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ASSESSMENT OF THE RESPONSE OF PATIENTS WITH CROHN'S DISEASE TO BIOLOGICAL THERAPY USING NEW NON-INVASIVE MARKERS: lactoferrin and calprotectin

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ABSTRACT – ***Context*** - The use of fecal markers to monitor Crohn's disease is crucial for assessing the response to treatment. ***Objective*** – To assess the inflammatory activity of Crohn's disease by comparing fecal markers (calprotectin and lactoferrin), colonoscopy combined with biopsy, and the Crohn's disease activity index (CDAI), as well as serum markers, before treatment with infliximab, after the end of induction, and after the end of maintenance. ***Methods*** – Seventeen patients were included who had been previously diagnosed with Crohn's disease and were using conventional treatment but required the introduction of biological therapy with infliximab. Each patient underwent a colonoscopy with biopsy, serum, and fecal (calprotectin and lactoferrin) tests to assess inflammatory activity, and CDAI assessments before treatment with infliximab, after induction (week 8), and after maintenance (week 32). ***Results*** - The calprotectin levels exhibited significant reductions ($P = 0.04$) between the assessment before treatment with infliximab and the end of induction, which did not occur after the end of the maintenance phase. Lactoferrin remained positive throughout the three phases of the study. Regarding the histological assessment, a significant difference was found only between the assessment before treatment and after the end of maintenance ($P = 0.036$), and 60% of the patients exhibited histological improvements after the completion of the follow-up period. The CDAI exhibited a significant difference between the assessment before treatment with infliximab and after induction, as well as before treatment and after maintenance ($P<0.01$). ***Conclusion*** – Calprotectin and lactoferrin are not useful for monitoring inflammatory activity in Crohn's disease patients who are subjected to biological therapy.

HEADINGS – Crohn disease. Biological markers. Biological therapy.

INTRODUCTION

The clinical presentation of Crohn's disease (CD) is complex and characterised by manifestations that are sometimes subclinical but sometimes severe, with extensive or multiple sites of stenosis, free perforation, intra-abdominal masses, or fistula formation being observed[9]. Due to this complexity, the diagnosis is difficult and requires clinical, endoscopic, and histopathological correlation[2].

After the diagnosis, it is crucial to monitor the disease via clinical, endoscopic, and laboratory indices of inflammatory activity. Among the indices of clinical activity, the CDAI (Crohn's disease activity index) and the Harey-Bradshow index (HDI) are the most widely used[13, 29].

The CDAI is considered the gold standard for assessing the activity of the disease because this validated index has been widely used for more than 25 years in clinical protocols and in studies seeking drug approval[26]. However, the CDAI has certain limitations, such as observer-dependent assessments of patient wellbeing and the intensity of their abdominal pain, which are subjective elements that are estimated based on information supplied by the patients regarding symptoms occurring 7 days earlier. In addition, the CDAI lacks precision in cases of fistula or stenosis and is useless in patients with extensive resections or stomas[29].

The Crohn's disease endoscopic index of severity (CDEIS) is the most widely used and validated endoscopic index. This index is widely used in therapeutic studies to assess mucosal healing and is considered the gold standard for endoscopic assessments[29]. The

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CDEIS is easy to calculate, addresses the factors that endoscopists rate as important during the study, and exhibits low interobserver disagreement, which makes it reproducible[19].

Unspecific laboratory assays measuring, for instance, the haemoglobin level, the C-reactive protein (CRP), and the erythrocyte sedimentation rate (ESR), are also widely used to assess inflammatory activity in patients with CD[35]. Because the measurement of fecal calprotectin and lactoferrin levels is simple and non-invasive, these proteins are considered to be promising markers for intestinal inflammation[1, 28].

The calprotectin concentration correlates with the excretion of indium-111-labelled granulocytes, which is considered to be highly sensitive in assessing inflammatory activity[23]. High levels of this calcium-binding protein are found in inflammatory bowel disease (IBD), colon cancer, and treatment with non-steroidal anti-inflammatory drugs (NSAIDs). Therefore, calprotectin is a sensitive, albeit non-specific, marker[10, 11].

Lactoferrin is an iron-binding protein found in the granules of neutrophils[15]. During intestinal inflammation, the polymorphonuclear cells infiltrate the mucosa, resulting in increased fecal lactoferrin concentrations[1].

