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Zonulin as a Mediator of Psychological Stress and Periodontal Disease

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Abstract

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The prevalence of chronic inflammatory diseases is on the rise. Some have posited the permeability of tight junctions in gastrointestinal epithelium tissues as a potential mechanism for precipitating inflammatory processes throughout the body. Zonulin is the only known modulator of tight junction permeability and has been implicated in numerous chronic inflammatory processes (e.g., proinflammatory cytokine production) and diseases, more generally. The role of zonulin in oral inflammation, however, has yet to be explored. Periodontal disease is the most common oral inflammatory condition and primary perpetrator of tooth loss. Periodontal disease also is associated with a number of other health problems including heart disease and stroke and has previously been associated with psychological stress. The exact mechanisms between stress and inflammation, including in the periodontium, however, are less known. Thus, this dissertation was aimed at exploring whether moderate-to-severe periodontal disease was associated with higher levels of zonulin when compared to mild periodontal disease or otherwise healthy gingiva. Second, this study evaluated the mediating role of zonulin in helping to explain the relation between psychological stress and cytokines in participants with periodontal disease. Participants included 114 individuals (50.9% women, M_{age} = 36.1 years, SD = 14.0) from greater Morgantown, West Virginia who were part of a larger research project examining biobehavioral contributors to oral inflammation. Participants provided demographic and basic health information, a serum sample, underwent a periodontal examination, and filled out a battery of psychosocial questionnaires. Results indicated a potential relation between zonulin and periodontal disease status. In particular, zonulin was a significant predictor of number of teeth with probing depths of 5 millimeters or more in non-smoking individuals (β = 0.25, p = 0.029). Zonulin also was associated with indicators of more acute stages of periodontal inflammation, including the number of teeth with a probing depth of 5mm or more in a stepwise regression that included many common risk factors of periodontal disease. Additionally, dietary factors such as B-vitamins were associated with serum zonulin levels. Psychosocial variables, however, were not associated with zonulin or periodontal variables as anticipated. Other exploratory analyses were indicative of potential relations with depressive symptoms and zonulin, along with periodontal parameters. The results of this study provide preliminary implications for the role of zonulin and gastrointestinal distress in oral inflammation.

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Zonulin as a Mediator of Psychological Stress and Periodontal Disease

Over the last two decades, there have been substantial advances in discovering and understanding the distinguishing characteristics of the protein *zonulin* and its role in inflammatory processes. Zonulin is a regulator of intestinal epithelial tissue permeability, which is a primary barrier to the outside world. Much of this prior work has focused on the functionality of increased zonulin being associated with a *leaky gut* in the pathogenesis of numerous chronic inflammatory diseases (Sturgeon & Fasano, 2016). No known study, however, has investigated the role of zonulin in oral inflammatory diseases, such as periodontal disease. Additionally, growing evidence has been suggestive of a relation between psychological stress and increased risk for periodontal disease (Genco & Borgnakke, 2013; Warren et al., 2014), yet the mechanism(s) involved in this association also are unclear. This dissertation examined the role of zonulin in the observed association between stress and periodontal inflammatory parameters.

Rise of Inflammation

The hygiene hypothesis has been one theory offered as an explanation for the steady increase in diseases rooted in inflammation since the 1950s (Bach, 2002). The hygiene hypothesis suggests that contemporary chronic inflammatory diseases may result from over-sanitation, which reduces early-life exposure to pathogens that typically program the developing immune system, resulting in a phenotype that is susceptible to autoimmunity, chronic inflammation, and allergic reactions (Bach, 2002). A second landmark article proposed the microflora hypothesis, claiming that the Western diet and medicinal practices (e.g., high antibiotic use) in industrialized countries have disrupted the microbiome, leading to increased risk for inflammatory disease (Noverr & Huffnagle, 2005). Although researchers continue to

consider many possible contributing factors, evidence supports the import of inflammatory processes in diseases, including emotional maladies (Bach, 2002; Noverr & Huffnagle, 2005; Segerstrom & Miller, 2004). Particularly with the decrease in infectious disease, exploring mechanisms of microbiome dysbiosis—in the gut, the oral cavity, and other bodily systems—remains vital to the understanding of diseases of the future (Fasano & Flaherty, 2021).

Importance of Gut in Development and Maintenance of a Functional Immune System

The digestive system, and more specifically the gastrointestinal (GI) tract, is one area generally affected by inflammatory processes. As a system tasked with numerous immune-related functions, the gastrointestinal tract encapsulates both the gates and gatekeepers to the rest of vital organ systems (Sturgeon & Fasano, 2016). Indeed, the digestive system is foundational to keeping an organism "safe" from toxins, while also allowing for necessary nutrients to pass into the bloodstream and body. This ability to allow the "good" to enter and keep the "bad" out is a complex physiological process of epithelial function and thereby, tissue permeability, and is well studied in microbiology (Arrieta, Bistritz & Meddings, 2006; Bischoff et al., 2014, Fasano & Shea-Donohue, 2005). Tissue permeability (i.e., the phenomenon that certain types of tissue are pervious and able to be passed through by certain substances) is facilitated by a number of microbiological processes not within the scope of this dissertation (see Bischoff et al., 2014). Suffice it to say, however, that these processes include understanding "tight junction" complexes, or the specific channels in the tissue, and their role in epithelial tissue being more or less permeable to either nutrients or toxins entering an organism (Sturgeon & Fasano, 2016). Increased tissue permeability, particularly in the gastrointestinal tract, has oft been described as leaky gut (Sturgeon & Fasano, 2016), depicting the idea that given the proper circumstances, tissues may allow toxins into the system, necessitating a defensive immune response.

Zonulin and inflammation. Discovered in the early 2000s, zonulin is the only known regulator of tight junction complexes embedded in epithelial tissues and is partly responsible for increased permeability of said tissues (Fasano, 2011; Fasano & Nataro, 2004; Sturgeon & Fasano, 2016). Though most well studied in the gut, zonulin may also affect the permeability of the blood-brain barrier (Sturgeon & Fasano, 2016) and vascular tissues (Ciccia et al., 2017). Within the gut, elevated levels of zonulin increase the permeability of the intestinal epithelial barrier, allowing larger molecules to leave the gut lumen and infiltrate the body. Zonulin upregulation results in the disruption of the gut microbiome (i.e., dysbiosis) and ultimately an increased activation of innate (e.g., short-term, initial) and adaptive (e.g., long-term, more targeted) immune processes, including the systemic release of immune mediators (Sturgeon & Fasano, 2016). These mediators, including pro-inflammatory cytokines, antibodies, or in some cases, autoantibodies, travel in the peripheral circulation and can impact distal organs (e.g., the brain).

Zonulin is a 47 kilodalton size protein regarded as a member of the complement-associated proteins family and is produced intracellularly within epithelial cells (Fasano, 2011). It has been found to be synonymous to pre-haptoglobin 2, which is the precursor to haptoglobin molecules (produced via genes *HP1* and *HP2* from chromosome 16) and primarily affects the upper gastrointestinal tract, meaning the jejunum and the distal ileum (Sturgeon & Fasano, 2016). Additional and comprehensive microbiological characterizations of zonulin are described elsewhere (Fasano, 2011; Surgeon & Fasano, 2016). Notably, the health significance of zonulin and intestinal permeability is, to date, best characterized in the autoimmune disorder, celiac disease. Here, gliadin (a protein found in wheat, barley, and rye) binds with CXCR3 receptors at a tight junction complex, which triggers zonulin release and results in increased intestinal

permeability (Sturgeon & Fasano, 2016). In genetically predisposed individuals (with gene variants *HLADQ2/DQ8*), this process triggers the development of autoantibodies that attack the small villi of the upper small intestine leading to diarrhea, malabsorption, and oral symptoms (among other extra-intestinal symptoms) such as chronic recurrent aphthous ulcers/canker sores and tooth enamel deficiencies (Malahias et al., 2010). Although not as well-characterized, zonulin also is believed to play a pathogenic role in a number of other chronic inflammatory conditions, including diabetes and other metabolic diseases, and autoimmune diseases such as multiple sclerosis, colitis, polycystic ovary syndrome, asthma, coronary artery disease, and HIV, among others (Sturgeon & Fasano, 2016; Zhang et al., 2014). Figure 1 displays a theoretical pathway, or directed acyclic graph (DAG), of zonulin and how it may contribute to inflammation.

Accumulating data have demonstrated the key roles that the gut and gut distress play in health and disease by communicating with the immune system and involvement in a variety of metabolic processes (Burcelin, 2016). By modulating intestinal barrier permeability, zonulin likely plays a pathogenic role in a range of inflammatory diseases (Sturgeon & Fasano, 2016). Periodontal disease also is a chronic inflammatory condition and co-occurs with other diseases linked to zonulin. For example, associations have been demonstrated between diabetes, oral microbiome disruption, and periodontal disease (Xiao et al, 2017). Though there is theoretical justification, to date, the possible role of zonulin and gut-related distress or dysbiosis in the pathogenesis of oral inflammation remains unknown. Zonulin-related diseases (e.g., celiac disease), however, do involve oral symptomology (e.g., canker sores, enamel deficiencies; Malahias et al., 2010). Patients with other oral inflammatory diseases (e.g., Sjogren's syndrome) also experience comorbidities with zonulin-related diseases (e.g., celiac disease; Kim-Lee et al.,

2015). Research assessing the association between celiac disease and periodontal disease specifically is only in its infancy, with only one known study examining the relations. Results were inconclusive due to methodological limitations (Spinell et al., 2017). Moreoever, zonulin levels affect vascular tissues which are densely distributed within the oral cavity (Ciccia et al., 2017). Thus, it is important to explore the potential role of zonulin in oral diseases either through gut permeability or potentially through oral tissue permeability.

Mouth as the Beginning of the Digestive System

While seen as a predominately gastrointestinal phenomenon, the digestive system truly begins with the oral cavity. In light of the existence of junctional epithelial tissues in the periodontium, it could be that zonulin directly affects the epithelial tight junction structures in the mouth, leading to "oral tissue permeability" and resultant inflammatory processes. Periodontal diseases have previously been characterized as biologically caused and host mediated (Tonetti, Greenwell, & Kornman, 2018), though the mechanism(s) by which bacteria is allowed to infiltrate junctional epithelial tissues within the gums are unknown. The idea of oral tissue permeability could implicate zonulin as a gatekeeper (in certain genetically predisposed individuals) that allow bacteria such as *p. gingivalis* to trigger an immune response with collateral damage such as breakdown of tooth structure and bone loss. Additional work is needed, though, to explore this area.

Alternatively, given that the gastrointestinal tract is the primary interface with the outside world for humans (Fasano & Flaherty, 2021) and, as stated previously, zonulin binds more heavily in the upper intestinal tract, most work has assumed an inflammatory response starting in the gut and traveling elsewhere throughout the body (Fasano, 2011; Sturgeon & Fasano, 2016). This could be the case for the role of zonulin in periodontal disease as well, meaning periodontal

inflammation could be a result of dysfunctional gut-immune activity. That is, perhaps increased tissue permeability in the gut leads to an inflammatory response in certain predisposed individuals who go on to develop periodontal inflammation or have inflammation manifest within the oral cavity.

Preliminary work with self-reported gut distress and periodontal disease has indicated a potential relation between the two, such that gut distress was positively associated with self-reported periodontal inflammation (Wright et al., 2021a). Similarly, other evidence has demonstrated a robust connection between the "gum-gut axis" (Byrd & Gulati, 2021). Whether zonulin affects local tissues or the broader system, no known data exists to understand the relation between zonulin and periodontal inflammation. Given that the most reliable method for quantifying zonulin is via serum accessed via the blood (as opposed to a saliva swab), this study started with and focused on the systemic route of action beginning in the gastrointestinal tract.

