Evaluation Of Intestinal Permeability With Plasma Zonulin Level In Patients With Vitiligo And Its Correlation With Inflammatory Cytokines And Oxidative Stress Markers

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Research Article

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Abstract

Background: It has been proven that there is an increase in intestinal permeability in some autoimmune diseases. In our study, we aimed to evaluate intestinal permeability in vitiligo disease by looking at zonulin levels. At the same time, we aimed to examine the correlation of inflammatory cytokines, oxidative stress markers and lipopolysaccharide (LPS) levels with zonulin.

Methods: Fourty one vitiligo patients and 41 healthy volunteers were included in our study. Venous blood samples were taken from the patient and control groups, and the levels of zonulin, LPS, Interleukin (IL)-6, Tumor necrosis factor (TNF)-α and oxidative stress markers were examined.

Results: The zonulin mean of the patient group was found to be statistically significantly higher than the control group ($p<0.05$). A positive and statistically significant correlation was found between zonulin level and IL-6, TNF-α and LPS levels ($p<0.05$). While the TNF-α and LPS levels of the patient group were found to be significantly higher than the control group, there was no significant difference in terms of IL-6 levels. The reduced glutathione, nitric oxide, total antioxidant status levels in the erythrocytes were lower and malondialdehyde levels were higher in the patient group compared to the control group.

Conclusion: We think that serum zonulin level increases and intestinal permeability increases in vitiligo disease.

Introduction

Vitiligo is a disease whose etiopathogenesis is unclear and presents with sharply depigmented or hypopigmented macules, patches clinically due to acquired loss of melanocytes [1]. Its prevalence ranges between 0.5% and 1% worldwide [2]. Its etiology has not been fully elucidated yet, and genetic and environmental factors play a role. Autoimmune, oxidative stress, biochemical, neural, and melanocyte separation theories have been suggested, with the most supported ones being autoimmune and oxidative stress theories [1, 3].

Recent studies have revealed that increased intestinal permeability has a substantial role in the development of autoimmune, allergic, and metabolic disorders [4]. The intestines play a key role in the digestion and absorption of nutrients, water, and electrolyte homeostasis, as well as preventing the entry of foreign environmental antigens into the body through the mucosal barrier [4]. Tight junctions (TJ) in the intestinal epithelium play a significant role in the paracellular transport in the intestinal epithelium [5]. Zonulin, a physiological modulator of tight junctions, plays a role in mucosal immunity. The problems that occur in the intestinal barrier, blood zonulin levels increase with the increased intestinal permeability [4]. Inappropriate production of increased amounts of zonulin results in a functional loss of barrier function, and subsequent passage of inappropriate and uncontrolled antigen across the mucosa initiates an innate immune response by submucosal immune elements. If this process continues, an adaptive immune response occurs and causes the production of proinflammatory cytokines, including Interferon gamma (IFN)-γ and Tumor necrosis factor (TNF)-α, which further opens the paracellular pathway for the

passage of antigens, creating a vicious circle. As a result of this process, immune tolerance is broken. Thus, the chronic inflammatory process begins [6].

The terms endotoxin and lipopolysaccharide (LPS) are frequently used interchangeably and form the primary component of the cell wall of gram-negative bacteria. Normally, LPS in the intestinal lumen does not pass through the healthy intestinal epithelium. In intestinal permeability disorders, the defective TJ barrier allows paracellular passage of LPS and other luminal antigens [7, 8, 9]. LPS induces disruption of the barrier formed by TJs between epithelial cells and epithelial cells through activating proinflammatory mediators [e.g., Interleukin (IL)-6, TNF-α] [10].

