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Predictive and Prognostic Value of Plasma Zonulin for Gestational Diabetes Mellitus in Women at 24–28 Weeks of Gestation

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ABSTRACT

Objective We aimed to examine the predictive and prognostic value of plasma zonulin for gestational diabetes mellitus (GDM) in women at 24–28 weeks of gestation.

Methods This retrospective study was carried out with pregnant women with GDM (n = 98) and normal glucose tolerance (control group) (n = 132). GDM was diagnosed according to American Diabetes Association (ADA) criteria with a one-step 75-g OGTT at 24–28 gestational weeks. Their serum zonulin levels measured during one-step 75-g OGTT and perinatal outcomes were compared, and the cut-off value of plasma zonulin for the prediction of GDM was calculated with receiver operating characteristic curve analysis.

Results Plasma zonulin level was significantly higher in women with GDM compared to controls (28.8 ± 24.9 and 7.3 ± 11.3 ng/mL, respectively). According to logistic regression analysis, plasma zonulin levels and GDM were statistically significant. The plasma zonulin cut-off value was > 45.2 ng/mL. The rate of cesarean section, the rate of meconium in the amniotic fluid, and the need for admission to the neonatal intensive care unit significantly differed between women with GDM and controls.

Conclusion In pregnant women with GDM, plasma zonulin increases, and with the cut-off level of > 45.2 ng/mL, it can predict GDM with values of sensitivity and specificity levels significantly higher in pregnant women with GDM, suggesting that it can be used as a tool for its screening and early diagnosis.

Introduction

Gestational diabetes mellitus (GDM) is a glucose intolerance disorder first diagnosed during pregnancy. It is one of the most common complications in pregnancy [1]. It is associated with increased fetal and maternal morbidity if it is not diagnosed early and ultimately treated. In addition, the mother and baby risk developing type 2 DM after birth [2]. Although many guidelines show different screening tests and risk factors, they agree that GDM is a public health problem due to the economic and medical problems caused by its complications [3]. According to the International Diabetes Federation (IDF) 2019 data, although the prevalence of GDM shows regional differences, one out of every six live births is exposed to hy-

perglycemia during pregnancy. It is predicted that 84 % of this group has GDM [4]. Different meta-analyses have indicated different risk factors for GDM. However, common risk factors are advanced maternal age, obesity, GDM history in a previous pregnancy, family history of DM, and ethnic origin [5]. In addition, the history of a macrosomic baby, excessive weight gain in the current pregnancy, polyhydramnios, glycosuria, polycystic ovary syndrome (PCOS), and sedentary lifestyle in childbearing age are among the criteria evaluated among the risk factors [6]. Possible mechanisms in GDM pathophysiology are insulin resistance and pancreatic B cell dysfunction. Indeed, each risk factor is directly or indirectly associated with impaired B cell function or insulin sensitivity [7].

There is ample evidence that the gut microbiome plays a role in the onset of many diseases. One of these diseases is thought to be GDM [8]. Passive material flow through the intestinal epithelial barrier is the dominant mode of action of the paracellular circulation. In this circulation, tight junction (TJ) regulation is the basic control step. Zonulin is a 47 kDa protein that manages intestinal permeability (IP) by controlling TJ functions in the intestine [9]. Human zonulin was discovered by demonstrating that anti-ZOT antibodies produced against Zonula occludens toxin, an enterotoxin secreted by Vibrio cholera, bind to human intestinal tissues [10]. Then, zonulin, which was found to be the precursor of haptoglobin 2 (pre-HP2), directly binds to epidermal growth factor (EGF) receptors or binds to proteinase-activating receptor 2 (PAR2), and so increases IP by indirectly causing TJ dysfunction through transactivation of EGF [9]. It has been shown that enterocytes are secreted primarily from the liver, heart, brain, lung, kidney, skin, and adipose tissue. The release is triggered by exposure to gliadin or the specific microbiome [11]. It has been proven that zonulin is overexpressed in autoimmune diseases where TJ dysfunction is evident, such as type 1 DM and celiac disease [12]. Zonulin is associated with an increasing number of autoimmune diseases. The main pathophysiology in the relationship of zonulin with diseases such as multiple sclerosis and rheumatoid arthritis is that more antigen is allowed to pass through the intestine due to the increase in IP caused by TJ dysfunction. Thus, the increased antigen load causes an immune response that can target any organ or tissue in genetically predisposed individuals [13].

