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The Relationship between Serum Zonulin and Innate Immunity in Patients with Inflammatory Bowel Disease

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Abstract: The aim of the study was to investigate the relationship between the serum zonulin and the cellular immunity in patients with ulcerative colitis (UC) and Crohn's disease (CD). The study included 97 patients, 13 (13.4%) patients with CD and 84 (86.6%) patients with UC. The concentration of zonulin in the serum was studied using the ELISA kits. The investigation of the circulated leukocyte subpopulation was carried out through flow cytometry. The functional activity of the circulating neutrophils was studied using the absorption capacity of the monodisperse polystyrene latex particles and indicators of oxygen-dependent metabolism in the nitroblue tetrazolium test. The serum zonulin concentration in CD patients was significantly higher compared with UC patients ($p = 0.003$). The zonulin concentration directly correlated with the functional activity of the circulating neutrophils. Patients with a zonulin concentration > 472.4 pg/mL had a significantly higher number of band neutrophils ($p = 0.0104$), CD3+CD8+ cells ($p = 0.0212$), NK cells ($p = 0.0161$), and lower–CD19+ cells ($p = 0.0034$). Among the IBD patients, zonulin was associated with IBD severity. An increase in the serum concentration of zonulin was associated with an increase in the functional activity of circulating neutrophils and an increase in the number of CD3+CD8+ cells, NK cells, and a decrease in the number of CD19+ cells.

Keywords: inflammatory bowel disease; ulcerative colitis; Crohn's disease; zonulin; intestinal permeability; innate immunity; neutrophils

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1. Introduction

Inflammatory bowel diseases (IBD) are currently attaining a level of prevalence where they can be characterized as becoming a global health problem. The spread of IBD is increasing worldwide and has grown from a figure of 3.7 million in 1990 to 6.8 million in 2017. The highest prevalence rates have been observed in North America, where there are 422.0 recorded cases per every 100,000 persons of the population [1]. With increasing urbanization, the number of cases is expected to increase in the rapidly developing countries of Asia, South America, and the Middle East. In a systematic review of 147 population-based studies on the incidence of ulcerative colitis (UC) and Crohn's disease (CD), the following trends have been noted: in Brazil, the annual percentage of CD increased by 11.1% (95% confidence interval (CI) 4.8–17.8) and of UC by 14.9% (95% CI 10.4–19.6), and in Taiwan, the increase in CD was 4% (95% CI 1.0–7.1) and in UC was 4.8% (95% CI 1.8–8.0) [2].

The following factors are involved and functionally integrated in the pathogenesis of IBD: the individual's genetic predisposition, specific environmental factors, the condition of the intestinal microbiome, and the individual's immune response. Currently, much attention is being paid to the role of intestinal permeability in the pathogenesis of IBD and the study of biomarkers that are used for the diagnosis and determination of UC and CD activity. One of these physiological biomarkers is zonulin, a haptoglobin precursor involved in the physiological regulation of both the epithelial and endothelial barrier functions, facilitated by modulating intercellular tight junctions [3]. Its discovery over 20 years ago by A. Fasano et al. has led to a better understanding of the complex mechanisms that regulate paracellular transport, as well as the role of intestinal permeability and the consistency of the intestinal barrier in maintaining homeostasis.

The current views on the biochemistry and pathophysiology of zonulin are reflected in works published earlier by A. Fasano [4,5]. In addition to IBD, the role of zonulin in the pathogenesis of a number of other diseases is also discussed, including multiple sclerosis, celiac disease, attention deficit hyperactivity disorder, non-alcoholic fatty liver disease, type 1 diabetes mellitus, ankylosing spondylitis, and others [5]. In the studies that have been conducted, zonulin in serum and feces has been determined to be useable to reliably assess intestinal permeability. The significance of the marker, depending on the biological environment where it has been determined to be, is due to its pathology.

To date, few studies are being conducted to investigate the relationship between the serum zonulin and the course of IBD. Additionally, the results of the studies that have been published are contradictory, while data concerning the interaction of zonulin with cellular immunity in patients are extremely limited. The aim of this study was to investigate the possible relationship between the level of the serum zonulin and the cellular immunity of patients with UC and CD.

2. Materials and Methods

A cross-sectional study was carried out which included patients that were hospitalized in the Chelyabinsk Regional Clinical Hospital in the period from September 2017 to January 2021. The study protocol was approved by the Ethics Committee of the South Ural State Medical University (Protocol No. 10 of 15.11.2019). All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all individual participants involved in the study.

The criteria for inclusion in the study were the following: an established diagnosis of UC or CD, an age of over 18, and the patient's written consent to participate in the study. The exclusion criteria for the study were the following clinical conditions: acute infectious diseases, active malignant neoplasms within the previous five years, and pregnancy. The diagnosis of UC and CD was carried out in accordance with current national clinical guidelines [6,7]. UC was divided into several categories: proctitis, left-sided colitis, pancolitis, CD–terminal ileitis, colitis, and ileocolitis, and depending on its presence, upper gastrointestinal tract (GI) lesions were also indicated. The severity of the IBD for UC was determined via the Truelove-Witts classification and for CD via the activity index (CDAI).