As fecal markers of inflammatory activity, calprotectin and lactoferrin appear to be highly useful due to their low cost and simplicity of measurement[1]. Both proteins are stable in the feces[16, 22], and in CD, these markers might serve to monitor the disease activity in addition to supplying prognostic information. Therefore, these putative markers might contribute to the assessment of the response to treatment and to the prediction of relapse in patients with sub-clinical inflammation[30].

The use of anti-tumour nuclear factor (anti-TNF) drugs is often necessary based on the severity of the CD. In such cases, the use of inflammatory markers—particularly, non-invasive markers—is crucial for assessing the response to treatment.

Therefore, the aim of the present study was to assess the inflammatory activities in patients with CD by comparing fecal markers (calprotectin and lactoferrin), colonoscopy with biopsy, and the CDAI, as well as serum markers before treatment, after infliximab induction, and after maintenance therapy.

METHODS

Patients

Seventeen patients who had previous diagnoses of CD that were confirmed by clinical examinations, as well as laboratory, endoscopic, histological, and imaging tests, who were using conventional treatments, and who were indicated for a change to anti-TNF drugs were included.

The inclusion criteria were as follows: CD diagnosis; age 16 years or older; naïve to treatment with biological agents; signing of an informed consent form; seronegative for hepatitis B and C, as well as for the human immunodeficiency virus (HIV); and tuberculosis ruled out by a previous chest radiograph and tuberculosis skin test (PPD).

Individuals were not included who had undergone a colectomy; patients who had severe heart disease; pregnant women; patients who were seropositive for hepatitis B, hepatitis C, or HIV; individuals who had active or latent tuberculosis; or patients with a previous history of colon cancer. During the study, patients who exhibited any intolerance to the drug or who did not match any of the indicated requisites were excluded.

The patients invited to participate were provided all of the information necessary to dispel their doubts regarding the study. The individuals who agreed to participate signed an informed consent form and were subsequently subjected to the following tests: complete blood count (CBC); ESR; CRP; alpha 1-acid glycoprotein; serology for hepatitis B, hepatitis C, and HIV; PPD test; chest radiograph; and colonoscopy. The participants were also asked to perform fecal collections. For this purpose, the feces had to be collected no later than the night before the medical visit and stored in a refrigerator until delivery on the morning of the medical visit.

The clinical activity was assessed via the CDAI in all the patients who were included in the protocol. Scores below 150 were rated as having no activity; 150 to 219, as mild activity; 220 to 450, as moderate activity; and above 450, as severe activity. All the tests were performed before the patients took their first dose of infliximab, according to the traditional phase of induction (5 mg/kg) on weeks 0, 2, and 6. On week 8 (i.e., at the end of induction), blood samples were collected for CBC, ESR, CRP, and alpha 1-acid glycoprotein determinations; colonoscopy was again performed; another fecal sample was requested; and the CDAI of the previous week was calculated. The patients were indicated for the conventional infliximab maintenance regimen and were subjected to a clinical assessment on the days the drug was administered (i.e., every 8 weeks until the end of the protocol on week 32). At the end of the treatment, the laboratory tests (CBC, ESR, CRP, and alpha 1-acid glycoprotein, as well as the final colonoscopy) were repeated, and an additional fecal sample was requested. The CDAI of the previous 7 days was also calculated during week 32. As in the initial stage, the patients were required to collect the fecal samples no later than the night prior to the medical visit and to store the samples in a refrigerator until delivery on the morning of the medical visit.

Fecal tests

The assessment of the fecal markers included two tests: a quantitative test to measure calprotectin (Phical® Calprotectin Elisa Kit, Immundiagnostik AG, Bensheim, Germany) and a qualitative test for detecting lactoferrin (IBD-CHEK®, Techlab, Blacksburg, VA, USA).

