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Original Article

# An Association Between the Intestinal Permeability Biomarker Zonulin and the Development of Diabetic Retinopathy in Type II Diabetes Mellitus

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## Abstract

**Objectives:** Increased intestinal permeability (IP) and gut microbiota dysbiosis have been implicated in low-grade chronic inflammation, which is an important factor in the pathogenesis of diabetic retinopathy (DR). This study aims to demonstrate the relationship between the IP biomarker zonulin and DR in patients with type 2 diabetes mellitus (T2DM).

**Materials and Methods:** This study was conducted with a total of 89 T2DM patients, including 33 non-DR, 28 with non-proliferative DR (NPDR), and 28 with proliferative DR (PDR), and 32 healthy controls. Zonulin levels were determined from blood samples using an enzyme-linked immunosorbent assay kit.

**Results:** There was no difference between the four groups in terms of age (p=0.236), gender (p=0.952), and body mass index (p=0.134) of the participants. Zonulin levels were significantly higher in the PDR group compared to the other three groups, as well as in the non-DR and NPDR groups compared to the control group. In multivariate logistic regression analysis, zonulin was found to be an independent predictor of DR (odds ratio: 1,781, 95% confidence interval: 1,122-2,829, p=0.014).

**Conclusion:** Our study showed that elevated zonulin levels may play a significant role in the development of DR, particularly during the transition to the proliferative stage. This suggests that regulation of IP could be one of the targets of DR treatment. More studies are needed to determine whether a eubiotic gut microbiota and IP have a direct relationship with DR.

**Keywords:** Diabetic retinopathy, inflammation, intestinal permeability, gut microbiota, zonulin

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## Introduction

Diabetic retinopathy (DR) is a multifactorial disease, and the pathogenetic mechanisms behind it are still unclear in certain regards. The complex interaction of immunological, vascular, neuronal, and low-grade chronic inflammation (LGCI)-related pathways plays a role in the development and progression of DR.[1,2] LGCI, one of these variables, has lately garnered increased scientific interest. The pathogenic processes involved in LGCI are thought to be just as responsible for the development of DR as hypertension and hyperglycemia.[3,4]

Recent studies have shown that LGCI-related diseases such as obesity, inflammatory bowel syndrome, liver cirrhosis, depression, type 1 diabetes mellitus (T1DM), and type 2 diabetes mellitus (T2DM) are associated with intestinal barrier dysfunction, increased intestinal permeability (IP) (leaky gut), and gut microbiota dysbiosis (GMD).[5,6,7] The LGCI response has been attributed to an inflammatory immune reaction triggered by antigens bypassing the intestinal barrier as a result of GMD and increased IP.[8] Thus, increased IP and GMD leading to LGCI via gut-derived endotoxins (metabolic endotoxemia) is believed to be an essential component of DR pathophysiology.[9,10]

Human zonulin, the eukaryotic counterpart of the zonula occludens toxin discovered during vaccination studies against *Vibrio cholerae*, is a protein weighing 47 kDa and the only physiological mediator known to regulate IP. This is accomplished by reversibly opening the intestinal tight junctions (TJs).[11] Serum zonulin levels correlate linearly with IP and can be utilized as a biomarker for intestinal barrier function.[12] There is significant evidence that the gut microbiota (GM) influences the barrier function of the intestinal mucosa, which regulates the permeability of the gastrointestinal tract.[13] As a result, serum zonulin levels are also an important indicator of GMD.[8,13]

Studies conducted to date have shown that zonulin is also associated with a wide range of diseases characterized by chronic inflammation and oxidative stress.[14,15,16] The purpose of this study was to evaluate the association between DR and serum levels of the IP biomarker zonulin.

