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GUT MICROBIOME: THE MISSING INGREDIENT IN CELIAC DISEASE?

ELENA F. VERDU

Farncombe Family Digestive Health Research Institute, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4LB, Canada

SUMMARY

Immune mediated diseases, including celiac disease, are increasingly prevalent affecting 1% of most populations worldwide. Celiac disease is characterized by a loss of oral tolerance to the prolamin family of proteins in wheat, rye, and barley, collectively called "gluten" in genetically susceptible individuals. The only available treatment is a strict gluten-free diet for life, which is difficult to adhere to and not always effective. Genes and gluten are necessary but insufficient to develop celiac disease, as a minority of those individuals genetically at risk will develop it. Microbes, both in the form of infections or alterations in the intestinal microbiome, are considered key additional environmental factors, but the mechanisms remain unclear. Gluten proteins are resistant to proteolysis by host enzymes. Thus, in this review we emphasize the metabolic capacity of the small intestinal microbiota and discuss how it can be altered to facilitate gluten degradation in patients with celiac disease. We also discuss the potential role of other metabolic alterations that can contribute to a proinflammatory environment, such as altered tryptophan metabolism and defective activation of the Aryl hydrocarbon receptor (AhR) pathway. Understanding precise microbiota-driven mechanisms in celiac disease will help develop adjuvant therapies to the gluten-free diet based on optimal microbial modulation of gluten protein metabolism or AhR activation.

A MODEL DISEASE TO UNDERSTAND HOW ENVIRONMENTAL FACTORS MODULATE AUTOIMMUNITY

Celiac disease is an autoimmune condition, triggered by the ingestion of gluten, the collective name given to storage proteins in wheat, rye, and barley. The disease is strongly associated with HLA-DQ, and almost all celiac patients carry the human leukocyte antigen HLA-DQ2.5, HLA-DQ2.2 or HLA-DQ8 (*Iversen* and *Sollid*, 2023). Celiac disease onset can occur at any age, and its presentation may relate to the genetic load, with DQ2.5 homozygosis associated with greater risk for childhood onset, and additional environmental triggers such as infections, antibiotics, especially if concomitant with high gluten exposure (*Verdu* et al., 2015). Once developed, autoimmune features include production of specific autoantibodies called anti-tissue transglutaminase (anti-tTG IgA), and immune-mediated killing of enterocytes. Pathologically, the hallmark lesion is the blunting of small intestinal villi and crypt hyperplasia (atrophy). Clinically, symptomatic celiac disease associates with nutritional deficiencies, and gastrointestinal symptoms, including changes in bowel habits and abdominal pain. However, patients, especially in adulthood, can present with extra-intestinal, and systemic manifestations such as

psychiatric and neurological symptoms, anaemia, infertility, osteoporosis, among others (*Catassi* et al., 2022). Potential celiac disease is characterized by activation of the adaptive immune response with persistently positive anti-tTG IgA antibodies and normal-appearing duodenal mucosa. Unfortunately, asymptomatic cases, despite the presence of small intestinal atrophy are common, and these patients are difficult to diagnose until complications arise related to micronutrient deficiencies (*Theethira* et al., 2014), bone fractures and even the higher incidence of certain cancers (*Catassi* et al., 2022). There is currently no pharmacological treatment for celiac disease, which is managed by a strict gluten-free diet (GFD) for life.

Compared with other autoimmune diseases, such as type 1 diabetes (T1D) or multiple sclerosis, celiac disease is the only one where the main necessary environmental driver, gluten, has been identified, and in which the autoimmune features such as gluten-specific $CD4^{+}T$ cells, anti-tTG antibodies and immune mediated epithelial cell killing, can be turned "on" or "off" by its ingestion or the GFD, respectively. Gluten is a mixture of proteins, that in wheat include gliadins and glutenins, secalins in rye, and hordeins in barley. All gluten proteins are resistant to complete degradation by mammalian enzymes, which results in the production of large peptides with immunogenic sequences, such as the 33-mer peptide, that trigger inflammation in celiac patients (*Shan* et al., 2022). Partially digested gluten peptides translocate the mucosal barrier and are deamidated by tTG (*Dieterich* et al., 1997). Gluten peptide deamidation increases their affinity to HLA-DQ2 or DQ8 in antigen-presenting cells, initiating the T-cell-mediated inflammation. HLA-DQ2 or DQ8 is therefore the second, necessary factor to develop celiac disease. About 30-40% of the worldwide population carry one, or a combination of these genes, and therefore are "at risk" to develop it when consuming a gluten. However only a minority of those at risk (3-4%), and consuming gluten, will develop celiac disease. This, together with the fact celiac disease prevalence has quadrupled in the past 40 years, in parallel with the increase in prevalence with other autoimmune conditions, such as T1D with which it associates (*Verdu* and *Danska*, 2018), suggests other environmental factors are at play.