The samples were stored at -20ºC for joint analysis. On the day of processing, the samples were thawed at room temperature according to the manufacturers' instructions. To measure calprotectin, 1 to 5 g of feces was taken from each sample; subsequently, a 100-mg aliquot was separated using a precision scale, placed in a test tube, mixed in a vortex for 30 seconds, and placed on a horizontal agitator (speed of 1,000 rpm) for 35 minutes. Approximately 1 to 2 mL of the supernatant was transferred to an Eppendorf tube and centrifuged at 10,000 g for 20 minutes. The extract was

diluted, and the ELISA test was performed in duplicate. An appropriate reader with a 450-nm filter was used. The optical density of all of the standards (included in the kit) was calculated, and a standard curve was obtained. The values corresponding to each sample were located on that curve, and the concentrations were calculated as ng/mL, which were multiplied by 2.5 to obtain the equivalents expressed as mg/kg. In this test, values up to 50 mg/kg of fecal calprotectin were considered to be normal, and values above 200 mg/kg defined inflammatory activity.

To perform the lactoferrin test, the samples were weighed because approximately 50 mg of each were required; subsequently, the samples were successively diluted 1:20 and 1:400 and were mixed in a vortex. The samples were subsequently placed in the reaction plate, conjugate was added, and this step was subsequently followed by a rinse, the addition of the substrate, another rinse, and subsequent steps. Duplicate samples, i.e., negative and positive controls, were used in each reaction. The measurement was performed using an ELISA reader with a 450-nm filter. The tests were considered to be negative if the optical density (OD) was lower than 0.200 and positive if the OD was above 0.200.

Colonoscopy

All the colonoscopy procedures were performed at the Endoscopy Unit of São Paulo Hospital, Federal University of São Paulo, SP, Brazil, always by the same team of endoscopists who were blinded to the results of the fecal and serum tests. Serial biopsies were performed, independent of the presence or absence of lesions, except for the cases in which stenosis was found. To standardise the endoscopic reports, the CDEIS was used, with scores lower than 3 being considered inactive disease; 3 to 9, mild disease; 9 to 12, moderate disease; and over 12, severe disease.

Histology

The following criteria were applied to the histological analysis: presence of neutrophils in the lamina propria, presence of erosion and/or ulceration, and crypt involvement[33]. The slides were analysed by a single pathologist who was blinded to the results of the colonoscopy, laboratory, and fecal tests.

Application of biological therapy

All the participants were administered infliximab at a dosage of 5 mg/kg on weeks 0, 2, and 6 (induction), and later every 8 weeks thereafter (maintenance). The induction therapy was performed at the Gastroenterology Clinic Ward of São Paulo Hospital, and the maintenance therapy was administered at the outpatient clinic under medical supervision. The drug was acquired by the patients through the high-cost pharmacy service of the State Health Secretary of São Paulo.

Statistical analysis

The analysis was performed using the statistical software SPSS 18 (IBM, Armonk, NY, USA) and Minitab 15 (Minitab, State College, PA, USA).

The patients were assessed for their clinical, serum, fecal, endoscopic, and histological activities before the onset of biological therapy (time 0), after induction (8 weeks), and after maintenance (32 weeks).

An ANOVA was used to compare the means of the 3 times that were assessed, and the chi-square test (x^2) was used to compare the frequencies. A P value <0.05 was considered statistically significant.

The Bonferroni test was used for pairwise comparisons of the groups that exhibited significantly different averages, i.e., 0 vs 8, 0 vs 32, and 8 vs 32 weeks.

The variables calprotectin, lactoferrin, the CDAI, the CDEIS, the histological score, the CRP, the ERS, alpha 1-acid glycoprotein, haemoglobin (Hb), the hematocrit (Ht), and the platelet and white cell count were correlated pairwise at the 3 times assessed using Pearson's correlation.

In the analysis of calprotectin, CD was considered active if the values were above 200 mg/kg.

For the analysis of correlation, the results of the histological examination (categorical variable) were translated into a numeric scale as follows: inactive = 0; mild = 1; moderate = 2; and severe = 3. A similar procedure was adopted in the case of lactoferrin: negative = 0 (absence of inflammation), and positive = 1 (presence of inflammation).

Ethics

All the participants signed an informed consent form, and the study was approved by the research ethics committee of the Federal University of São Paulo (UNIFESP 1726/07).