Oxidative stress is considered one of the potential pathogenic events in the loss of melanocytes in vitiligo. It is an argued view in vitiligo that an autoimmune response occurs due to faulty protein production caused by the effects of oxidative stress [11]. Various indicators are utilized to determine the oxidative stress state. The decreased reduced glutathione (GSH) is an indication of increased oxidative stress [12]. Moreover, Total Antioxidant Status (TAS) showing antioxidant capacity and Total Oxidant Status (TOS) showing oxidant capacity are significant markers. Malondialdehyde (MDA) is the end product of lipid peroxidation and is an important indicator of increased oxidative stress. Studies have revealed that MDA levels increase in patients with vitiligo [13, 14, 15]. Besides, elevated circulating levels of myeloperoxidase (MPO), primarily expressed on neutrophils and monocytes, are associated with inflammation and increased oxidative stress [16]. Nitric oxide (NO) is a free radical produced from L-arginine by nitric oxide synthase (NOS) enzymes in cells. It has been demonstrated that abnormal in vivo production of NO species reduces the binding of melanocytes to the extracellular matrix and may contribute to depigmentation [17]. One of the biochemical markers utilized to identify oxidative stress and inflammation is dynamic thiol/disulfide balance. The role that plasma thiols play in physiologically scavenging free radicals and acting as antioxidants by various mechanisms is widely recognized [18].

In our study, we aimed to investigate intestinal permeability in vitiligo disease by blood zonulin level and to shed light on the etiopathogenesis of the disease and novel treatment options that can be developed. Furthermore, we aimed to investigate the correlation of zonulin level with some proinflammatory cytokines, such as IL-6, TNF-α, LPS, a bacterial endotoxin, and some markers of oxidative stress, which are considered involved in the pathogenesis of vitiligo.

Material And Method

In the present study, 82 participants, 41 patients who applied to the polyclinic in the Department Dermatology between October 2020 and August 2020, and 41 age- and sex-matched healthy volunteers constituting the control group, were included. This study was conducted under the Declaration of Helsinki. To implement this study, ethical approval was obtained from the local ethics committee. All participants filled out the informed consent form. To be included in this study, the patient group had to be diagnosed with vitiligo, and the control group had to have no vitiligo at any time in their life and be healthy in other regards (in accordance with the exclusion criteria).

For both groups, being under the age of 18, presence of autoimmune disease affecting any organ or system, presence of chronic kidney or liver disease, diabetes mellitus, inflammatory or infectious bowel disease, hypertension, dyslipidemia, cardiovascular disease, malignancy, pregnancy, breastfeeding, chronic alcohol consumption, use of systemic immunosuppressive or immunomodulatory medications, and for the patient group, systemic treatment for vitiligo within the last three months were the exclusion criteria of this study. In addition, patients and healthy volunteers with active systemic disease and medication use were excluded from this study.

The detailed anamnesis of the volunteers evaluated in the patient group was taken, and the diagnosis of vitiligo was made clinically by physical examination and Wood's light examination. Patients' age, sex, disease duration, family history of vitiligo, treatments used, and Vitiligo Area Severity Index (VASI) scores were noted down.

Venous blood samples were taken from patients with vitiligo and the control group into a heparinized tube. The blood was centrifuged at 2000 rpm for 10 minutes and the plasma was separated (supernatant). Then, the lower phase containing the erythrocyte pack was rinsed 2–3 times with physiological saline to obtain red blood cell packs. Both plasma and red blood cell packs were placed in storage tubes and stored in a deep freezer at -80°C until studied.

Plasma zonulin, IL-6, LPS, and TNF-α levels of the patients were determined via the enzyme-linked immunosorbent assay (ELISA) technique. In accordance with the kit procedure, at the end of this study, the samples were read in an ELISA reader (BioTek Synergy H1, BioTek Instruments) and their absorbances were recorded. Using the data analysis program (Gen5, BioTek Instruments), the concentration values of the samples were determined from the generated standard curve with the help of the standards whose absorbance was read and whose values were known.

In addition, TAS, TOS, Oxidative Stress Index (OSI), MDA, GSH, myeloperoxidase, NO levels in erythrocytes of blood samples were studied. TAS, TOS, OSI, MDA, Total Thiol, Native Thiol, Disulfide levels were analyzed in plasma.

