay with optical measurement, a point-of-care (POC) test (Quantum Blue Calprotectin rapid test, Buhlmann Laboratories AG, Switzerland). This assay included RFID (Radio Frequency Identification) chip and required a Reading system (Quantum Blue Reader).
3. Quantitative enzyme immunoassay test (Ridascreen calprotectin, R-Biopharm, Germany)
4. Automated quantitative enzyme immunoassay test (Calprotectin, Alegria, Orgentec).

Fecal samples were processed according to the extraction protocol applied to the kit. All methods for FC testing were carried out in the Laboratory of Clinical Immunology, University Hospital "St. Ivan Rilski", Sofia, strictly following the instructions of the manufacturer.

Statistical methods

Statistical analysis of the raw data was performed with the software package for statistical analysis (SPSS®), IBM 2009, version 19 (2010). We accepted the results for significant if $p<0.05$. Diagnostic specificity, sensitivity, positive and negative predictive value were calculated by adapted formulas.

Results

Comparison of technical and performance characteristics of the tests

The technical and performance aspects of the four methods used for FC testing are presented in **Table 1**. All tests used precise FC identification by monoclonal antibodies against a specific epitope of its molecule. One-step card test is a qualitative test that gives a "positive" or "negative" result, while the other three tests give quantitative results. However, all four tests use the same cut-off to distinguish between positive and negative results - 50 mg/kg (μg/mg). The fastest and easiest method for FC determination was the One-step card test which allowed measuring a single sample "next to the patient's bed" for about 15 minutes. The Quantum Blue system was shown as a relatively quick method (about 25 minutes per sample) but required specialized equipment. The most time-consuming tests requiring also qualified staff, and specialized laboratory equipment were the two immunoenzyme methods **(Table 1)**. However, the latter are the most economical and suitable for a series of samples testing.

Table 1: Technical and performance characteristics of One-step card test, Quantum Blue, a standard and an automated ELISA.

Parameters	One-step card test Cer test	Quantum Blue	Ridascreen ELISA	Alegria Orgentec ELISA
Type of method	Chromatographic	Lateral flow	Enzyme immunoassay	Enzyme immunoassay
Measurement	Qualitative	Quantitative	Quantitative	Quantitative
Units	Positive/negative	µg/mg (=mg/kg)	mg/kg (=µg/mg)	mg/kg (=µg/mg)
Cutt off	>50 mg/kg	>50 µg/mg	>50 mg/kg	>50 mg/kg
Interval	Positive/negative	30-5000	0 - 2500	0 - 1000
Assay time	10-15 min/test	20-30 min/test	240-300 min/series	90-120 min/series
Qualified personnnel	Not required	Not required	Required	Required
Technics requiremen	None	Quantum Blue reader	ELISA Reader	Automated ELISA Alegria Orgentec

Diagnostic sensitivity, specificity, positive and negative predictive value of the tests

Our data showed that the Quantum Blue system possessed the highest diagnostic sensitivity to determine IBD patients, for both UC and CD entities, followed by the immunoenzyme methods (with the superiority of Alegria ELISA) **(Table 2)**. The lowest sensitivity was displayed by the One-step card test. The One-step card assay, the Quantum Blue system, and Alegria ELISA showed 100% specificity and 100% positive predictive value (PPV), whereas the Ridascreen ELISA method - 92% and 94-97%, respectively **(Table 3)**. The best negative predictive value (NPV) belonged to the Quantum Blue system; the other three tests showed similar NPVs. The diagnostic sensitivity and specificity of the four presented tests were comparable with those provided by the manufacturers.

Coincidence and correlations between the tests

On **Table 4** we presented the percent of coincident and non-coincident results between the four assays. The coincidence varied between 85.36-97.56% among tests with the maximum coincidence of the results for both ELISA methods (40/41 IBD patients with equal results after ELISA FC testing).