## Materials and Methods

This research was carried out in the Ophthalmology department of the Ordu University Training and Research Hospital between November 2019 and May 2020. Individuals diagnosed with T2DM who applied to the internal medicine clinic were referred to the ophthalmology clinic for an eye and vision examination, and eligible patients were included in the research. The control group was recruited from healthy individuals referred for routine eye examination following an assessment at the internal medicine clinic. The criteria for the diagnosis of T2DM are based on American Diabetes Association guidelines.[17] All participants underwent a complete ophthalmologic examination and assessment of retinopathy status by fundus photography, fluorescein angiography, and optical coherence tomography. Guidelines on Diabetic Eye Care were used for DR diagnostic criteria and grading.[18] Non-proliferative DR (NPDR) was identified based on the presence of any characteristic lesion such as microaneurysms, hemorrhages, hard exudates, cotton wool spots, intraretinal microvascular abnormalities, and venous beading. PDR was identified based on the presence of neovascularization, vitreous hemorrhage, or tractional retinal detachment.

The exclusion criteria for the study were as follows: T1DM, pregnancy, acute or chronic infectious disease, severe hypertension, heart failure, liver or kidney disease, cancer, body mass index (BMI) percentile over 95%, and other long-term complications of diabetes. All study procedures were carried out following the Helsinki Declaration. The study was approved by the Ordu University Faculty of Medicine Ethics Committee (decision no: 2019-160) and informed written consent was obtained from all participants.

Eighty-nine participants with T2DM (32 men, 57 women) and 32 age-, gender-, and BMI-matched adult healthy controls without pre-existing ocular disease (11 men, 21 women; group 1) were included in the study. Participants with T2DM were divided into three groups according to clinical ocular examination findings: 33 in the non-DR group (group 2), 28 in the NPDR group (group 3), and 28 in the PDR group (group 4). Clinical and demographic information was recorded and BMI was calculated for each participant.

Blood samples were taken from all subjects after an overnight fast. After blood samples were centrifuged at 3,000 rpm for 10 minutes at 4 °C, the serum was separated and stored at -80 °C until the time of testing. Glycated hemoglobin (HbA1c), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and creatinine levels in the studied samples were recorded. Zonulin levels were measured using a human zonulin enzyme-linked immunosorbent assay (ELISA) kit (Sunred®, China, 201-12-5578). All samples were tested by the same researcher blinded to the clinical trial.

### Statistical Analysis

The IBM SPSS for Windows (v 23.0, IBM Corp, Armonk, NY, USA) package program was used for statistical analysis. Power analysis for parametric and non-parametric tests was performed using G*power software (d=0.5, $\alpha$=0.05, power=80%). Therefore, the study was conducted with a sufficient sample size. The Shapiro-Wilk test was used to evaluate the distribution of data. Numerical variables were expressed as mean and standard deviation and categorical variables as numbers. One-Way analysis of variance (ANOVA) and Kruskal-Wallis variance analysis were used for multi-group comparison of continuous variables. Tukey's test was used to make comparisons between groups in post-hoc analysis. Logistic regression analysis was used to calculate predictors of DR. Receiver operating characteristic (ROC) analysis was used to determine the sensitivity and specificity of zonulin for DR.

## Results

The mean age of the participants was 62.10±10.0 years in group 1, 60.24±8.72 years in group 2, 62.77±5.88 years

in group 3, and 64.42±9.51 years in group 4. There was no difference between the four groups in terms of the age (p=0.236), gender (p=0.952), and BMI (p=0.134) of the participants. There were also no significant differences in TG, TC, HDL-c, LDL-c, and creatinine between the groups. There was no statistically significant difference in metformin usage or HbA1c levels among the T2DM groups (groups 2, 3, and 4). The demographic and clinical features of the participants are given in **Table 1.**

The groups differed significantly in terms of T2DM duration and serum zonulin levels. The mean duration of T2DM was 7.58±2.75 years in group 2, 9.94±4.77 years in group 3, and 11.85±8.87 years in group 4. The mean zonulin level was 4.65±0.85 ng/mL in group 1, 5.33±1.58 ng/mL in group 2, 5.26±0.67 ng/mL in group 3, and 11.85±6.87 ng/mL in group 4 (**Table 2**). In post-hoc analysis, disease duration was significantly longer in group 4 than groups 2 and 3 but did not differ significantly between groups 2 and 3. Serum zonulin level was significantly higher in group 4 than in the other groups and in groups 2 and 3 compared to the control group. Again, there was no significant variation in zonulin levels between groups 2 and 3. **Figure 1** shows the distribution of serum zonulin concentrations in the groups.