When analyzing the findings obtained in this study, the IBM SPSS Statistics 22 software was used for statistical analysis. Whether the parameters fit the normal distribution or not was analyzed via the Shapiro Wilks test. When analyzing the study data, in addition to descriptive statistical methods (mean, standard deviation, frequency), Student's t-test was used for comparisons of normally distributed parameters between two groups, and Mann-Whitney U test was used for comparisons of non-normally distributed parameters between two groups. Besides, Yates's correction for continuity was used to compare qualitative data. Pearson correlation analysis was used to analyze the correlations between parameters conforming to the normal distribution, and Spearman's rho correlation analysis was used to analyze the correlations between the parameters not conforming to the normal distribution. The most appropriate cut-off point was chosen based on the ROC curve analysis. The results were considered significant at $p < 0.05$.

Results

The cases were evaluated under two groups as “Patient” and “Control,” consisting of 41 individuals. The ages of the patients in the patient group ranged from 18 to 64, with 21 (51.2%) male and 20 (48.8%) female. The mean age was 37.73 ± 11.37 years. The ages of the cases in the control group ranged from 18 to 60, with 18 (43.9%) males and 23 (56.1%) females. The mean age is 32.98 ± 11.45 years (Table 1). No significant difference was found between the groups regarding mean age and distribution of sex ($p > 0.05$).

Table 1
Evaluation of groups in terms of age and gender

	Patient	**Control**	**P**
Age $_{Mean±SD}$	37.73 ± 11.37	32.98 ± 11.45	^{1}0.063
Gender $_{n\ (\%)}$			
Male	21 (%51.2)	18 (%43.9)	^{2}0.658
Female	20 (%48.8)	23 (556.1)	
[1]Student t test [2] Continuity (yates) fix			

The disease duration of the patients in the patient group ranged from one to 26 years, with a mean of 5.71 ± 5.12 years and with a median duration of five years. VASI scores ranged from 1 to 80, with a mean of 15.24 ± 19.97 and a median score of 8.4 cases (9.8%) had a family history.

The mean zonulin (plasma) of the patient group was significantly higher than the control group (p:0.005; $p < 0.05$). Moreover, the MDA (erythrocyte) mean, LPS (plasma) mean, and TNF-α (plasma) mean of the patient group were significantly higher than the control group ($p < 0.05$). The mean GSH (erythrocyte), mean NO (erythrocyte) and mean TAS (erythrocyte) values of the patient group were significantly lower than the control group ($p < 0.05$) (Table 2).

Table 2
Comparison of zonulin, LPS, IL-6, TNF-α and oxidative stress markers between groups

	Patient	Control	
	Mean ± SD	**Mean ± SD**	**P**
Erythrocyte GSH (µmol/g)*	16.37 ± 2.59	17.8 ± 2.92	0.021***
MDA (mmol/g)*	1.92 ± 0.33	1.64 ± 0.35	0.001***
NO (µmol/g)*	4.99 ± 0.76	5.44 ± 0.77	0.008***
MPO (U/g)*	8.93 ± 3.07	9.92 ± 3.07	0.147
Total Oxidant Status (µmol H2O2 Equiv/L)*	92.54 ± 4.86	94.66 ± 5.28	0.062
Total Antioxidant Status (mmol Trolox Equiv./L)*	2.57 ± 0.12	2.7 ± 0.14	0.001***
Oxidative Stress Index (mmol Trolox/mmol H2O2)*	36.14 ± 2.34	35.14 ± 2.41	0.061
IL-6 (ng/L)**	225.27 ± 67.97	207.45 ± 55.37	$^{+}$0.216
LPS (pg/mL)**	259.75 ± 65.24	231.68 ± 53.83	$^{+}$0.011***
TNF-α (µg/L)**	8.58 ± 1.75	7.78 ± 1.6	$^{+}$0.006***
Zonulin (ng/mL)**	2.02 ± 0.93	1.61 ± 0.47	$^{+}$0.005***
MDA (mmol/L)**	2.82 ± 0.28	2.86 ± 0.26	0.487
Total Oxidant Status (µmol H2O2 Equiv/L)**	5.36 ± 1.13	5.06 ± 0.88	0.184
Total Antioxidant Status (mmol Trolox Equiv./L)**	1.09 ± 0.16	1.04 ± 0.19	0.218
Oxidative Stress Index (mmol Trolox/mmol H2O2)**	4.94 ± 0.88	4.99 ± 1.14	0.829
Total Thiol (umol/L)**	320 ± 40.35	322.24 ± 49.24	0.822
Native Thiol (umol/L)**	180.02 ± 34.52	175.89 ± 32.89	0.580
Disulphide (umol/L)**	70.77 ± 10.29	71.28 ± 11.52	0.833