Although animal and human studies show the relationship of zonulin with DM, evidence-based studies on its relationship with GDM has not yet been clarified. The objective of this study was to examine the predictive and prognostic value of plasma zonulin for GDM in women at 24–28 weeks of gestation.

Materials and Methods

Patient selection and antenatal management

The study was conducted retrospectively in the obstetrics unit of the Samsun Training and Research Hospital. The study group consisted of pregnant patients who applied to the obstetrics unit of our hospital and followed up until their delivery. The study was approved by the Institutional Ethical Committee (ECASM-AIMS-2021–126) and informed written consent was obtained from all women. All the patient details, antenatal and postnatal evaluation, surgical details, and neurological outcomes were obtained from the hospital information system.

By examining the files of 230 pregnant women selected by computer randomization among the patients who were followed up, 98 patients diagnosed with GDM according to the results of a 75-g oral glucose tolerance test (OGTT) performed between 24–28 weeks were determined as the GDM group and 132 patients with normal results were determined as the control group. Multiple pregnancies; pregnant women with a history of allergic disease, dermatological disease, epilepsy, pregestational diabetes mellitus and GDM in their previous pregnancy; a diagnosis of chronic hypertension, pregnancy-related hypertension and preeclampsia; a history of thromboembolic disease, thyroid dysfunction, chronic liver, biliary and kidney disease, or hyperemesis gravidarum; a diagnosis of rheumatological disease; or women to be followed for fetal chromosomal aneuploidy and/or malformations, and smoking were determined as exclusion criteria and not included in the study.

The ethics committee approved our institution's study with the application number 2021/97 and the date of February 25, 2021, and all patients were informed about the study, and their consent was obtained. Our study was designed following the Declaration of Helsinki. The diagnosis of GDM was made with an OGTT performed between 24 and 28 weeks of gestation and following the American Diabetes Association (ADA) guidelines [14]. Accordingly, a one-step 75-g OGTT was used for subjects who were at 24–28 weeks of pregnancy and had no history of GDM. All patients were warned to avoid excessive physical activity one day before the blood sample was taken for biochemical analysis. Fasting plasma glucose level was measured after a fasting period of at least 8 hours and one hour later after a 75-g glucose administration. Plasma glucose was measured spectrophotometrically using Abbott Aeroset 2.0 (Abbot Diagnostics, Abbott Park, IL, USA). If one of the following criteria was positive, a diagnosis of GDM was made: fasting blood plasma glucose above 92 mg/dL and a first-hour fasting plasma glucose above 180 mg/dL. Weight and body mass index (BMI) values of study groups were recorded during the single-step 75-g OGTT.

Pregnant women diagnosed with GDM and immediately referred to a dietitian were given a balanced diet of 40 kilocalories per kilogram for a BMI below 22, 24 kilocalories per kilogram for a BMI of 22–29, and 15 kilocalories per kilogram for a BMI of 30 and above. At the same time, an informative interview was held for exercise, and they were advised to create daily exercise programs. All pregnant women diagnosed with GDM were provided with a glucometer device, and they were asked to check fasting capillary blood glucose (FCG) three times a day before meals, and seven times a day, including postprandial blood glucose (PPG) in the 1st hour and before going to bed. After two weeks of follow-up, those with FCG above 95 mg/dl and those with PPG above 140 mg/dl in the first hour despite diet and exercise were hospitalized to diagnose unregulated GDM and included in the insulin therapy program for regulation of blood glucose. Other patients continued to diet and exercise and were monitored at subsequent visits.

Insulin doses were calculated as 0.8 IU per kilogram between 24–28 weeks of gestation, 0.9 IU per kilogram between 28–36

weeks of gestation, and 1 IU per kilogram between 36–40 weeks of gestation. Thirty percent of the total dose was planned as neutral protamine hagedorn insulin, the rest as crystallized insulin. The patients were again monitored by measuring capillary blood glucose seven times daily. During the hospitalization period, embolism prophylaxis was performed by administering subcutaneous 0.4 IU low-molecular-weight heparin (LMWH) to pregnant women weighing less than 90 kilograms and administering a single dose of subcutaneous 0.6 IU LMWH to pregnant women over 90 kilograms once a day. Those whose blood glucose was regulated were discharged after injection training. The average length of stay in the hospital was one week. After the regulation, the patients were followed up every two weeks until the 32nd week of pregnancy and weekly after the 32nd week of pregnancy. A consultation was obtained from the endocrinology clinic for blood glucose regulation when necessary.