In multivariate logistic regression analysis, zonulin was found to be an independent predictor of DR (odds ratio: 1.781, 95% confidence interval: 1.122-2.829, p=0.014) (**Table 3**).

ROC curve analysis for zonulin is shown in **Figure 2**. The area below the ROC curve of zonulin for distinguishing DR was 0.657 (p=0.003). The optimal cut-off value was 10.27, with a sensitivity of 65.2% and a specificity of 58.3%.

## Discussion

Zonulin, the precursor to haptoglobin (HP) 2, leads to epidermal growth factor receptor activation through PAR2. Similar to the modulating effect of epidermal growth factor in actin cells in the intestines, zonulin has a regulatory effect on the TJs, enabling them to open and thereby increasing IP.[19] Circulating zonulin is considered a biomarker of IP and GM.[8] Increased IP and GMD have recently been shown to play a role in the development of many diseases, including T2DM.[5,6,7] Jayashree et al.[16] showed that increased serum lipopolysaccharide (LPS) and zonulin levels were associated with T2DM. LPSs produced by intestinal bacteria pass into the systemic circulation as a result of increased IP. LPS is thought to be a significant contributor to LGCI.[16] LGCI and concomitant blood cell aggregation in the tissue are believed to contribute to neurodegeneration and microvascular damage in DR, although the exact processes are not fully known.[20] According to Simonsen et al.,[21] serum LPS levels were associated with severe DR in T1DM patients, and bacterial endotoxemia was a risk factor for DR. Studies in humans and animals conducted in recent years have suggested that changes in the GM and increased IP may pose a new risk factor for the development of DR.[9,10]

The main findings of this study were that there was a strong association between PDR and high serum zonulin levels, and T2DM patients with and without NPDR had considerably higher serum zonulin levels than the healthy control group. There was also a significant difference between the non-DR T2DM group (group 2) and the control group (group 1), consistent with previous studies.[22] Zonulin levels were also shown to be linked to NPDR, but there was a similar relationship in the non-DR T2DM group. High zonulin levels were found to be an independent predictor of DR using multivariate logistic regression analysis in this study. However, its sensitivity and specificity are relatively low according to our ROC curve analysis, and further studies are required to understand whether it will prove beneficial for the prediction of DR in routine use. These findings suggest that T2DM patients with high zonulin levels are strong candidates for progression to the proliferative stage of DR. Also, it points out that increased IP

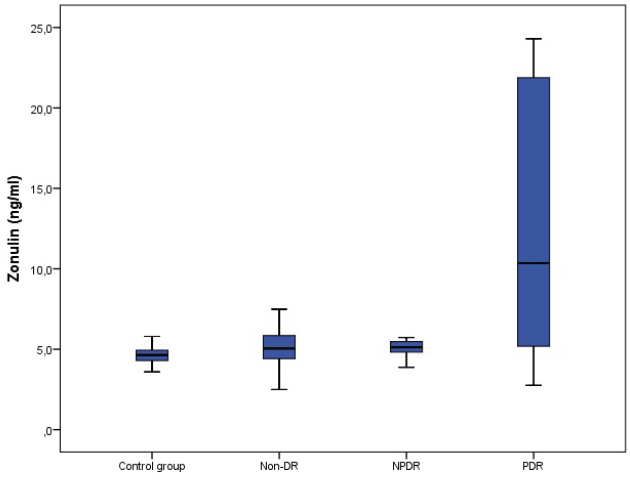


**Figure 1.** The distribution of serum zonulin concentrations in the study groups
*Non-DR: No diabetic retinopathy, NPDR: Non-proliferative diabetic retinopathy, PDR: Proliferative diabetic retinopathy*

