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# Sex differences in zonulin in affective disorders and associations with current mood symptoms

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ABSTRACT

*Introduction:* The bidirectional connection between the brain and the gut within psychiatric entities has gained increasing scientific attention over the last years. As a regulator of intestinal permeability, zonulin acts as a key player on the interface of this interplay. Like several psychiatric disorders, intestinal permeability was associated with inflammation in previous findings.

*Methods:* In this study we explored differences in zonulin serum levels in currently depressed ($n = 55$) versus currently euthymic ($n = 37$) individuals with an affective disorder. Further, we explored sex differences and possible influences on zonulin and affective symptoms like medication, age, body mass index, and smoking status.

*Results:* Serum zonulin was significantly higher in females than in men independent from affective status ($z = -2.412$, $p = .016$). More specifically, females in the euthymic subgroup had higher zonulin levels than euthymic men ($z = -2.114$, $p = .035$). There was no difference in zonulin serum levels in individuals taking or not taking a specific psychopharmacotherapy. We found no correlation between zonulin serum levels and depression severity.

*Discussion:* Increased serum zonulin levels as a proxy for increased intestinal permeability in women may indicate a state of elevated susceptibility for depression-inducing stimuli.

## 1. Introduction

The bidirectional connection between brain and gut has developed into a field of high scientific interest over the last years. The gut is not only responsible for digestion as it possesses millions of neurons as well as substantial parts of the immune system and affects emotional and cognitive processes (Bonaz et al., 2018). Not only stress-related changes in bowel movement – and therefore alterations in gut microbiota composition – via the hypothalamus-pituitary-adrenal (HPA) axis, but also effects of the gut on central nervous signaling could be shown. The mechanisms by which these influences are exerted include vagal stimulation, release of inflammatory and anti-inflammatory compounds, and modification of intestinal permeability, and therefore, concentrations of circulating agents in the bloodstream (Bonaz et al., 2013). Inflammatory processes are known to be involved in the etiology of affective disorders as unipolar depression and bipolar disorder. The enormous socio-economical influence of these diseases is not only highlighted by annual treatment costs for unipolar depression exceeding 4,5 billion euros in Germany alone (Friemel et al., 2005) but is also represented by a shortening of life expectancy between 10 and 15 years for bipolar disorder and unipolar depression (Kessing et al., 2015, Laursen et al., 2016).

Within the gut, its microbial colonization, the so-called gut microbiota, has gained increasing attention in the last years. It interacts with and influences our body's immune system, digestion and nutrition regulation (Michielan and D'Inca, 2015). In addition, recent research suggests that microbiota were found to be associated with microglial function, behavior, affect, motivation and cognitive functions in animals as well as in individuals with and without psychiatric diseases (Ait-Belgnaoui et al., 2014, Alam et al., 2017, Frohlich et al., 2016, Hoban

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et al., 2017, Lowry et al., 2016, Painold et al., 2019).

These findings highlight the importance of the intestinal mucosal barrier as a regulating interface to this highly active and interacting (eco-)system. This barrier is formed by epithelial cells and a mucus layer (Madara, 1998). These cells are conjunct via tight junctions which prevent and control paracellular passing of agents. In contrast to the former assumption that tight junctions are impenetrable, they are now understood as a complex interacting system of proteins, which regulates intestinal permeability (Turner, 2009). Fasano et al. (Fasano et al., 2000) discovered pre-haptoglobin 2 as the first member of a family of structurally and functionally related endogenous proteins, which they named zonulin (Fasano, 2020), with the ability to open tight junctions and to consecutively increase intestinal permeability. Another member of this family is properdin (Scheffler et al., 2018). Zonulin is synthesized by the intestinal mucosa and exerts its loosening effect on tight junctions through internalization and subsequent actin polymerization (Fasano et al., 1991, Fasano, 2011). It is therefore a key element in paracellular permeability (see Fig. 1).

In line with the assumption that inflammatory processes increase intestinal permeability and are involved in the pathogenesis of depression, a recent study could show that psychological stress in healthy subjects led to an increase of paracellular permeability in the small intestine (Vanuytsel et al., 2014), allowing the suggestion that zonulin might be involved in the mechanism through which this effect is mediated. Proinflammatory cytokines like tumor-necrosis-factor α and interferon γ may cause an increase in intestinal permeability (Turner, 2009, Zufferey et al., 2009). This may lead to the perpetuation of a process in which agents and possible toxins pass the intestinal barrier unregulated, causing an inflammatory response which, in turn, causes the further disintegration of the intestinal barrier, resulting in a vicious circle (Fasano, 2011). Interestingly, alterations in intestinal permeability in type-1-diabetes were shown prior to the onset of autoimmune disease activity in humans as well as in an animal model (Carratu et al., 1999, De Magistris et al., 1996, Meddings et al., 1999). This led to the hypothesis that inflammatory activity might be rather a consequence of intestinal barrier deregulation than its cause.