In line with this are the following results regarding statistical correlations between tests. We found that there were statistically significant correlations between the results of FC determination by all four methods. The correlation between the two ELISA methods (Ridascreen/Alegria) was evaluated as very strong (Pearson`s rho=0.970, p<0.001), between the One-step Card test and ELISA methods - moderate to strong (Spearman`s rho=0.780, p<0.001); between the One-step card test and Quantum Blue – moderate (Spearman`s rho=0.629, p<0.001); and between Quantum Blue and ELISA methods – moderate to strong (Pearson`s rho=0.790, p<0.001). **Figure 1** shows the latter correlation. It is visible that the association is more linear at the lower FC values measured by both methods.

Clinical usefulness of the tests: diagnosing and clinico-laboratory associations

In **Figure 2** we present receiver operating characteristic (ROC) curve analysis of Quantum Blue and ELISA methods (Ridascreen and Alegria) for diagnosis of UC and CD patients. For UC patients **(Figure 2A)** it is visible that the area under the curve (AUC) for both tests is approximately 0.94, i.e., 94% of patients with UC will be diagnosed correctly as UC patients, regardless of which method for FC is assigned (p<0.001). On the neighboring graph **(Figure 2B)**, we observed an excellent performance of the Quantum Blue test for diagnosing CD patients with AUC=0.993, and ELISA methods with AUC=0.896 (p<0.001).

Table 5 showed some associations between clinico-laboratory findings and FC assessed by the four different methods. We documented a significant relationship between the duration of the disease in years and the increased FC (r=0.369-0.395): patients with FC level above the cut-off had longer disease duration than those who had normal FC levels. We also found that the presence of complications (intestinal and/or extraterrestrial) correlates weakly (r=0.395, p=0.018) with FC levels – patients with elevated FC showed the presence of complications more frequently. We also obtained a significant association between FC levels and the type of treatment with the highest levels of FC measured in patients without therapy (r=0.535, p=0.008).

According to laboratory and FC, our results also showed that elevated FC levels correlated significantly with the increased platelet counts in IBD patients **(Table 5)**. The presence of high FC exerted a correlation with ESR (p=0.019), and a reversal correlation with the serum iron (p=0.031). We did not observe correlations between FC levels and CRP, hemoglobin, leukocytes, and albumin levels.

Discussion

To reduce the time of diagnosing IBD patients and the number of invasive procedures, many efforts are given on seeking simple, non-invasive, fast, cheap and reliable methods of assessing intestinal inflammation and mucosal recovery after successful therapy. Of the markers studied in recent years, the most promising was the neutrophil derive FC [6].

In practice, there are already several different methodological approaches to protein research in feces [2]. To evaluate the possibilities of clinical application - accessibility, accuracy and reliability, sensitivity and specificity of the different methods, we have approached and implemented four methods for FC testing.

We found that, independently of the FC determination method, measured FC levels were significantly higher in IBD patients than in the healthy control group, which coincided with the literature data [10]. The diagnostic sensitivity of all four methods for FC testing in IBD patients in our study was very similar: the highest for the Quantum Blue (90%), followed by the ELISA tests (80-82.9%), whereas the lower sensitivity was showed for the One-step card test - 78%. The diagnostic specificity for the differentiation of IBD patients from healthy subjects for all methods was 100%, except the Ridascreen ELISA. The specificity of the latter immunoassay was 92%, as one of the healthy subjects showed a low positive result. More extensive studies on healthy individuals for FC levels

Table 2: Fecal calprotectin positive (>50 mg/kg) and negative results of IBD, UC, and CD patients, and healthy persons. The results are presented as number (%).

Parameters		IBD patients n=41	UC patients n=20	CD patients n=21	Healthy persons n=12
Positive result	One-step card test	32 (78%)	15 (75%)	17 (81%)	0
	Quantum Blue	37 (90%)	17 (85%)	20 (95.2%)	0
	Ridascreen ELISA	33 (80%)	15 (75%)	18 (85.7%)	1 (8.33%)
	Alegria ELISA	34 (82.9%)	16 (80%)	18 (85.7%)	0
Negative result	One-step card test	9 (21.9%)	5 (25%)	4 (19%)	12 (100%)
	Quantum Blue	4 (9.75%)	3 (15%)	1 (4,8%)	12 (100%)
	Ridascreen ELISA	8 (19.5%)	5 (25%)	3 (15%)	11 (91.67%)
	Alegria ELISA	7 (17.1%)	4 (20%)	3 (14.3%)	12 (100%)

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn`s disease

Table 3: Diagnostic specificity and sensitivity, positive and negative predictive value of One-step card test, Quantum Blue, a standard and an automated ELISA.