RESULTS

The characteristics of the patients are shown in Table 1. Patients were assessed for clinical infammatory activity,

TABLE 1. Characteristics of the patients

VARIABLE	Frequency	Percentage (%)
GENDER		
Male	8	47.1
Female	9	52.9
ETHNICITY		
East Asian	1	5.9
Pardo	5	29.4
White	11	64.7
SMOKING		
No	16	94.1
Yes	1	5.9
EXTENT *		
L1	2	11.8
L2	7	41.2
L3	8	47.1
BEHAVIOUR *		
B1	9	52.9
B2	4	23.5
B3	4	23.5
EXTRA-INTESTINAL SYMPTOMS		
No	11	64.7
Yes (joint pain**)	6	35.3
SURGERY		
No	8	47.1
Yes	9	52.9

* Montreal classification L1-ileum; L2-colon; L3-ileum-colon; B1-non-stenosing/non-fistulising; B2-stenosing; B3-fistulising

** one patient with joint pain and erythema nodosum

serum, fecal, endoscopy and histological before biological therapy (time 0), after induction (time 8) and after maintenance (time 32). Therefore, all comparative analyzes were performed taking into account the three phases of the study.

Calprotectin

Upon comparing the calprotectin levels at the three assessed times, we found a significant difference between times 0 and 8, with a reduction in the calprotectin fecal levels, thus indicating an improvement in the inflammation ($P = 0.03$). However, the levels of this marker increased again on week 32, thus denoting a worsening of the inflammatory process, as shown in Table 2.

Based on the comparison of the average calprotectin levels at times 0, 8, and 32, pairwise comparisons were performed via the Bonferroni test, as described in Table 3. This analysis revealed a significant difference only between times 0 and 8 ($P = 0.041$).

Lactoferrin

Lactoferrin was positive at all of the assessed time-points without significant differences among them, as described in Table 4.

Histology

Comparisons of the histological exams among the 3 assessed times revealed significant differences ($P = 0.021$). As in the case of the calprotectin levels, the histology also improved at 8 weeks when most of the patients exhibited mild inflammatory activities. On week 32, most of the patients exhibited mild-to-moderate activity, as described in Table 5.

Upon pairwise comparisons, we found a significant difference only between times 0 and 32 ($P = 0.036$).

Pairwise comparisons

Time 0 vs time 8: (x^2): $P = 0.121$
Time 0 vs time 32: (x^2): $P = 0.036$
Time 8 vs time 32: (x^2) : $P = 0.189$

Table 6 describes the histological progression of the disease after treatment.

CDEIS

There was no difference between the averages throughout the three phases of the study as shown in Table 7.

CDEIS exhibited a significant correlation with lactoferrin only on week 0, with histology only on week 8 and with

TABLE 2. Comparison of the average calprotectin values at the three treatment times assessed in the study

TIME	N	Mean	Standard deviation	Standard error	95% CI lower	95% CI upper	Minimum	Maximum
0	17	682.5294	294.32043	71.38319	531.2038	833.8550	202.00	1199.00
8	16	414.5000	230.41007	57.60252	291.7231	537.2769	93.00	935.00
32	15	646.6667	364.63223	94.14764	444.7401	848.5933	83.00	1130.00

ANOVA: $P = 0.030$

TABLE 3. Pairwise comparison of calprotectin among the groups via the Bonferroni test

(I) time	(J)time	Mean difference (I-J)	Standard error	95% CI lower	95% CI upper	*P*
0	8	268.0294	104.4135	8.3759	527.6829	0.041
0	32	35.86275	106.1913	-228.211	299.9373	1.000
8	32	-232.1666	107.7356	-500.081	35.7482	0.110

TABLE 4: Comparison of the frequency of lactoferrin positivity at the three treatment times assessed in the study

	Time 0 n (%)	Time 8 n (%)	Time 32 n (%)
Negative	1 (5.9%)	4 (25%)	4 (26.7%)
Positive	16 (94.1%)	12 (75%)	11 (73.3%)
Total	17 (100%)	16 (100%)	15 (100%)

x^2: $P = 0.189$

TABLE 5: Comparison of the frequency of characteristics upon the anatomopathological analysis at the three treatment times assessed in the study

	Time 0 n (%)	Time 8 n (%)	Time 32 n (%)
Inactive	0 (0.0%)	0 (0.0%)	3 (18.75%)
Mild	4 (25.0%)	8 (50.0%)	5 (31.25%)
Moderate	3 (18.75%)	3 (31.25%)	6 (37.50%)
Severe	9 (56.25%)	3 (18.75%)	2 (12.50%)
Total	16 (100%)	16 (100%)	16 (100%)

x^2: $P = 0.021$

TABLE 6. Histological progression

		Number	Percentage
Histological progression	No change	5	33.3%
	Improvement	9	60.0%
	Worsening	1	6.7%