2.1. Laboratory Examination

For immunological examination, venous blood was used, which was collected into 6 mL lithium-heparin test tubes. The investigation of the circulated leukocyte subpopulation was carried out through flow cytometry on a Navios 6/2 flow cytometer (Beckman Coulter, Brea, CA, USA). The following monoclonal antibody conjugates were used for the phenotyping of leukocyte subpopulations: CD3, PE-eFluor 610 (eBioscience, San Diego, CA, USA); CD19, PE (eBioscience, San Diego, CA, USA); CD56, PE (eBioscience, San Diego, CA, USA); CD4, APC (eBioscience, San Diego, CA, USA); CD8, PE-Cy5.5 (Invitrogen, Waltham,

MA, USA); CD25, PE-Cy7 (eBioscience, San Diego, CA, USA); CD127, FITC (eBioscience, San Diego, CA, USA).

The functional activity of the peripheral blood neutrophils was studied using the indicators of the absorption capacity of monodisperse polystyrene latex particles and the indicators of the oxygen-dependent metabolism in the nitroblue tetrazolium test (NBT). To assess phagocytic function, 200 μL of a cell suspension was mixed with 20 μL of a polystyrene latex particle suspension. After a one-hour incubation at 37 °C, cell preparations were prepared, dried, fixed with methanol and stained using the Romanowsky method. The following parameters were evaluated when assessing the absorption capacity of the monodisperse polystyrene latex particles: the number of latex particles absorbed per phagocyte (neutrophil phagocytosis, conventional units), the percentage of phagocytes capturing at least one latex particle (phagocytosis activity, %), and the number of latex particles absorbed per every 100 cells (phagocytosis intensity, %). For the NBT, 0.1 mL of a 0.2% nitroblue tetrazolium solution in a 0.1 mL phosphate buffer (pH 7.4) was added to tubes containing 0.2 mL of cell suspension. After a 30 min incubation at 37 °C, 3 mL of 0.1 n hydrochloric acid solution was added to the reaction mixture to stop the reaction. The tubes were centrifuged at 1000 rpm for 5 min. The supernatant was drained and the sediment was used to prepare smears. After drying, the preparations were fixed with methanol and stained with a 0.1% aqueous safranin solution. A polystyrene latex at a concentration of 108 particles/mL, with a particle diameter of 1.7 μm, was used as a stimulant for the induced test. The reactions were recorded using light immersion microscopy (Zeiss Primo Star, Oberkochen, Germany) at a magnification of 90 × 10 × 1.5. The percentage of formazan-positive neutrophils per every 100 cells in the spontaneous (activity, %) and induced (activity, %) versions of the NBT was assessed. The lysosomal activity was determined through the luminescence intensity of lysosomes treated with acridine orange [8].

The concentration of zonulin in serum was studied using the ELISA kits (Blue Gene Biotech, Shanghai, China, catalog number E01Z0004). The concentration of the enzyme peptidyl-arginine deiminase type 4 (PAD4) in serum was evaluated using the ELISA kits (Wuhan Fine Biotech, Wuhan, China, catalog number EH3496). This study was carried out on an enzyme immunoassay analyzer Personal LAB (Adaltis, Rome, Italy).

2.2. Statistical Analysis

Statistical data processing was carried out using the MedCalc software (v.20.216). Qualitative variables were described in absolute and relative frequencies (percentages). Quantitative variables were described by a median (Me) indicating the interquartile interval [lower quartile (LQ); upper quartile (UQ)]. To determine the interrelationships of the indicators, Spearman's correlation analysis was used. In order to assess the significance of the differences between the two groups, the Mann–Whitney criterion was used. To assess the significance of the differences between the three groups, the Kruskal–Wallis criterion was used, followed by a pairwise comparison using Dunn's test. Pearson's criterion χ^2 was used to compare qualitative data. The differences were considered statistically significant at a critical significance level of 0.05. The graphs were created using OriginPro 2024 (OriginLab Corp., Northampton, MA, USA) software.

3. Results

The study included 97 patients, 13 (13.4%) patients with CD and 84 (86.6%) patients with UC. The characteristics of the patients included in the study are presented in Table 1.

Table 1. Clinical characteristics of the patients with IBD.