*Student t test^{+} Mann Whitney U Test *Erythrocyte **Plasma ***p < 0.05*

ROC curve was generated for zonulin in the diagnosis of vitiligo. The area under the curve was 0.678, with a standard error of 0.06. The area under the ROC curve was significantly higher than 0.5 (p:0.004; p < 0.05). The cut-off point for zonulin in the diagnosis of vitiligo was > 1.88. The sensitivity of this value was 60.9% and the specificity as 85.4% (Fig. 1).

When age, disease duration, VASI score, and zonulin correlation were assessed in the patient group, no significant correlation was found between age, disease duration, VASI score, and zonulin (p > 0.05) (Table

3). When the patient group was analyzed regarding genders, no significant difference was found between the zonulin levels of males and females ($p > 0.05$).

Table 3
Correlation of age, disease duration and VASI score with zonulin in the patient group

	Zonulin	
	R	**p**
$^{+}$Age	-0.076	0.497
$^{++}$ Disease duration	-0.051	0.754
$^{++}$VASI	-0.026	0.873
$^{+}$ Pearson correlation analysis $^{++}$ Spearman's rho correlation analysis		

When the correlations between zonulin and other study parameters were evaluated in the patient group (Table 4), a positive, strong (84.9%) and significant correlation was found between zonulin level and IL-6 (p:0.001; $p < 0.05$) (Fig. 2). Likewise, a positive, strong (77.7%) and significant correlation was found between zonulin level and LPS (p:0.001; $p < 0.05$) (Fig. 3). Moreover, a positive, strong (83.1%) and significant correlation were found between zonulin level and TNF-α (p:0.001; $p < 0.05$) (Fig. 4).

Table 4
Correlations of zonulin and study parameters in the patient group

	Zonulin (ng/mL) Plasma	
Patient	**R**	**P**
Erythrocyte GSH (µmol/g)*	0.069	0.666
MDA (mmol/g)*	-0.135	0.401
NO (µmol/g)*	0.023	0.885
MPO (U/g)*	0.053	0.741
Total Oxidant Status (µmol H2O2 Equiv/L)*	-0.295	0.061
Total Antioxidant Status (mmol Trolox Equiv./L)*	-0.051	0.752
Oxidative Stress Index (mmol Trolox/mmol H2O2)*	-0.214	0.178
+IL-6 (ng/L)**	0.849	0.001***
+LPS (pg/mL)**	0.777	0.001***
+TNF-a (µg/L)**	0.831	0.001***
MDA (mmol/L)**	-0.174	0.275
Total Oxidant Status (µmol H2O2 Equiv/L)**	-0.093	0.563
Total Antioxidant Status (mmol Trolox Equiv./L)**	-0.095	0.555
Oxidative Stress Index (mmol Trolox/mmol H2O2)**	-0.035	0.827
Total Thiol (umol/L)**	-0.045	0.779
Native Thiol (umol/L)**	0.072	0.653
Disulphide (umol/L)**	0.160	0.319