Periodontal Disease

Nearly half (45.9%) of the United States adult population of those 30 years and older, or about 64 million Americans, suffer from periodontal disease (Eke et al., 2015). Periodontal disease is a chronic inflammatory disease typically measured via probing into the pocket between the tooth and adjacent gingival tissue (i.e., sulcus) of multiple sites per tooth, combined with any recession present to equate a measure of attachment loss, in addition to radiographic imaging (Tonetti et al., 2018). These various clinical parameters, such as probing depth and attachment loss, in addition to bleeding on probing, plaque scores, tooth mobility, among others, each provide a nuanced perspective to an otherwise etiologically and ontologically complex array of conditions (i.e., periodontal diseases). That is, probing depth and bleeding on probing typically are indicators of acute inflammation (Eke, Borgnakke, & Albandar, 2021). Parameters

such as attachment loss, however, provide a measure of how much the gingival tissues and supporting structure (i.e., alveolar bone) have resorbed and are signs of progression and later stages of periodontal disease (Eke et al., 2021; Tonetti et al., 2018). Moreover, bone loss, or the degradation of the alveolar bone, also is possible, further destabilizing the tooth structure, ultimately leading to tooth loss, and is seen via radiograph, which can be the final outcome or endstage of periodontitis (Eke et al., 2021; Tonetti et al., 2018).

Classification, diagnosis, and treatment of periodontal conditions. The official classification of periodontal diseases has been altered from time to time (see Highfield, 2009; Tonetti et al., 2018), with the latest classification shifting to a staging and grading system (Tonetti et al., 2018). One of the most commonly used classifications has been a combined effort between the Centers for Disease Control and the American Academy of Periodontology (CDC/AAP). The CDC/AAP classification was based on a combination of probing depth and attachment loss parameters. Other periodontal disease classification or measurement efforts have been tested, including an attempt to identify phenotypic profiles based on clinical parameters (Periodontal Profile and Tooth Profile Classes; Morelli et al., 2017) or through patient-report questionnaires (Blicher, Joshipura, & Eke, 2005; Chatzopoulos, Tsalikis, Konstantinidis, & Kotsakis, 2016; Wright, Heaton, & McNeil, 2021b), which have advantages related to being cost-effective, efficient to administer, and scalable to population groups (DeVellis, 2017).

In general, periodontal disease exists on a continuum, ranging from a mild form—gingivitis—to a more destructive form—aggressive periodontitis (National Institutes of Health, 2019). Gingivitis is inflammation of the gingiva, or gums, which normally are prevented via basic dental hygiene practices such as brushing, flossing, regular dental cleaning appointments (National Institutes of Health, 2019). Periodontitis is “inflammation around the tooth” (National

Institutes of Health, 2019, p. 3), and can progress to the surrounding tissues such as ligaments or connective bone, and is related to serious long-term implications.

Treatment for periodontal disease varies depending on severity of inflammation and attachment. Both non-surgical (e.g., scaling and root planing, medications) and surgical (e.g., flap procedures, grafts) treatments have demonstrated efficacy in ameliorating periodontal disease and reducing inflammation (National Institute of Dental and Craniofacial Research, 2017). Scaling and root planing involve scraping away any plaque, tartar, and calculus that has built up in the pockets and around the periodontal tissues, as well as smoothing any rough spots that may collect bacteria, thus alleviating the inflammation caused by the bacteria and resultant collateral damage (National Institute of Dental and Craniofacial Research, 2017). Flap procedures or grafts include alterations to gingival tissue such as cutting away tissue or displacing it for better access as a way to remove calculus or to actually replace lost soft tissue (National Institute of Dental and Craniofacial Research, 2017). Reversing bone loss as a result of periodontal disease, or growing new bone, still remains a challenge in treatment. For those who lose teeth due to periodontal disease, if the supporting structures allow, implant technologies are available.

Risk factors of periodontal disease. Inflammation from periodontitis has suspected systemic effects and has been linked to increased risk for a number of aging-related diseases (e.g., stroke, myocardial infarction; Eke et al., 2016; Gargano & Hughes, 2014) and to aging itself (Ebersole et al., 2016). Both biological (e.g., genetic) and behavioral/psychological factors (e.g., diet, stress, depression) have been associated with periodontal disease (Genco & Borgnakke, 2013; Kinane et al., 2017; Warren et al, 2014; Wright et al., 2017). Fewer studies, however, have examined the role of psychological variables in the pathogenesis of periodontal

disease. Nevertheless, stress has been associated with altering gut processes related to intestinal permeability (Lyte et al., 2010; Moloney et al., 2015) which, as mentioned previously, has been implicated in other inflammatory diseases. Tissue permeability may be one way to explicate the relation between stress and the pathogenesis of periodontal inflammation.

Psychological Stress and Periodontal Disease

Common risk factors such as the presence of *p. gingivalis* and smoking status predict some but not all of the variance in periodontal diseases. That is, periodontal disease is biologically caused, and host mediated (Tonetti et al., 2018). Given this mediation by host factors, psychological variables have been implicated as potential risk factors, though less is known as to how they may be associated. More broadly, the association of psychological stress with increased susceptibility to inflammatory processes is well known, including the complex relation between stress, cytokine expression, and different components of the immune system, including temporal variegations of stress such as acute versus chronic forms (Segerstrom & Miller, 2004; Slavich & Irwin, 2014).

In the case of periodontal disease, psychological stress is related to the development of pathological processes (Aleksejunienė et al., 2002; Dumitrescu, 2006; Genco et al., 1999; Hildebrand et al., 2000; Ng & Keung Leung, 2006; Warren et al., 2014). Although most of these studies report that psychosocial factors (including life stress) are positively associated with periodontal disease, more work needs to be done in this area (Genco & Borgnakke, 2013). It remains unclear whether psychological stress directly affects biological mechanisms involved in disease development or whether people who are stressed make life choices that affect their biology (e.g., smoking, poor diet) and lead to periodontal disease (Dumitrescu, 2006). In addition to the evidence suggesting the role of gut distress and periodontal inflammation mentioned

earlier, this work also has demonstrated the role of gut distress as a mediator in the relation between psychological stress and self-reported periodontal inflammation (Wright et al., 2021a). Thus, one way to better understand the role of psychological stress in periodontal disease is to know whether zonulin, a biomarker of gut distress, mediates basic stress and inflammatory indicators.

As already mentioned, prior work has shown how zonulin also plays a role in the pathogenesis of inflammation (Sturgeon & Fasano, 2016). Once zonulin breaks down the tight junction complex in epithelial tissues and the barrier function of the mucosa is compromised, toxins and antigens can more readily access the body. The role of zonulin in relation to psychological stress and ensuing pro-inflammatory cytokines is, however, largely unknown. Though some work has shown a relation between stress and intestinal permeability (Lyte et al., 2010), only one known preliminary rodent study has examined the role of stress and its effects on zonulin, in which it was found that exercised (i.e., "stress-induced") rats had higher levels of zonulin than sedentary rats (Holland et al., 2015).

Acute and chronic stress in relation to the immune system. Psychological stress is contextualized primarily in terms of temporality, meaning whether it is experienced acutely or chronically (Felicione, Blank, Wright, & McNeil, 2021). Differences in immune function are associated with various types of stress and the literature describing these functions—psychoneuroimmunology—is complex (Segerstrom & Miller, 2004). Broadly stated, immune responses in human organisms are described in terms of the innate immune response and the adaptive immune response. The innate immune response is a broader, less targeted reaction to potential threats within the body (Segerstrom & Miller, 2004). Immune cells react in such a way that is more immediate, but due to the less targeted approach, can be less effective at eliminating

the threat, though act as “first responders” at the scene until the more targeted and powerful immune response can be activated (Segerstrom & Miller, 2004). This more targeted system is the adaptive immune reaction, in which cells tailor their attack based on the intruder (Segerstrom & Miller, 2004).

Concerning the latter response, namely adaptive (i.e., the more long-term and targeted) immune processes, consistent evidence shows that chronic psychological stress relates to a down-regulation of immune function, leaving the body vulnerable to infection. A different pattern is observed, however, when innate (i.e., initial and more broad) immune processes are examined. Here, chronic stress is associated with increased susceptibility to prolonged inflammation that may play a role in the etiology of diseases (Slavich & Irwin, 2014), such as periodontal disease.

Acute and more chronic types of stress are associated with increased circulating levels of pro-inflammatory mediators, such as the cytokine, interleukin (IL)-6 (Segerstrom & Miller, 2004). Since IL-6 is a pro-inflammatory cytokine, it is typically associated with greater levels of systemic inflammation, which is associated with comorbidities of periodontal disease such as heart disease, diabetes, and osteoporosis (Segerstrom & Miller, 2004). IL-6 also is one of three proinflammatory cytokines with a substantial role in the breakdown of tissues that are a main symptom of periodontal disease (Nikolopoulos et al., 2008; Tawfig, 2016). Others have noted the importance of IL-6 in both acute and chronic inflammation, making it important to understand in the etiology and development of periodontal disease (Tawfig, 2016).

Statement of the Problem

The oral cavity is the beginning of the digestive system or process (e.g., to extract nutrients and protect the organism) and include complex interactions among the oral

microbiome, the act of chewing, salivary functions, and immune responses, among others. The human digestive tract is similar in function to skin, in that its purpose is to protect the host from harmful organisms, while still absorbing important nutrients (Fasano & Nataro, 2004; National Institute of Diabetes and Digestive and Kidney Diseases, 2013). Little work has been done, however, to examine the role of gastrointestinal functions and factors in influencing or being associated with the oral cavity and its processes. Specifically, no known study has examined zonulin, a regulator of gut permeability, in oral diseases or in the relation of behavioral factors, such as stress, to oral inflammatory processes.

Conducting this study aids in the declared vision of the National Institute of Dental and Craniofacial Research (NIDCR), namely helping to understand "a world where dental, oral and craniofacial health and disease are understood in the context of the whole body" (Somerman, 2017). It further builds interdisciplinary efforts to understanding oral disease, including how behavior interacts with basic underlying biological processes (National Institute of Dental and Craniofacial Research, 2014-2019).

Given the exploratory nature of this study, the *a priori* theoretical model hypothesizing the role of zonulin in periodontal disease was twofold. First, prior literature implicating zonulin in extraintestinal diseases suggest it starts in the gut and then inflammatory processes go elsewhere, as is depicted in Figure 1, with "chronic inflammation" alluding to inflammation anywhere in the body (Sturgeon & Fasano, 2016). This is one theoretical starting point for periodontal disease such that zonulin affects the gut and thereby settles in the oral cavity and is mediated by other risk factors such as stress, diabetes, body mass index (BMI), and other covariates. It also could be, however, that zonulin directly effects the oral cavity via junctional

epithelial tissues and is moderated via interacting covariates such as BMI, diabetes, or oral hygiene factors.

The purpose here was to explore the role of zonulin in periodontal disease more generally, and well as the role of zonulin as a mediator of psychological stress and inflammation in periodontal disease patients. In the preliminary study mentioned previously, self-reported visceral sensitivity (gut anxiety/distress) mediated the relation between self-reported psychological stress and a self-report measure of periodontal disease while accounting for age, sex, education, and income (Wright et al., 2021a). Given the results of this preliminary study and the literature examining zonulin, gut-related permeability, and inflammation (Fasano, 2011), two *a priori* hypotheses were evaluated in this dissertation, and several exploratory *post hoc* hypotheses also were considered.

First, given the association between zonulin and numerous chronic inflammatory diseases, it was hypothesized that patients with moderate to severe periodontal disease would have higher levels of zonulin. That is, patients with moderate-to-severe periodontal disease would have zonulin levels that were significantly higher than those with little to no disease.

Second, because other mechanism(s) potentially explain the association between psychological stress and cytokine levels are less understood, it was hypothesized that zonulin levels would mediate the relation between stress and IL-6 levels. That is, it was hypothesized that greater psychological stress would be associated with greater zonulin levels, which would in turn be associated with greater IL-6 levels. Likewise, it was anticipated that an indirect effect would exist such that the relation between stress and IL-6 levels would be partially explained by zonulin levels, all while controlling for covariates such as gender, age, smoking status, diabetes, and BMI.