THE EMERGING ROLE OF THE MICROBIOME IN CELIAC DISEASE PATHOGENESIS

Both viral and bacterial infections have been associated with higher celiac disease risk, and this phenomenon has been reviewed extensively elsewhere (*Caminero* et al., 2019). In the past decade, microbiome alterations have been described in celiac patients (*Verdu* and *Schuppan*, 2021), but the mechanisms through which changes in human-associated microbial communities influence disease pathogenesis have been elusive.

ROLE OF MICROBIAL METABOLISM IN CELIAC DISEASE

We have shown that duodenal bacteria participate in gluten metabolism, and that depending on the type of bacteria present, microbial enzymes may yield peptides with enhanced or reduced immunogenicity. We found that elastase

from the opportunistic pathogen *Pseudomonas aeruginosa* degrades gluten, producing immunogenic peptides that better translocate the epithelial barrier, and stimulate gluten-specific T cells from patients with celiac disease. On the other hand, *Lactobacillus* species, isolated from a healthy control, further detoxify the *P. aeruginosa* generated immunogenic peptides (*Caminero* et al., 2016). These findings are supported by our follow-up study, where we showed that although duodenal microbiota composition in active CeD patients differs from that in non-celiac controls, the changes are specific to localized regions of the small proximal intestine, namely first, second and third portion of the duodenum. Moreover, duodenal microbiota from celiac patients also had an altered predicted gluten proteolytic profile, which was location specific. The altered proteolytic profile translated to functional differences *in vivo*, as mice colonized with duodenal microbiota from active celiac patients had impaired capacity to degrade gluten, while mice colonized with duodenal microbiota from control subjects, including those with genetic predisposition for celiac disease, effectively degraded gluten (*Constante* et al., 2022). Thus, taken together, these results indicate that microbial gluten metabolism is a) location specific and, b) associated with the condition of active celiac disease. It is possible altered microbial "glutenasic" function is both driver and consequence of inflammation in the small intestine. This insight has clear preventive and therapeutic implications. This mechanism could be targeted for preventive or therapeutic purposes, based on increasing the effectiveness of gluten detoxification by microbes adapted to survive in the duodenum.

In addition to microbial gluten metabolism, other pathways of altered microbial metabolism could be at play in celiac disease. Tryptophan is an essential amino acid provided by foods such as poultry and cruciferous vegetables. After digestion, tryptophan becomes available for further metabolism by the host through the kynurenine or serotonin pathway, or by certain gut microbes, such as lactobacilli. Microbial metabolism of tryptophan results in the production of indole and its derivatives (indole-3-aldehyde (IAld), indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-acetaldehyde (IAAld), indole-3-lactic acid (ILA) and indole-acrylic acid), as well as tryptamine, many of which are ligands for the aryl hydrocarbon receptor (AhR) (*Agus* et al., 2018). AhR signalling contributes to maintaining intestinal homeostasis through its role in epithelial renewal and maintaining barrier integrity. Thus, the indole pathway, mainly dictated by microbial metabolism of tryptophan, is beneficial for the host. Because host and microbial tryptophan metabolic pathways are interconnected and in equilibrium, a reduction in the microbial metabolic pathway of tryptophan could provide more substrate to the remaining two metabolic pathways. Indeed, this has been shown in several chronic inflammatory diseases, where an increase in the kynurenine pathway, has been demonstrated and proposed to be proinflammatory (*Tennyson* et al., 2016; *Natividad* et al., 2018). However, a recent paper has suggested some kynurenine metabolites could play an anti-inflammatory role in IBD (*Michaudel* et al., 2023). Thus, interventions that target tryptophan metabolic pathways need to consider the complexity of its metabolism, the location of disease and thus dietary supplementation may be an attractive approach, especially for small intestinal conditions, as most microbe-dietary interactions (except for fermentable fibres) will occur in the upper gastrointestinal tract.