*Pearson correlation analysis + Spearman's rho correlation analysis *Erythrocyte **Plasma ***p < 0.05*

Discussion

Recently, many diseases have been shown to be associated with increased intestinal permeability. Zonulin, one of the markers showing intestinal permeability, has been associated with various diseases, such as autoimmune diseases [Type-1 diabetes mellitus (DM), celiac disease, inflammatory bowel diseases, ankylosing spondylitis, systemic lupus erythematosus], some tumors or cancers [brain, breast, ovary, pancreas, lung, cancer], neurodegenerative or psychiatric diseases (multiple sclerosis, schizophrenia) [19]. In the literature, there are papers on the relationship between intestinal permeability and zonulin in some dermatological diseases, such as psoriasis, atopic dermatitis, rosacea, dermatitis

herpetiformis [20, 21, 22, 23]. In a study by Richetta et al., consisting of 50 psoriasis patients and 32 healthy controls, serum zonulin and LPS concentrations were analyzed to evaluate intestinal permeability. They found that serum zonulin and LPS levels were significantly higher in the patient group than the control group. In addition, they also found a linear correlation between zonulin and LPS. Based on these data, they concluded that there was increased intestinal barrier permeability in psoriasis and that TJ-mediated passage of antigens across the intestinal barrier may play a role in triggering inflammation in the skin [24].

In our study, we compared plasma zonulin levels of patients with vitiligo and healthy volunteers to investigate whether there is an increased intestinal permeability due to elevated zonulin expression in vitiligo disease. Plasma zonulin level in the patient group (2.02 ± 0.93 ng/ml) was higher than the zonulin level in the control group (1.61 ± 0.47 ng/ml). This difference was significant (p:0.005; $p < 0.05$). This finding obtained in our study suggests that the elevation of zonulin, which is a remarkable biomarker indicating the increased intestinal permeability, may be responsible for the increased intestinal permeability in the etiopathogenesis of vitiligo disease.

There are also studies on LPS levels, which can both increase intestinal permeability and trigger systemic inflammation [25, 26, 27]. Jayashree et al. investigated zonulin, LPS, IL-6, and TNF-α levels in their study with 45 patients with Type-2 DM and the same number of healthy control groups. They found that these parameters were significantly higher in the patient group than the control group [26]. Loffredo et al., on the other hand, aimed to assess serum LPS and oxidative stress in patients with peripheral artery disease (PAH) and control group in their study consisting of 40 patients with PAH and 40 healthy controls. They found zonulin, LPS, and oxidative stress markers to be higher in the PAH group than the control group. They found a linear correlation between LPS and zonulin as well as between LPS and oxidative stress markers [27]. Similar to the results of this study, we also found a positive, strong (64.8%) and significant correlation between zonulin level and LPS in our study (p:0.001; $p < 0.05$). Given these findings, we are of the opinion that LPS may play a role in the increased intestinal permeability in vitiligo and that the increased level of LPS in the circulation may also play a role in the formation of the immune response that causes the destruction of melanocytes in vitiligo disease. Besides, the high level of LPS indicates that there might be a possible intestinal dysbiosis in patients with vitiligo.

Studies have demonstrated that there is an imbalance between pro- and anti-inflammatory cytokines in the skin and serum of patients with vitiligo. Singh et al., in their study with 80 patients with vitiligo and healthy controls, investigated the levels of IL-6, IL-2, TNF-α, and IFNγ in the serum of patients and healthy groups to investigate the role of cytokines in the pathogenesis of vitiligo. The mean serum IL-6 and IL-2 levels in the patient group were significantly higher than the normal controls, whereas the mean serum IFNγ level in patients with vitiligo was significantly lower than the control group, and they did not detect a significant difference in serum TNF-α levels between patients with vitiligo and healthy controls [28]. Yang et al., in their study with patients with vitiligo and healthy volunteers, found that the expression levels of IL-6, IL-17, and TNF-α in the blood of the volunteers in the healthy control group were significantly lower than in the vitiligo group, and the differences were significant [29]. In our study, we investigated IL-6 and