TABLE 7. Comparison of the averages analysis at the three treatment times assessed in the study

	Time 0 Mean	Time 8 Mean	Time 32 Mean
CDAI	231.5606	123.6306	104.8750
CDEIS	8.0000	5.6250	6.1250
PCR	15.7712	8.6324	5.8337
VHS	28.7647	21.6471	23.9375
Alpha 1-acid glycoprotein	98.5412	79.8353	79.8813
HB	12.6235	12.8882	13.4375
HT	38.6529	38.9000	40.0250
Platelet	344.4118	316.2941	303.1875

calprotectin only on week 32, as described in Table 8, 9 and 10, respectively.

The most relevant information was that high calprotectin levels on week 0 correlated with endoscopic worsening on week 32, as shown in Table 11.

Other parameters

Upon comparisons among the CDAI and CDEIS averages and the serum (CRP, ERS, and alpha 1-acid glycoprotein) and laboratory (Hb, Ht, platelet, and white blood cell count) measurements of inflammatory activity, only the CDAI exhibited a significant difference, as shown in Tables 7 and 12.

The CDAI exhibited significant differences (P<0.001) between times 0 and 8 and between times 0 and 32, indicating the clinical remission of the disease.

The Tables 8 and 10 describe the correlation between lactoferrin and the measure variables and calprotectin and the measure variables respectively.

Correlation between calprotectin, lactoferrin and histology

Based on multiple linear regression model, calprotectin and lactoferrin are not able to predict histological improvement/worsening, as shown Table 13.

TABLE 8. Correlation analysis between lactoferrin and variables (Pearson correlation)

	Lactoferrin 0 Pearson correlation/*P*	Lactoferrin 8 Pearson correlation/*P*	Lactoferrin 32 Pearson correlation/*P*
CDAI	-0,158/0,545	-0,189/0,483	0,124/0,659
CDEIS	-0.474/0,054	0,183/0,514	0,368/0,177
Histology	0,491/0,054	-0,208/0,456	0,193/0,490
PCR	-0,772/0,000	0,231/0,388	0,198/0,479
VHS	-0,562/0,019	-0,187/0,489	-0,233/0,403
Alpha 1-acid glycoprotein	-0,857/0,000	0,116/0,670	0,554/0,032
Hb	0,152/0,560	0,115/0,672	-0,024/0,932
HT	0,170/0,515	0,219/0,415	0,097/0,730
Platelet	-0,347/0,172	-0,254/0,342	0,110/0,698

TABLE 9. Correlation analysis between CDEIS, CDAI and histology (Pearson correlation) at the three treatment times

	CDEIS 0 Pearson/*P* correlation	CDEIS 8 Pearson/*P* correlation	CDEIS 32 Pearson/*P* correlation
Histology	0,167/0,535	0,507/0,045	0,445/0,084
CDAI	0,289/0,261	-0,097/0,722	0,367/0,163

TABLE 10. Correlation analysis between calprotectin and variables (Pearson correlation)

	Calprotectin 0 Pearson/*P* correlation	Calprotectin 8 Pearson/*P* correlation	Calprotectin 32 Pearson/*P* correlation
CDAI	0,094/0,721	-0,288/0,280	0,003/0,991
Lactoferrin	0,421/0,093	0,420/0,105	0,789/0,000
CDEIS	0,255/0,323	0,032/0,910	0,597/0,019
Histology	0,471/0,066	-0,262/0,346	0,416/0,123
PCR	-0,305/0,234	0,357/ 0,174	0,188/0,503
Alpha 1-acid glycoprotein	-0,386/0,126	0,428/ 0,098	0,385/0,156
Hb	0,089/0,734	0,085/0,754	0,126/0,654
Ht	0,104/0,691	0,110/0,686	0,231/0,408
Platelet	-0,068/0,796	-0,165/0,541	0,144/0,610

TABLE 11. Multiple linear regression model to assess calprotectin and lactoferrin time 0 in the condiction endoscopic after 32 weeks