Pregnant women with normal blood glucose levels and no problems detected in their obstetric follow-ups were followed up to the 40th week of pregnancy and for spontaneous vaginal delivery. Pregnant women carrying a fetus with an expected birth weight exceeding 4000 g and pregnant women with presentation anomalies were delivered by elective cesarean section at 39 weeks of gestation. Conditions that develop acutely and make normal delivery impossible, such as cephalopelvic disproportion in labor, and acute fetal bradycardia, were accepted as emergency cesarean section indications. All demographic data of the patients, weeks of birth, delivery methods, birth weights of babies, meconium-stained amniotic fluid, Apgar scores at 1 and 5 minutes, and intensive care needs were recorded.

Measurement of plasma zonulin levels

Fasting blood samples of all pregnant women taken on the day of one-step 75-g OGTT measurement at 24–28 weeks of gestation were processed in a centrifuge at 3000 rpm and stored at −80 °C until analysis.

Commercially available enzyme-linked immunosorbent assay kits (Cat No. 201–12–5578; Sun-Red Bio Company, Shanghai, China) were used to measure the concentrations of human zonulin in sera. This kit uses a double-antibody sandwich enzyme-linked immunosorbent assay to assay the level of human zonulin. The enzymatic reactions were quantified in an automatic microplate photometer. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to human zonulin. Then, standards or samples are added to appropriate microtiter plate wells with a biotin-conjugated antibody preparation for human zonulin. Next, streptavidin-horseradish peroxidase is added to each microplate well and incubated to form an immune complex. Only wells containing human zonulin, biotin-conjugated antibody, and enzyme-conjugated avidin will exhibit a color change after adding chromogen solutions. The addition of sulfuric acid solution terminates the enzyme-substrate reaction, and the color change is measured spectrophotometrically at a wavelength of 450 nm. Then, the concentration of human zonulin in samples is determined by comparing the optic density of the samples to the standard curve. The human zonulin levels were expressed as ng/mL. The sensitivity of this assay is 0.223 ng/mL, and the assay range is between 0.25 ng/mL and 70 ng/mL. All assays were conducted according to the manufacturer's instructions. The samples that showed shown higher concentrations were diluted and measured in duplicate.

Statistical analysis

IBM SPSS v25 for Windows (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Descriptive statistics, frequency, and percentage were used to summarize the characteristics of the study population. The normality of continuous variables was evaluated using the Kolmogorov-Smirnov test, Shapiro-Wilk test, and Z score [−3, +3]. Mean and standard deviations were used for variables normally distributed; median value and range were used for continuous variables not normally distributed. The independent sample t-test and paired sample t-test were used for normally distributed variables; the Mann-Whitney U and Kruskal-Wallis tests were used for non-normally distributed variables; subsequently, pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons. Logistic regression analysis was performed to ascertain the effects of variables and receiver operating characteristics (ROC) curves to calculate the cut-off points. A p-value of less than 0.05 was considered statistically significant for all analyses. A priori power analysis was conducted using G-Power Version 3.1.9.7 to test the difference between two independent groups using a two-tailed test, a medium effect size (d = 0.50), and an alpha of 0.05. Results showed that a sample of 210 participants with two equal-sized groups of 105 was required to achieve a power of 0.95.

Results

Our study included 230 pregnant women with GDM and controls. ▶ **Table 1** displays the clinical and laboratory variables of the study groups. There was a statistical significance in median ages of the two groups but not with the patients ≥ 35 years old. Also, there was no significant difference between the two groups concerning gravida and gestational age at delivery. The median values of BMI, birth weight, fasting glucose, one-hour post-75 g glucose load glucose, and two-hour post-75 g glucose load glucose of the gestational diabetes group were significantly higher than those of the control group ($p < 0.001$). Also, the cesarean delivery, NICU rates, and meconium-stained amniotic fluid were significantly higher in the gestational diabetes group than in the control group (p:0.003, $p < 0.001$, p:0.028, respectively). While the number of neonates with a low Apgar score at 1 minute was higher in the GDM group (p:0.001), there was no statistically significant difference with a low Apgar score at 5 minutes (p:0.426).