Characteristics	CD (n = 13)	UC (n = 84)	Total (n = 97)	*p*
Male (n, %)/Female (n, %)	2 (15.4%)/11 (84.6%)	41 (48.8%)/43 (51.2%)	43 (44.3%)/54 (55.7%)	0.024
Age, years (Me, [LQ; UQ])	40 (32.5; 64.0)	45 (34.3; 56.8)	45 (34.5; 57.0)	0.987
Age of IBD onset, years (Me, [LQ; UQ])	33.0 (29.0; 54.5)	37.0 (28.0; 47.5)	36.0 (28.0; 48.0)	0.983
IBD severity:				
Mild (n, %)	2 (15.4%)	19 (22.6%)	21 (21.6%)	0.556
Moderate (n, %)	3 (23.1%)	24 (28.6%)	27 (27.8%)	0.681
Severe (n, %)	8 (61.5%)	41 (48.8%)	49 (50.6%)	0.393
CD localiztion:				
Isolated upper disease (n, %)	1 (7.7%)			
Ileal (n, %)	2 (15.4%)			
Ileocolonic (n, %)	6 (46.2%)			
Colonic (n, %)	5 (38.4%)			
UC localization:				
Proctitis (n, %)		4 (4.8%)		
Left-sided colitis (n, %)		19 (22.6%)		
Pancolitis (n, %)		61 (72.6%)		

Comments: IBD = inflammatory bowel disease; CD = Crohn's disease; UC = ulcerative colitis; LQ = lower quartile; UQ = upper quartile.

As shown in Table 1, it was found that the CD was less common in men, while an equal assessment of the severity of IBD did not reveal significant differences between males and females. The evaluation of the functional activity of the neutrophils and the subpopulation composition of the leukocytes is presented in Figures 1 and 2.

