

## Serum zonulin in patients with inflammatory bowel disease: a pilot study

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## ABSTRACT

**BACKGROUND:** In recent years it has been supposed that impaired intestinal permeability represents an early event preceding the onset of inflammatory bowel disease (IBD). Since zonulin has been proposed as a biomarker of intestinal permeability, we investigated its role in patients with IBD and the correlation between serum and fecal zonulin.

**METHODS:** A total of 118 IBD patients (86 Crohn's disease [CD] and 32 ulcerative colitis [UC]) and 23 healthy controls (HC) were prospectively enrolled. A serum sample was collected for all the subjects included in the study. A stool specimen collected in the same day of blood drawing was available for a subgroup of 33 IBD patients. Serum and faecal zonulin were tested by ELISA. Non-parametric statistical tests were used for data analysis.

**RESULTS:** Serum zonulin concentration was higher in IBD patients compared to HC (34.5 [26.5-43.9] ng/mL vs. 8.6 [6.5-12.0] ng/mL,  $P < 0.001$ ) showing an area under the curve of 0.98 for their discrimination. No difference in serum zonulin concentration was observed between patients with CD and those with UC ( $P = 0.074$ ). An inverse correlation was observed between serum zonulin concentration and disease duration ( $r_s = -0.30$ ,  $P = 0.001$ ); no correlation was observed between serum and faecal zonulin ( $r_s = 0.15$ ,  $P = 0.394$ ).

**CONCLUSIONS:** Serum zonulin is highly sensitive for the evaluation of intestinal permeability in IBD patients. There is no correlation between zonulin values in serum and feces.

**Key words:** biomarker - Crohn's disease - intestinal permeability - ulcerative colitis - zonulin

Pathogenesis of inflammatory bowel disease (IBD) is multifactorial with immunological, genetic, microbial and environmental factors contributing to the onset of the disease.<sup>1,2</sup> Since intestinal epithelial barrier plays a key role in regulating the interaction between antigens from the intestinal lumen and the mucosal immune system, intestinal permeability represents an additional piece of this intricate puzzle.<sup>3,4</sup>

Zonulin is a 47 KDa protein involved in intestinal tight junction modulation; increased zonulin activity can enhance antigens delivery through the paracellular pathway of intestinal epithelial leading to the abrogation of immune tolerance and in turn to an abnormal immune response.<sup>5</sup> A direct role of zonulin in the pathogenesis of several autoimmune diseases has been already proven.<sup>6,7</sup>

In the setting of IBD, it is supposed that impaired intestinal permeability represents an early event preceding disease onset.<sup>8</sup> The passage of non-self-antigens in the lamina propria may trigger the immune response mediated by cytokines such as interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  which in turn may perpetuate the increased intestinal permeability, starting a vicious circle.<sup>9</sup>

Since it has been hypothesized that a loss of intestinal barrier function is involved in IBD development and maintenance, the aims of the present pilot study were to investigate the role of serum zonulin as a potential biomarker of intestinal permeability in patients with IBD and to assess the correlation between serum and fecal zonulin.

## **Materials and methods**

### *Patients*

All consecutive patients with a definite diagnosis of IBD, in follow up at the outpatient clinic of the Division of Gastroenterology, AOU Città della Salute e della Scienza di Torino – San Giovanni Antica Sede Hospital, were invited to join the present prospective, single-centre, observational study between April 2017 and June 2017. In addition, 23 blood donors recruited from

the Blood Bank, AOU Città della Salute e della Scienza di Torino – Molinette Hospital were included as healthy controls (HC).

Concerning IBD patients, inclusion criteria were age >18 years old and a definite diagnosis of Crohn's disease (CD) or ulcerative colitis (UC) according to ECCO criteria.<sup>10,11</sup> All patients underwent a physical examination and inflammatory markers testing (i.e. white blood cell [WBC] count and fecal calprotectin [FC])<sup>12</sup>. During the scheduled visit, a full medical history was obtained, including data regarding sex, age, smoking habit, type and duration of disease, disease activity, previous and ongoing therapy. According to Montreal's classification,<sup>13</sup> CD was classified as follows: L1, disease confined to distal ileum; L2, exclusively to the colon; L3, ileocolonic location. UC was classified according to the segment involved: E1, ulcerative proctitis; E2, left sided colitis; E3, pancolitis. Disease activity was calculated with the Harvey-Bradshaw index for CD and with the partial MAYO score for UC.<sup>14,15</sup>

The study protocol was compliant to the Declaration of Helsinki and was approved by the Institutional Ethics Committee (0056924). All subjects signed a written informed consent prior to recruitment.