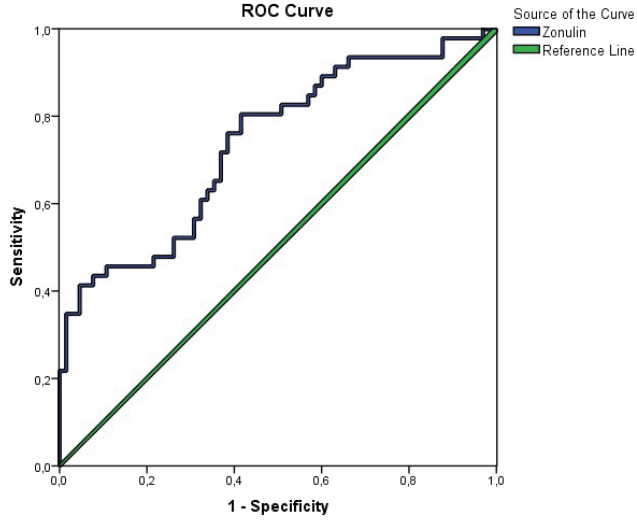


**Figure 2.** Receiver operating characteristic (ROC) curve analysis of zonulin as a predictor of diabetic retinopathy

**Table 1. Clinical features and demographic data of the participants**

| | Control group n=32 | Non-DR group n=33 | NPDR group n=28 | PDR group n=28 | p value |
|---|---|---|---|---|---|
| Age (years) | 62±10.0 | 60.2±8.7 | 62.7±5.8 | 64.4±9.5 | 0.236 |
| Gender (female/male) | 21/11 | 20/13 | 17/11 | 17/11 | 0.952 |
| BMI (kg/m$^2$) | 29.8±6.3 | 32.7±4.1 | 31.8±4.3 | 30.6±3.4 | 0.134 |
| Duration of DM (years) | - | 7.58±2.75[a] | 9.94±4.77[a] | 11.85±8.87[b] | **0.002*** |
| Use of metformin (using/not using) | - | 9/24 | 2/16 | 7/21 | 0.401 |
| HbA1c [mmol/mol (%)] | - | 59.0±30.1 (7.58±2.75) | 64.0±13.6 (8.02±1.24) | 66.0±18.1 (8.22±1.66) | 0.189 |
| Triglycerides (mg/dL) | 144.8±79.3 | 167.7±67.9 | 159.8±61.5 | 168.7±86.0 | 0.313 |
| HDL-c (mg/dL) | 51.5±15.4 | 45.7±10.7 | 44.5±14.4 | 47.6±12.5 | 0.284 |
| LDL-c (mg/dL) | 129.5±32.8 | 122.7±39.6 | 110.5±27.3 | 127.0±35.4 | 0.425 |
| Creatinine (mg/dL) | 0.82±0.19 | 0.79±0.14 | 0.96±0.42 | 0.91±0.34 | 0.365 |

Data presented as mean ± standard deviation or number. BMI: Body mass index, DM: Diabetes mellitus, HDL-c: High-density lipoprotein cholesterol, LDL-c: Low-density lipoprotein cholesterol, Non-DR: No diabetic retinopathy, NPDR: Non-proliferative diabetic retinopathy, PDR: Proliferative diabetic retinopathy
*Matching letters indicate no significant difference, different letters indicate a significant difference between the groups

**Table 2. Comparison of serum zonulin levels between groups**

| | Control group n=32 | Non-DR group n=33 | NPDR group n=28 | PDR group n=28 | p value |
|---|---|---|---|---|---|
| Zonulin (ng/mL) | 4.65±0.85 | 5.33±1.58[a] | 5.26±0.67[a] | 11.85±8.87[b] | **0.001*** |

Non-DR: No diabetic retinopathy, NPDR: Non-proliferative diabetic retinopathy, PDR: Proliferative diabetic retinopathy
*Significant difference between the groups in one-way ANOVA. [a]Zonulin level is significantly higher in groups 2 and 3 than the control group in post-hoc analysis ($p<0.05$). There is no difference between groups 2 and 3 ($p>0.05$). [b]Zonulin level is significantly higher in group 4 than the other three groups in post-hoc analysis ($p<0.05$)

**Table 3. Logistic regression analysis to identify possible factors associated with diabetic retinopathy**

| | Odds ratio | 95% CI | p |
|---|---|---|---|
| Age (years) | 0.924 | 0.887-1.016 | 0.813 |
| BMI (kg/m$^2$) | 0.948 | 0.911-1.128 | 0.902 |
| HDL-c (mg/dL) | 0.968 | 0.933-1.005 | 0.092 |
| Zonulin (ng/mL) | 1.781 | 1.122-2.829 | **0.014** |

BMI: Body mass index, HDL-c: High-density lipoprotein cholesterol, CI: Confidence interval

should be considered in the pathogenesis of DR. We identified only one study in the literature comparable to ours. Sirin et al.[23] examined the relationship between zonulin and DR but observed no association, contrary to our results. Additionally, the time interval after DM diagnosis did not differ significantly between non-DR and NPDR but was significant for the development of PDR in the current study. This indicates that longer diabetes duration is related to high zonulin levels. Prolonged diabetes is likely to have effects on GM that are difficult to reverse.