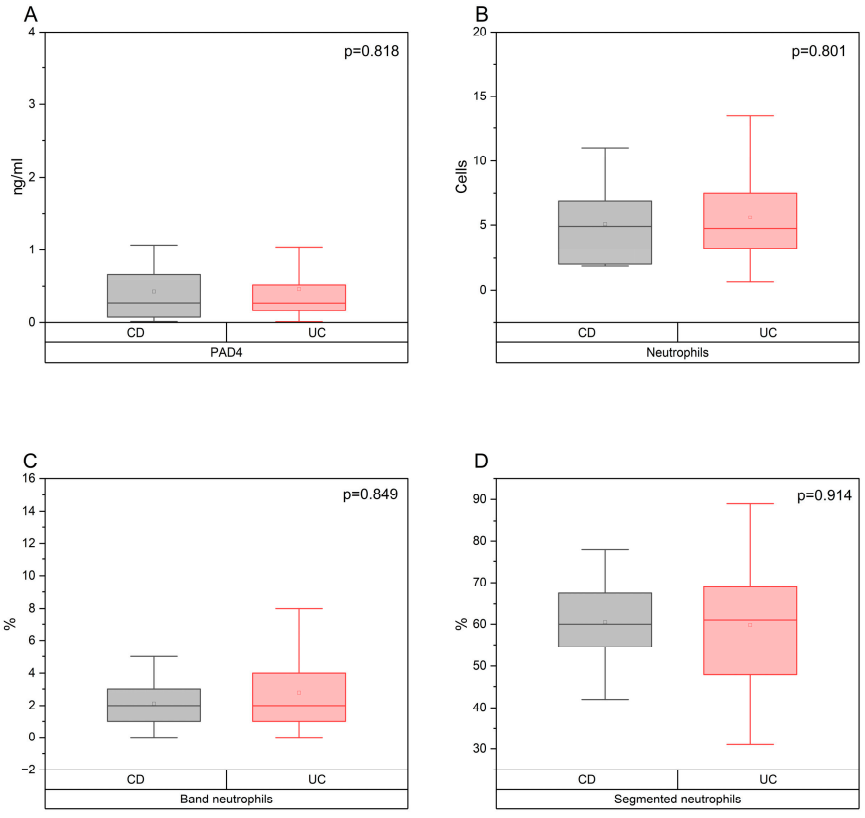

Figure 1. *Cont.*

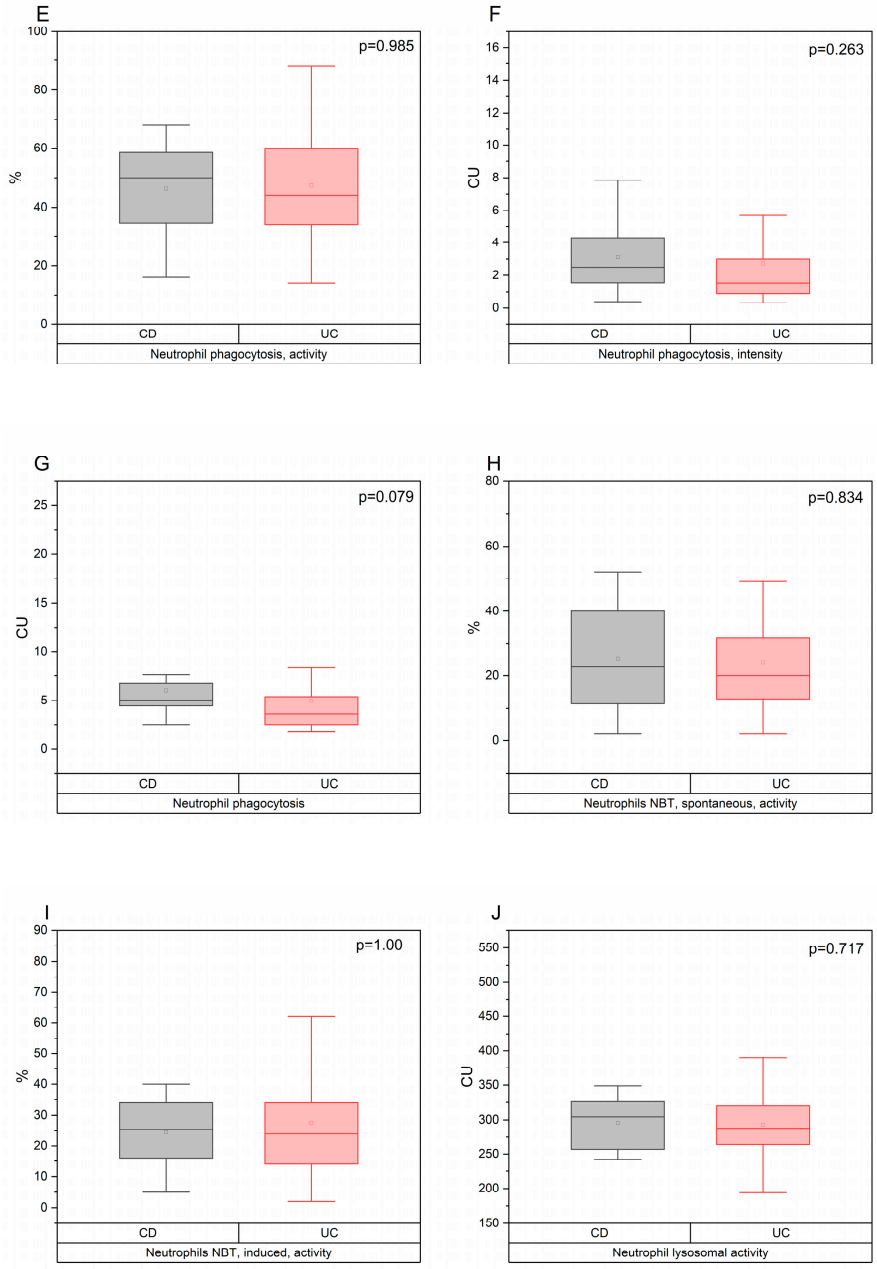

Figure 1. Evaluation of neutrophil subpopulation's composition (**B**–**D**) and their functional activity (**A**,**E**–**J**). Comments: CD = Crohn's disease; UC = ulcerative colitis; CU = conventional units; NBT = nitroblue tetrazolium test; PAD4 = peptidyl arginine deiminase 4.