### *Methods*

Serum and stool specimens for zonulin determination were collected from all patients and stored at -20°C until the time of measurement. Laboratory personnel performing analysis was blinded to clinical diagnosis and details regarding patients' clinical histories. Serum and fecal zonulin were assessed by competitive ELISA method (IDK® Zonulin ELISA Kit, Immunodiagnostik AG, Germany). Optical density was determined with a microplate reader (Model 680, Bio-Rad Laboratories Inc., Berkeley, CA, USA) at 450 nm against 620 nm as reference. Results were calculated using a 4-parameter algorithm with linear ordinate for absorbance and logarithmic abscissa for concentration. Zonulin results were given in ng/mL. Based on

manufacturer's declaration, intra-assay and inter-assay coefficient of variation were 3.4% and 13.3%, respectively.

### *Statistical analysis*

Data were tested for normality using the D'Agostino-Pearson test. Since data were not normally distributed, age was reported as median (range) whereas all other continuous variables as median (interquartile range [IQR]). Categorical variables were reported as number (%). Comparison between groups was performed by non-parametric Mann-Whitney test for continuous variables, whereas Fisher exact test or  $\chi^2$  test for trend were used to analyse categorical variables. Spearman's coefficient of correlation ( $r_s$ ) was calculated to analyse the correlation between variables. The correlation analysis between serum and fecal zonulin concentration was performed on a subgroup of 33 patients, for whom a stool specimen collected at the same day of serum sampling was available. To evaluate diagnostic performance of serum zonulin, area under the curve (AUC), sensitivity (Se) and specificity (Sp) were assessed by using receiver operating characteristic (ROC) curve analysis.

All statistical analyses were performed by using MedCalc software v.14.8.1 (Ostend, Belgium) and a  $P \leq 0.05$  was considered statistically significant.

### **Results**

A total of 129 consecutive patients with IBD were proposed to participate to the study: 9 patients refused to sign informed consent; 2 patients were excluded due to difficulty in finding a vein to draw blood. Overall, 118 patients with IBD (49 [18-77] years, 63 males and 55 females) and 23 HC (50 [26-63] years, 10 males and 13 females) were finally included in the study. No differences were observed in age ( $P=0.110$ ) and gender ( $P=0.086$ ) between IBD patients and HC. The clinical characteristics and laboratory parameters of included patients are reported in Table I. Serum zonulin concentration was significantly higher in patients with IBD compared to HC (34.5 [26.5-43.9] ng/mL vs. 8.6 [6.5-12.0] ng/mL,  $P<0.001$ ) (Figure 1), whereas no difference was

observed between CD and UC patients (33.5 [26.1-43.5] ng/mL vs. 40.3 [29.9-47.6] ng/mL,  $P=0.074$ ). ROC analysis showed a high diagnostic accuracy for distinguishing patients with IBD from healthy controls (AUC=0.98, 95% confidence interval [CI]: 0.94-0.99) (Figure 2); a serum zonulin value of 14.2 ng/mL showed Se=91% and Sp=100% for IBD detection.

Serum zonulin concentration was inversely correlated with disease duration ( $r_s=-0.30$ , 95% CI: -0.46--0.12,  $P=0.001$ ). No correlation was observed between serum zonulin concentration and clinical activity index ( $r_s=0.07$ , 95% CI: -0.12-0.25,  $P=0.478$ ), WBC count ( $r_s=0.13$ , 95% CI: -0.08-0.33,  $P=0.211$ ) and FC values ( $r_s=0.27$ , 95% CI: -0.04-0.54,  $P=0.083$ ).

In the subgroup of 33 IBD patients in which the stool sample was collected in the same day of blood drawing, median fecal zonulin was 112.8 (67.9-182.9) ng/mL. In those patients, no correlation was observed between serum and fecal zonulin ( $r_s=0.15$ , 95% CI: -0.20-0.47,  $P=0.394$ ) (Figure 3).

## Discussion

Zonulin has been associated with different chronic inflammatory diseases; many of them have unknown etiologies and increased intestinal permeability appears to precede disease onset.<sup>16</sup> In the present study, we observed that patients with IBD had higher serum zonulin concentration in comparison to HC. Furthermore, serum zonulin showed an excellent diagnostic accuracy (AUC=0.98) for IBD detection.