The zonulin level was higher in GDM than in the control group at 24–28 weeks of pregnancy (28.84 ± 24.89 and 7.35 ± 11.3, respectively), with a statistically significant difference of $p < 0.001$ (95 % CI: −0.84 to −0.58) (▶ **Fig. 1**). Logistic regression was performed to ascertain the effect of 24–28 weeks of pregnancy zonulin level on the likelihood that patients had GDM. The logistic regression model was statistically significant; for every 1 ng/ml increment of zonulin, the risk of GDM increased by 8 % [OR 1.08 (95 % CI 1.05–1.1) ($p < 0.001$)]. The model explained 34.7 % (Nagelkerke R^2) of the variance in GDM and correctly classified 73.5 % of cases. The area under the ROC curve was 0.853 ($p < 0.001$, 95 % CI 0.804–0.902), an excellent level of discrimination. When the cut-off value

for plasma zonulin was 45.17, sensitivity was 25.5 %, specificity was 99.2 %, positive predictive value was 96.1 %, and negative predictive value was 64.2 %. Increasing zonulin level was associated with an increased likelihood of GDM (▶ **Fig. 2**). The mean zonulin level of all pregnant women with and without NICU needs at the 24–28 weeks of pregnancy were 42.2 ± 29.06 and 13.37 ± 17.71 (p<0.001, 95 % CI: –0.81 to –0.34) (▶ **Fig. 3**).

Discussion

In our study, pregnant women diagnosed with GDM and those without GDM were compared, and BMI, birth weights, cesarean section rates, and low Apgar scores at 1 minute were statistically significant in favor of GDM, as expected, in demographic data antepartum findings. In comparing the zonulin levels of patients with GDM and those without a diagnosis of GDM, the primary subject of our study, zonulin levels, were statistically significantly higher in patients with GDM. In addition, a significant curve was obtained in terms of screening with the ROC curve in our study, and when the cut-off value for plasma zonulin was taken as 45.17 ng/ml, it was found to be a statistically significant cut-off value. Above this value, the diagnosis of GDM was considered statistically significant. Considering the relationship between NICU need and zonulin, our secondary research parameter, neonatal intensive care, increases as the zonulin level increases.

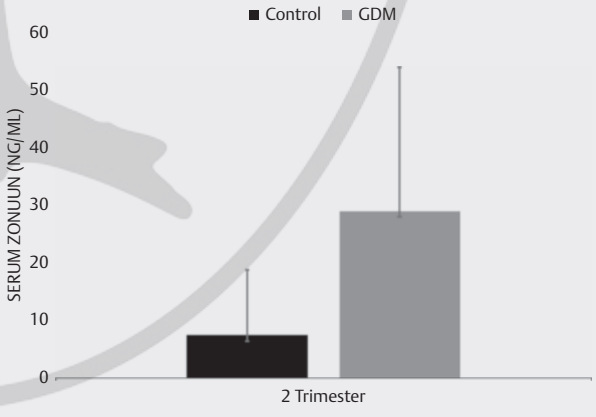
The intestinal epithelium provides the largest mucosal barrier between the internal host and the external environment. Epithelial tight junctions act as the main gateway through which macromolecules can cross the intestinal barrier. Recent studies show that immune system flare-ups caused by TJ dysfunction occur in various autoimmune diseases, including type 1 diabetes mellitus and celiac disease [15, 16]. With its intact TJ, healthy and mature intestinal mucosa serves as the main defense organ against foreign antigens, toxins, and macromolecules entering the host via the oral/enteral routes. TJ permeability increase was observed in the type 1 diabetes picture created in the studies performed in rats, and zonulin level was detected up to 35 times higher in this group than in non-diabetic rats. This situation led to the formation of pancreatic autoantibodies, which were also detected [17]. Although the pathway between activation of the gastrointestinal tract immune system and pancreatic beta-cell destruction has not been fully defined, current data suggest that antigens are paracellularly presented to gut-associated lymphoid tissue (GALT) [18]. It has been observed that gluten triggers the autoimmune mechanism in celiac disease by increasing zonulin release. In case of its restriction, the zonulin level decreases, and the active disease picture regresses [19].

Zonulin can also be used as a predictive biomarker of TJ and GDM in the pathogenesis of GDM for the same reasons. The possible mechanism involving zonulin in developing gestational diabetes mellitus is interference with insulin receptors' action and inflammation stimulation. Moreover, when the intestinal barrier is breached, infectious agents and dietary antigens against mucosal immune elements gain access to the body. As a result, the microbiota changes, leading to increased immune reactions and a possible increase in cytokine production by destroying the pancreas' beta cells. Therefore, an increase in zonulin levels is expected to increase pro-inflammatory adipocytokines such as leptin [8]. In ad-