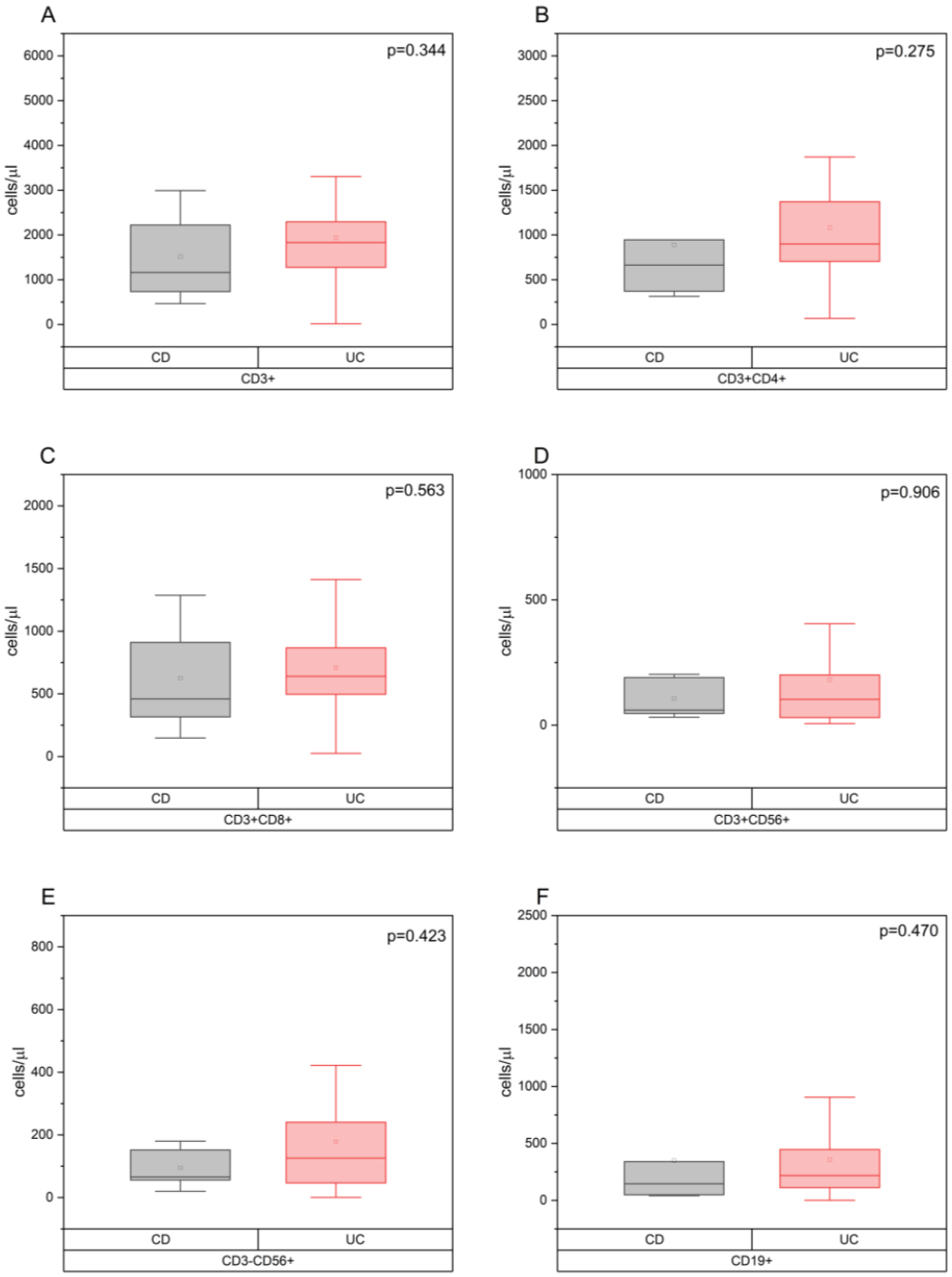

Figure 2. Evaluation of the subpopulation composition of circulating leukocytes. The number of T-lymphocytes (**A**–**C**), TNK cells (**D**), NK cells (**E**) and B cells (**F**) in patients with CD and UC.

3.1. Concentration of the Serum Zonulin in Patients with CD and UC, Depending on the Severity of IBD

Comparing the serum level of zonulin in patients with UC and CD, it was found that its concentration in patients with CD was significantly higher, 509.3 (367.9; 757.0) ng/mL compared to 338.4 (236.8; 446.8) ng/mL (see Figure 3).

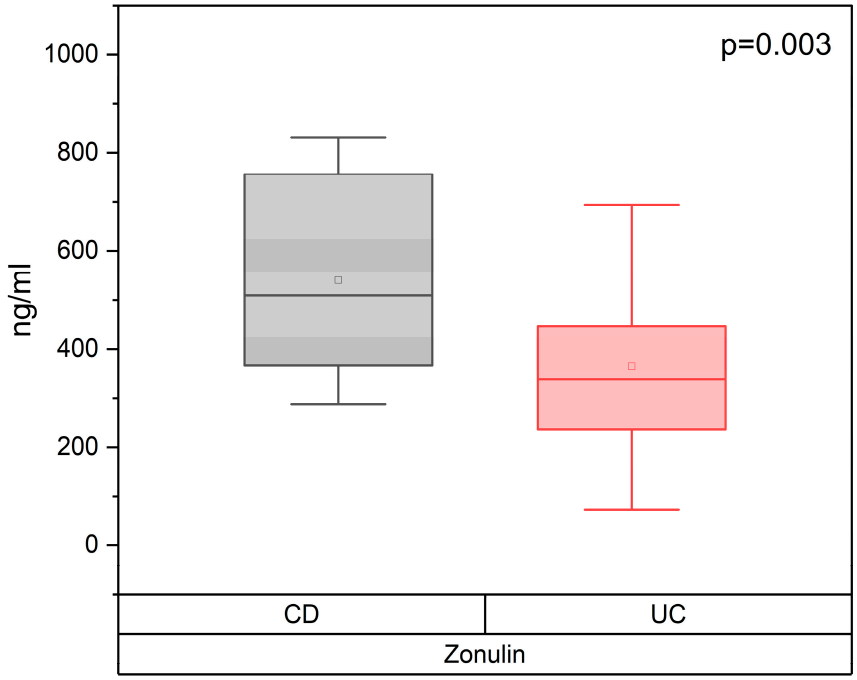

Figure 3. Serum zonulin levels in patients with UC and CD.

After that, the concentration of zonulin in the blood serum was analyzed depending on the severity of the IBD (see Figure 4).