TNF-α levels in the patient and control groups. While we did not detect a significant difference between the groups regarding IL-6 levels, we found that the TNF-α level of the patient group was significantly higher than the control group (p:0.006; $p < 0.05$). We found a positive and significant correlation between zonulin level and IL-6 and TNF-α levels. Similar to the previous studies [29, 30, 31], we found the TNF-α level to be higher in patients with vitiligo. We consider that the positive correlation between zonulin level and IL-6 and TNF-α is an indicator that antigen translocation that occurs with increased intestinal permeability in vitiligo disease triggers the immune response. The improvement in vitiligo disease with TNF-α inhibitors also supports our study. However, varying outcomes have been obtained in the treatment with TNF-α inhibitors in vitiligo disease. In this study [32], in which studies on the effectiveness of TNF-α inhibitors in vitiligo disease were reviewed, the researchers revealed that TNF-α inhibitors achieved successful outcomes in patients with active generalized vitiligo, but that worsening of vitiligo can be seen in those with autoimmune disease, and de novo vitiligo may develop in those who receive TNF-α inhibitors for other diseases. Despite successful results with TNF-α inhibitors, worsening of vitiligo or development of de novo vitiligo suggest that TNF-α may have other functions that are not yet known, in addition to its proinflammatory and melanogenesis inhibition effects.

There are studies investigating the relationship between oxidative stress markers and vitiligo. In a study conducted with sixty patients with vitiligo and 62 healthy controls, TAS, glutathione peroxidase activity (GPX activity) was compared in plasma. They found lower TAS and GPX activity levels in all patients with vitiligo than the control group [33]. Akoğlu et al. investigated TAS, TOS, and OSI in blood samples taken from patients with vitiligo and healthy controls in their study. Significantly lower TAS and higher TOS and OSI values were observed in patients with vitiligo than controls [34]. Yıldırım et al. reported that serum NO levels were higher in patients with vitiligo [35]. In another study, total thiol and native thiol levels were higher in patients with vitiligo than the control group [36].

In this study, we examined TAS, TOS, OSI, GSH, NO, MPO, MDA levels in erythrocytes and TAS, TOS, OSI, MDA, Total Thiol, Native Thiol, and Disulfide values in plasma to determine the oxidative stress level in the patient and healthy control group. We did not find a significant difference in the level of oxidative stress markers in the plasma of the patient and control groups. The GSH, NO, TAS levels were lower and MDA levels to be higher in the erythrocytes in the patient group than the control group, whereas we did not detect a significant difference in terms of MPO, TOS, and OSI levels. Based on these results, we consider that oxidative stress has a crucial role in the pathogenesis of vitiligo; compensation is achieved regarding oxidative stress in plasma in vitiligo, which is a chronic disease, whereas the oxidant-antioxidant balance in erythrocytes shifts in favor of oxidative stress and compensation is not achieved. Furthermore, in our study, we could not find a significant correlation between zonulin level and oxidative stress markers in the patient group. We think that these values should be studied at the tissue level to more clearly reveal whether oxidative stress has an impact on intestinal permeability since this balance has shifted in favor of oxidative stress in erythrocytes, albeit the balance in terms of oxidative stress has been achieved in the plasma.

The fact that patients with vitiligo were selected without considering segmental and nonsegmental subgroups in the patient group and the small sample size can be considered as limitations of our study.

In conclusion, our study is important as, to our knowledge, it is the first study in the literature to evaluate intestinal permeability in vitiligo disease. In our study, we revealed that the level of zonulin increased in patients with vitiligo; thus, intestinal permeability might be increased. Besides, the high level of LPS suggests that there might be a potential intestinal dysbiosis in patients with vitiligo.