These findings led to the consideration of potential triggers of zonulin activation within the gut. Fasano et al. identified not only gluten but also small intestinal bacteria exposure as potent stimuli for zonulin secretion (El Asmar et al., 2002, Fasano et al., 2000). The finding of dysbiosis in stool samples of coeliac disease patients by De Palma et al. (De Palma et al., 2010) further highlighted the role of dysbiosis and microbial interaction with the zonulin regulation system. Beside this, zonulin levels were found to be associated with age and body mass index (BMI) (Del Chierico et al., 2018, Qi et al., 2017).

Intestinal permeability is not only negatively influenced by microbial presence, though. Improvements in barrier integrity mediated by probiotic bacteria were shown both in human and animal models (Ulluwishewa et al., 2011). Pediatric Crohn's disease patients receiving a probiotic for six months showed significantly reduced intestinal permeability (Gupta et al., 2000). This effect may be due to an increase of tight junction protein expression, which was shown in human cell cultures treated with probiotics and probiotic metabolites (Anderson et al., 2010, Ewaschuk et al., 2008).

Moreover, several neuropsychiatric disorders, like autism spectrum disorder, were shown to be associated with disruption of intestinal permeability (de Magistris et al., 2010). In retrospect, indications for the involvement of intestinal barrier impairment in schizophrenia date back to the 1950s (Julio-Pieper et al., 2014). Further, zonulin was associated with butyrate-producing gut bacteria in a sample containing women with anorexia nervosa (Mörkl et al., 2018).

Zonulin activity may exert an influence on the central nervous system through two main mechanisms. The first is through regulation of the intestinal barrier alone, allowing metabolites and microbiota to access enteric neurons which in turn may lead to vagal afference stimulation. This stimulation happens through short chain fatty acids, metabolites produced by some microbiota, or indirectly through toll-like-receptor mediation (Bonaz et al., 2018). Consecutively, afferent vagal firing is suspected to alter central nervous neurotransmitter concentrations (Ressler and Mayberg, 2007), constituting the last link of the chain through which microbiota exert central nervous influences. The second mechanism consists of loosening of the intestinal barrier, allowing translocation of microbiota and potentially harmful agents into the systemic bloodstream. By additional alteration of the blood-brain-barrier, zonulin may be directly involved in the crossing of systemic agents into the central nervous system, allowing them to exert their influence on the brain after also passing the blood-brain barrier. Its potential role in the regulation of blood-brain-barrier permeability is depicted in Fig. 2.

Arguments for this consideration are the high similarity in molecular composition between tight junctions of the gut epithelial and the blood-brain-barrier (Daneman and Rescigno, 2009) as well as the identification of zonulin receptors within the human brain (Lu et al., 2000).

Despite the knowledge that disruption in intestinal permeability may be involved in the development and perpetuation of psychiatric disorders, there is a lack of knowledge in this field. There are, to our knowledge, no studies measuring zonulin as a discriminating factor in mood disorders as well as in dependence of acute mood symptoms. Furthermore, there is a lack of literature regarding potential interactions between zonulin and psychopharmacological treatment in psychiatric entities. Antipsychotics were associated with changes in the gut microbiome (Bahr et al., 2015). Further, anti-inflammatory properties could be shown for several antipsychotics (Haring et al., 2015) and antidepressants (Kenis and Maes, 2002, Kubera et al., 2001). Considering this,

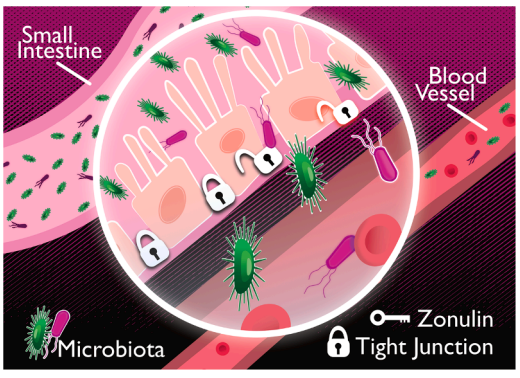


**Fig. 1.** Role of zonulin in the intestinal barrier.
Note. Secretion of zonulin opens tight junctions, allowing luminal bacteria and metabolites to enter the bloodstream (own depiction).