Model	Coefficient	Coefficient standard error	*t*
Constant	3.768	5.567	.677
calprotectin 0	.011	.005	2.154
lactoferrin 0	-5.553	6.235	-.891

TABLE 12. Pairwise comparison of the CDAI among the groups via the Bonferroni test

(I) time	(J) time	Mean difference (I-J)	standard error	95% CI lower	95% CI upper	*P*
0	8	107.93000*	23.70159	49.0862	166.7738	<0.001
0	32	126.68559	24.06908	66.9294	186.4418	<0.001
8	32	18.75559	24.06908	-41.0006	78.5118	1.000

TABLE 13. Model of multiple binary logistic regression to assess the calprotectin and lactoferrin effect at time 0 in improvement histological after 32 weeks

Model	Coefficient	Standard error	*P*	Odds ratio
calprotectin 0	0.02	.002	.337	1.002
lactoferrin 0	20.778	40192.982	1.000	1.056E9
Constant	-21.634	40192.982	1.000	.000

DISCUSSION

Although endoscopy with biopsy is considered to be the best method for assessing the localisation, extent, and severity of inflammation, it is an invasive method that is prone to risks and complications[8, 12, 33].

The participants in the present study were subjected to three endoscopic exams with biopsy at 3 different times. This protocol allowed a comparison of the histological and endoscopic findings with the clinical, serum, and fecal results.

CDEIS

In the present study, the CDEIS exhibited a significant correlation with the histological score only on week 8; by contrast, there was no significant correlation between weeks 0 and 32, although significant differences have been described in two studies performed by Sipponen et al.[27, 28].

Another interesting analysis was that high calprotectin levels on week 0 were correlated with worsening endoscopy on week 32 therefore a inflammation predictor.

CDAI

From the clinical perspective, the CDAI is widely used to assess the degree of CD activity and the response to treatment[20]. In the present study, no significant correlation was found between the endoscopic, histological, and fecal variables at any time, but the CDAI exhibited a significant difference between weeks 0 and 8 and between weeks 0 and 32, indicating that the clinical status of the patients had improved. After induction (week 8) and after maintenance (week 32) of treatment, the CDAI exhibited a remarkable reduction, reaching levels associated with clinical remission of the disease, in spite of the persistence of the endoscopic, histological, serum, and fecal indicators of inflammation. That the intestinal mucosa might remain inflamed in patients with CD in a state of clinical remission is a well-known fact. For this reason, inflammation of the intestinal mucosa is not the only parameter that is used to determine if the disease is inactive. In addition, a portion of the items assessed in the CDAI are subjective, i.e., they depend on each patient's individual perception of his or her own disease[26]. Another criticism of the CDAI is the fact that previous surgical procedures and the presence of fistulas and fibrotic stenosis, which are not inflammatory processes, influence the score[32].

Serum markers

Regarding the serum markers, the CRP exhibited a significant correlation with lactoferrin the week before induction (week 0) and with the ERS and alpha 1-acid glycoprotein during weeks 0 and 32. The average CRP value the week before induction (week 0) was 15.7 and decreased to 8.6 on the week after induction (week 0) and to 5.8 after the end of maintenance (week 32). Although we did not find a significant correlation between the CRP and the histological activity, the latter improved, and the former decreased on weeks 8 and 32.

Serum measurements of the activity, such as the ERS and the platelet and white cell count, among others, are considered to be unspecific and might be affected by a wide range of non-intestinal diseases. Consequently, these measurements are unable to measure the intestinal inflammatory activity directly. Patients with the active disease might exhibit normal levels of the serum markers, whereas patients with the quiescent disease might exhibit abnormal values[32].

Calprotectin

Roseth et al.[23] validated calprotectin as a marker of intestinal inflammation by comparing it to the excretion of indium-111-labelled neutrophils during 4 days in patients with CD and by finding a significant correlation between the markers. Consequently, several other authors suggested the calprotectin assay as a non-invasive test for assessing intestinal inflammation in patients with IBD[17, 18].

Tibble et al.[32] observed that patients in clinical remission exhibit low calprotectin levels, whereas the patients with high levels exhibit an increased risk of relapsing within 1 year, suggesting that calprotectin might be used not only as a marker of clinical remission but also to predict relapse[5]. Thus, calprotectin might serve both as a marker of mucosal healing and as a sign of future inflammatory relapse[24].

