

岩手医科大学  
審査学位論文  
(博士)

## Clinical Trials Study

**Effect of tumor necrosis factor- $\alpha$  antagonists on oxidative stress in patients with Crohn's disease**

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**Abstract**

**AIM:** To investigate changes in oxidative stress in Crohn's disease (CD) before and after anti-tumor necrosis factor (TNF)- $\alpha$  treatment.

**METHODS:** A total of 42 patients with active CD, who were scheduled to be treated by anti-TNF- $\alpha$  antibodies, were enrolled. Serum levels of diacron-reactive oxygen metabolites (d-ROM), biological antioxidant potential (BAP), and modified ratio of oxidative stress and antioxidant capacity (m-OA) were measured using the Free Radical Analytical System before and 8 wk after induction of therapy with infliximab or adalimumab. The values for oxidative stress were correlated with disease activity and clinical response as determined by the CD activity index (CAI) at 8 and 54 wk after the therapy.

**RESULTS:** Prior to treatment, d-ROM showed significant correlations with CAI ( $r = 0.42$ ,  $P < 0.01$ ). There was a significant negative correlation between m-OA and CAI before and after treatment ( $r = -0.48$  vs  $r = -0.42$ ,  $P < 0.01$ ). CAI and d-ROM had decreased significantly by 8 wk after treatment (CAI;  $223.3 \pm 113.2$  vs  $158.3 \pm 73.4$ ,  $P < 0.01$ , d-ROM;  $373 \pm 133$  vs  $312 \pm 101$ ,  $P < 0.05$ ). However, neither BAP nor m-OA had changed significantly. In patients who had responded to the treatment at 8 wk, d-ROM, BAP, and m-OA levels before treatment did not differ significantly between patients with and without loss of response.

**CONCLUSION:** Anti-TNF- $\alpha$  therapy decreases oxi-

oxidative stress in patients with CD, but does not alter the production of antioxidants. Dysregulation of antioxidants may be associated with the disease.

**Key words:** Crohn's disease; Severity; Oxidative stress; Anti-tumor necrosis factor- $\alpha$  antibody

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**Core tip:** We measured serum markers of oxidative stress (d-ROM) and antioxidant potential (BAP) prior to and 8 wk after the initial administration of infliximab or adalimumab in CD. As a consequence, d-ROM decreased significantly after treatment. However, BAP and the ratio of oxidative stress and antioxidant potential remained unchanged. Anti-tumor necrosis factor- $\alpha$  therapy decreases oxidative stress, but does not alter the production of antioxidants. Dysregulation of antioxidants may be associated with CD.

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## INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory condition of the intestinal tract. While the etiology is unknown, it is commonly thought that oxidative stress underlies the persistence of a chronic inflammatory process in the gut<sup>[1]</sup>. Oxidative stress is generally defined as an imbalance between pro-oxidant and anti-oxidant systems<sup>[2]</sup>. The oxidative stress consists of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Excessive ROS attack target molecules such as lipids, proteins, and nucleic acids, and as a consequence can cause protein denaturation, inactivation of enzymes, and modifications to DNA<sup>[1,3,4]</sup>. Since CD is a disease characterized by neutrophil infiltration in the intestinal wall, there is a large degree of tissue damage because of the release of ROS<sup>[1,5]</sup>.

To date, the influence of tumor necrosis factor (TNF)- $\alpha$ , which is the most effective treatment for CD, on oxidative stress, has not been much investigated<sup>[6]</sup>. In 1999, Cesarone *et al*<sup>[7]</sup> described a simple procedure for measuring oxidative stress by means of quantification of serum diacron-reactive oxygen metabolites (d-ROM). d-ROM is a marker of hydroperoxide originating from ROS<sup>[8-11]</sup>. Biological antioxidant potential (BAP) is an antioxidant marker, which is representative of serum antioxidant capacity<sup>[12,13]</sup>. d-ROM and BAP can be readily measured

**Table 1** Clinical characteristics of patients *n* (%)

Characteristics	All patients ( <i>n</i> = 42)
Sex	
Male	30 (71)
Female	12 (29)
Age (yr), median	31.9 (17-57)
Disease duration (yr), median	6.2 (0-16)
Disease type	
Ileal	11 (26)
Ileo-colonic	26 (62)
Colonic	5 (12)
Patients with concomitant medication	
5-aminosalicylic acid	41 (98)
Prednisone	9 (21)
Azathioprine	7 (17)
Enteral nutrition	24 (57)
Previous segmental resection	8 (19)
Infliximab	10 (24)
Adalimumab	32 (76)

by the Free Radical Analytical System 4 (FRAS4) (H&D srl, Parma, Italy)<sup>[13,14]</sup>. Furthermore, a modified ratio of oxidative stress to antioxidant capacity (m-OA), calculated from d-ROM and BAP, is believed to indicate the degree of resistance to oxidative stress.