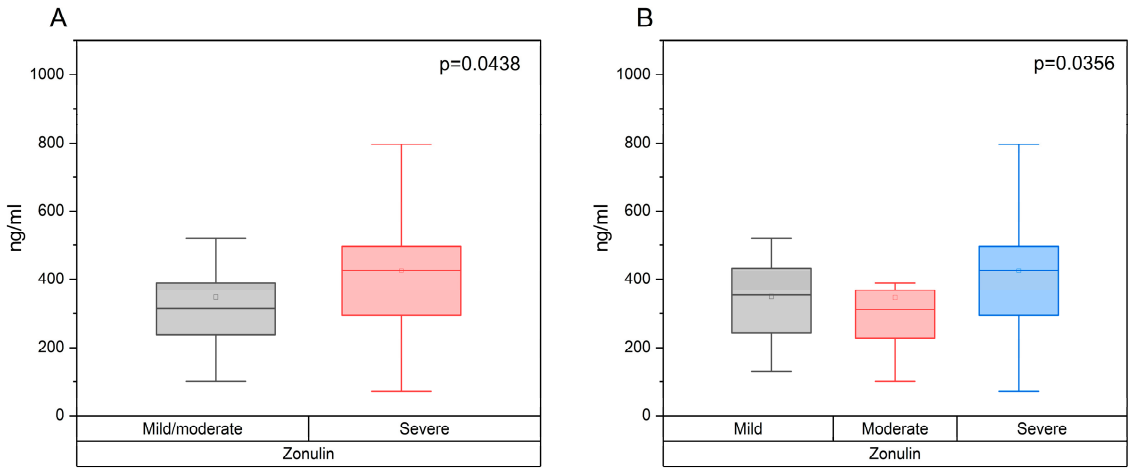

Figure 4. Serum zonulin concentration depending on the severity of IBD: (**A**) when comparing the three severity groups; (**B**) when comparing the severe group with the mild/moderate group.

When comparing the zonulin concentration in the three severity groups of IBD, a statistically significant change in its concentration was found ($p = 0.0356$); however, no intergroup differences were revealed in a post hoc pairwise comparison using Dunn's criterion with the Bonferroni correction ($p < 0.0166$ was considered significant), resulting in the difference becoming unsignificant. On the other hand, when the sample group was divided into two subgroups depending on the severity of the IBD (mild/moderate versus severe), the zonulin concentration was significantly higher among patients with severe IBD.

3.2. The Relationship between the Concentration of Zonulin in the Blood Serum and the Indicators of Cellular Immunity

The concentration of zonulin directly correlated with the functional activity of the circulating neutrophils (see Figure 5A,B).

