HYPOTHESIS
Insights & Perspectives

From celiac disease to coccidia infection and vice-versa: The polyQ peptide CXCR3-interaction axis

Martin A. Lauxmann¹,²  Diego S. Vazquez³,⁴  Hanna M. Schilbert⁵,⁷  Pia R. Neubauer⁵  Karen M. Lammers⁶  Veronica I. Dodero⁵

¹ Institute for Biochemistry, Brandenburg Medical School (MHB) Theodor Fontane, Germany  
² Department of Nephrology, Campus Clinic Brandenburg, Brandenburg Medical School (MHB) Theodor Fontane, Germany  
³ Grupo de Biología Estructural y Biotecnología (GBEyB-IMBICE), Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Bernal, Buenos Aires, Argentina  
⁴ Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Autónoma de Buenos Aires, Argentina  
⁵ Department of Chemistry, Organic Chemistry OCIII, Universität Bielefeld, Universitätsstraße 25, Bielefeld, Germany  
⁶ Tubascan Ltd., Amsterdam, Netherlands  
⁷ Genetics and Genomics of Plants, Center for Biotechnology (CeBiTec) & Faculty of Biology, Universitätsstraße 25, Bielefeld 33615, Germany

Correspondence
Veronica I. Dodero, Department of Chemistry, Organic Chemistry OCIII, Universität Bielefeld, Universitätsstraße 25, 33615 Bielefeld, Germany.  
Email: veronica.dodero@uni-bielefeld.de

Martin A. Lauxmann and Diego S. Vazquez contributed equally to this work.

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Abstract
Zonulin is a physiological modulator of intercellular tight junctions, which upregulation is involved in several diseases like celiac disease (CeD). The polyQ gliadin fragment binds to the CXCR3 chemokine receptor that activates zonulin upregulation, leading to increased intestinal permeability in humans. Here, we report a general hypothesis based on the structural connection between the polyQ sequence of the immunogenic CeD protein, gliadin, and enteric coccidian parasites proteins. Firstly, a novel interaction pathway between the parasites and the host is described based on the structural similarities between polyQ gliadin fragments and the parasite proteins. Secondly, a potential connection between coccidial infections as a novel environmental trigger of CeD is hypothesized. Therefore, this report represents a promising breakthrough for coccidial research and points out the potential role of coccidian parasites as a novel trigger of CeD that might define a preventive strategy for gluten-related disorders in general. Also see the video abstract here: https://youtu.be/oMaQasStcFI

KEYWORDS
Chemokine receptor CXCR3, coccidiosis, Eimeria, gliadin, gluten-related disorders, polyQ sequence, zonulin

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INTRODUCTION

Zonulin is a physiological modulator of intercellular tight junctions involved in intestinal macromolecule trafficking and regulation of the intestinal barrier function connected with several autoimmune diseases, including celiac disease (CeD). The ongoing CeD disease process gives rise to a variable degree of intestinal damage. The mucosa progresses from intraepithelial and lamina propria villous infiltration of inflammatory cells (intraepithelial lymphocyte) to villous atrophy, crypt hyperplasia, and flattening after the ingestion of gluten. Gluten is a complex protein mixture present in wheat (gliadins), rye (secalins), barley (hordeins), and some varieties of oats (avenins). Those intestinal epithelial changes have been systematically classified (Marsh stages I through III) to diagnose the disease. Recently, it has been reported that non-celiac wheat sensibility (NCWS) is also characterized by an increase in intestinal permeability independently from the existence of CeD; however, the molecular mechanism is unknown. It has been uttered that wheat gliadin is handled by the host as if it were a microorganism, for instance, because of the early innate immune activation it induces. With regard to increased permeability, pathogenic bacteria have been shown to exert this effect, for instance, Salmonella typhimurium and Vibrio cholerae. Moreover, chronic inflammatory bowel diseases, ulcerative colitis, and Crohn’s disease, which often occur following an episode of traveler’s diarrhea, show increased intestinal permeability and are thought to reflect misbalances in the microflora or loss of its tolerance.

The traveler’s diarrhea is associated with human-related coccidian parasites mainly, cyclosporiasis, and is reported worldwide. In general, coccidian parasites are responsible for diseases targeting the digestive tract of vertebrates causing acute enteritis of varying severity. The most well-studied host-parasite system is chicken coccidiosis because it has a substantial adverse economic impact and an estimated global cost of more than 2 billion US dollars per year. Interestingly, Chen et al. have reported that broiler chickens subjected to a rye-wheat-barley diet experienced a clear gut barrier failure with higher ileal mucosal zonulin gene expression compared to those fed with maize, but the reason remains unclear. Besides these findings, CeD and coccidiosis share many similarities summarized in Table 1.