Parameters		One-step card test Cer test	Quantum Blue	Ridascreen ELISA	Alegria Orgentec ELISA
Diagnostic sensitivity	IBD	78%	90%	80%	82.90%
	UC	75%	85%	75%	80%
	CD	81%	95.20%	85.70%	85.70%
	Manufacturer*	>94%	84,4%	95.00%	97%
Diagnostic specificity	IBD	100%	100%	92%	100%
	UC	100%	100%	92%	100%
	CD	100%	100%	92%	100%
	Manufacturer*	93%	94.50%	93%	95%
Positive predictive value	IBD	100%	100%	97,4%	100%
	UC	100%	100%	94,7%	100%
	CD	100%	100%	95,2%	100%
Negative predictive value	IBD	60%	75%	50%	63.15%
	UC	66,7%	80%	61,1%	75%
	CD	85,7%	92%	73,3%	80%

*Manufacturer`s sensitivity and specificity for the test to detect intestinal inflammation. IBD: Inflammatory Bowel Disease; UC: Ulcerative Colitis; CD: Crohn`s Disease

Table 4: Coincidence and non-coincidence between test results for fecal calprotectin in IBD patients assessed by One-step card test, Quantum Blue, a standard and an automated ELISA.

	One-step card test Cer test	Quantum Blue	Ridascreen ELISA	Alegria Orgentec ELISA	
One-step card test Cer test		37 (90.2%)	38 (92.68%)	39 (95.12%)	Coincidence (number, %)
Quantum Blue	4 (9.75%)		35 (85.36%)	38 (92.68%)	
Ridascreen ELISA	3 (7.3%)	6 (14.6%)		40 (97.56%)	
Alegria Orgentec ELISA	2 (4.87%)	3 (7.3%)	1 (2.44%)		
	Non-coincidence (number, %)				

Table 5: Correlations between some clinico-laboratory findings and level of fecal calprotectin, assessed by One-step card test, Quantum Blue, a standard and an automated ELISA.

	One-step card test Certest	Quantum Blue	ELISA methods (Ridascreen/Alegria)
C- reactive protein, mg/dl	NS	NS	NS
Hemoglobin, g/l	NS	NS	NS
ESR, mm/h	NS	r=0.595, p=0.019	NS
Albumin, g/l	NS	NS	NS
Leucocytes, G/l	NS	NS	NS
Thrombocytes, G/l	r=0.514, p=0.007	r=0.390, p=0.015	r=0.357, p=0,003
Serum iron, µmol/l	r=0.525, p=0.031	NS	NS
Duration of the disease (years)	r=0.369, p=0.027	NS	r=0.395, p=0.016
Presence of complications	NS	r=0.395, p=0.018	NS
Therapy administered	NS	r=0.535, p=0.008	NS

in fecal samples for the Bulgarian population were made by Nakov et al. in 2009 [11]. If we judge our data against their established cut off (70 mg/kg), then none of our healthy individuals would be with elevated FC. Thus, the diagnostic specificity of the Ridascreen ELISA test would be 100%. The data of Nakov et al. for adult patients with IBD showed the sensitivity of 88.2% and specificity of 83.3% [11], which are slightly lower than our results, but the differences may be related to the different number of examined patients or the commercial ELISA used.

The differences between our results regarding specificity and sensitivity might be explained in two ways. Firstly, the cited by manufacturer data was related to the ability of the test kits to detect intestinal inflammation. Secondly, the discrepancies might