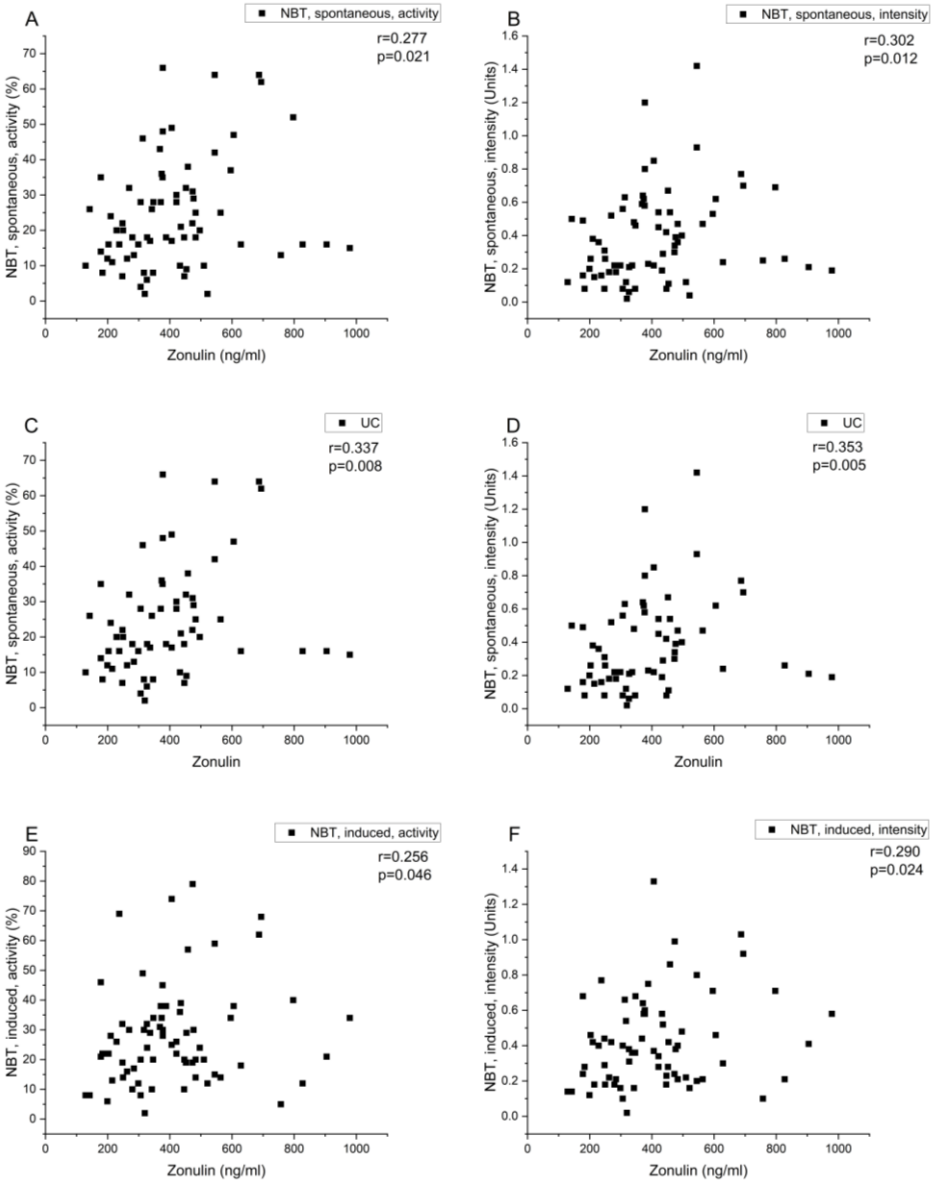

Figure 5. Relationships between the serum zonulin concentration and circulating neutrophil activity (explained in the text). Relationships of spontaneous NBT parameters with serum zonulin concentrations in patients with UC and CD (**A**,**B**), and separately in the subgroup of UC patients only (**C**,**D**), relationships of induced NBT parameters with serum zonulin concentrations in patients with UC and CD (**E**,**F**).

These correlations were observed mainly in the subgroup of patients with UC, and became stronger after the exclusion of patients with CD (see Figure 5B,C). There were also significant associations of zonulin with the activity and intensity in the induced NBT test (see Figure 5D,E).

Following these tests, the features of the circulating immune cells were analyzed depending on the concentration of zonulin in the blood serum. Thus, patients with a zonulin concentration corresponding to the fourth quartile (>472.4 pg/mL) had a significantly higher relative number of circulating band neutrophils (3.50% vs. 1.0.0%, $p = 0.014$), a relative number of CD3+CD8+ cells (33.7% vs. 27.5%, $p = 0.0212$), a relative number of NK cells (10.6% vs. 5.12%, $p = 0.0161$), a lower absolute (103.0 cells/μL vs. 240.5 cells/μL, $p = 0.0442$) and a relative number (5.43% vs. 12.9%, $p = 0.0034$) of CD19+ cells. No correlation was found between the concentration of PAD4 and zonulin, nor with any other indicators of cellular immunity.

4. Discussion

Among the patients with IBD in our study, the concentration of the serum zonulin was significantly higher in patients with CD compared to UC. The data obtained do not contradict the study conducted by Malíčková K et al., where serum/faecal zonulin levels were elevated only in CD, but not in UC patients [9]. It can thus be assumed that in CD the zonulin level is higher than in UC, due to the signaling cascade after the binding of one of the trigger proteins leading to the release of zonulin–gliadin, with the chemokine receptor CXCR3. This has CXCR3 being activated via the interaction with MyD88 (a key adapter molecule in the toll-like receptors signaling pathway) which leads to a subsequent increase in permeability both in the cell culture models and in ex vivo studies of intestinal tissues. The CXCR3 receptor is overexpressed on the apical surface of enterocytes in patients with CD, which may explain the increase in zonulin expression [10]. There are also suggestions that there are permeability anomalies in CD compared to UC due to the presence of certain registered genetic associations in CD, for example, mutations in C-terminal caspase recruitment domain 15 (3020insC) [11]. G.P. Caviglia et al. found that the serum zonulin concentration was higher in those with both diseases compared to healthy controls and observed an inverse correlation between the serum zonulin concentration and the disease duration [12]. X. Wang et al. included 130 women (51–61 years old) from the WHILA (The Women's Health in the Lund Area) study, of which 18 cases of IBD were diagnosed at the time of inclusion and 47 cases of IBD were diagnosed during the following 17 years of follow-ups. The authors showed that the serum zonulin is not a predictor of IBD onset, but is associated with markers of metabolic risk, such as the body mass index, triglycerides, and cholesterol [13].

In the last few years, the potential role of zonulin as a predictor for the development of various autoimmune diseases has been actively discussed [14,15]. It is assumed that an increase in the serum zonulin, indicating a persistent violation of the integrity of the intestinal barrier, may also indicate an excessive antigen passage from the small intestine into systemic circulation. This, in turn, contributes to the inappropriate activation of innate and adaptive immunity. Consequently, the persistent increase in the serum zonulin observed in IBD may also represent the activation of the immune response.