A weak inverse correlation between serum zonulin concentration and disease duration was found; this finding is consistent with the concept that altered intestinal permeability is present in IBD patients from the beginning of the disease, being this condition a necessary but not sufficient pathogenic element for disease development.<sup>17, 18</sup> As a matter of fact, Sturgeon *et al* using a zonulin transgenic mice model showed that increased intestinal permeability precluded the development of colitis only in the presence of an inflammatory trigger.<sup>19</sup>

To date, only few studies investigated serum and fecal zonulin concentration in patients with IBD. Malickova *et al* reported higher serum and fecal zonulin values in patients with active CD compared to UC.<sup>20</sup> Our results do not support this observation; moreover, we did not find any correlation between serum and fecal zonulin concentration. In agreement with our findings, Ohlsson *et al* reported no correlation between zonulin concentration in serum and feces; in addition, these authors did not find any correlation between serum zonulin and fecal calprotectin.<sup>21</sup> Considering that zonulin is a marker of intestinal permeability and fecal calprotectin a marker of intestinal inflammation,<sup>22</sup> we can suppose that in the setting of an established disease, these two mechanisms follow different behaviors according to clinical, genetic and environmental features. Finally, Frin *et al* investigated fecal zonulin as a potential predictor of response to infliximab therapy in patients with UC, but a low accuracy was observed.<sup>23</sup> Likely, the measurement of zonulin in serum rather than in stool may be of greater clinical interest.

The present study could be limited by the size and the heterogeneity of the population analyzed that makes difficult to highlight a clear message for clinicians regarding the potential use of serum zonulin in the management of patients with IBD. Nonetheless, our results could represent the starting point for further studies aiming at defining the clinical role of serum zonulin in such patients.

In conclusion, this pilot study demonstrated the usefulness of serum zonulin measurement for the evaluation of intestinal permeability in IBD patients. According to previous findings, no correlation was observed between zonulin measurement in serum and in feces. Further studies are needed to investigate the dynamic of serum zonulin in the course of IBD and to explore novel therapeutic approaches targeting not only inflammatory response but also other pathogenic factors in order to obtain the recovery of the barrier function of the intestinal mucosa and thus disease control.

*Conflict of interest.*

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Table I. *Clinical characteristics and laboratory parameters of the whole cohort of IBD patients and comparison between CD and UC patients.*

Patients' characteristics	Overall	CD	UC	P
Patients (n)	118	86	32	
Familiarity for IBD	8 (7%)	8 (9%)	0	0.106
Duration of disease (years)	9 (2-19)	12 (3-20)	4 (2-14)	0.012
Extent of disease				
CD (L1, L2, L3)	-	40, 11, 35	-	-
UC (E1, E2, E3)	-	-	13, 6, 13	-
Disease activity				
Remission	24 (20%)	19 (22%)	5 (16%)	
Mild	47 (40%)	31 (36%)	16 (50%)	0.393
Moderate	42 (36%)	32 (37%)	10 (31%)	
Severe	5 (4%)	4 (5%)	1 (3%)	
Medication				
No treatment	22 (19%)	14 (16%)	8 (25%)	0.118
5-ASA	73 (62%)	54 (63%)	19 (59%)	0.825
Antibiotics	41 (35%)	32 (37%)	9 (28%)	0.497
Immunosuppressors	14 (12%)	12 (14%)	2 (6%)	0.511
Topical steroids	23 (19%)	15 (17%)	8 (25%)	0.280
Systemic steroids	23 (19%)	18 (21%)	5 (16%)	0.791
Anti-TNF	23 (19%)	21 (24%)	2 (6%)	0.066
Surgical resection				
No	62 (53%)	41 (48%)	21 (66%)	
1	39 (33%)	28 (32%)	11 (34%)	0.012
>1	17 (14%)	17 (20%)	0	
Current smoker (Yes)	30 (25%)	27 (31%)	3 (9%)	0.017
WBC (x 10 <sup>3</sup> /μL)	7.6 (6.3-9.5)	7.7 (6.3-9.2)	7.3 (6.3-10.5)	0.928
FC (μg/g)	345 (162-613)	321 (101-883)	349 (266-516)	0.862

Continuous variables are reported as median (interquartile range) while categorical variables are reported as absolute number (percentage).

5-ASA: 5-aminosalicylic acid; CD: Crohn's disease; FC: faecal calprotectin; n: number; TNF: tumor necrosis factor; UC: ulcerative colitis; WBC: white blood cells.

Figure 1. Comparison of serum zonulin concentration in HC and patients with IBD.

HC: healthy controls; IBD: inflammatory bowel disease.

Figure 2. ROC curve reporting the AUC value of serum zonulin for the discrimination between patients with IBD and HC.

AUC: area under the curve; ROC: receiver operating characteristic.

Figure 3. Correlation between serum and faecal zonulin.

$r_s$ : Spearman's correlation coefficient.