In the present study, we measured d-ROM and BAP in CD patients before and after induction of remission by anti-TNF- $\alpha$  therapy to investigate whether oxidative stress is associated with disease activity and efficacy of anti-TNF- $\alpha$  antibodies. We also studied whether oxidative stress is associated with the long-term efficacy of the treatment.

## MATERIALS AND METHODS

### Patients

This was a pilot study of 42 patients with active CD, who were scheduled to be treated with anti-TNF- $\alpha$  antibodies. The demographics of the patients appear in Table 1. There were 30 males and 12 females with a mean age of 32 years. Eleven patients had ileal disease, 26 had ileo-colonic disease, and 5 had colonic disease. The duration of the disease ranged from 0 to 16 years with a mean of 6 years. Eight patients had a prior history of intestinal resection. Ten patients were treated with infliximab (IFX), while the remaining 32 patients were treated with adalimumab (ADA).

### Treatment and follow-up

IFX was administered at a dose of 5 mg/kg intravenously at 0, 2 and 6 wk as induction therapy. Subsequently, IFX at a dose of 5 mg/kg every 8 wk was administered as maintenance therapy. ADA was administered subcutaneously at a dose of 160 mg at wk 0 and 80 mg at wk 2, followed by scheduled maintenance therapy at a dose of 40 mg every other week. Treatment responses were determined by physical examination and the CD activity index (CDAI) before and 8 wk after the initial administration of anti-

**Table 2** Biochemical tests for C-reactive protein, albumin, and white blood cell count

Biochemical tests	Before	8 wk	P value
CRP (mg/dL)	2.4 ± 2.6	1.1 ± 1.6	< 0.01
Alb (g/dL)	3.4 ± 0.75	3.7 ± 0.84	NS
WBC (per $\mu$ L)	7339 ± 2484	7278 ± 2532	NS

CRP: C-reactive protein; WBC: White blood cell count; Alb: Albumin; NS: Not significant.

TNF- $\alpha$  antibodies. Peripheral blood samples were also collected before and 8 wk after starting treatment. White blood cell count (WBC) and serum levels of C-reactive protein (CRP) and albumin were measured. CDAI was assessed at 8 wk and every 4 wk thereafter up to 54 wk after initial administration of anti-TNF- $\alpha$ .

### Assessment of disease activity

Patients were assessed for response to treatment as defined by a decrease in the CDAI score of 70 points or more from the baseline value, and at least a 25% reduction in the total score after 8 wk of treatment. In patients who showed a clinical response to induction therapy, those who fulfilled any one of the following criteria were regarded as having a loss of response (LOR): (1) an increase in CDAI of at least 70 points from the score at 8 wk, with a total score of at least 175; (2) an increase in CDAI of 35% or more from the baseline value; or (3) the introduction of a new treatment for active CD<sup>[15]</sup>.

### Measurement of oxidative stress

Oxidative stress was measured as serum d-ROM, BAP and m-OA. The stress was assessed before and 8 wk after the initial administration of anti-TNF- $\alpha$ . Blood samples were stored on ice after collection, and centrifuged to separate the serum. The serum was then stored at -80 °C until analysis.

Immediately prior to measurement, the samples were defrosted at room temperature and vortexed. To measure d-ROM levels, the FRAS4 system was used. For the measurement of d-ROM, 20  $\mu$ L serum and 1 mL buffered solution (R2 reagent of the kit, pH 4.8) were mixed in a cuvette, and then 10  $\mu$ L chromogenic substrate (R1 reagent) was added. After mixing and centrifugation for 60 s, the cuvette was incubated in a thermostatic block for 5 min at 37 °C. Then, the absorbance at 505 nm was recorded. Measurements were expressed as Carr U. Reference values measured by the manufacturer were indicated as being from 250 to 300 Carr U. Values greater than 300 Carr U suggest oxidative stress<sup>[7-9]</sup>.