Method

Participants and Procedure

As part of a larger cross-sectional project comprised of a number of biological and psychosocial variables, this dissertation included a cross-sectional sample of 114 adult community participants between the ages of 18 and 77 years old with varying levels of periodontal disease, or the absence of disease, recruited from greater Morgantown, West Virginia. The larger project was originally targeted to explore biobehavioral correlates of oral inflammation and included the collection of various biological (e.g., whole blood, serum, saliva, gingival crevicular fluid) and psychosocial (e.g., anxiety, depression, disgust, diet) data, not all of which are included in the aims of the dissertation. Participants were recruited through a convenience sampling method including distributing advertisements in dental clinics or non-profit organizations, word of mouth, and from periodontal patients in the faculty practice at the West Virginia University School of Dentistry. Eligible participants with periodontal disease or no disease were included, unless they were currently undergoing periodontal treatment. Those with past treatment were still included in the study. Utilizing the 2012 CDC/AAP case definitions (see Table 1), a target distribution was anticipated to be and was similar to that of other studies in other population groups (i.e., 15-20% severe, 30-40% moderate, 10% mild, and 15-20% little to no disease). Data collection and management was facilitated using REDCap (Harris et al., 2009). All procedures were conducted in accordance with West Virginia University's Institutional Review Board (Protocol #1902473418) requirements and procedures.

To narrow the scope and focus of the study on inflammation-related etiology of periodontal disease, individuals experiencing other oral inflammatory conditions (e.g., herpes, aphthous ulcers or canker sores, Steven-Johnson condition, oral lichen planus), pregnant women,

and individuals on anti-inflammatory medications, and participants with fewer than eight of their natural were excluded from participation. Likewise, as with NHANES (National Health and Nutrition Examination Survey, 2013) periodontal examinations, individuals with a history of a heart transplant, artificial heart valve, heart disease necessitating prophylactic antibiotics, or bacterial infection of the heart were excluded. Other disease information was collected as part of the demographic data to gather information about other chronic inflammatory diseases such as lupus or celiac disease. After expressing interest in the study, potential participants were screened via telephone or email to ensure eligibility. Upon arrival to the study site (i.e., West Virginia University Suncrest Towne Center Dental Clinic), participants completed the informed consent process, and provided demographic data including self-reported health information.

The first participant was enrolled on September 18, 2019. After the onset of the COVID-19 pandemic, after enrolling 51 participants, recruitment was paused from March 14, 2020 until September 16, 2020, when data collection resumed. Additional safety protocols were initiated including research staff wearing additional personal protective equipment, calling participants the night before their appointments to screen for COVID-19 symptoms, as well as re-asking the COVID-19 questions and taking a temperature before allowing the participant to enter the facility. Participants had to endorse they were not experiencing COVID-19 symptoms and to have a temperature of less than 100.4 degrees Fahrenheit in order to participate. After enrolling 34 participants and after another spike in new cases in the Morgantown, WV area, recruitment was again paused from December 5, 2020 until February 27, 2021, when the study was resumed, and another 29 participants were recruited. The last day of data collection was March 19, 2021.

Biospecimen collection. After consenting and the demographic and health history data were collected, a trained phlebotomist conducted a screening for the blood draw that included

questions about current medications, and illnesses/vaccinations/tattoos in the last two weeks. Next, the phlebotomist conducted a standard venipuncture blood draw of 8 milliliter (mL), including one 5mL vacutainer for serum (yellow cap with separating gel) and one 3mL vacutainer (lilac cap) for whole blood to be used for derivation of biological variables. After resting for approximately 15 minutes to allow for coagulation, the 5mL vacutainer was placed in centrifuge for 10-15 minutes and the resultant serum was aliquoted into three 500 microliter Eppendorf tubes. Eppendorf tubes were then immediately placed in a freezer (-80°C) and later transported to another such freezer on the Health Sciences Campus of West Virginia University for long-term storage. To determine the quantitative value (ng/mL) of zonulin present in the serum, the second aliquot of serum for each participant was used and an ELISA (Immundiagnostik) procedure was conducted. A reference range by Immundiagnostik (*n* = 40*)*, the company that produces the kit included a median value of 34 ng/ml (*SD* = 14 ng/ml). Moreover, because of its relevance in systemic inflammation and its key role in the destruction of periodontal tissues (Tawig, 2016), the quantitative value of interlukin-6 (IL-6) also was determined using ELISA (R & D Systems). In the protocol, R&D systems stated in a normative sample of 40 participants, 33 samples had IL-6 values of less than 3.13 pg/mL and 7 samples were between 3.13 and 12.5 pg/mL. Standard procedures and protocol for both ELISA kits, including running samples in duplicates, were followed by an outsourced laboratory (i.e., Dr. Jennifer Franko at West Virginia University).

Periodontal examination and assessment. Following the blood draw, a periodontal examination was conducted by one of two trained and calibrated dental examiners, including one board-certified periodontist or a periodontics resident. Repeat examinations on about 10% of the participants were conducted in order to determine intra- (i.e., compared to self) and inter- (e.g.,

periodontist repeat of resident) examiner reliability. All study-related periodontal examinations were conducted according to the NHANES (National Health and Nutrition Examination Survey, 2013) oral health examination procedures.

The examiner probed six sites per tooth (excluding third molars), using a periodontal probe graduated from 2 to 12 mm. First, the distance between the free gingival margin (top of gum) and the bottom of the sulcus (i.e., pocket depth) was measured. Next, a measure of the distance between the free gingival margin and the cemento-enamel junction (CEJ) was taken. For cases in which the gingival margin was below the CEJ (i.e., measure of gingival recession), recession was documented. Each of these measures was taken from the mesial, mid, and distal sites on both the buccal (cheek) and lingual (tongue) sides of each tooth (i.e., six sites per tooth). Each measurement was called out by the examiner to a trained research assistant and recorded on a printed paper chart. Bleeding on probing was recorded for each site probed, and plaque scores were recorded for the mesial, mid, and distal sites of the buccal side and the mid site on the lingual (i.e., total of four sites per tooth). Number of missing teeth also was recorded. If data were collected from one of the two examiner's patients recruited in the periodontics specialty practice, their initial examination data were utilized by the dental provider giving the charted numbers (deidentified from HIPPA-related information) to a research staff member.

Pen-and-paper-recorded values were then entered into an excel spreadsheet that used various formulas to calculate and provide a number of other periodontal parameters. Clinical attachment loss was calculated as the sum of the probing depth and recession in millimeters if the gingival crest was below the CEJ. If above the CEJ, attachment loss was calculated by subtracting the distance from the CEJ to the gingival crest from the probing depth. Additionally, average probing depth, average attachment loss, number of missing teeth other than third molars,

the percent of sites that bled on probing, the percentage of sites with plaque, and the number of teeth with probing depths greater than or equal to 4 millimeters (mm) or 5 mm, as well as the number of teeth with clinical attachment loss greater than or equal to 3mm, 4mm or 6mm were calculated. Using these parameters, the excel sheet calculated the data and determine the CDC/AAP status categorizations (e.g., no disease, mild, moderate, severe), and are described in Table 1.

Psychosocial and diet assessments. Following the periodontal examination (or in some cases, while waiting for the periodontal examination), a battery of psychosocial questionnaires on REDCap was administered via a computer tablet. As a part of the greater battery of questionnaires, the two primary measures initially completed included the Perceived Stress Scale (PSS-10; Cohen & Williamson, 1988) and the Life Events List (LEL; Cohen, Tyrrell, & Smith, 1991). The PSS-10 is a well-established, reliable, and valid assessment designed to measure everyday perception of stress. It includes 10 items and is a standard measure of overall stress (Cohen, Kamarck, & Marmelstein, 1983; Cohen & Williamson, 1988). The PSS-10 has shown strong psychometric properties including good internal consistency ($\alpha = 0.78$-0.91; Lee, 2012), and good evidence for convergent and discriminant validity (Lee, 2012). Normative data have previously been published, including means ranging from 11.41-20.21 depending on the sample and demographic information (e.g., 1983 vs. 2009; employed vs. unemployed; Cohen & Janicki-Deverts, 2012).

The LEL (Cohen et al., 1991) was originally derived, in part, from the List of Recent Experiences (Henderson et al., 1981). This instrument has evidence for validity (Cohen et al., 1991; Cohen, Tyrrell, & Smith, 1993), though no known evidence has been provided for internal consistency or other reliability measures. The LEL was used, though, because it can be

administered in one sitting and takes a number of more common stressful events into account than other competing measures such as the Life Events Checklist (Weathers et al., 2013), which is more trauma focused. The LEL includes a number of items and can provide a metric of stressors and takes into account whether it was a positive or negative valence (e.g., ending a relationship, but an abusive one) and was employed to provide additional perspective about varying types of stress (e.g., chronic, acute, time-limited, naturalistic, positive or negative stressors).

The other psychosocial variables collected as part of the larger project were included in post-hoc exploratory analyses and are described here. The Center for Epidemiologic Studies Depression (CESD; Radloff, 1977) scale includes 20 items targeted at assessing depressive symptomology. It has been well-validated and utilized broadly (Radloff, 1977).

The Fear of Pain Questionnaire-9 (FPQ-9; McNeil et al., 2018) is a nine-item short-form of its parent measure, the Fear of Pain Questionnaire-III (FPQ-III; McNeil & Rainwater, 1998), and has demonstrated evidence for reliability and validity (McNeil et al., 2018). The FPQ-9 measures the fear associated with painful experiences such as falling down concrete steps, getting a paper cut, or injections as part of a medical or dental procedure. Fear of pain has been implicated in pain perception (McNeil et al., 2011), poorer medical or dental procedure and utilization outcomes (Chen et al., 2019; McNeil et al., 2018), and varies with age (Wright & McNeil, 2021).

The Anxiety Sensitivity Index-3 (ASI-3; Taylor et al., 2007) includes 18 items that assess the fear of anxiety sensations. Well-validated and used broadly, the ASI-3 assesses differences in anxiety proneness, particularly as they relate to physiological, cognitive, and social anxiety symptoms (Taylor et al., 2007).

A similar measure targeted on gut-specific anxiety, the Visceral Sensitivity Index (VSI; Labus et al., 2004) targets sensations and anxiety symptoms (e.g., looking for a restroom at new restaurants, worry about bloating) often experienced by those with gastrointestinal distress (Labus et al., 2004; Labus et al., 2007). It has demonstrated evidence for reliability and validity, particularly when used with individuals that have irritable bowel syndrome or other gastrointestinal complaints (Labus et al., 2004; Labus et al., 2007; Wright et al., 2020).

The Periodontal Disease Self-Report (PDSR; Wright et al., 2021b). includes items that participants indicate both functional and physiological symptomology as it relates to periodontal inflammation. Example items include “I can feel my teeth move when I chew” or “My gums often bleed.” Psychometric analyses were used and provided good evidence of reliability and factor validity in the initial validation of the measure (Wright et al., 2021b).

The Automated Self-Administered 24-Hour Dietary Assessment Tool (ASA24® 2018) was used to collect diet data. The ASA24 is a self-administered 24-hour diet recall measure created by a collaboration of various institutes at the National Institutes of Health. It is administered via a web-portal and is free to use for researchers. Participants using the ASA24 are asked to search a database and enter the foods, drinks, and supplements they consumed over the prior 24 hours from the time of filling out the measure. The system then asks details about the meals including where the food was acquired, with whom was it consumed, and other details. The data from the tool is vast and includes information about quantities of vitamins, minerals, various food groups, energy, and more. Prior versions of the measure have been evaluated and validated (ASA24, 2021; Kipnis et al., 2003; Moshfegh et al., 2008); it is widely used in diet and nutrition studies. Additional information about the ASA24 is available at https://epi.grants.cancer.gov/asa24/.

Data Management and Statistical Analyses

Psychosocial data from REDCap, diet data from the NIH-supported web-portal, periodontal parameters from excel sheets, and biospecimen data all were merged into one master dataset. The quality of data was screened for linearity, independence, normality, equality of variance, and to determine the degree of multicollinearity (Keith, 2014). No models included non-linear relations, data were independent (from only one time point). Data were normal based on what would be expected or consistent with norms. That is, variables that were skewed lower such as IL-6 or current smoking status were expected to be based on norms. Age was somewhat skewed younger. The CDC/AAP status was skewed in the less pathological direction. Residual plots were within normal limits with all of the variables from the first hypothesis. Equality of variance was tested as part of the ANOVA conducted and age, smoking status, and BMI were significant ($p < 0.05$) for Levene's test, but when a Welch test was conducted (to account for non-equal variance), results were essentially the same. In terms of multicollinearity, VIF scores were checked for regression models and there was no evidence for issues related to multicollinearity.