Up to now, bacterial infections and gliadin proteins are known powerful triggers of the zonulin release leading to intestinal permeability, however, it is observed in other many diseases. The upregulation of zonulin facilitates the paracellular translocation of diverse stressors (dietary) antigens and pathogens from the intestinal lumen into the lamina propria leading to immune response, inflammation (tissue damage), and possibly immune dysfunction. During the early stages of CeD, zonulin is released from the epithelium via gliadin binding to apically expressed CXCR3 chemokine receptors. At least two different 20-mer gliadin fragments were shown to bind to CXCR3 and increase intestinal permeability. Among them, the polyglutamine (polyQ) P4022 peptide fragment has been reported. Importantly, the CXCR3 chemokine receptor is luminal abundantly upregulated in active CeD, but the underlying reasons are not clear. However, after the implementation of a gluten-free diet, CXCR3 expression returns to basal levels. Additionally, since the CXCR3 is an interferon-inducible chemokine receptor expressed on epithelial cells and various immune cell types, it is hypothesized that this receptor is originally designed for innate defense against microorganisms.

By a stringent Basic Local Alignment Tool search (BLAST) of polyQ P4022 against the non-redundant protein database excluding the Gramineae family, we found high sequence identity (near 90% amino acid identity) and significance (E-value of 7e−10) of the α-gliadin polyQ peptide with proteins from different species of the enteric protozoa Eimeria related to coccidian parasites in chicken, and other vertebrates. More than 100 different polyQ-containing proteins were

| TABLE 1 | Similarities between celiac disease and coccidiosis |
|----------------|-----------------------------------------------------|
| **Similarities between coccidiosis and celiac disease** | **Route through body** |
| | Targeting the intestinal lumen (epithelial cells) through oral intake. Gastrointestinal digestion resistance, and detection in excrements, urine and faeces. |
| **Localization in the gastrointestinal tract** | Intestinal epithelium and ability to reach the lamina propria. |
| **Immune response** | Induction of an inflammatory response activates an innate and adaptive immune response. |
| **Involved cells and molecules** | Involvement of NK cells, DC, epithelial cells, heterophils, neutrophils, and macrophages. Activation of TLR receptors (TLR4). Production of IFN-γ, TNF-α, TGF-β, and cytokines IL-2, IL-6, IL-8, IL-15, and IL-16. Eosinophilia and decrease in the level of anti-IgA. Upregulation of CxCL9-11 chemokines. |
| **Gastrointestinal, metabolic disturbances** | Loss of epithelial cells, villous atrophy, crypt destruction, infiltration of lamina propria with inflammatory cells, and gut barrier failure. |
| **Symptoms** | Fever, malaise, abdominal pain, diarrhea, steatorrhea, and weight loss. |
| **Prevalence** | CeD pooled global prevalence is 1.4%. In endemic countries, Cyclospora infection has an average rate of 1.7%. |

Abbreviation: TLR, Toll-like receptor; NK, natural killer cell; DC, dendritic cell; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin.
Summary of the proposed hypothesis. (A) Normal epithelial cells with low expression of CXCR3 chemokine. (B) Upregulation of zonulin expression and its release is triggered by binding PolyQ proteins to the CXCR3, thus contributing to the parasite’s early entrance into lamina propria (pathway 2) accompanied by the other components of the intestinal milieu, leading to immune response, inflammation (tissue damage) and priming. A plausible mechanism is that CXCR3 binding leads to zonulin activation via the recruitment of MyD88 and the physical association of CXCR3 with this adaptor protein. MyD88 is originally associated with TLRs and IL-1R family, and thus with NfKB signaling. MyD88 has also been associated with the Interferon-gamma Receptor, which induces a pro-inflammatory response. Furthermore, zonulin has an EGF-like motif in it and transactivates EGFR via PAR2. Both PAR-2 and EGFR cooperate in opening of the tight junctions leading to the observed increase of intestinal permeability. (C) As innate response of the host towards coccidial infection the CXCR3 remains highly overexpressed in the epithelial cells after the coccidiosis episode. (D) Gliadin PolyQ sequences present in the diet bind to the highly expressed CXCR3 receptors, starting zonulin mediated increase of the permeability, leading to the massive flux of the intestinal milieu in lamina propria which results in immune response, inflammation, tissue damage and in the case of susceptible individuals to immune dysfunction leading to CeD or the different gluten-related disorders. Here, the presence of other toxic and immunodominant gliadin fragments activate the innate and adaptive response observed in CeD.[22] Created with BioRender.com