To measure BAP levels, the BAP test was performed using the same analyzer. In brief, 50  $\mu$ L R2 reagent (ferric chloride) was added to a cuvette containing R1 reagent (thiocyanate derivative). The absorbance was measured and the reagent blank value subtracted. Then, 10  $\mu$ L serum sample was added to the cuvette.

After incubation for 5 min at 37 °C, the absorbance at 505 nm was recorded. The BAP levels were expressed as  $\mu$ mol/L. Reference values provided by the manufacturer were greater than 2200  $\mu$ mol/L. Values lower than 2200  $\mu$ mol/L suggest a reduction of antioxidant capacity<sup>[8,12]</sup>. The modified ratio of oxidative stress to antioxidant capacity (m-OA) was calculated as BAP/d-ROM/7.541<sup>[16]</sup>.

### Statistical analysis

Data are expressed as mean  $\pm$  SD, or as median (25<sup>th</sup>–75<sup>th</sup> percentiles). Values were compared between groups using the paired *t*-test or Student *t*-test, as appropriate. Correlation coefficients were assessed by linear regression analysis. *P* < 0.05 was considered to be statistically significant.

## RESULTS

### Efficacy of anti-TNF- $\alpha$ antibody

After 8 wk, 32 (76%) of the patients showed a response to treatment. At 54 wk, 22 patients (52%) were in remission, while 6 patients (14%) had dropped out, and 4 (15%) had shown LOR. CDAI decreased significantly 8 wk after treatment initiation (223.3  $\pm$  113.2 vs 158.3  $\pm$  73.4, *P* < 0.01). Changes in serum albumin, white blood cell count (WBC), and CRP are shown in Table 2. CRP values decreased significantly, while there was no statistically significant change in serum albumin or WBC.

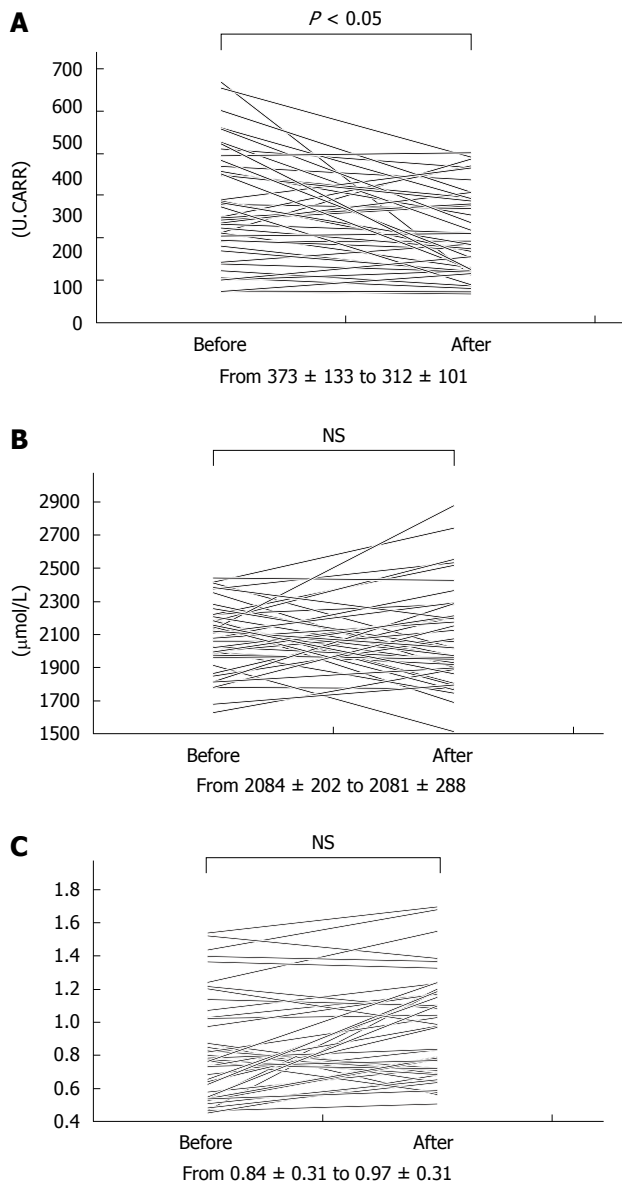
### d-ROM, BAP and m-OA levels

Figure 1 illustrates changes in d-ROM, BAP, and m-OA before and 8 wk after induction of therapy. After 8 wk, d-ROM had decreased significantly (373  $\pm$  133 vs 312  $\pm$  101, *P* < 0.05) (Figure 1A). The decrease was statistically significant in each type of regional involvement (ileal, ileocolonic, or colonic disease) (data not shown). However, neither BAP nor m-OA changed significantly after 8 wk (Figure 1B, 1C).