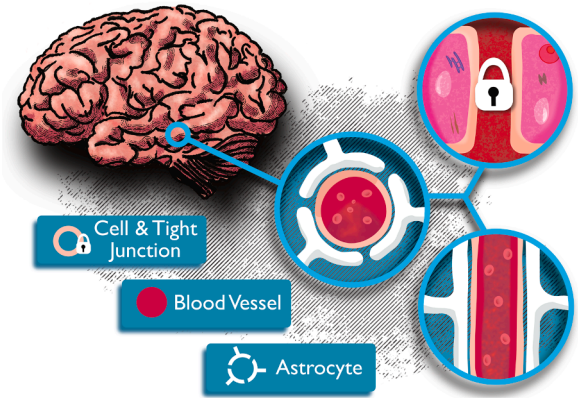


**Fig. 2.** Potential role of zonulin in blood-brain-barrier regulation.
Note. Zonulin may influence blood-brain-barrier integrity through loosening of tight junctions (own depiction).

possible interactions of these medications and the zonulin regulation system could be expected.

We hypothesized that current mood symptoms were significantly positively associated with zonulin in our sample. In the present proof of principle study, we therefore determined and compared the levels of zonulin concentration in fasting blood in individuals with affective disorders. We divided the group into currently euthymic and currently depressed individuals. We further explored differences in zonulin serum levels within patients currently taking antidepressants and antipsychotics and those without. Additionally, we divided the affective subgroups by sex. Furthermore, we associated zonulin levels with current affective symptoms. The main hypothesis was that euthymic individuals have lower zonulin levels compared to unipolar and bipolar depression.

## 2. Methods

Subjects were in- and out-clinic patients of the Department of Psychiatry and Psychotherapeutic Medicine of the Medical University Graz, Austria. Exclusion criteria were inability for informed consent, age under 18, severe addiction (alcohol, benzodiazepines, morphines), severe affective episode (Hamilton Depression Scale (Hamilton, 1960) (HAMD) > 30), severe brain organic disease (epilepsy, brain tumor), status post severe traumatic brain injury or brain surgery, known malignancy, intellectual disability, dementia (Mini Mental Status < 20), severe autoimmune disease or immunosuppression, chronic laxative abuse, acute infectious diarrhea, antibiotic treatment within the last month, and regular intake of probiotic or butyrate-containing dietary supplements.

All participants included took part in a study approved by the local ethics committee of the Medical University of Graz (EK-number EK- 28-413 ex 15/16; EK- 28-413 ex 15/16; EK 29-235 ex 16/17). All subjects gave written informed consent before being enrolled.

Afterwards, fasting blood was collected between 08:00 and 09.00 a.m. either on the same or the following day and immediately frozen and stored at -80°Celsius. Participants underwent a clinical interview to assess potential comorbidities and previous course of disease as well as anthropometric data. Furthermore, cognitive testing was performed, and subjects filled out questionnaires concerning current and previous medication, and other factors relevant for the disorder like lifestyle and hereditary background. Psychiatric diagnosis of bipolar disorder or unipolar depression was evaluated by a psychiatrist using the Mini International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998). Additionally, mood assessment via the HAMD was performed.

Zonulin concentration was measured with the IDK® Zonulin ELISA assay from Immundiagnostik AG (Bensheim, Germany). Briefly, samples were incubated with a biotinylated Zonulin family peptide (ZFP) tracer. In the second incubation step, peroxidase-labelled streptavidin binds to the biotinylated ZFP tracer. Following a washing step to remove unbound components the peroxidase substrate tetramethylbenzidine was added. Then the enzyme reaction was stopped by the addition of acid. The resulting chromogenic compound was measured photometrically at 450 nm. The intensity of the color was inversely proportional to the concentration of the measured analytes.

All statistical analysis was performed using SPSS 25, the level of significance was set to $p < .05$. As data was not parametric, group comparisons were done with the Mann-Whitney-U test, while correlations were performed using Spearman correlation.

## 3. Results

Study participants were divided into two different groups according to their current state of mood (euthymic or depressed) at the time of inclusion in the study. We used the HAMD score to distinguish between currently euthymic individuals (HAMD < 10) and those with currently mild to moderate depression (HAMD 10 - 30). Our sample consisted of 121 subjects, with 45 individuals considered euthymic and 76 considered depressed. Further group characteristics can be found in table 1.

Groups did not differ significantly regarding sex ($z = -1.480$, $p = .139$), current use of antipsychotic medication ($z = -1.201$, $p = .230$), current use of antidepressants ($z = -1.587$, $p = .112$), and smoking status ($z = -.257$, $p = .797$).