All of the patients who were invited to participate in the present study had been indicated for infliximab and thus were considered to have a more aggressive form of the disease, which was confirmed in most of these subjects by their high calprotectin levels and positive lactoferrin tests. The reduction of the calprotectin levels and the negative lactoferrin results on week 8 did not correlate with the decrease in histological inflammation on week 32. In some of the patients who exhibited a remarkable reduction of calprotectin and had negative lactoferrin tests on week 8, the calprotectin levels had risen, and lactoferrin had become positive again by week 32. Therefore, in our study, there was no significant correlation between calprotectin and the histological scores during the last two phases of the study, but there was a significant correlation on week zero. In addition, high calprotectin levels before induction (week 0) did not correlate with a histological deterioration after the maintenance (week 32). Despite having submitted calprotectin on week 8 a drop in their levels ($P = 0.041$) but did not achieve remission rates or close to it perhaps explained by the fact that 50% of patients had moderate to severe histological activity. Furthermore, some patients had high calprotectin levels (up to 1000 mg/kg) before induction and others showed no change in profile. This feature was not observed in the studies reviewed.

Several studies have revealed a satisfactory correlation between calprotectin and the endoscopic and histological findings, such as the extent and degree of inflammation, unlike the indices of clinical and laboratory activity. In addition, measurement of calprotectin is inexpensive, non-invasive, sensitive, and rather easy to perform[11, 27].

In the present study, as already mentioned, high calpro-

tectin levels on week 0 correlated with poorer endoscopic findings on week 32 and were thus predictive of inflammation. Calprotectin and the histological scores exhibited a significant correlation only before induction (week 0). In addition, between weeks 0 and 8, the calprotectin levels exhibited a significant difference with a remarkable reduction, and the histological score improved.

The correlation found in several studies between calprotectin levels and the degree of inflammation suggests that calprotectin levels are due to inflammation at the tissue level. Several investigations have demonstrated that fecal calprotectin correlates in particular with the histology, rather than the colonoscopy findings, which means that in many cases, inflammation might not be macroscopically assessed[4].

In the present study, this correlation occurred significantly only on week 0. Tables 2 and 5 indicate high calprotectin levels at week 0 when more than 50% of the patients exhibited severe inflammation on the histological assessment. During week 8, the calprotectin levels exhibited a significant reduction, which correlated with a histological improvement in which 50% of the patients exhibited mild degrees of inflammation; however, this correlation was not statistically significant. During week 32, the calprotectin again increased and reached levels similar to those of week 0. Moreover, from the histological perspective, 50% of the patients still exhibited moderate-to-severe inflammation; however, this correlation was not statistically significant.

Some studies have been questioning that there is a better correlation of fecal markers with colonoscopy and histology in UC than CD because the presence of restricted lesions in the small intestine could influence the dosage of fecal markers[5, 7, 31]. However, in a study by Bunn et al.[4] observed that calprotectin also correlated with disease in the small intestine through its correlation with technetiun-labelled neutrophils. In another study made by Jensen et al.[14] also observed that calprotectin is sensitive in assessing disease in small intestine. The majority of the studies use as population patients with Crohn's disease with involvement of the small bowel and colon which was not different to this study.

Lactoferrin

Like calprotectin, lactoferrin is also a non-invasive fecal marker of intestinal inflammation. Lactoferrin has been used to assess the clinical response to treatment with infliximab in children with severe CD, and the response to treatment has been demonstrated by clinical improvement (Pediatric CDAI – PCDAI) and reduction of the lactoferrin levels. These results suggest that lactoferrin might serve as a marker for the response to treatment with infliximab[3]. A study conducted by Kane et al.[15] revealed that the lactoferrin concentration is significantly higher among patients with active or inactive IBD compared with healthy controls and patients with irritable bowel syndrome. In addition, the patients with active IBD exhibit higher lactoferrin concentrations compared to the patients with inactive disease, suggesting that lactoferrin is a sensitive and specific marker for inflammation in IBD[15, 34]. Our study used a qualitative lactoferrin kit, rather than the quantitative kit that is also commercially available; thus, we could not assess the correlation between the lactoferrin level and the degree of inflammation. Lactoferrin remained positive in most of the patients during the three stages of the study. Perhaps, lactoferrin did not become negative because the test used was qualitative, and most of the patients exhibited continuous inflammatory activity on histological assessment.