In our study, zonulin concentration directly correlated with the functional activity of circulating neutrophils, and patients with a zonulin concentration > 472.4 ng/mL differed significantly with regard to the relative number of circulating band neutrophils that were found. In the work of K. Gokulakrishnan et al., who included 60 patients with schizophrenia, an increase in the serum zonulin was associated with an increase in the neutrophil-to-lymphocyte ratio [16]. Data on the study of the relationship between the zonulin levels of patients with IBD and their neutrophil activity are currently limited. Thus, in a study by Qi You et al., among patients with type 1 diabetes mellitus, direct correlations were found between the concentration of zonulin and the NET-associated proteins, such as neutrophil elastase and PAD4 [17]. It is interesting to note that in our study there were no correlations between PAD4 and indicators of neutrophil oxidative explosion. However, despite the fact that direct mechanisms linking the formation of PAD4-dependent NETs with the cytosolic "machinery" of oxidative explosion have been identified in experimental studies, there are no clinical studies on this issue [18,19].

The studies addressing the subpopulation composition of circulating leukocytes in patients with IBD compared to healthy volunteers are also limited. Thus, for example, A. Tkachev et al. demonstrated an increase in the total number of T-lymphocytes and B-lymphocytes in 44 patients with UC compared with 20 healthy volunteers [20]. In a study by Y. Long et al., patients with UC also differed from healthy participants in that there was an increase in their number of peripheral helper T cells [21]. In another more recent study, UC patients had lower proportions of CD4+T cells and higher proportion of CD8+T cells than healthy controls, and there was also evidence of the potential diagnostic value of CD3+HLA-DR+ T cells as a marker of UC severity [22].

The relationship of the zonulin concentration in the blood with other indicators of systemic inflammation has also been the subject of only a small number of studies. Thus, in a study involving 36 patients with IBD, zonulin levels directly correlated with serum concentrations of IL10, IL17, and IL22 [23]. S. Arslan et al., in a sample of patients with vitiligo, demonstrated the direct correlations between the level of zonulin and IL6, TNFa, and lipopolysaccharide [24]. On the contrary, in a study by Lacombe L.A.C. et al., there were no associations between the zonulin levels and the circulating cytokines (IL17A, IL10, IL6, IL4, IL2, TNF, IFNγ) in the serum of patients with IBD [25]. It is likely that zonulin not only represents changes in the cellular immunity of patients with increased intestinal permeability but is in fact one of the drivers of these changes.

In an experimental study by N. Tajik et al., the effect of the zonulin antagonist (larazotide acetate, AT-1001) in connection with the development of arthritis was studied in a mouse model of collagen-induced arthritis [26]. Among other things, it was found that the use of larazotide acetate led to a decrease in the severity of intestinal inflammation, as well as a change in the T cells' homeostasis in the spleen and their shift towards an anti-inflammatory phenotype. Thus, an increase in the serum zonulin concentration reflects not only the dysregulation of the barrier function of the small intestine, but also the associated systemic inflammation and changes in the homeostasis in various immune niches, which, in turn, plays an important role in the progression of IBD and the development of extra-intestinal manifestations [24,26–28].

The main limitation of the current study was the small sample size. Thus, in order to correctly perform the correlation analysis presented in this paper, the number of patients required ranged from 60 to 117. It should be noted, in addition, that there was a marked asymmetry of the sample with a small number of patients with CD. However, this affects only one of the findings of the current study. Further studies with a large number of patients are required to clarify the relationship between zonulin levels and the immune parameters of patients with IBD. There is also a limitation associated with the method of measuring blood zonulin, namely the commercially available ELISA kit, which quite likely detects not only zonulin but also other unknown representatives of the zonulin family [3]. In connection with these data, the use of improved laboratory kits with a more highly developed detection of specific and reliable monoclonal antibodies to zonulin is required in the future [13].

5. Conclusions

Among IBD patients, zonulin was significantly higher in CD patients compared to UC patients and was associated with IBD severity. An increase in the serum concentration of zonulin was associated with an increase in the functional activity of circulating neutrophils and an increase in the number of CD3+CD8+ cells, NK cells, and a decrease in the number of CD19+ cells.

Author Contributions: Conceptualization, G.K., O.A. and A.D.; data curation, G.K., O.A. and A.D.; formal analysis, G.K. and V.G.; investigation, G.K., V.G., A.K., K.N., A.S., O.A. and A.D.; methodology, G.K., O.A. and A.D.; project administration, O.A. and A.D.; supervision, O.A. and A.D.; writing—original draft, G.K., V.G., A.K., K.N., A.S., O.A. and A.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments. The study protocol was approved by the Ethics Committee of South-Ural State Medical University (Protocol No. 10 of 15 November 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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