We demonstrated that patients with active celiac disease have decreased AhR expression in the duodenum (*Natividad* et al., 2018). We also showed that the microbiota of active CeD have impaired capacity to metabolize tryptophan to produce AhR ligands, resulting in reduced capacity of the microbiota to activate the AhR pathway, and reduced expression of host genes related to AhR activation. Lower abundance of lactobacilli, known AhR ligand producers, has been described in the small intestine and faeces of patients with active celiac disease. AhR ligand production, AhR activity, and expression of AhR pathway genes were only partially restored 2 years after a GFD. We also demonstrated that in mice expressing the celiac disease risk gene, DQ8, a diet supplemented with 1% tryptophan increased lactobacilli abundance and indole production while ameliorating gluten immunopathology. Finally, in a mouse model, tryptophan supplementation increased endogenous lactobacilli, increased AhR ligands (indoles) and increased AhR activation in the small intestine (*Natividad* et al., 2018). Moreover, tryptophan supplementation improved gluten immunopathology in mice expressing celiac disease risk genes. A clinical trial is underway (https://clinicaltrials.gov/study/NCT03566238) to study the efficacy of tryptophan supplementation in celiac patients that are non-responsive to a gluten-free diet for more than 1 year. We hypothesize that tryptophan supplementation will lead to resolution of persistent symptoms in celiac disease, while increasing taxa that metabolize tryptophan and restore AhR signalling.

LIMITATIONS OF THE GFD AND NEW THERAPIES IN CED

The only treatment for celiac disease is a strict gluten-free diet (GFD) and dietary compliance is essential, not only for intestinal mucosal recovery and alleviation of symptoms, but also for the prevention of complications such as anaemia, osteoporosis, and small bowel lymphoma (*Michaudel* et al., 2023). However, a GFD is an imperfect treatment, as it is difficult to follow and expensive, resulting in high non-adherence rates. Accidental contamination is common and small amounts of gluten (~50 mg) cause inflammation (*Lindfors* et al, 2019; *Lamas* et al., 2020). Mucosal recovery after starting a GFD is slow, and more than 60% of patients have persistent mucosal inflammation even after 5 years of a GFD (*Silvester* et al., 2020). This is clinically important because long-term, low-grade mucosal injury increases bone fracture risk and nutritional deficiencies. In addition, a large proportion of celiac disease patients are non-responders to a GFD or become symptomatic after initial response (*Catassi* et al., 2007; *Rubio-Tapia* et al., 2010; *Oza* et al., 2016; *Stasi* et al., 2016). Annual health care costs are higher in celiac disease than in non-celiac patients, mainly related to poorly controlled disease. Celiac disease therefore has a significant health, social, and economic burden, highlighting the need for novel or adjuvant therapeutics in addition to GFD. Importantly, celiac disease patients have a very high perceived burden of treatment and desire an adjuvant treatment to the GFD (*Leffler* et al., 2007; *See* et al., 20156; *Pinto-Sanchez* et al., 2015). This reality presents a clearly unmet need for patients with celiac disease which is also evidenced by the intense research and investment into the development of adjuvant therapies to the GFD.