There was a significant difference between groups regarding HAMD ($z = -9.182$, $p < .001$), age ($z = -2.068$, $p = .039$), and BMI ($z = -2.944$, $p = .003$). Euthymic individuals displayed higher age and BMI but lower HAMD scores.

We found no significant difference in zonulin serum levels between the groups ($z = -.950$, $p = .342$).

Further, we found no difference in zonulin serum levels between subjects currently taking antipsychotic medication and those without, neither in the whole sample ($N = 92$, $z = -.325$, $p = .745$) nor in the euthymic ($N = 36$, $z = -.127$, $p = .900$) and depressed ($N = 56$, $z = -.459$, $p = .647$) subgroups.

There was no significant difference serum zonulin levels between individuals currently taking antidepressants and those without, neither in the whole sample ($N = 92$, $z = -.855$, $p = .393$), nor in the euthymic ($N = 36$, $z = -.420$, $p = .674$) and depressed ($N = 56$, $z = -.260$, $p = .795$) subgroups.

Independent of affective subgroup, we found zonulin to be significantly higher in women compared to men within the whole sample ($N = 93$, $z = -2.412$, $p = .016$). Women also displayed higher zonulin serum levels than men in the euthymic subgroup ($N = 37$, $z = -2.114$, $p = .035$). There was no significant difference in zonulin concentration between sexes in the depressed subgroup ($N = 56$, $z = -.833$, $p = .405$).

We found no significant correlation between HAMD and zonulin serum levels within our sample ($N = 93$, $p = .479$) or in the affective subgroups (euthymic, $N = 37$, $p = .324$; depressed, $N = 56$, $p = .377$).

## 4. Discussion

The aim of our study was to determine, whether serum zonulin, as a marker of intestinal permeability, was elevated in currently affective states of subjects with affective disorders compared to euthymia. Further, we aimed to create knowledge regarding the role of previously described influences on zonulin concentrations in the interaction between this biomarker and the course of affective disorders. Lastly, we explored the relation between psychopharmacological treatment and zonulin as well as sex differences for zonulin in our sample.

We found no significant difference in serum zonulin levels between euthymic and depressed individuals with bipolar disorder or unipolar depression. Furthermore, we found no significant difference in zonulin serum levels between subjects taking antipsychotic medication and those currently without. Additionally, there was no significant

**Table 1**
Group description.

| | Euthymic ($n = 37$) M (+/- SD) | Depressed ($n = 55$) M (+/- SD) |
|---|---|---|
| Age [Years] | 48.04 (13.67) | 42.43 (12.31) |
| BMI | 28.36 (6.57) | 27.81 (5.87) |
| HAMD | 3.84 (3.24) | 16.25 (4.90) |
| Zonulin [ng/ml] | 63.67 (45.24) | 51.62 (18.01) |
| Sex: female [%] | 37.80 | 25.00 |
| Smoking: yes [%] | 42.90 | 45.30 |
| Antipsychotics: yes [%] | 50.00 | 38.70 |
| Antidepressants: yes [%] | 70.50 | 82.90 |
| Bipolar Disorder: yes [%] | 81.10 | 14.30 |

Abbreviations: M: mean, SD: standard deviation, BMI: body mass index, HAMD: Hamilton Depression Scale.

correlation between depressive symptom severity and serum zonulin levels. These findings, at first, appeared inconclusive. When considered with pre-existing literature, however, our findings fit in with the current scientific discourse. There are contradictory and inconsistent reports on zonulin and its role within psychiatric entities. A recent study found zonulin to be decreased in depressed individuals who had recently attempted suicide, even though another marker for intestinal permeability, the intestinal fatty acid binding protein (FABP) was elevated in this subgroup. Moreover, zonulin correlated negatively with interleukin 6, a pro-inflammatory cytokine in this study (Ohlsson et al., 2019). Another recent study found both zonulin and FABP to be elevated in patients with depression and anxiety compared to healthy controls (Stevens et al., 2018). Mechanisms tightly intertwined with permeability regulation are immunity and inflammation. There already are several inflammatory diseases for which an association with intestinal permeability and zonulin could be shown, including inflammatory bowel disease, rheumatoid arthritis, coeliac disease, type-1-diabetes, multiple sclerosis, and schizophrenia (De Palma et al., 2010, Edwards, 2008, Fasano, 2008, Fasano, 2011, Mäkelä et al., 2006, Ochoa-Reparaz et al., 2009). Still, the heterogeneity and inconsistency in results concerning the role of zonulin in intestinal permeability changes due to inflammatory processes, highlights the need for further research to create a deeper understanding of its implication. It was hypothesized that a zonulin-mediated opening of the intestinal barrier may represent a defense mechanism to flush out microorganisms (El Asmar et al., 2002), but findings of bacterial translocation and endotoxins in the bloodstream provide arguments for the involvement of intestinal barrier decrease in the pathophysiology of several entities (Carratu et al., 1999, Fasano et al., 1991, Forsyth et al., 2011, Maes et al., 2008, Maes et al., 2012).