Missing data analyses were then conducted to test whether the data were missing completely at random (MCAR) or missing at random (MAR) and multiple imputations were used to account for missingness. Little's MCAR test was run on the variables included in the main hypotheses. Results indicated that the data are missing completely at random (MCAR; $p = 0.755$). Overall, six participants were missing zonulin and IL-6 data due to an inability to draw blood, while two participants had fewer than eight teeth and did not undergo the periodontal examination. BMI was calculated using height and weight, and due to missingness in the height variable, seven participants did not have a BMI calculated. Overall, 93 participants had complete

data across all variables. Given the data were MCAR, no imputations were conducted, and analyses were conducted using pairwise deletion and listwise deletion in the PROCESS mediation analyses. Missingness in all primary variables is depicted in Table 2.

First, to test the hypothesis of whether periodontal inflammation is associated with zonulin levels, CDC/AAP periodontitis categories were collapsed across cases of moderate and severe periodontal disease and assessed as a binary variable (0/1) as an attempt to present a parsimonious result. This new variable was included in a multiple linear regression model with individual zonulin levels as the outcome variable and accounting for the covariates of gender, age, smoking status, BMI and participant diabetes status. While other covariates could have been included in the models, the covariates were selected because of their relative importance in prior literature studying risk factors in periodontal disease. Moreover, additional covariates would have required more power which was balanced with feasibility of completing the overall project. Analyses were conducted using SPSS 27 (IBM, 2020).

An *a priori* power analysis was conducted using G*Power 3.1 (Faul et al., 2009) to determine the appropriate number of participants to detect a meaningful result for differences in zonulin levels between those with moderate-to-severe periodontal disease and those who do not meet the threshold of moderate disease using a regression model for analysis. To detect a small effect size of 0.15 with a power set at 0.80, and a regression including 6 predictors (binary periodontal status variable, and covariates of age, sex, diabetes, BMI, and smoking status) would require 98 participants. Figure 2 depicts the analysis for hypothesis one.

To test the second hypothesis, a multiple linear regression mediation model was conducted to assess the relations among psychological stress, serum zonulin, and serum cytokine levels across all CDC/AAP disease categories using PROCESS in SPSS 27 (Hayes, 2013; IBM,

2020). The predicting variable in the model was participant stress levels and the outcome variable was IL-6 cytokine levels. Quantified zonulin (ng/mL) levels were included as the mediator and age, gender, smoking status, BMI, and diabetes as covariates.

As with the first hypothesis, an *a priori* power analysis was conducted using G*Power 3.1 (Faul et al., 2009) to determine the sample size needed for the mediation analysis. In order to detect an effect size of 0.15 with power set at 0.80, and a regression including 8 predictors (perceived stress, zonulin levels, and covariates of age, gender, diabetes, BMI, and smoking status), an effective sample size would require 109 participants. Figure 3 depicts the mediation model for hypothesis two.

Results

Demographic Data and Sample Characteristics

The average age of participants was 36.1 years old (*SD* = 14.0, Range = 18-77). The sample consisted of 58 (50.9%) women and 50 (43.9%) men, 3 (2.6%) identifying their gender as "other" and 3 (2.6%) were missing or did not respond. In terms of race/ethnicity, the sample consisted of 86 (75.3%) White individuals, 24 (21.1%) Black or African American, and one (0.9%) Asian, one (0.9%) American Indian/Native American, one individual (0.9%) with a race identity of "other," and on (0.9%) was missing or did not respond. Participants' average years of education was 15.2 (*SD* = 2.9, Range = 8-21). The mean body mass index (BMI) was 28.2 (*SD* = 8.2, Range = 15.8-68.7). Regarding relevant medical conditions, four participants (3.5%) had diabetes, including two with type I and two with type II diabetes. In terms of smoking status, 25 (21.9%) participants reported currently smoking some days or every day. Demographic information are summarized in Table 2, including gender, age, race/ethnicity, household income,

education, body mass index, number of cigarettes smoked per day, and whether the participant had a diabetes diagnosis.

The board-certified periodontist completed 97 (85.1%) of the examinations, the periodontics resident completed 15 (13.2%), and two individuals were not examined due to missing teeth (1.8%). The periodontist completed 11 (11.3%) repeat examinations and was reliable in terms of intra-examiner agreement between the initial probing depth and the repeat probing depth; exact agreement was 79.0% and agreement within plus or minus 1 millimeter was 99.9%. Intraclass correlation coefficient (ICC) also was calculated using a two-way mixed model and absolute agreement type in SPSS. A two-way mixed model was selected given its amenability to greater statistical power and likelihood of approximating the results of a random effects model. The average measures ICC for probing depth was 0.92. For the measures of the free gingival margin, exact agreement was 84.4%, and agreement within plus or minus 1 millimeter was 99.9%. The average measures ICC was 0.96. No repeat examinations were completed for the periodontics resident, but interrater reliability scores were conducted by repeating one examination where the periodontist and the resident were compared with each other and demonstrated evidence of reliability. The exact agreement for both probing depths and free gingival margin measurements between the periodontist and the resident was 64.3%, and the agreement within 1 millimeter was 98.2%. The ICC average measures coefficient was 0.63. Kappa scores also were calculated to determine reliability of CDC/AAP categorizations. The periodontist intra-examiner assessments were identical in terms of CDC/AAP status and thus resulted in reliable results ($\kappa = 1.00$). Similarly, when examining the periodontist compared to the resident, the categorization for that one participant was identical and thus reliable ($\kappa = 1.00$).

According to the CDC/AAP criteria for periodontal disease, 53 (46.5%) participants presented with no disease, 14 (12.3%) with mild disease, 39 (34.2%) with moderate disease, and 6 (5.3%) with severe disease status. The average of all mean probing depths across all sites was 2.3 (*SD* = 0.5, *Range* = 1.4-4.0) and the average of all mean clinical attachment loss was 0.8 (*SD* = 1.1, Range = 0.0-6.6). The mean number of teeth with interproximal probing depth of 4mm or more was 6.3 (*SD* = 4.5, *Range* = 0-26), and with 5mm or more was 2.2 (*SD* = 4.1, Range = 0-22).

Mean number of teeth with interproximal clinical attachment loss of 3mm or more was 4.5 (*SD* = 7.2, Range = 0-28), with 4mm or more was 2.3 (*SD* = 5.0, Range = 0-25), and with 6mm or more was 0.6 (*SD* = 2.5, Range = 0-19). Given the CDC/AAP categorization, only clinical attachment loss of 3mm, 4mm, and 6mm are reported. The mean number of missing teeth (not including third molars/wisdom teeth) was 1.5 (*SD* = 2.5, Range = 0-12). The mean percentage of sites that bled on probing was 20.9% (*SD* = 17.5, Range = 1.0-79.0); the mean percentage of sites with plaque was 72.5% (*SD* = 27.9, Range = 1.0-100.0).

The mean Perceived Stress score was 17.2 (*SD* = 7.1 Range = 0-35) and the average number of stressful life events over the last year was 3.8 (*SD* = 3.0, Range = 0-15). Mean serum zonulin for all participants was 22.8 ng/mL (*SD* = 5.7, Range = 12.8-38.5) and mean serum IL-6 was 3.2 (*SD* = 3.5, Range = 0.1-21.6). The total number of teeth with a probing depth of 4mm or more was significantly correlated with zonulin levels (Pearson $r = 0.24$, $p = 0.014$), as was total of number of teeth with a probing depth of 5mm or more (Pearson $r = 0.24$, $p = 0.014$). IL-6 levels also were correlated with number of teeth with an interproximal probing depth of greater than 4mm (Pearson $r = 0.21$, $p = 0.033$) or greater than 5mm (Pearson $r = 0.22$, $p = 0.025$),

respectively. Correlations coefficients for all variables of interest related to hypothesis one and two are displayed in Table 3.

CDC/AAP Periodontal Status and Zonulin

Related to hypothesis one, zonulin levels—when compared using ANOVA—did not differ based on the four CDC/AAP status categorizations (n = 107; $F(3,103) = 0.673$, $p = 0.570$). When examining covariates across CDC/AAP status, the only one that differed significantly across categorizations was participant age (n = 112; $F(3, 108) = 6.600$, $p < 0.001$). Participants with severe periodontal disease ($M = 52.5$, $SD = 13.2$) were older on average when compared to participants with no disease ($M = 31.2$, $SD = 10.7$) or mild disease ($M = 35.9$, $SD = 11.6$). Means, standard deviations, counts and percentages for each variable in hypothesis one and two are displayed in Table 4.

Those with moderate-to-severe periodontal disease were expected to have higher levels of zonulin than those with otherwise healthy gingiva when accounting for the covariates. Before including covariates in the model, a simple regression was conducted with the dichotomous CDC/AAP variable of none-to-mild disease and moderate-to-severe disease predicting zonulin levels. The model was not statistically significant (n = 107; $F(1,105) = 1.41$, $p = 0.238$). When including the covariates of gender, age, smoking status, diabetes, and BMI, the overall regression model also was not statistically significant (n = 101; $F(6,94) = 1.86$, $p = 0.095$) and results indicating BMI as a significant predictor of zonulin, are therefore not trustworthy. Similarly, when CDC/AAP status was treated as a continuous variable, the regression model was non-significant (n = 101; $F(6,94) = 1.78$, $p = 0.111$). Regression results, including standardized beta coefficients for both the dichotomous and the continuous models, are displayed in Table 5.

As a result of these initial findings, CDC/AAP status was replaced in the model with the number of teeth with interproximal probing depth of 5mm or more as a predicting variable and all other variables remaining the same; the model was statistically significant (n = 101; $F(6,94) = 2.38$, $p = 0.035$). In this model, (as it was in the non-significant/non-interpretable CDC/AAP models), BMI was the only significant variable ($\beta = 0.28$, $p = 0.011$), suggesting a positive association between BMI and zonulin when accounting for the other variables. Number of teeth with interproximal probing depth of 5mm or more was non-significant at the $p < 0.05$ level ($p = 0.069$). When examining the change in r-square, BMI accounted for an additional change in variance ($R^2_{\text{Change due to BMI}} = 0.062$) for serum zonulin levels. If BMI was removed from the models, they became non-significant. Table 6 displays the results of the regression model.

Because of the potentially unique association of BMI to zonulin, a stratified analysis was conducted with two models. One model included only those with high BMI (i.e., at or above the mean) and the other model included those with BMI below the mean. The high BMI model was non-significant ($n = 11$; $F(6,4) = 0.65$, $p = 0.695$) and no predictors were significantly associated with zonulin. Likewise, the low BMI model was non-significant ($n = 84$; $F(6,77) = 1.34$, $p = 0.251$).

Psychological Stress, Zonulin, and IL-6

In terms of hypothesis two, PROCESS handles all missing data via listwise deletion (i.e., participant is not included if missing data on any variable in the model). Thus, 93 participants had complete data for the variables to be included. The total effect model was significant ($n = 93$; ($F(6,86) = 4.20$, $p = 0.001$), though the total effect of psychological stress on IL-6 was not ($\beta = 0.08$, $p = 0.481$). Similar to the prior hypothesis, the covariate of BMI was a significant predictor of IL-6 ($\beta = 0.38$, $p < 0.001$). PROCESS was unable to converge and would not

produce indirect effects in this model. Upon further exploration, there was not enough variability in the diabetes variable, and when it was removed, the model converged, producing indirect effects. The overall findings remained the same with BMI as a significant predictor of IL-6. In both models (with and without the diabetes variable), zonulin was not a significant predictor of IL-6 levels in the presence of the other variables (β = -0.05, p = 0.655). Without diabetes as a variable, the indirect effect was calculated and psychological stress via zonulin levels was not significant. That is, zonulin levels did not mediate the relation between PSS-10 scores and IL-6 levels.

Similarly, when the PSS-10 scores were replaced with total life events (LEL), the total effect model was significant (n = 93; (F(6,86) = 4.10, p = 0.001) and BMI was the only significant predictor of IL-6 (β = 0.38, p < 0.001). Also, when diabetes status was removed for convergence purposes, zonulin levels did not mediate the relation between LEL scores and IL-6 levels. LEL scores were likewise not predictive of IL-6 levels. When BMI was removed, the model was not significant (n = 98; (F(5,92) = 2.00, p = 0.086).