### Correlations between oxidative markers and clinical parameters

Table 3 shows the correlations between oxidative markers and clinical parameters. Before treatment, d-ROM showed statistically significant correlations with CRP (*r* = 0.64) and CDAI (*r* = 0.42) (Figure 2A). The correlation between d-ROM and CRP was significant even after induction therapy (*r* = 0.53). d-ROM showed no significant correlation with the other parameters either before or after treatment. We failed to find any significant correlation of BAP with CRP, CDAI, WBC, or serum albumin.

There were statistically significant negative correlations between m-OA and CDAI both before (*r* = -0.48) and after (*r* = -0.42) treatment (Figure 2B, 2C). There were negative correlations between m-OA and CRP before (*r* = -0.63) and after (*r* = -0.45) treatment. Significant correlations were also observed



**Figure 1** Changes in oxidative stress markers before and 8 wk after starting anti-TNF- $\alpha$  treatment in patients with Crohn's disease. A: d-ROM decreased significantly 8 wk after treatment; B: BAP did not change significantly after anti-TNF- $\alpha$  treatment; C: m-OA did not change significantly after anti-TNF- $\alpha$  treatment.

between d-ROM and CRP. Furthermore, we found a positive correlation between m-OA and serum albumin before ( $r = 0.45$ ) and after ( $r = 0.47$ ) treatment.

#### Correlation between efficacy of anti-TNF- $\alpha$ and oxidative stress

We then compared d-ROM, BAP, and m-OA between responders and non-responders after 8 wk of treatment. d-ROM, BAP, and m-OA were not significantly different between the 2 groups of patients either before or after treatment. When we compared d-ROM, BAP, and m-OA before and after the therapy between patients with and without LOR, we found no significant differences, suggesting that these parameters of oxidative stress are not predictive of

**Table 3** Correlations between d-ROM, BAP, m-OA, CDAI, serum CRP, WBC, and serum albumin in Crohn's disease patients treated with anti-TNF- $\alpha$

Oxidative markers	Clinical parameters	<i>r</i>	<i>P</i> value
d-ROM	CDAI	Before	0.42 < 0.01
		After	0.33 0.03
	CRP	Before	0.64 < 0.01
		After	0.53 < 0.01
	WBC	Before	0.12 0.47
		After	0.03 0.84
Alb	Before	-0.33 0.03	
	After	-0.38 0.01	
BAP	CDAI	Before	-0.17 0.28
		After	-0.18 0.24
	CRP	Before	0.03 0.85
		After	0.12 0.44
	WBC	Before	-0.04 0.79
		After	0.01 0.95
Alb	Before	0.24 0.13	
	After	0.14 0.38	
m-OA	CDAI	Before	-0.48 < 0.01
		After	-0.42 < 0.01
	CRP	Before	-0.63 < 0.01
		After	-0.45 < 0.01
	WBC	Before	-0.20 0.21
		After	-0.01 0.73
Alb	Before	0.45 < 0.01	
	After	0.47 < 0.01	