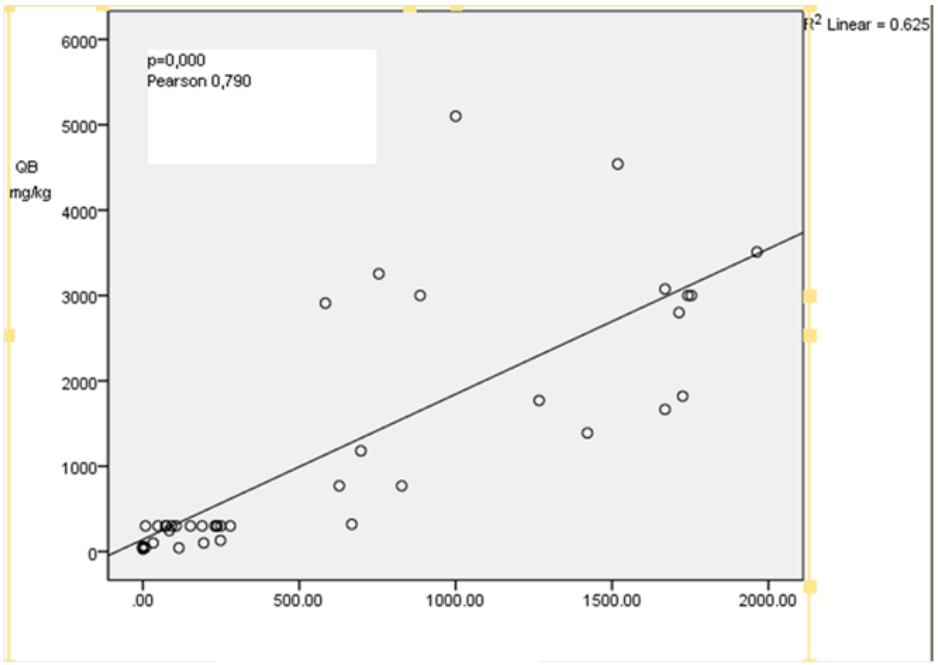

Figure 1: Correlation between test results obtained by Quantum Blue and Ridascreen ELISA.

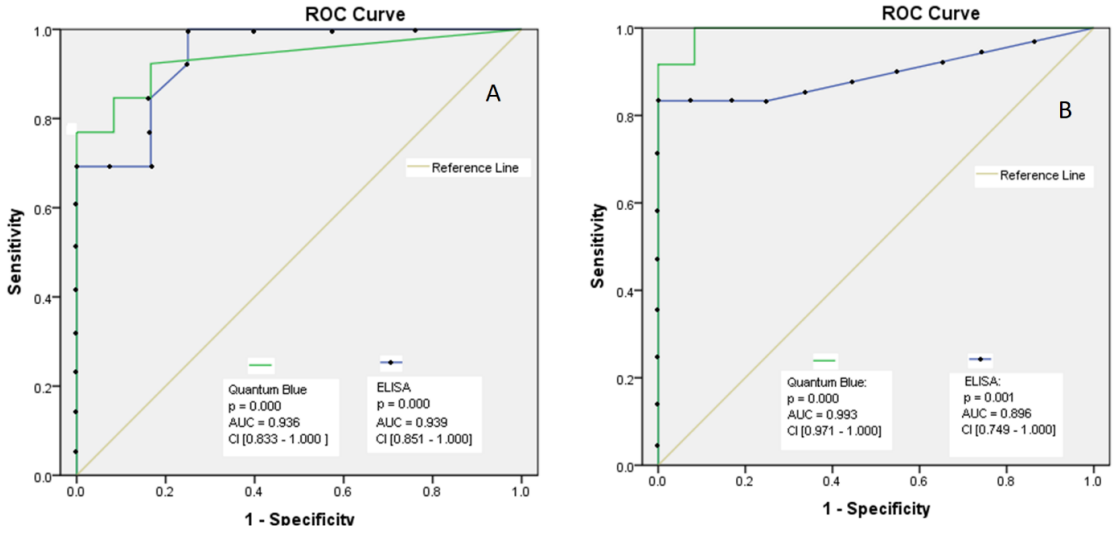

Figure 2: Receiver operating characteristic (ROC) curve analysis of Quantum Blue and ELISA methods (Ridascreen and Alegria) for diagnosis of ulcerative colitis (A) and Crohn`s disease (B) patients.

be due also to the relatively lower number of healthy individuals surveyed by us.