Additional Exploratory Analyses

As a follow-up to the two *a priori* hypotheses, a number of *post hoc* exploratory analyses were conducted. The purpose of the exploratory analyses was to examine potential relations between variables that explore alternative theoretical routes of association. For example, it could be that the categorization of CDC/AAP status does not capture early versus late state periodontal inflammation or perhaps other negative affective constructs such as depression would be associated with zonulin levels. Moreover, given the connection of zonulin to dietary factors, additional analyses were conducted to explore potential connections to nutritional and diet variables.

Acute stage versus more advanced periodontal disease parameters. First, given that the number of teeth with interproximal probing depths of greater than 5mm as a replacement to CDC/AAP status produced a significant model, but was not a significant predictor of zonulin, exploratory analyses were conducted with it as a dependent variable. Number of teeth with interproximal probing depths of 5mm or more is a measure of more acute inflammatory processes in periodontal conditions. Thus, the same model as with hypothesis one was conducted, only with zonulin as a predictor and number of teeth with probing depth of 5mm or more as the dependent variable. All other covariates remained the same (i.e., gender, age, current smoking status, diabetes status, and BMI). The overall model was significant (n = 101; (F(6,94) = 4.10, p = 0.001). Zonulin was not a significant predictor of the periodontal probing outcome, but current smoking status was significantly associated. Those with greater current smoking levels had a greater number of teeth with deeper pockets (β = 0.28, p = 0.004). BMI, diabetes status, gender, and age were non-significant. Because periodontal disease is a disease of aging (Ebersole et al., 2016), another model was tested with age as a dichotomous variable, with 0 associated with the lowest three quartiles and 1 linked with upper most quartile. This age variable was still not a significant predictor. Results for the regression model with age as a continuous variable are displayed in Table 7.

A stratified analysis was again conducted to examine one model of non-current smokers and another with current smokers. The model with those endorsing not currently smoking was significant (n = 79; (F(5,73) = 3.11, p = 0.013) and interestingly, zonulin was a significant predictor of periodontal probing depth of 5mm or more (β = 0.25, p = 0.029) such that greater zonulin levels were associated with number of teeth with deeper probing depths. Age and diabetes status also were significant predictors (β = 0.23, p = 0.034; β = 0.25, p = 0.042) such

that increased age and having diabetes were associated with number of teeth with deeper probing depths.

Next, to assess whether zonulin predicted more advanced stages of periodontal disease, the number of teeth with interproximal clinical attachment loss of 3mm, 4mm, or 6mm (the primary parameters used in deciding CDC/AAP categorizations) variables were used in three separate models, again including the original covariates. The overall model for more teeth with clinical attachment loss of greater than 3mm was significant (n = 101; ($F(6,94) = 5.77$, $p < 0.001$). Zonulin levels, gender, BMI, and diabetes status were not significant. Age ($\beta = 0.41$, $p < 0.001$) and current smoking ($\beta = 0.27$, $p = 0.004$) were significant, suggesting those who are older and those who are currently smoking some days, or most days had more attachment loss (i.e., 3mm or greater). Results were similar and the findings the same for a model with clinical attachment loss of 4mm or more. The model with number of teeth with clinical attachment loss of 6mm or more was not significant and thus uninterpretable. Results for all three models are displayed in Table 8.

Additionally, as an exploratory method to identify other potentially relevant variables not initially included in the model, a stepwise regression analysis was used. Stepwise regression is an exploratory analysis, it cannot replace theory, and cannot help to explain relations between variables like experimental or prospective studies might (Keith, 2014). It can be useful, though, in understanding potential predictors while elevating the variance explained of any given outcome. Here, a number of variables that have been shown to play a role in periodontal outcomes were included in a model to predict number of teeth with a probing depth of greater than 5mm and variables are systematically selected based on which one would contribute the most to significantly explaining the variance of said outcome. Potential predicting variables

included zonulin, education level, gender, age, BMI, household income, diabetes status, current smoking status, perceived stress, and depression scores. The regression resulted in a significant model (n = 101; (F(4,96) = 6.94, $p < 0.001$). The four variables that were significant predictors of number of teeth with an interproximal probing depth of 5mm or more included current smoking status, serum zonulin level, age, and household income. As current smoking ($\beta = 0.23$, $p = 0.014$), zonulin ($\beta = 0.23$, $p = 0.015$), and age ($\beta = 0.25$, $p = 0.009$ increased, number teeth with deeper pockets increased. Those with higher incomes had fewer deep periodontal pockets ($\beta = -0.22$, $p = 0.025$) and those with lower incomes more. The R-square for this model was 0.22, suggesting this model accounts for 22 percent of the variance in number of teeth with probing depths of 5mm or more. Results are displayed in Table 9.

Differences across outcomes related to CDC/AAP status. Building on the alternative analyses assessing different stages or parameters of periodontal disease, additional analyses were conducted to explore other variable differences based on the CDC/AAP categorization. Using an Analysis of Variance (ANOVA), basic differences in zonulin levels, perceived stress, life events, IL-6, depression scores, fear of pain, and visceral sensitivity were explored based on CDC/AAP periodontal disease status categories. No significant differences were noted, except in the case of IL-6 (F(3,103) = 3.84, $p = 0.012$, $\eta^2 = 0.101$). Post-hoc analysis (i.e., Tukey Honestly Significant Difference Tests) determined the difference in CDC/AAP status was such that those in the severe disease category had significantly higher serum IL-6 levels ($M_{\text{severe disease}} = 7.4$; $SD = 3.5$), and those with no disease ($M_{no\ disease} = 2.6$; $SD = 3.8$; $p = 0.008$) or those with mild disease ($M_{mild\ disease} = 2.6$; $SD = 3.1$; $p = 0.026$) lower levels. Those with moderate disease did not differ from the other CDC/AAP categories ($M_{moderate\ disease} = 3.6$; $SD = 3.0$; $p = 0.062$). Results of the ANOVA are displayed in Table 10.

Relation of zonulin to self-reported periodontal symptomology. As another way of assessing at indicators of periodontal inflammation, PDSR scales were explored. The PDSR scores were first correlated to clinical periodontal disease parameters including CDC/AAP status, percent of sites with bleeding on probing, percent of sites with plaque, number of teeth with interproximal probing depths of 4mm and 5mm or more, number of teeth with interproximal clinical attachment loss of 3mm, 4mm, and 6mm or more, and number of missing teeth other than the third molars. PDSR total scores were positively correlated with all clinical parameters ($0.36 < r < 0.53$; $p < 0.001$). The Physiological and Functional symptom subscales were likewise correlated with all parameters, though only the Functional symptoms subscale was associated with number of missing teeth ($r = 0.37$, $p < 0.001$). Zonulin was not associated with any of the PDSR scales.

Correlations among other psychosocial or diet variables and zonulin. Given the lack of association between the stress variables, other psychosocial variables and their associations with zonulin were explored. Psychosocial, diet, and periodontal disease variables were examined in a correlation table to explore potential relations to zonulin levels. None of the psychosocial variables were significantly correlated with serum zonulin. In terms of the diet variables, a number of vitamin or mineral consumption amounts were negatively correlated with zonulin including iron, thiamin, niacin, vitamin B-6, folate, and hexadecenoic acid. As the consumption of each of these minerals and vitamins increased, zonulin levels decreased. Similarly, total grain intake, along with red meat intake (e.g., beef, veal, pork, lamb, game meat) was negatively correlated with serum zonulin. After these correlations were discovered, periodontal disease parameters were added to the correlation table. Almost all of diet variables that were significantly correlated with zonulin also were correlated with CDC/AAP status or number of

teeth with probing depth of greater than 5mm. The zonulin, dietary/nutritional factors, and periodontal disease parameter correlations are displayed in Table 11.

Discussion

The primary aims of this study were to explore potential relations between zonulin and periodontal disease status, as well as the potential mechanistic role of zonulin in the association between psychological stress and inflammatory markers such as IL-6. Those with moderate-to-severe periodontal disease were expected to have higher levels of zonulin than those with otherwise healthy gingiva, after accounting for covariates. This would support the hypothesis that moderate-to-severe periodontal disease may be related to elevated zonulin levels and thereby possible intestinal permeability. Results from this study did not, however, support this hypothesis in terms of CDC/AAP moderate-to-severe periodontal disease status being related to serum levels of zonulin, after controlling for all covariates.

Nevertheless, basic correlations indicated a significant association between increased number of deeper probing depths and increased serum zonulin, which may suggest it plays a role in periodontal diseases. The model tested in hypothesis one was significant when the number of teeth with interproximal probing depths of 5mm or more was used as a periodontal disease parameter instead of CDC/AAP status, though in the presence of other variables, it did not predict zonulin levels. Moreover, when the number of probing depths variable was included with age, gender, diabetes status, and current smoking status, BMI was the only significant predictor and contributed nearly half of the accounted variance in the model. This finding, however, was complicated when dropping the two highest BMI participants due to being outliers, resulting in BMI no longer being a significant predictor. Prior literature that suggests a role of elevated zonulin levels in obesity and metabolic diseases (Aasbrenn, Lydersen, & Farup, 2020; Moreno-

Navarrete et al., 2012; Zak-Gołąb et al., 2013), which would support the finding of BMI associated with zonulin in this study. A novel finding here, however, is the potential relative weight of BMI in its accounting for the variance of serum zonulin. Elevated BMI, and potentially obesity, when in the presence of other variables such as age, gender, current smoking status, and diabetes status was predominant. That is, BMI may be a common predisposing and precipitating factor together with zonulin in the manifestation of inflammatory states (Wright et al., 2019).

Interestingly, the exploratory results of the stepwise regression replicated important predictors of periodontal disease predominately demonstrated in epidemiologic literatures. That is, smoking and household income are well-known risk factors of periodontal disease and was indicated in the results of this analysis. Noteworthy, however, was that zonulin also was included as a potential predictor accounting for variance. Moreover, in the stratified analysis of non-current smokers, zonulin also was a significant predictor of number of teeth with deeper probing depths, along with age and diabetes status. This work fits with prior known risk factors of periodontal disease yet provides some suggestive evidence of zonulin as a potential novel contributor to the disease. Given the exploratory nature of such an analysis, and in light of zonulin not being associated in other analyses, more work is needed to explicate the nature of the relation of zonulin to periodontal inflammation. As mentioned elsewhere, perhaps examining zonulin in a more proximal location to the inflammation (e.g., gingival crevicular fluid) would elicit different results.

Results of pilot work leading to this study demonstrated the potential mediating role of gut-related distress (i.e., gut-specific anxiety) in self-reported periodontal inflammation (Wright et al., 2021a). Based on these prior data, it was expected that psychological stress and cytokine levels would be positively associated with zonulin mediating the relation, though this relation

was not supported by the current study. Even when BMI was taken out of the model, no significant relations were found. Psychological stress remains a complex construct to study given it is often used interchangeably with other negative affective terms such as anxiety, fear, or even pain (Felicione et al., 2021). None of the psychosocial variables were correlated with serum zonulin levels, suggesting such variables may not have to do much with systemic zonulin or vice versa, but additional work is needed given this is the first study to examine these relations. The lack of an association could be related to an overall baseline increase in stress levels resulting from the COVID-19 pandemic occurring during much of the data collection. Mean PSS-10 levels were slightly higher than previously published normative data (Cohen & Janicki-Deverts, 2012), but some of that normative data used in that study are nearly 40 years old. Additional work is needed to ascertain the effects of COVID-19 on perceived stress and health outcomes. Furthermore, the complex nature of the relation between acute versus chronic stress and the immune system cannot be overstated (Segerstrom & Miller, 2004). The PSS-10 and the LEL used in this study assess the effect of stress or life events over the last month or 12 months, respectively. It could be that more immediate or short-term stress plays a role in the zonulin pathway. Additional studies, perhaps experimental in nature, could test the production of zonulin when a participant undergoes an acute stress task. This study also only assessed for one cytokine, IL-6. Many other pro-inflammatory and other inflammatory markers could be examined that also are associated with psychological stress and could be mediated by zonulin.