The main goal of treatment in IBD is to achieve remission of the disease (improvement of the signs and symptoms, as well as healing of the mucosa). Consequently, biological agents have been the focus of much attention because they induce rapid improvement of CD symptoms and mucosal healing 4 weeks after administration[25].

In our study, during the phase prior to treatment with infliximab, all of the patients exhibited a degree of histological activity (mild, moderate, or severe). After the period of maintenance treatment (week 32), histological improvement was detected in 60% of the patients. However, this result was not confirmed by the calprotectin and lactoferrin levels.

The major surprise in the present study was the high number of patients who relapsed during week 32, exhibiting an increase in the calprotectin levels and a positive lactoferrin test. Furthermore, these findings were not confirmed at the histological level because although most of the patients continued exhibiting histological activity, improvement at the microscopic level was remarkable (60%).

The statistical analysis revealed that in the present study, calprotectin and lactoferrin did not act as markers of remission and relapse in CD patients who were subjected to biological therapy. Although the high calprotectin levels during week 0 were predictive of the poorer endoscopic findings on week 32, this correlation was not found regarding the histological score. Therefore, because we consider the combination of endoscopy and histology to be the gold standard for assessing the inflammatory activity, we do not recommend that calprotectin and lactoferrin be used as markers to monitor CD patients who are undergoing biological therapy.

CONCLUSIONS

The major limitation of the present study was the small number of participants associated with losses. Although smaller samples are associated with greater inaccuracies in the results, we can often accept results that are indicative of a conclusion, rather than the conclusion itself.

Only a small number of investigations have utilised endoscopy and histology to compare calprotectin and lactoferrin levels with the inflammatory activities in CD patients subjected to biological therapy. The studies that we found also included UC or otherwise associated another biological therapy. Therefore, studies with larger numbers of patients, longer follow-up periods, and the inclusion of control groups are necessary.

Nogueira IM, Miszputen SJ, Ambrogini Jr. O, Artigiani Neto R, Carvente CT, Zanon MI. Avaliação da resposta de pacientes com doença de Crohn ao tratamento biológico, através de novos marcadores não invasivos: lactoferrina e calprotectina. Arq Gastroenterol. 2013,50(2):130-37.

RESUMO – ***Contexto*** - O uso de marcadores fecais para a monitorização da doença de Crohn é muito importante para a avaliação da resposta ao tratamento instituído. ***Objetivo*** – Avaliar a atividade inflamatória da doença de Crohn comparando os marcadores fecais (calprotectina e lactoferrina), colonoscopia com biópsias, Crohn's Disease Activity Index (CDAI) e marcadores séricos antes do uso do Infliximabe, após a fase de indução e após a fase de manutenção. ***Método*** – Foram incluídos 17 pacientes com diagnóstico prévio de doença de Crohn, que faziam uso da terapia convencional, mas que necessitaram da introdução da terapia biológica: Infliximabe. Esses pacientes realizaram colonoscopias com biópsias, exames de atividade inflamatória sérica, fecal (calprotectina e lactoferrina) e análise do CDAI nas fases pré Infliximabe, pós indução (semana 8) e pós manutenção (semana 32). ***Resultados*** – Houve queda significativa (P = 0,04) da calprotectina entre as fases pré Infliximabe e pós indução, o mesmo não ocorrendo após a fase de manutenção. A lactoferrina manteve-se positiva nas três fases do estudo. Na análise histológica, houve diferença significativa apenas entre as fases pré Infliximabe e pós manutenção (P = 0,036), com 60% dos pacientes apresentando melhora histológica após o período de acompanhamento. O CDAI apresentou diferença significativa entre as fases pré Infliximabe e pós indução e entre as fases pré Infliximabe e pós manutenção (P<0,01). ***Conclusão*** – A calprotectina e a lactoferrina não foram capazes de monitorizar a atividade inflamatória nos pacientes com doença de Crohn em uso de terapia biológica.

DESCRITORES – Doença de Crohn, Marcadores biológicos. Terapia biológica.

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