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Figures

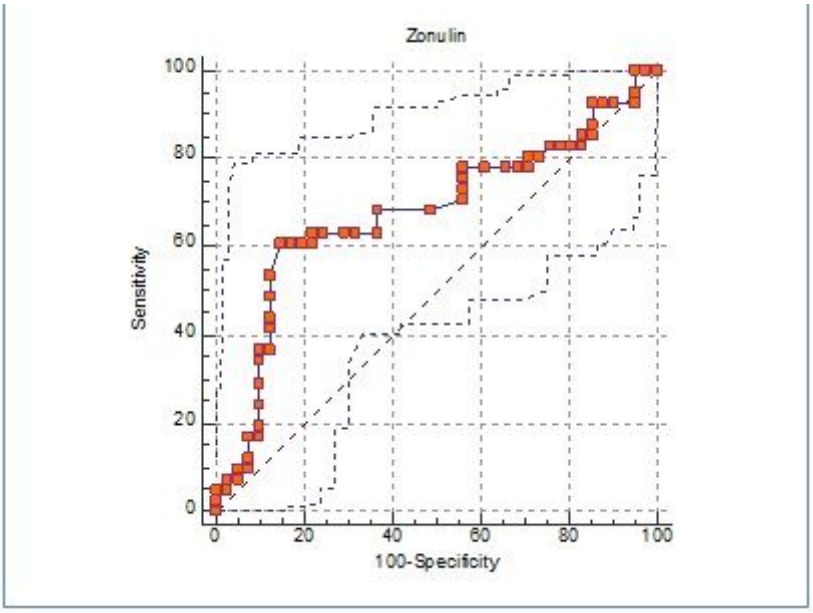

Figure 1

ROC curve for zonulin in the diagnosis of vitiligo

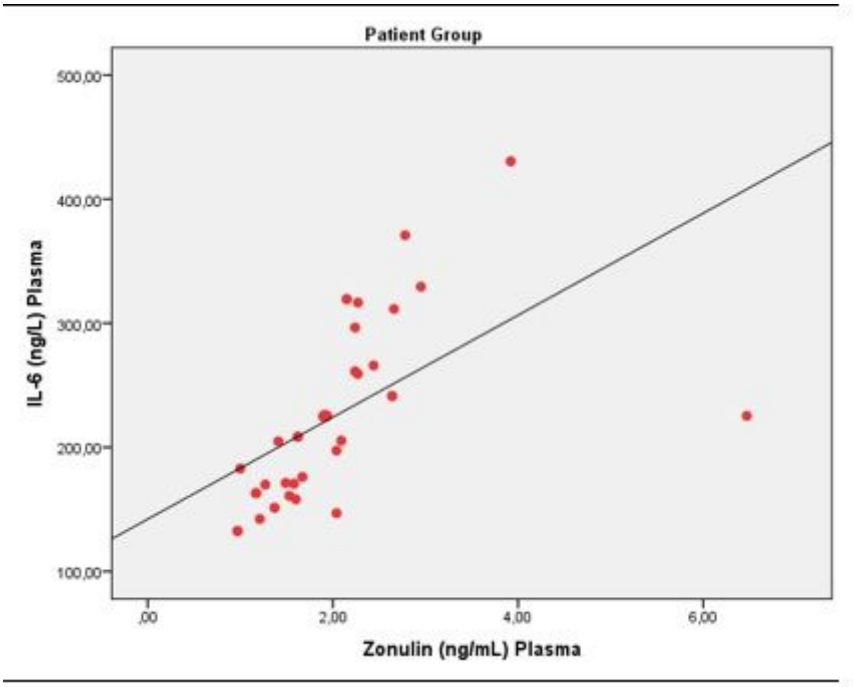

Figure 2