Our finding that serum zonulin was significantly higher in women with bipolar and unipolar depression during euthymia is potentially intriguing. Given the higher prevalence of unipolar depression among women (Möller et al., 2011), one might speculate as to what extent zonulin might be involved in a possible explanation for this sex difference. One might speculate, that increased permeability may pose a state of elevated susceptibility for depression-inducing stimuli. Regarding this hypothetical concept, zonulin's possible effect on the blood-brain-barrier is of special interest. The identification of zonulin receptors in human brain tissue (Lu et al., 2000) underpinned the hypothesis that zonulin may not only act as a regulator of intestinal permeability but could also influence passage through other epithelial barriers.

Disruption of blood-brain-barrier function was shown for brain edema, brain tumor, traumatic brain injury and multiple sclerosis (Lu et al., 2000). For the latter, an elevation of zonulin serum levels in patients with relapsing remitting and secondary progressive multiple sclerosis compared to controls could be shown (Waubant, 2006). Alterations of serum zonulin in women were described in several publications before (Demir et al., 2019, Mörkl et al., 2018), but our study is, to our best knowledge, the first report of a sex difference in affective disorder patients. For depression and anxiety, data regarding intestinal permeability is sparse to date. Maes et al. (2008) found elevated lipopolysaccharide antibodies in depressed patients, suggesting an inflammation-associated impairment of the intestinal barrier. They later could confirm their results, when they found lipopolysaccharide antibodies to be higher in depressed patients than in healthy controls, with chronically depressed subjects showing the highest serum levels (Maes et al., 2012). Research on psychological stress showed an association between stress and relapse in patients with Crohn's disease, which is characterized by deregulated intestinal permeability (Camara et al., 2009). Still, further research to assess zonulin's potential role in blood-brain-barrier regulation, especially in women, is needed.

## 5. Limitations

An important limitation to our results is the fact that the euthymic subgroup was significantly older and had a higher BMI than currently depressed individuals. Both parameters were shown to be associated with elevated zonulin levels in previous publications (Del Chierico et al., 2018, Qi et al., 2017). Within our sample, however, we found no significant association between age ($p = .704$), BMI ($p = .171$) and serum zonulin. Another limitation is the overrepresentation of men within our sample ($N = 88$). However, subgroups did not differ significantly regarding sex distribution ($z = -1.537$, $p = .124$). Further, our sample consisted of subjects suffering from either unipolar or bipolar depression. Still, bipolar and unipolar subjects did not differ regarding zonulin levels ($z = 1.016$, $p = 0.310$). To shed some light on the implications of zonulin for different psychiatric entities, further studies using matched samples to compare subjects suffering from bipolar disorder and major depression. The lack of healthy controls is an important weakness of our study. As this was a proof of principle study, further research, comparing our findings to a healthy cohort, is necessary. Lastly, the cross-sectional design of our study allows no interpretation of potential changes of zonulin in the course of disease.

## 6. Conclusion

Epithelial permeability poses an interesting potential factor in neuropsychiatric entities. Further research should aim to evaluate the therapeutic potential of epithelial barrier amelioration. The role of zonulin as a valid biomarker in affective disorders remains unclear due to partly inconclusive findings. Future research should focus on the comparison of zonulin with other promising biomarkers like FABP. To assess time-dependent dynamics in the interaction of those biomarkers and the course of disease, longitudinal study designs should be preferred.

## Conflict of Interest

This study was supported by funding from Institut Allergosan (Graz, Austria). No funding bias has been associated with this study. Funding parties did have no influence on study design, analysis, or interpretation of results.

### *Author Contributions*

Maget A was responsible for the writing of the manuscript, data collection and statistical analysis, Dalkner N and Lenger M were involved in statistical analysis and manuscript improvement, Hamm C, Bengesser SA, Fellendorf F, Platzer M, Queissner R, Birner A, Mörkl S, and Reininghaus EZ were responsible for data collection and manuscript improvement. Kohlhammer-Dohr A, Rieger A, Seidl M, Mendel L, Färber T, Wetzlmair L, Schwalsberger K, Amberger-Otti DV, Schöggl H, Lahousen T, Leitner-Afschar B, and Unterweger R were involved in data collection. Zelzer S, and Mangge H were responsible for chemical analysis and manuscript improvement.

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