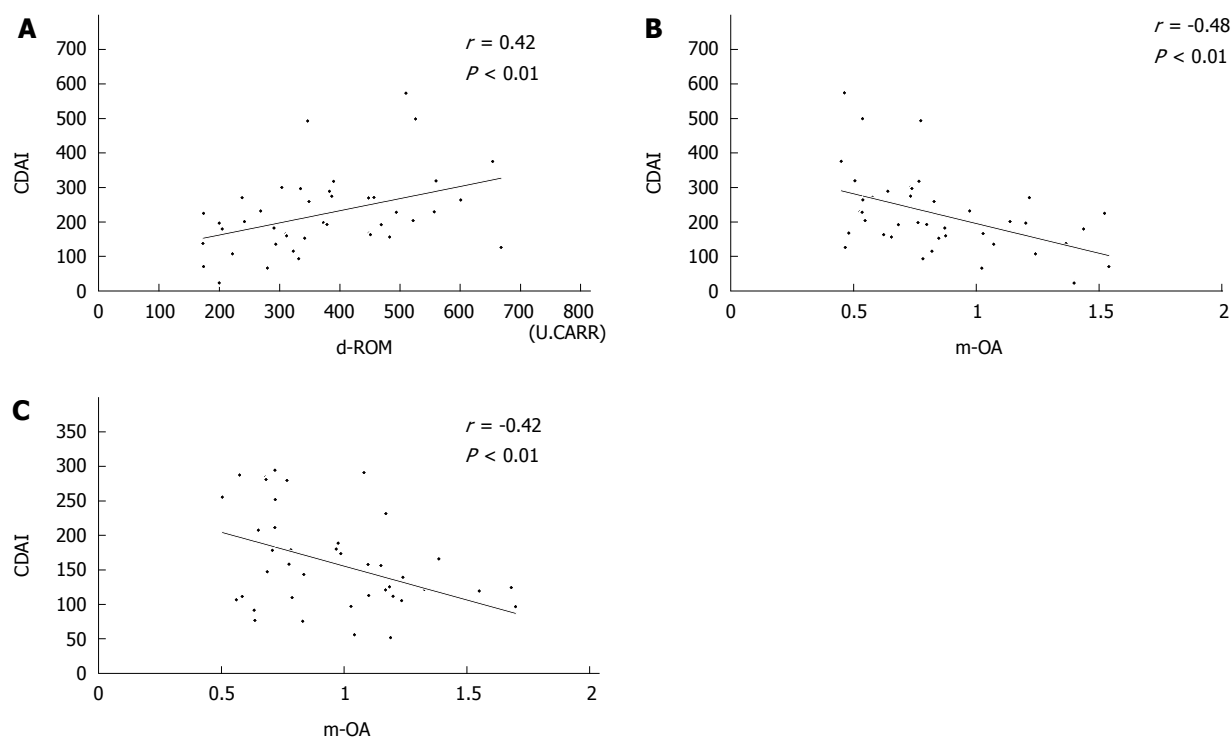
TNF: Tumor necrosis factor; m-OA: Modified ratio of oxidative stress and antioxidant capacity; BAP: Biological antioxidant potential; d-ROM: Diacron-reactive oxygen metabolite; CDAI: Crohn's disease activity index.

LOR.

## DISCUSSION

Increases in oxidative stress have been demonstrated in the serum and intestinal mucosa of patients with CD<sup>[17-20]</sup>. More recently, Kupčova *et al*<sup>[21]</sup> described a reduction of oxidative stress in patients with CD after administration of anti-TNF- $\alpha$  antibody. In the present investigation, we found that serum oxidative stress decreased in patients with CD after anti-TNF- $\alpha$  treatment. We also showed that the ratio of antioxidant activity vs oxidative stress calculated as m-OA was negatively associated with the activity of CD even after anti-TNF- $\alpha$  treatment. These findings strongly suggest that a decrease in antioxidant activity is characteristic of CD, and that m-OA may be one of the specific biomarkers for patients with the condition.

d-ROM and BAP levels have been used to assess



**Figure 2** Correlation of d-ROM and m-OA with Crohn's disease activity index. A: d-ROM showed a significant correlation with CDAI before anti-TNF- $\alpha$  treatment ( $r = 0.42$ ,  $P < 0.01$ ); B, C: m-OA showed significant correlations with CDAI both before (B) and after (C) the induction of therapy.

oxidative status, and their significance as clinical markers has been described in several fields. The 2 parameters have been studied in particular in cardiovascular disease, central nervous system disease, and lifestyle-related illnesses, and it has been shown that increases in reactive oxygen metabolites are associated with an increased risk of vascular endothelial damage<sup>[22-26]</sup>. Furthermore, Sugiura *et al.*<sup>[22]</sup> measured serum d-ROM levels in patients at high risk of cardiovascular disease and found that the d-ROM level coupled with serum high-sensitivity CRP was predictive of cardiovascular events. In our patients with CD, we found that d-ROM, serum CRP, and CDAI decreased significantly during induction therapy. Although we have not presented the data, there was no difference in the decrease in d-ROM between patients treated with IFX and those with ADA. Thus it seems likely that anti-TNF- $\alpha$  therapy suppresses the production of oxidative stress, thereby resulting in clinical benefit. In consideration of the biologic activity of anti-TNF- $\alpha$ , a decrease in the activity of circulating neutrophils seems to have made an important contribution to the decrease in oxidative stress measured as d-ROM.