According to prior research, several variables, including age, obesity, and dyslipidemia, have an influence on serum zonulin levels.[24,25] The average BMI of all groups in our study shows that the participants were overweight and obese. However, as there was no significant difference in BMI between groups, including the control group, this factor is not expected to affect the study results.

Rahman et al.[26] reported that there was a direct association between the blood-brain barrier (BBB) and zonulin via the TJs, like in the intestine. Subsequently, zonulin has been associated with a wide range of central nervous system diseases and psychiatric diseases. As in the BBB, TJs are also found in the basic skeleton of the retina-blood barrier (RBB), and there is evidence that vascular endothelial growth factor-mediated pathological mechanisms contribute to the development of DR by disrupting the structure of TJs.[27,28] Accordingly, it is also possible that circulating zonulin has a direct effect on the retina in the transition from NPDR to PDR, acting on TJs in the RBB structure. Nevertheless, this association needs to be supported with more evidence based on controlled studies.

Although zonulin is still regarded as the best measure of IP in most recent studies,[29,30] some researchers have reported contradictory information concerning zonulin. When Scheffler et al.[31] conducted their study on obese individuals, they divided them into groups based on the genes encoding HP. Naturally, they did not expect to detect zonulin in individuals carrying the homozygous HP1 allele. After detecting zonulin in these patients' blood, the researchers deepened their studies. They eventually determined that commercial ELISA kits did not measure zonulin, but rather a structurally identical protein family. However, Scheffler et al.[31] found zonulin to be upregulated in diabetic and obese patients, as in previous studies. Other studies also warn researchers that what is measured with commercial zonulin ELISA kits is not zonulin.[32,33] There are also claims that zonulin does not accurately reflect IP.[34] Power et al.[35] investigated the correlation between lactulose/mannitol ratio and zonulin in first-degree relatives of Crohn's patients known to have increased IP. As a result, they found that there was no correlation between lactulose/mannitol ratio, which is a good indicator of IP, and zonulin. Consequently, it is a fact that zonulin is a target molecule in LGCI-related diseases, although there are conflicting findings.

### Study Limitations

There are some limitations to this study. One is that it was conducted with a small group. In addition, because there is a wide range of drug groups and diseases that can cause GMD and increased IP, it was not possible to include all of them in the exclusion criteria. Moreover, the individuals' eating habits were not documented in this study. Dietary records in may offer new insights in future GMD and IP studies. Also, the participants were limited to a single ethnic group, and the results may not be valid for other ethnic groups. HP genotyping would have been useful in our study because of the controversy surrounding zonulin kits, but we were unable to do this. Although the use of zonulin as an indicator of IP is controversial, for many researchers zonulin is still the most important indicator of IP.

## Conclusion

The results of this study showed that participants with T2DM had high levels of serum zonulin. Moreover, serum zonulin levels were much higher in participants with PDR than in participants with NPDR and those without DR. Accordingly, IP regulation and GM remodeling may be one of the main goals in the treatment of DR. In addition, zonulin or a structurally similar protein family, as claimed in some studies, may serve as a direct target molecule in the treatment of DR. More studies are needed to determine whether there is a direct association between a eubiotic GM and IP or between zonulin and DR.

### Ethics

**Ethics Committee Approval:** Ordu University Clinical Research Ethics Committee (decision no: 2019-160).

**Informed Consent:** Written consent for the study was obtained from all participants.

### Authorship Contributions

Surgical and Medical Practices: B.E., S.Y., Y.K., Concept: B.E., Design: B.E., Y.K., Data Collection or Processing: T.R.K., S.Y., Analysis or Interpretation: B.E., Y.K., Literature Search: B.E., Writing: B.E., Y.K.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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