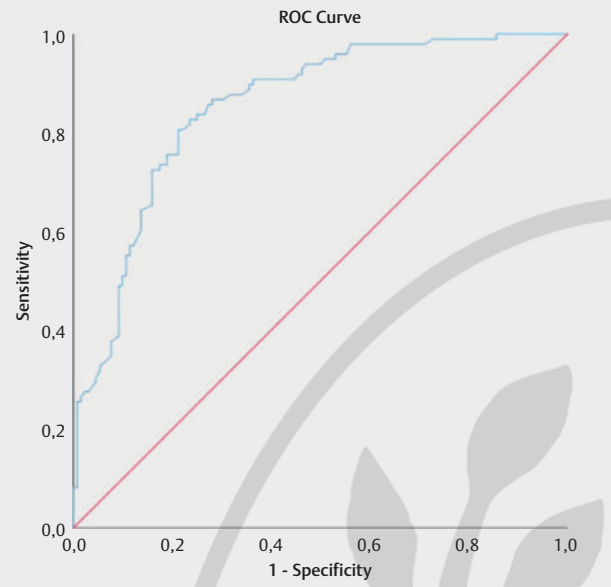
▶ **Table 1** Clinical and laboratory variables of study groups.

	Control Group (n:132)	GDM (n:98)	p-value
Age (y)	26 (19–44)	27 (19–42)	**0.046**
≥ 35 years	6 (4.5 %)	9 (9.2 %)	0.159
Gravida (n)	2 (1–6)	1 (1–5)	0.239
BMI (kg/m^2)	26 (22.1–42.1)	28.6 (22.1–38.1)	**<0.001**
Birth weight (g)	3485 (1400–4400)	3705 (1080–5800)	**<0.001**
Gestational age at delivery (w)	39 (29–41)	39 (27–40)	0.513
Type of delivery, n (%)			**0.003**
Vaginal delivery	111 (84.1 %)	66 (67.3 %)	
Cesarean delivery	21 (15.9 %)	32 (32.7 %)	
Admission to NICU, n (%)	6 (4.5 %)	19 (19.4 %)	**<0.001**
Meconium-stained amniotic fluid, n (%)	5 (3.8 %)	11 (11.2 %)	**0.028**
Low Apgar score at 1 min	1 (0.8 %)	10 (10.2 %)	**0.001**
Low Apgar score at 5 min	0 (0 %)	1 (1 %)	0.426
75 g OGTT (mg/dL)			**<0.001**
Fasting	89 (66–92)	98 (73–201)	
1 hour	155 (82–179)	204 (77–387)	
2 hours	142 (73–151)	166 (84–290)	

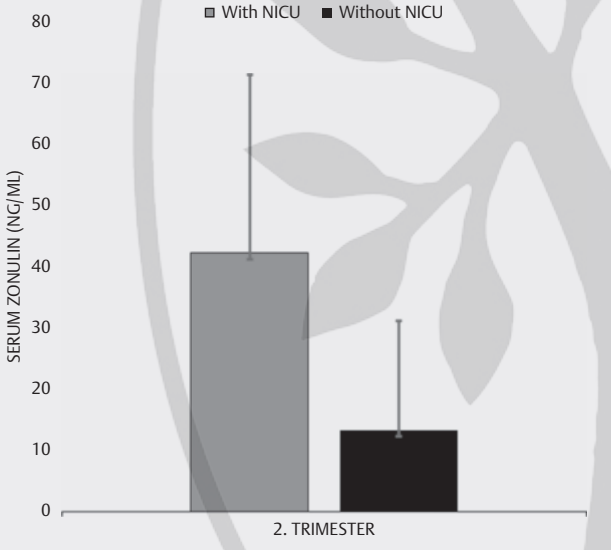
Data are presented as median with range and number (%) as appropriate. Statistical analysis was performed with Mann-Whitney or chi-square tests. Low Apgar score was defined as < 7. BMI, body mass index; NICU, neonatal intensive care unit; OGTT, oral glucose tolerance test.



▶ **Fig. 1** Comparison of mean plasma zonulin level of women with GDM and controls.



▶ **Fig. 2** ROC curve of second trimester zonulin level for the diagnosis of GDM.