Based on these findings and an extensive literature search, we present here two novel scenarios that may open new research areas in coccidian research and in gluten-related disorders treatment and prevention:

1. Coccidian parasites are a new trigger of zonulin upregulation and release via the polyQ gliadin-CXCR-type chemokine pathway, thus representing an underappreciated mechanism of coccidial infection (Figure 1 A-C).
2. Human coccidian parasites are an environmental trigger or predisposing factor for CeD and other gluten-related disorders by the upregulation of CXCR3 (Figure 1 A-D).

Importantly, the coccidiosis episode as a predisposing factor to CeD, may explain the fact that only 1.4% of the general population develop CeD although that HLA-DQ2 and/or HLA-DQ8 haplotype is common in the general population (30%) which shows that gluten consumption alone is essential but not sufficient to develop CeD.

DISCLOSING THE ROLE OF POLYQ GLIADIN PEPTIDE/CXCR3 INTERACTION AS NOVEL PATHWAY IN COCCIDIOSIS

Gliadin polyQ sequence is found in proteins of the enteric protozoa Eimeria and Cyclospora

We performed a BLASTP search against the non-redundant protein database, excluding the Gramineae family (taxid: 4479).[47] It was expected to find several polyQ sequences since such imperfect polyQ
TABLE 2  BLASTP-search hits for the polyQ P4022 sequence (120QQQQQQQQQQLQQ139) of γ-gliadin

| Hit # | Description | Species               | NCBI access number | Matching sequence | Subcellular /topological localization | Topology a |
|-------|-------------|-----------------------|--------------------|-------------------|---------------------------------------|------------|
| 1     | tRNA-splicing endonuclease positive effector | E. brunetti          | CDJ52926.1         | 422QQQQQQQQQQLQQ | T (double-pass)/E                        | I/I        |
| 2     | Hypothetical protein                        | E. maxima             | XP_013334286.1     | 924QQQQQQQQQQLQQ | T (single-pass)/I                       | I/I        |
| 3     | Hypothetical protein                        | E. mitis              | XP_013355649.1     | 26QQQQQQQQQQLQQ | I/I                                   | I/I        |
| 4     | Hypothetical protein EPH                    | E. praecox            | CDI76686.1         | 87QQQQQQQQQQLQQ | I/E                                   | E/E        |
| 5     | Translation initiation factor 3 subunit 10  | E. maxima             | XP_013336659.1     | 264QQQQQQQQQQQQVQQ282 | I/I                                     | I/I        |

Panel 1: Eimeria proteins associated with chicken coccidiosis

Panel 2: Proteins associated with human coccidiosis

| Hit # | Description                  | Species              | NCBI access number | Matching sequence | Subcellular /topological localization | Topology a |
|-------|------------------------------|----------------------|--------------------|-------------------|---------------------------------------|------------|
| 1     | Plectin                      | C. cayetanensis      | XP_026192064.1     | 124QQQQQQQQQQLQQ | E/E                                   | I/I        |
| 2     | Transcription factor kayak   | C. cayetanensis      | XP_026189818.1     | 258QQQQQQQQQIQQQ | E/E                                   | I/I        |
| 3     | Myb-like protein P           | C. hominis           | OLQ19473.1         | 2230QQQQQQQQQQLQQ | I/I                                   | I/I        |
| 4     | Myb-like DNA-binding domain  | C. hominis TU502     | XP_665879.1        | 1851QQQQQQQQQQLQQ | I/I                                   | I/I        |
| 5     | Hypothetical protein         | C. parvum Iowa II    | QOY40252.1         | 2284QQQQQQQQQQLQQ | I/I                                   | I/I        |
| 6     | Ubiquitin C-terminal hydrolase| C. parvum Iowa II    | XP_626187.1        | 660QQQQQQQQQQLQQ | I/I                                   | I/I        |

Note: Panel 1: Best five output proteins (≥ 90% amino acid identity, E-value < 1e−9) retrieved from a stringent BLASTP[47] search against the non-redundant protein database excluding the Graminaceae family (taxid: 4479) using the P4022 sequence as a query in non-gluten related proteins. P4022-matching sequences are depicted indicating the flanked amino acids (uppercase numbers) and mismatch residues (underlines). Panel 2: Two best proteins > 85% amino acid identity, E-value < 7e-10) retrieved from a stringent BLASTP[47] search against the non-redundant protein database excluding the Graminaceae family (taxid: 4479) using the P4022 sequence as a query in non-gluten related proteins. P4022-matching sequences are depicted indicating the flanked amino acids (uppercase numbers) and mismatch residues (underlines).