Discussion of Exploratory Analyses

"Mode of action". When considering additional findings outside of the initially proposed hypotheses, something could be said about the potential "mode of action" that zonulin might play in periodontal inflammation. Results indicated zonulin was more highly associated with

probing depth, thus substantiating literature outside of periodontology that indicate zonulin contributes to the innate immune response (Sturgeon & Fasano, 2016). Zonulin similarly could be operating in the acute inflammatory stages of periodontal disease. That is, exploring the role of zonulin in periodontal disease could be time dependent, meaning zonulin could be playing a role in the acute inflammation, and less so in advanced stages of disease that include more attachment loss or edentulism.

For example, when CDC/AAP status was replaced with number of teeth with interproximal probing of 5mm or more, the overall model with zonulin as an outcome became statistically significant. Additionally, the correlation analysis showed a significant association between serum zonulin levels and the number of teeth with probing depths of 5mm or more. Probing depths of greater than 5mm alone are indicative of more acute inflammation and "puffy gums" often seen in gingivitis (Eke et al., 2021), whereas severe attachment loss or even tooth loss tend to be indicative of more severe periodontal disease (Eke et al., 2021). Given this study measured systemic zonulin (i.e., via serum), it would be important to examine zonulin at a local level including in the gingival crevicular fluid to understand whether zonulin is directly contributing to increased inflammation. Also, given the relative weight of BMI, additional work could be conducted in large population studies to see whether zonulin contributes much to oral inflammation at a system level.

When considering the *a priori* theoretical assumptions of this study, this "mode of action" information could help in establishing a more complete theoretical model in the future. While it is still possible that zonulin affects the gut and "settles" in the oral cavity, after this study it seems more likely that a more targeted, oral cavity-focused mode of action is at play. With what is known, it is likely that zonulin plays a role in the inflammatory processes of

periodontal disease, more locally, and are moderated by relevant covariates, especially BMI, and less so by age or others, though more work is needed to fully explicate these associations. Figure 4 is a directed acyclic graph (DAG) which provides a working theoretical model to help guide future work elucidating the role of zonulin in periodontal disease etiology and maintenance.

Differences in variables based on CDC/AAP status. The six participants in the severe periodontal disease status categorization of the CDC/AAP status did not differ from moderate, mild or no disease groups in key outcome variables such as zonulin, stress, depressive symptoms, fear of pain, or visceral sensitivity, although it must be noted that few participants are included in the severe group. Participants with severe periodontal disease based on CDC/AAP criteria did, however, have significantly higher levels of IL-6 and those with no disease or mild disease had lower levels. This finding supports prior literature demonstrating the role of IL-6 in periodontal disease (Irwin & Myrillas, 1998; Nikolopoulos et al., 2008; Tawfig, 2016). IL-6 is a pro-inflammatory cytokine and aligns with the finding that severe cases have greater levels of serum level cytokines than those with mild, but not moderate disease. Moderate disease status is achieved in multiple routes including having two or more interproximal probing depths (not on the same tooth) of 5 millimeters or more. Severe cases are required to have at least 1 probing site of 5 or more, in addition to significant attachment loss (National Health and Nutrition Examination Survey, 2013). Deeper pocket depths often are associated with greater rates of active inflammation (Eke et al., 2021). Thus, having greater active inflammation is likely to result in greater system-level (i.e., serum) pro-inflammatory cytokines, such as IL-6.

Zonulin and self-reported periodontal inflammation. This was the first study to compare the PDSR (Wright et al., 2021b) with clinical parameters of periodontal disease. Results help to provide evidence of concurrent validity for the PDSR, further establishing it as a possible

easy-to-use and low-cost alternative to some more time intensive clinical measures. Especially interesting was how the Physiological symptoms subscale was not associated with tooth loss, but the Functional symptoms subscale was, substantiating the nature of each subscale. Neither the total score nor the subscale scores were associated with zonulin despite deep pocket depth being correlated with zonulin levels.

Diet relations to zonulin and periodontal disease. Overall, there were several associations between serum zonulin and diet or nutritional factors, including grain intake, red meat consumption, and a number of vitamins and minerals. Given that intake of gluten (a protein found in wheat, barley, and rye) and its mechanistic role in zonulin production (Sturgeon & Fasano, 2016), it was surprising that increased total grain intake was associated with less serum zonulin. While gluten is more prevalent in breads and grains, so too are minerals and vitamins such as iron, thiamin, niacin, vitamin B-6, folate, and hexadecenoic acid (Harvard School of Public Health, 2021), which also were associated with lower levels of zonulin in this study. B-complex vitamins typically are more prevalent in grain and red meat-rich diets (Najeeb et al., 2016), which may partially explain the association among these variables in the current study. Prior work examining the associations between vitamins and serum zonulin has been sparse, though one study found similar results as the present study, including higher levels of thiamin, niacin, and other B-vitamins in those with low levels of serum zonulin (Mokkala et al., 2016). No known work has examined the association between hexadecenoic acid and zonulin, although some have explored its roles in combating inflammation and metabolic disorders (de Souza et al., 2018). Understanding the role of zonulin in this way could be a rich area for further exploration.

Similar associations were found between increased levels of the vitamins or minerals also associated with zonulin and decreased indication of periodontal disease. These findings are

consistent with existent literature. Members of the vitamin B complex family such as thiamin, niacin, vitamin B-6, and folate have demonstrative effects on periodontal health (Najeeb et al., 2016; Varela-López et al., 2018). Greater level of B-vitamins has been indicative of less problems with periodontal probing depth and clinical attachment loss (Najeeb et al., 2016), as well as improvement in periodontal wound healing (Najeeb et al., 2016; Neiva et al., 2005). Moreover, individuals who smoke—a known predictor of periodontal disease—also tend to have lower levels of serum folic acid (i.e., synthetic folate; Erdemir & Bergstrom, 2006). Iron rich diets also play a protective role in periodontal disease due to their anti-oxidative properties (Chakraborty et al., 2014; Najeeb et al., 2016). Given the commonalities found among the B-vitamins, zonulin production, and periodontal disease characteristics, additional work should be conducted to elucidate whether there are mechanisms that may contribute to increases in zonulin and play a potential mediating or moderating role between zonulin and periodontal disease outcomes.

Additionally, while more work is needed in this area, lesser amounts of zonulin have been shown to attenuate the effects of those diseases that it has been associated with, including celiac disease. Indeed, some have tested the effects of a zonulin antagonist (i.e., Larazotide acetate) in improving symptoms in patients with celiac disease (Leffler et al., 2015). Perhaps more work could test interventions that target the effects of increased B-vitamins in reducing zonulin levels and thereby a host of chronic inflammatory ailments.

Limitations

This sample included a disproportionate amount of no disease participants and could have benefitted from more severe cases. Given the limitations with recruitment during COVID-19, additional severe cases were difficult to obtain. The lack of statistical differences or larger effects

could be due, in part, to the small sample of severe cases in comparison to the others. Having more severe cases could have provided additional power potentially needed to see a greater effected related to zonulin levels.

Representation in terms of race/ethnicity was good in terms of the number of individuals who identified as Black or African American, compared to the demographics of West Virginia. There was less representation in other minority status groups, however, such as those who identify as Asian, American Indian/Native Alaska, or Latinx. Given work demonstrating disparities in periodontal disease and oral health more broadly, future work would do well to examine the role of zonulin and psychological factors among racial and ethnic minority populations.

The younger mean age of this sample likely contributed to the disproportionate number of healthy or no disease participants. Periodontal disease is a disease of aging and future work would do well to include older participants. This was a limitation potentially due to the cross-sectional and convenience sampling method of recruiting in a college town where the mean age in the region is naturally lower. The analyses, however, examining the prediction of zonulin and covariates in relation to periodontal disease parameters typically associated with more advanced disease (i.e., number of teeth with clinical attachment loss of 3mm, 4mm, and 6mm ore more) indicated no association with zonulin, but did with age and smoking status. Age was not a significant predictor of more acute periodontal disease (i.e., number of teeth with deep pockets). The comparison of these two findings—age being associated with clinical attachment loss and late-stage disease, and not with acute inflammation more typical of gingivitis—does indicate that even though the mean age was potentially skewed to be younger, age still played a role.

For the hypotheses one and two, 101 and 93 participants had complete data, respectively. For the mediation analysis, it could be that it was underpowered given the *a priori* power analysis. Given the relative weight of BMI in this model, however, it is unlikely that the overall conclusions would have changed. MPlus was used with a full information likelihood estimator (FIML) and without diabetes status as a way to provide complete data for 114 participants. The overall findings did not change.

Similarly, the interpretation of the stepwise regression results should be tempered. Stepwise regression leans heavily on mathematics and computation to determine the relative "importance" of any given variable and does not replace good theory (Keith, 2014). That being said, the results of the analysis in this study were consistent with other risk factors in periodontology such as age, smoking status, and household income. Such consistency may give additional credence to the finding of zonulin also being included in this model and the need for further experimental and microbiological work in understanding the etiological role of zonulin in periodontal inflammation and disease.

In terms of the self-report periodontal measure (PDSR), a potentially methodological confound could be that the PDSR was administered after the clinical periodontal examination, potentially influencing and inflating responses. That is, the participants would be thinking more attentively to their gum health than the general population. Future work should counter-balance and account for this potential study effect by having individuals complete the PDSR prior to undergoing a specialized periodontal examination that could influence their responses.

Directions for Future Research

In terms of future work related to zonulin and its potential association with periodontal inflammation and/or disease, this study provides preliminary data that makes it logical to conduct

further microbiological work. That is, microbiologists and cell biologists could benefit this literature by explicating the role of zonulin in cell-cell adhesion not only in the gastrointestinal tract, but also the junctional epithelium within the periodontium. Similarly, this study focused on the systemic associations of zonulin to periodontal disease, which is "downstream" or distal to where the actual inflammation is occurring. Others should examine a more proximal view by examining whether zonulin is present in the gingival crevicular fluid, or even saliva. Doing so could provide evidence that zonulin is operating at a local level as well.

This study did not provide much evidence in terms of the association between stress or other psychosocial variables and zonulin. This could have been due to the focus on more temporally distant stress (i.e., PSS-10 measuring stress in last month and the LEL stressors over the last 12 months). As mentioned previously, additional exploration could be done by conducting experimental studies with more immediate stressors. This study seemed to indicate a stronger presence of zonulin in acute inflammation, which may coincide with psychological variables with more acute inflammatory effects. That being said, this study has implications for another psychological or behavioral construct, namely diet. This work confirmed some of the broad associations between B-vitamins and zonulin, and additional work could be done to understand why these associations and the related mechanisms. Given diet is heavily influenced by behavior, additional exploration into developing behavioral interventions should be conducted, particularly those that might influence the B-vitamin pathway and thereby potentially reduce inflammation that is a result of increased zonulin level.

Summary and Conclusions

This study provided some evidence to suggest a contributing role of zonulin to periodontal inflammation and disease. As this is the first such study to suggest that zonulin might

be related to oral inflammation, it potentially has important implications for understanding a complex disease. This study also suggests little association between psychosocial variables and zonulin level, except when considering dietary behavior. Other exploratory analyses replicate findings from prior work including the role of IL-6, smoking status, and age in periodontal disease, as well as BMI in zonulin. Furthermore, this study demonstrates the importance of considering the gastrointestinal-periodontal health pathway. Better elucidating how the oral cavity is affected by other bodily systems will provide new insights into the etiology of oral diseases, as well as in innovating new treatments. Given the complexity of such a task, team science across multiple disciplines using transdisciplinary approaches may provide needed unique perspectives and prompts new ways of asking questions.

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Tables

Table 1

Criteria for classifying periodontitis according to 2012 CDC/AAP recommendations, as well as number and percentage of sample in each class.

Class of Periodontitis	Criteria
Severe (*n* = 6 [5.3%])	≥2 interproximal sites (not on the same tooth) with AL ≥ 6mm **AND** ≥1 interproximal site with PD ≥5mm.
Moderate (*n* = 39 [34.2%])	≥2 interproximal sites (not on the same tooth) with AL ≥ 4mm, **OR** ≥2 interproximal sites with PD ≥ 5mm.
Mild (*n* = 14 [12.3%])	≥2 interproximal sites with AL ≥3mm, **AND** ≥2 interproximal sites with PD ≥ 4mm (not on the same tooth), **OR** 1 site with PD ≥5mm
No Disease (*n* = 53 [46.5%])	No evidence of mild, moderate, or severe periodontitis

Note: *n* = 112 (2 [1.8%] participants did not undergo periodontal examination due to complete edentulism.)