Unlike d-ROM, we failed to show any changes in BAP, which represents antioxidant capacity, in our patients with CD even after anti-TNF- $\alpha$  treatment. While we did not include healthy controls in this investigation, the values of BAP in our patients were generally low, with more than half of the patients having values less than 2200  $\mu\text{mol/L}$ . In previous

studies, a reduction in serum antioxidant capacity was found in patients with inflammatory bowel disease (IBD)<sup>[17,27,28]</sup>. On the other hand, evidence for enhancement of antioxidant capacity was also found in biopsy specimens from IBD patients<sup>[19]</sup>. However, since the latter investigation included both patients with ulcerative colitis and with CD, it is uncertain that the antioxidant capacity was actually increased in CD. From our present results, it seems likely that the impairment of antioxidant capacity is specific to CD. This may be a consequence of a genetically determined disturbance in autophagy, which does not seem to be increased by any therapeutic strategy<sup>[29]</sup>.

Normally, cells handle oxidative stress by mechanisms that include the production of antioxidant agents. These compounds have a limited capacity to buffer against an increase in oxidative stress and prevent its toxic consequences. There are several naturally occurring antioxidant defenses in the human intestine that serve to protect cell membranes from lipid peroxidation, protein oxidation, and enzyme inactivation<sup>[18]</sup>. In this regard, m-OA has been considered to be a candidate serum marker for the balance between oxidative stress and antioxidant capacity. In the present investigation, m-OA showed the strongest and negative correlations with CDAI and CRP. Furthermore, the correlations were significant even after induction of anti-TNF- $\alpha$  antibody treatment. These observations strongly suggest that m-OA is a marker of persistent chronic inflammation of the intestine, and may be one of the surrogate biomarkers for patients

with CD. While we could not show any significant utility of m-OA for predicting the short-term response to anti-TNF- $\alpha$  treatment or LOR in our CD patients, the potential role of the marker warrants further study.

There are some limitations to this study. First, as we used frozen samples of serum, we may have underestimated the concentrations of d-ROM, BAP, and m-OA. Second, as we did not enroll healthy controls, we could not determine the reference values of the concentrations of the markers for oxidative stress, especially BAP, in the Japanese population. Third, since our investigation was a pilot study with a relatively small number of patients, we could not make strong conclusions regarding the roles of d-ROM, BAP, and m-OA in the prediction of the response to treatment or the long-term clinical course of CD. This was especially the case for the lack of data regarding serum concentrations of and serum antibodies to IFX and ADA. Furthermore, we could not apply practical intestinal damage, namely mucosal healing, as a parameter of disease activity<sup>[30]</sup>. These issues are under investigation in another prospective study.

In conclusion, we have shown that anti-TNF- $\alpha$  therapy decreases oxidative stress in patients with CD without affecting the production of antioxidants. In addition, m-OA, a marker for the balance between oxidative stress and antioxidant capacity, showed significant correlations with other parameters of disease activity in CD. Therefore, we propose that impairment of antioxidant activity may be associated with intractability of CD.

## COMMENTS

### Background

While the etiology of Crohn's disease (CD) is unknown, it is commonly thought that oxidative stress underlies the persistence of a chronic inflammatory process in the gut.

### Research frontiers

The authors investigated whether oxidative stress is associated with disease activity and efficacy of anti-tumor necrosis factor (TNF)- $\alpha$  antibodies in patients with CD.

### Innovations and breakthroughs

Serum oxidative stress decreased after anti-TNF- $\alpha$  treatment, while antioxidant capacity remained unchanged. The ratio of antioxidant activity vs oxidative stress (m-OA) negatively correlated with disease activity prior to and after anti-TNF- $\alpha$  treatment. Dysregulation of antioxidants may be associated with CD.

### Applications

The ratio of antioxidant activity vs oxidative stress measured by m-OA may be one of the specific biomarkers for patients with CD.

### Peer-review

This paper is interesting to know a relationship between oxidative stress and CD. The authors have necessary additional investigation in order to adjust for certain confounder in the correlation with oxidative markers.

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