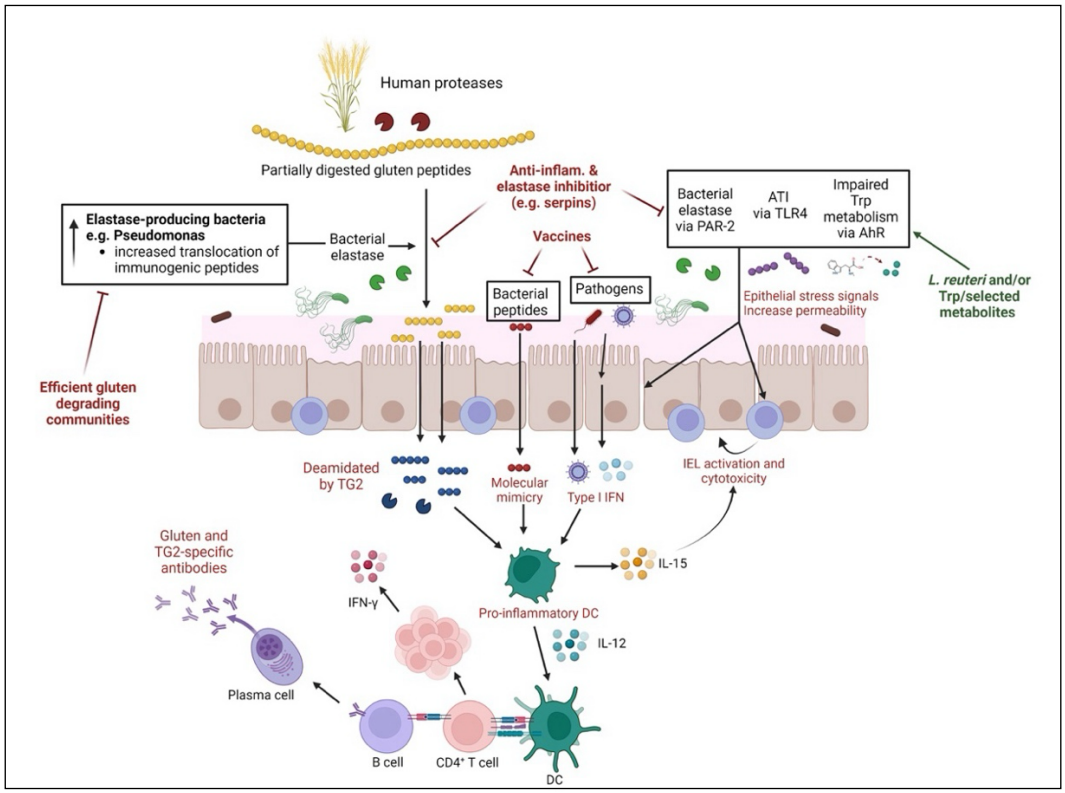

Figure 1: Potential Microbial Therapeutic targets for celiac disease. Owing to the detailed knowledge of celiac immunobiology and microbe-gluten interactions, several druggable targets have emerged. These include, development of gluten degrading communities that effectively detoxify gluten immunogenic sequences, inhibitors of bacterial elastase (e.g. serpins), pre- or probiotic combinations of tryptophan metabolite producers and vaccines aiming at preventing viral infections that could act as additional triggers of autoimmunity. (Adapted from: *Verdu* and *Schuppan*, Nat. Struct. Mol. Biol. 27, 5-7, 2020).

Major pharmaceutical companies have launched pipelines to develop adjunct therapies to the GFD (*Guandalini* et al., 2016) that currently include tTG inhibitors (*Pinto-Sanchez* et al, 2021), TAK-062 a computationally designed enzyme that targets 2 gluten proteins (*McCarville* et al, 2015), TAK-101 a gluten protein shielded within a polymer-based nanoparticle that aims to restore tolerance to gluten (*Schuppan* et al., 2021), and anti-IL-15 antibodies to limit intestinal epithelial cell destruction and for the treatment of refractory celiac disease (*Pultz* et al., 2021) in clinical trials. The use of probiotics for the treatment of celiac disease have previously been proposed (*Cellier* et al., 2019; *Murray* et al., 2023) and shown to be safe if the probiotic is certified gluten free, but this application has not been based on deep knowledge of mechanisms of action, and currently no recommendations of specific probiotics can be made (*De Angelis* et al., 2006). Based on our basic and translational results, we propose to target dysregulated microbial metabolism by developing and characterizing duodenal communities that efficiently degrade gluten or enhance AhR activation, through the supplementation of tryptophan or specific indole metabolites (Figure 1).

MAIN CONCLUSIONS

The small intestinal microbiome has emerged in the last decade as a recognized cofactor in celiac disease pathogenesis. Celiac disease is a unique autoimmune disorder, with a *known* environmental trigger (gluten) that is responsible for the generation of $CD4^+$ T cell mediated inflammation and development of autoantibodies in genetically at-risk individuals. Immune mediated killing of intestinal epithelial cells contributes to intestinal atrophy, and cofactors that are independent of gluten may modulate risk. Thus, celiac disease is an ideal model disease to advance microbiomics and therapeutic microbiology towards clinical applications by targeting precise microbial mechanisms that impact key steps in its pathogenesis.

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