Table 2

Descriptive characteristics of sample (N = 114).

	Mean / N	*SD / %*
Age (years)	36.1	14.0
Gender		
Female	58	50.9%
Male	50	43.9%
Other	3	2.6%
Missing	3	2.6%
Race/Ethnicity		
White	86	75.3%
Black/African American	24	21.1%
Asian	1	0.9%
American Indian/Native American	1	0.9%
Other	1	0.9%
Missing	1	0.9%
Education (Years)*	15.2	2.9
Income		
Less than $10,000	17	14.9%
$10,000-$14,999	20	17.5%
$15,000-$24,999	16	14.0%
$25,000-$34,999	20	17.5%
$35,000-$49,999	8	7.0%
$50,000-$74,999	18	15.8%
$75,000-$99,999	3	2.6%
$100,000-$149,999	4	3.5%
$150,000-$199,999	6	5.3%
Missing	2	1.8%
Body Mass Index (BMI)*	28.2	8.2
Diabetes Status		
Yes	4$^{\Phi}$	3.5%
No	108	94.7%
Missing	2	1.8%
Current Smoker		
Not at all	88	77.2%
Some days	9	7.9%
Every day	16	14.0%
Missing	1	0.9%

Notes: *=7 missing Education; 7 missing Body Mass Index; $^{\Phi}$=2 type II and 2 type I diabetes.

Table 3
Correlation table of all primary variables using Pearson correlation coefficients.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Zonulin	-																
2 IL-6	0.03	-															
3 CDC/AAP Status	0.10	**0.25***	-														
4 # teeth with PD 4+	**0.24***	**0.21***	**0.68+**	-													
5 # of teeth with PD 5+	**0.24***	**0.22***	**0.68+**	**0.86+**	-												
6 # of teeth with CAL 3+	0.10	**0.31+**	**0.71+**	**0.69+**	**0.70+**	-											
7 # of teeth with CAL 4+	0.08	**0.32***	**0.62+**	**0.69+**	**0.74+**	**0.90+**	-										
8 # of teeth with CAL 6+	0.06	0.17	**0.42+**	**0.53+**	**0.64+**	**0.55+**	**0.74+**	-									
9 % sites with BOP	0.02	0.12	**0.37+**	**0.55+**	**0.48+**	**0.30+**	**0.37+**	**0.38+**	-								
10 % sites with plaque	0.09	0.21	**0.29+**	**0.41+**	**0.33+**	**0.28+**	**0.30+**	0.18	**0.43+**	-							
11 PSS	-0.02	0.07	-0.04	-0.09	-0.09	-0.04	-0.04	0.04	-0.08	0.08	-						
12 LEL	-0.17	-0.10	-0.13	-0.04	-0.04	-0.11	-0.10	-0.10	-0.04	0.05	**0.43+**	-					
13 Age	0.10	0.19	**0.37+**	0.12	**0.23***	**0.43+**	**0.38+**	**0.21***	0.04	-.07	**-.23***	-0.18	-				
14 Gender	*0.10*	*0.06*	*0.14*	***0.22****	***0.22****	*0.10*	*0.12*	*0.01*	*0.14*	*0.19*	***-.29+***	*-0.02*	*0.18*	-			
15 Current Smoker	0.08	0.17	**0.27+**	**0.28+**	**0.36+**	**0.31+**	**0.26+**	**0.23***	0.07	**0.28+**	**0.23***	**0.20***	0.05	0.10	-		
16 BMI	**0.25***	**0.41+**	0.13	0.18	0.15	0.14	**0.19***	0.07	0.17	0.09	-0.04	-0.06	0.12	0.05	0.09	-	
17 Diabetes	*-0.03*	***0.22****	*0.04*	*-0.04*	*0.03*	*0.11*	*0.11*	*0.10*	*0.04*	*0.17*	*0.07*	*-0.02*	*0.11*	*0.02*	*0.13*	***0.26+***	-

*Note*s: * = $p < .05$. + = $p < .01$. Italics indicates Spearman's rho was used instead of Pearson *r*. Bold indicates statistically significant.

Table 4

Mean scores for variables included in hypothesis one and two by CDC/AAP status.

	CDC/AAP Status - *M (SD) / n* (%)				
	No disease (*n* = 53)	Mild (*n* = 14)	Moderate (*n* = 39)	Severe (*n* = 6)	*p*-value
Zonulin (*n* = 107)	22.5 (4.9)	21.6 (7.5)	23.4 (6.1)	24.9 (6.3)	0.570
IL-6 (*n* = 107)	2.6 (3.8)	2.6 (3.1)	3.6 (3.0)	7.4 (3.5)	**0.012**
PSS Total (*n* =111)	17.5 (7.4)	17.4 (7.5)	16.8 (6.6)	16.7 (8.8)	0.974
# of life events (*n* = 112)	4.1 (3.0)	4.2 (2.9)	3.5 (3.1)	2.3 (2.7)	0.475
Age (*n* = 112)	31.2 (10.7)	35.9 (11.6)	39.6 (15.9)	52.5 (13.2)	**< 0.001**
Gender (*n* = 106)					0.126
Women (*n*/% of total)	33 (63.5%)	4 (30.8%)	19 (52.3%)	2 (33.3%)	
Men (*n*/% of total)	19 (36.5%)	9 (69.2%)	16 (45.7%)	4 (66.7%)	
Smoking status (*n* = 111)	0.2 (0.6)	0.3 (0.7)	0.5 (0.8)	0.8 (1.0)	0.071
Diabetes (*n* = 110)					0.317
Yes (*n*/% of total)	2 (3.8%)	0 (0%)	1 (2.7%)	1 (16.7%)	
No (*n*/% of total)	51 (96.2%)	14 (100%)	36 (97.3%)	5 (8.3%)	
BMI (*n* = 106)	27.4 (7.8)	29.0 (12.6)	28.5 (6.3)	33.7 (10.1)	0.350

Notes: *p*-values determined by ANOVA in case of continuous variables and chi-square for categorical variables. Bold indicates statistical significance. PSS = Perceived Stress Score; BMI = Body mass index.

Table 5

Regression results of CDC/AAP status dichotomized and as continuous predicting zonulin levels and accounting for covariates.

	b	*S.E.*	*β*	*t*	*p* - value
Intercept	15.802	2.453		6.442	< .001
CDC/AAP dichotomous	.927	1.226	.080	.756	.452
Gender (Male)	1.159	1.120	.102	1.026	.308
Age	.013	.043	.033	.314	.754
Current smoking status	-.002	.811	.000	-.003	.998
BMI	.206	.074	.297	2.778	.007
Diabetes (Yes)	-5.205	3.296	-.170	-1.579	.118
	b	S.E.	β	t	p - value
Intercept	15.783	2.466		6.400	< .001
CDC/AAP continuous	.233	.611	.042	.381	.70
Gender (Male)	1.119	1.135	.098	.987	.326
Age	.018	.043	.043	.405	.687
Current smoking status	.059	.816	.007	.073	.942
BMI	.206	.074	.297	2.768	.007
Diabetes (Yes)	-5.223	3.304	-.170	-1.581	.117

Notes: b = unstandardized coefficient and *β* = standardized coefficient. S.E. = standard error. CDC/AAP dichotomous was coded as 0 = no disease or mild disease and 1 = moderate or severe disease status. CDC/AAP continuous was coded as 0 = no disease, 1 = mild disease, 2 = moderate disease, and 3 = severe disease status.

Table 6

Regression results with number of teeth with interproximal probing depth of 5 or more millimeters predicting zonulin levels while accounting for covariates.

	b	*S.E.*	*β*	*t*	*p* - value
Intercept	16.429	2.446		6.717	.000
# of teeth w/ interproximal probing depth of 5mm+	.268	.146	.195	1.839	.069
Gender (Male)	.792	1.130	.069	.701	.485
Age	.009	.041	.022	.218	.828
Current smoking status	-.299	.814	-.038	-.368	.714
BMI	.191	.074	.276	2.600	.011
Diabetes (Yes)	-4.866	3.254	-.159	-1.495	.138

Notes: b = unstandardized coefficient and *β* = standardized coefficient. S.E. = standard error.

Table 7

Regression results with zonulin predicting the number of teeth with the of number interproximal probing depth of 5 or more millimeters as outcome and while accounting for original covariates.

	b	*S.E.*	*β*	*t*	*p* - value
Intercept	-4.781	2.006		-2.383	.019
Zonulin	.129	.070	.178	1.839	.069
Gender (Male)	1.175	.777	.142	1.512	.134
Age	.051	.028	.173	1.843	.069
Current smoking status	1.591	.541	.276	2.939	.004
BMI	.038	.053	.076	.725	.470
Diabetes (Yes)	-.793	2.284	-.036	-.347	.729

Notes: b = unstandardized coefficient and *β* = standardized coefficient. S.E. = standard error.

Table 8

Regression results with zonulin predicting the number of teeth with an interproximal clinical attachment loss of 3 millimeters, 4 millimeters, or 6 millimeters as outcome and while accounting for original covariates, respectively.

# of teeth w/CAL of 3mm+	b	*S.E.*	*β*	*t*	*p* - value
Intercept	-6.906	3.353		-2.060	.042
Zonulin	.046	.118	.036	.387	.700
Gender (Male)	.221	1.299	.015	.170	.865
Age	.210	.046	.406	4.518	< .001
Current smoking status	2.666	.905	.266	2.946	.004
BMI	.061	.088	.069	.692	.491
Diabetes (Yes)	.238	3.818	.006	.062	.950
# of teeth w/CAL of 4mm+	b	*S.E.*	*β*	*t*	*p* - value
Intercept	-5.168	2.392		-2.160	.033
Zonulin	.017	.084	.020	.204	.839
Gender (Male)	.250	.927	.025	.270	.788
Age	.126	.033	.353	3.811	< .001
Current smoking status	1.409	.646	.203	2.183	.032
BMI	.063	.063	.104	1.003	.318
Diabetes (Yes)	2.111	2.724	.079	.775	.440
# of teeth w/CAL of 6mm+	b	*S.E.*	*β*	*t*	*p* - value
Intercept	-1.348	1.266		-1.064	.290
Zonulin	.015	.044	.035	.343	.733
Gender (Male)	.177	.491	.036	.361	.719
Age	.032	.018	.179	1.811	.073
Current smoking status	.817	.342	.238	2.389	.019
BMI	-0.0001	.033	.000	-.002	.999
Diabetes (Yes)	.885	1.442	.067	.613	.541

Notes: b = unstandardized coefficient and *β* = standardized coefficient. S.E. = standard error. CAL = clinical attachment loss. Model with number of teeth with CAL of 6mm+ was non-significant.

Table 9

Results of an exploratory stepwise regression that included series of potential variables.

Model 1	b	*S.E.*	*β*	*t*	*p* - value
Intercept	1.561	.443		3.520	.001
smokenow	1.728	.552	.300	3.131	.002
Model 2	b	*S.E.*	*β*	*t*	*p* - value
Intercept	-2.298	1.604		-1.433	.155
smokenow	1.709	.538	.297	3.177	.002
Zonulin	.169	.068	.233	2.498	.014
Model 3	b	*S.E.*	*β*	*t*	*p* - value
Intercept	-4.046	1.778		-2.276	.025
Current smoking	1.656	.529	.288	3.130	.002
Zonulin	.155	.067	.213	2.310	.023
Age	.058	.027	.196	2.124	.036
Model 4	b	*S.E.*	*β*	*t*	*p* - value
Intercept	-3.535	1.756		-2.014	.047
Current smoking	1.348	.536	.234	2.516	.014
Zonulin	.163	.066	.225	2.486	.015
Age	.074	.028	.250	2.670	.009
Household income	-.398	.175	-.218	-2.269	.025

Notes: b = unstandardized coefficient and *β* = standardized coefficient. S.E. = standard error.

Table 10

Results of exploratory ANOVA assessing the differences in listed variables by CDC/AAP periodontal disease status.