▶ **Fig. 3** Comparison of all pregnant women's mean zonulin levels with/without NICU need.

dition, the abnormal gut microbiome formed during pregnancy may affect the metabolic state and thus the formation of GDM. Studies have shown that the gut microbiota composition in the third trimester is similar to that of non-pregnant adults with metabolic syndrome. This microbiota consists of many Actinobacteria, Proteobacteria, and a small amount of Faecalis components [20]. This microbiota is similar to the microbiota observed in GDM, gestational hypertension, and dyslipidemia [21, 22]. Conversely, supplementation of Lactobacillus rhamnosus HN001 altered the composition and function of the gut microbiota in favor of improved insulin sensitivity and inflammation in the host, reducing the propensity for GDM [23]. A clinical study in Finland showed that a probiotic supplement containing Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 taken from the first trimester of pregnancy reduced the prevalence of GDM [24].

Besides the studies on the relationship between GDM and zonulin, there is no proven screening and diagnosis method for GDM. In this case, there is still a debate about whether it causes anxiety and unnecessary testing in patients [1, 5]. There is no proven gold standard screening test worldwide, and diagnostic tests are difficult and sometimes confusing. It seems unlikely that a consensus will emerge on this issue soon [25], making it more essential to use a non-invasive biomarker for screening. Previously, several other serum markers identified in early pregnancy have been studied for use in predicting GDM with varying success. Measuring fasting plasma glucose provided 47 % sensitivity and 77 % specificity [26]. In addition, HbA1c predicted GDM with 19 % sensitivity and 95 % specificity, fructosamine 12 % sensitivity and 95 % specificity, and hs-CRP with 37 % sensitivity and 87 % specificity [27, 28]. A recent meta-analysis found that the most recently studied biomarker, adiponectin, predicted GDM in the first trimester with a sensitivity of 60 % and a specificity of 81 % [29].

In the first study conducted with a small group on the use of zonulin as a biomarker in the prediction of GDM, zonulin was found to be 53.4 (14.3) ng/mL in the group with GDM and 45.2 (9.7) ng/mL in the control group in the measurements made at mid-pregnancy. The cut-off value was 43.3 ng/mL, the positive predictive value of this value was 29 %, and the negative predictive value was 94 % [8]. In another zonulin study conducted on 85 patients with GDM, 20 ng/ml was accepted as the limit according to the ROC curve, and this value was reported to be found with 98.8 % sensitivity and 100 % specificity [30]. In the study by Mokkala et al., the mean age of the study group was 30.1 years, and the mean BMI was determined as 30.8 kg/m^2 [8]. Eighty-five pregnancies were studied in the GDM group with an average age of 31.3 years and a BMI of 29.4 kg/m^2 [30]. The mean BMI of our study group was 28.6 kg/m2, and the mean age was 27 years. In our study, zonulin was measured as 28.9 ± 24.9 ng/mL in the GDM group and 7.3 ± 11.3 ng/mL in the normal group, forming a prediction consistent with previous studies. In addition, when used as a biomarker for early diagnosis, the cut-off level was found to be 45.2 ng/mL with 25.5 % sensitivity and 99.2 % specificity. The positive predictive value of this value is 96.1 %, and the negative predictive value is 64.2 %. It was observed that these values were higher than the previously studied markers. An important aspect of our study is that our study group was the non-obese and young patient group compared to other groups since zonulin increases with increasing BMI and age [8, 31]. In another study conducted with zonulin levels taken from obese patients (BMI 34.2 ± 4.1) at the end of the first trimester, the cut-off value was 47.5 ng/ml with 80.95 % sensitivity and 80.41 % specificity. This study calculated that obese pregnant women with high zonulin levels have a 109 times higher risk of GDM than pregnant women with low normal BMI zonulin levels [31]. In our study, a high

level of zonulin was correlated with the increased need for neonatal intensive care. However, this statistical ratio is common since the patient group had GDM.

In conclusion, our study strengthened our opinion that plasma zonulin level, associated with many autoimmune diseases and diabetes, is also significantly higher in pregnant women with GDM and can be used for screening and early diagnosis.

Strengths and limitations

As the strengths of our study, it is important to provide new and fresh data to the literature since there are very few and a limited number of patient groups in the literature on this subject. In addition, the study examined younger and weaker patients as a patient group, contributing to more accurate values regarding the relationship of zonulin levels with GDM. The limitations were that due to its retrospective design, measuring and comparing zonulin levels in each trimester and comparing it with the values of the patient group in the postpartum period could provide healthier results. In addition, it would be more appropriate to work in a more extensive series. Controlling and standardizing patients' diets may provide results that shed light on the relationship between zonulin and the gut microbiome. Inhibition of the zonulin system could represent an innovative therapeutic tool for preventing and possibly treating all autoimmune diseases, including type 1 diabetes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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