Zonulin and IL-6 correlation graph

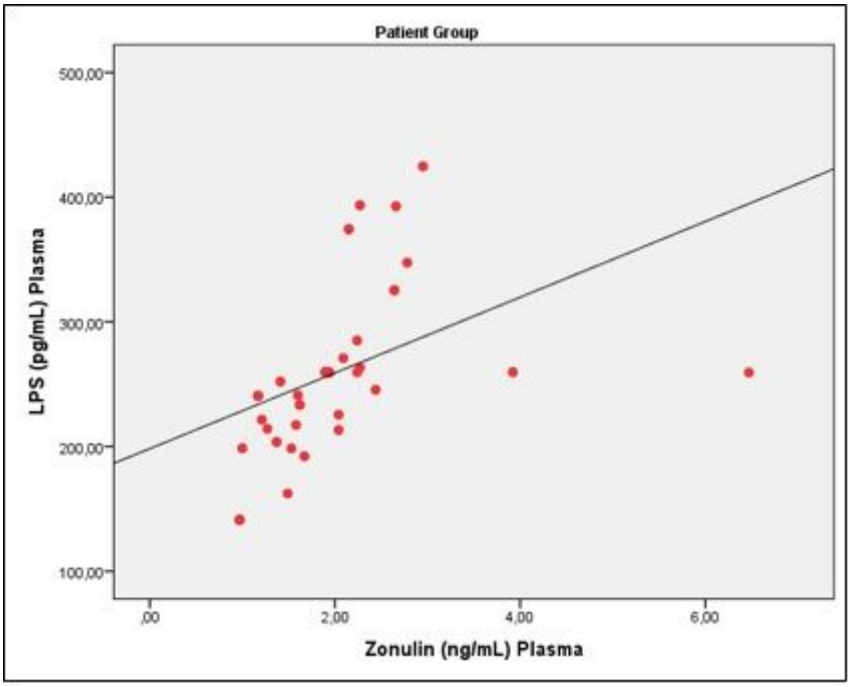

Figure 3

Zonulin and LPS correlation graph

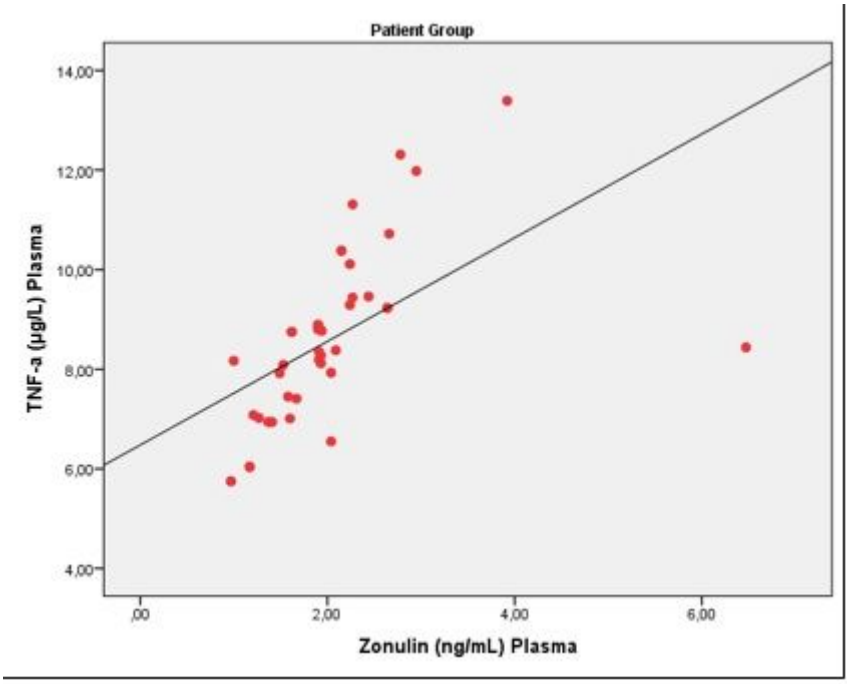

Figure 4

Zonulin and TNF-α correlation graph