		Sum of Squares	*df*	Mean Square	*F*	*P*-value
PSS Total	Between Groups	11.56	3	3.85	.074	.974
	Within Groups	5555.47	107	51.92		
	Total	5567.03	110			
LEL Total Events	Between Groups	23.21	3	7.74	.840	.475
	Within Groups	994.21	108	9.21		
	Total	1017.42	111			
Zonulin (ng/mL)	Between Groups	66.86	3	22.29	.673	.570
	Within Groups	3409.97	103	33.11		
	Total	3476.83	106			
Serum IL-6	Between Groups	134.80	3	44.93	3.844	**.012**
	Within Groups	1203.84	103	11.69		
	Total	1338.63	106			
CESD Total	Between Groups	282.08	3	94.03	.678	.567
	Within Groups	13999.88	101	138.61		
	Total	14281.96	104			
FPQ9 Total	Between Groups	17.68	3	5.90	.097	.961
	Within Groups	6256.19	103	60.74		
	Total	6273.87	106			
VSI Total	Between Groups	15.90	3	5.30	.024	.995
	Within Groups	22336.06	101	221.15		
	Total	22351.96	104			

PSS = Perceived Stress Scale; LEL = Life Events List; IL-6 = interlukin-6; CESD = Center for Epidemiologic Studies Depression; FPQ9 = Fear of Pain Questionnaire-9; VSI = Visceral Sensitivity Index

Table 11

Pearson correlation table of zonulin, IL-6, and dietary/nutritional factors.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 Zonulin ng/mL	--																	
2 Serum IL-6	.03	--																
3 CDC/AAP	.10	.25**	--															
4 PD 5mm+	.24*	.22*	.68**	--														
5 Iron	-.27**	-.06	-.17	-.18	--													
6 Thiamin (mg)	-.28**	-.09	-.22*	-.24*	.78**	--												
7 Niacin (mg)	-.21*	-.07	-.01	-.07	.62**	.72**	--											
8 Vitamin B6 (mg)	-.25*	-.10	.01	-.02	.48**	.59**	.83**	--										
9 Folate	-.23*	-.03	-.25*	-.24*	.75**	.76**	.58**	.38**	--									
10 Folate DFE	-.23*	.01	-.21*	-.23*	.74**	.73**	.57**	.36**	.98**	--								
11 Fatty acids	-.19	-.08	-.25**	-.21*	.53**	.56**	.46**	.35**	.44**	.39**	--							
12 H. acid	-.21*	-.07	-.25**	-.21*	.57**	.61**	.52**	.39**	.49**	.44**	.98**	--						
13 H. acid, undiff.	-.30**	-.02	-.08	-.14	.53**	.47**	.56**	.40**	.40**	.38**	.78**	.78**	--					
14 Total Grains	-.20*	-.06	-.26**	-.23*	.70**	.74**	.49**	.22*	.70**	.69**	.51**	.57**	.33**	--				
15 Red Meats	-.20*	-.05	.15	.09	.39**	.25**	.39**	.46**	.07	.05	.15	.14	.30**	-.01	--			
16 CAL 3mm+	.10	.31**	.71**	.70**	-.18	-.18	-.07	.03	-.23*	-.20*	-.29**	-.29**	-.18	-.31**	.13	--		
17 CAL 4mm+	.08	.32**	.62**	.74**	-.16	-.22*	-.14	-.04	-.20*	-.17	-.26**	-.27**	-.20*	-.28**	.07	.90**	--	
18 CAL 6mm+	.06	.17	.42**	.64**	-.09	-.18	-.14	-.09	-.17	-.16	-.14	-.13	-.15	-.13	.01	.55**	.74**	--

Notes: *=Correlation is significant at the 0.05 level (2-tailed). **=Correlation is significant at the 0.01 level (2-tailed). 4=# of teeth with interproximal sites of 5mm or more. H.acid=Hexadecenoic acid. H. acid, undiff=Hexadecenoic acid, undifferentiated. 16, 17, 18=# of teeth with interproximal sites of clinical attachment loss of 3mm, 4mm, or 6mm or more, respectively.

Figures

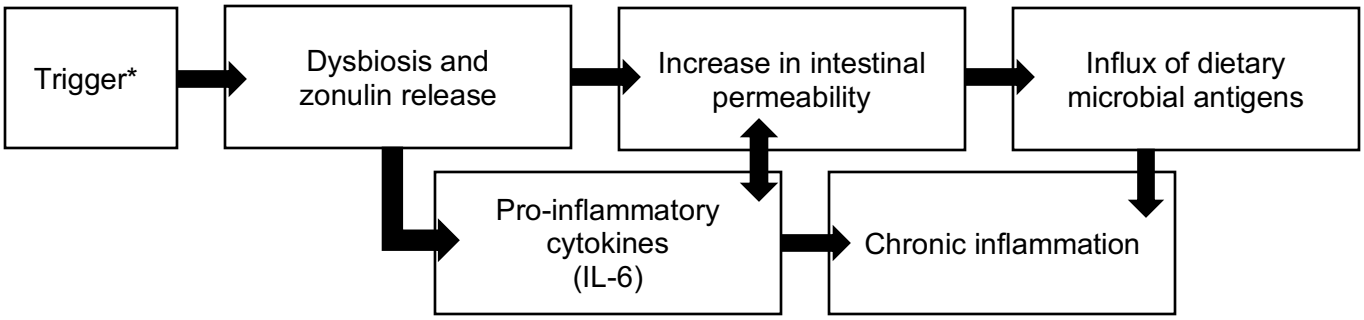

Figure 1. Model of zonulin pathway adapted from Sturgeon and Fasano (2016). *In Celiac disease, the specific trigger is gliadin (a peptide found in gluten). In other diseases, the trigger is not necessarily known.

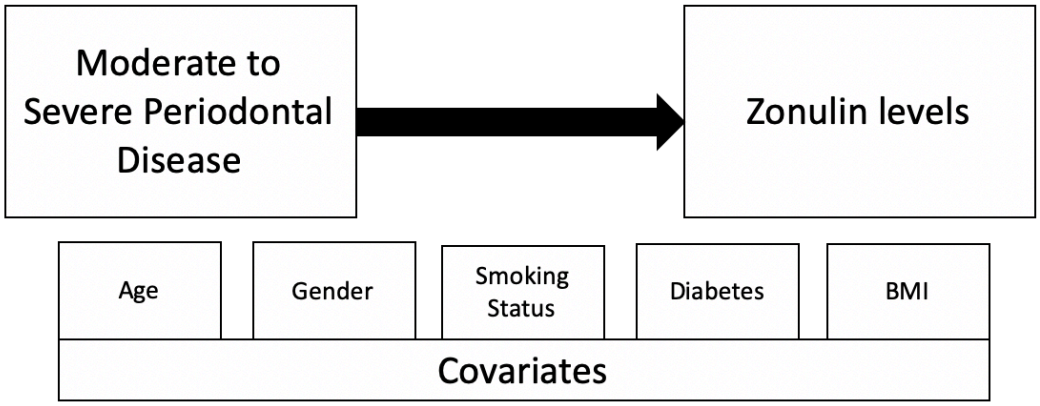

Figure 2. Model depicting hypothesis one. Those with moderate to severe periodontal disease are anticipated to have higher zonulin levels compared to those with no disease or mild disease, all while accounting for covariates.

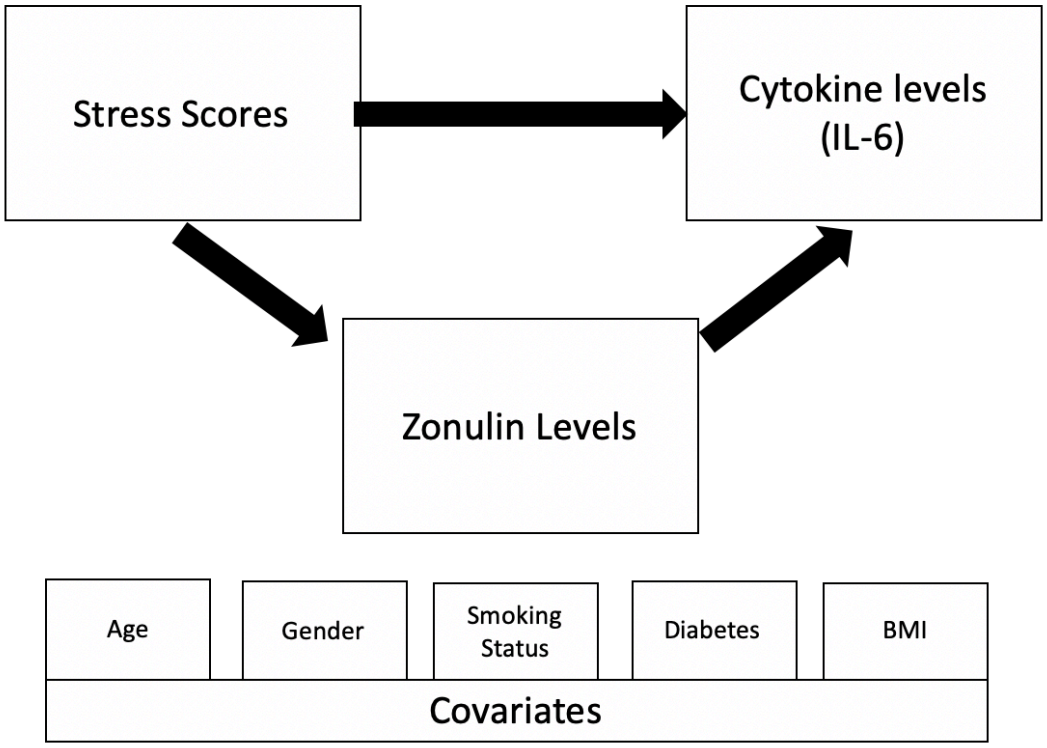

Figure 3. Model depicting hypothesis two. Zonulin is anticipated to meditate the relation between psychological stress and cytokine levels such that increased stress will lead to increased zonulin and thereby, increased cytokines while accounting for covariates.

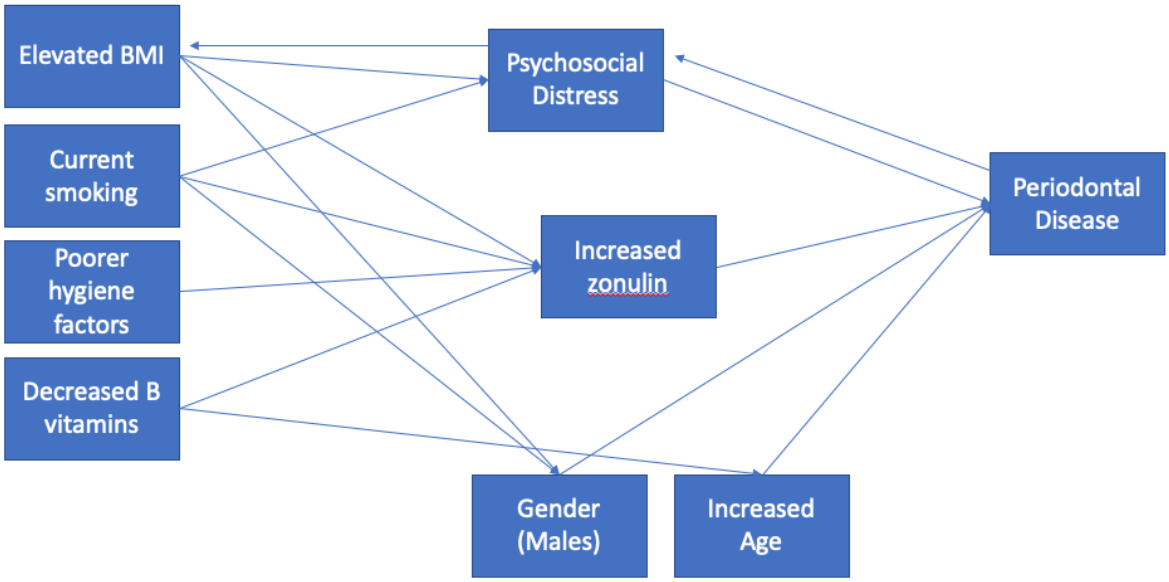

Figure 4. Directed acyclic graph (DAG) depicting theoretical model of the role of zonulin in the etiology and maintenance of periodontal disease that could be used in future work.