The high sensitivity and specificity of FC are remarkable given the diverse and complex antigens in the feces. In various other studies, the sensitivity of the test for IBD patients ranged between 63-100% and the specificity was 80-100% [3,7,12-16]. Apparently, our data is within these limits. In healthy subjects, FC values obtained by the two quantitative methods remained below the manufacturers` cut off (50 mg/kg) - 41.3 μg/mg mean level obtained from Quantum Blue system and 3.20-6.77 mg/kg for the ELISA methods (data not shown). With the Ridascreen ELISA, we had one patient with FC level over 50 mg/kg but less than 70 mg/kg (a cut off limit accepted in some literature to distinguish between negative and positive samples) [11]. If we agree on this limit, none of our healthy controls exerted higher than normal FC result.

We obtained PPVs of the methods ranged between 97.4-100%. These high PPVs determine the high probability that patients with increased FC levels would have the diagnosis IBD. On the other hand, the NPVs of the tests range between 50-75% for IBD patients. It is well-known that the NPV is related to excluding the diagnosis in a person with a negative result. The NPVs of the four methods were better for excluding CD patients than UC patients. These results were also confirmed by the ROC curve analysis **(Figure 2)**. Analyses from different studies determined median values of 89% (70-100%) for PPV and 81% (51-91%) for NPV in diagnosing IBD

patients [14-16]. Our PPV and NPV data, determined by the four tests, fit within the leading world research. We can also conclude that the negative FC result obtained by Quantum Blue system, can exclude IBD to the highest degree. However, it is important to have in mind, that high levels of FC in the feces were also observed in patients with colorectal carcinoma [4], patients on NSAID therapy, necrotizing enterocolitis, diverticulosis, microscopic colitis [3], as well as other illnesses, but in lower ranges - cystic fibrosis, rheumatoid arthritis, bacterial infection [12]. All these conditions served as excluding criteria in our study by routine clinical, laboratory and endoscopic examinations.

We also found that the results of the four tests corresponded with each other to a high degree. Similar results for correlation between the Quantum Blue and an ELISA method have also been demonstrated by other authors [14-21]. We also compared the Quantum Blue and both ELISA assays by superimposing their ROC curves for determining the UC and CD patients, respectively. The two tests showed similar performance for patients with UC, but regarding the CD patients distinguishing, the Quantum Blue was better represented with an AUC of 0.993 ($p<0.001$).

Since the process of inflammation in the intestine begins locally, and then often gives a systemic reflection in the body, we also questioned the correlations between FC levels and some clinico-laboratory findings. We observed a significant association between FC levels and the type of therapy being conducted [22]. We did find significantly higher FC levels in IBD patients without therapy ($p<0.001$). Other authors also noticed a decrease in FC in IBD patients after corticosteroid and probiotic administration, as well as in patients with chronic enterocolitis following treatment with probiotics [23]. In our studies, we obtained a significant correlation between FC levels and platelet count in the blood. Patients with increased FC also had a higher level of blood platelets. Many authors report such a correlation regardless of duration or form of the disease [24]. This background raises the issue of underestimating the platelet count as a useful marker of active inflammation [24]. Nevertheless, patients with inflammatory conditions often have various hemostatic disorders. On the other hand, FC levels showed a reverse correlation with the serum iron ($p=0.031$) and ESR ($p=0.019$). We did not observe any statistical relationship between FC levels and the C-reactive protein or leukocyte count, unlike other authors [23-25].

Limitations

Our study had some flaws, including a relatively small sample size of patients and healthy persons. We used a convenient sample of IBD patients and a caution must be applied, as the findings might not be transferable to large sample size. However, we have included both children and adults to avoid bias regarding age.

Conclusion

After verification and comparison of four tests for FC testing – One-step card test, Quantum Blue, a standard ELISA and automated ELISA, our data showed that all methods exhibited comparable results, making them acceptable and convenient for clinical use. Furthermore, these results were obtained in a mixed cohort of both adult and children patients. By our results, we can conclude that the One-step card test could be useful for FC screening. Then, if the result is positive, we suggest testing with Quantum Blue (at the clinical site) or ELISA method (at the immunological laboratory).

Conflict of Interests

The authors have no competing interests to declare. Declarations of interest: none.

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