Abbreviation: T: Transmembrane; E: extracellular; and I: intracellular.

aSubcellular localization prediction was performed with Protter.[61]

repeats have been found in at least 17 eukaryotic proteomes.[53] The five top hits with high significance (E-value < 1e−9) and high sequence similarity (> 85% identity) to the celiac γ-gliadin polyQ P4022 peptide included proteins from pathogenic organisms and are presented in Table 2, Panel 1. Interestingly, all best five BLASTP-output proteins with up to two mismatches in the P4022-matching sequence belong to the genus of protozoa Eimeria, phylum Apicomplexa. As of September 2020, other proteins were also retrieved from the BLASTP search, not only from Eimeria species but also from other organisms. The ones reported here were consistently ranked as top 5 hits over several BLASTP searches performed over time.

Coccidia of the family Eimeriidae, such as Eimeria species, are monoxenes (one-host parasites), a group of obligate intracellular parasites of great interest in vertebrates causing acute enteritis and coccidiosis.[25–28] Seven Eimeria species are recognized to affect chicken: E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox, and E. tenella.[54] The different Eimeria species have distinctive characteristics in prevalence, pathogenicity, infection in the intestine, and oocyst morphology.[28,55] Eimeria tenella affects the paired caeca, leading to extensive bleeding. The presence of lesions due to the second generation of schizonts deeply compromises the intestinal epithelium within the lamina propria.[56] Eimeria maxima infects the mid-small intestine, leading to a thickening of the intestinal lining accompanied by a mucoed to bloody exudate. Eimeria mitis and E. praecox both infect the upper small intestine. Eimeria brunetti and E. necatrix affect the distal small intestine and the colon, being able to cause severe pathology.[28]

From the BLASTP query, only the tRNA-splicing endonuclease positive effector (Hit 1) and the eukaryotic translation initiation factor 3 (Hit 5) have known biological functions. The tRNA-splicing endonuclease positive effector contains a domain belonging to the P-loop containing nucleoside triphosphate hydrolases (InterPro Domain: IPR027417), which are DEAD-like helicases involved in ATP-dependent RNA or DNA unwinding.[57] Moreover, the tRNA-splicing endonuclease positive effector bears two DEXXQ-box helicase domains of the RNA/DNA helicase senataxin (SETX). SETX is involved in transcription, neurogenesis, and antiviral response. Mutations in SETX have been linked to two neurodegenerative disorders: ataxia with oculomotor apraxia type 2, and amyotrophic lateral sclerosis type 4.[58] The eukaryotic translation initiation factor 3, subunit 10 is a component of the eukaryotic translation initiation factor 3 (eIF-3) complex. It participates in several steps of the initiation of protein synthesis.[59] It may regulate cell cycle progression and cell proliferation[60] which
may be important steps to intervene in the parasite infection. Up to now, no pathogenic role of the identified proteins has been reported. However, it requires to be investigated, considering that only their gene sequences were uploaded to the GenBank in October 2013 as part of a genomic analysis that studies the causative agents of coccidiosis in chickens.

Until now, only four coccidian parasites have been reported to infect humans: *Cystoisospora belli*, *Cyclospora cayetanensis*, and both *Cryptosporidium hominis* and *Cryptosporidium parvum*. *Cyclospora cayetanensis* is currently considered the “human Eimeria” that causes human coccidiosis. Accordingly, based on a reevaluation of the parasite molecular taxonomy, it has been suggested that the human-associated Cyclospora is closely related to *Eimeria* species, and it has to be considered as a mammalian *Eimeria* species and associated with traveler’s diarrhea. Cyclosporiasis has been reported worldwide in both developed and developing countries, but it is most common in tropical and subtropical areas with a high prevalence in Turkey 5.7% and Peru 4.3%. In 2010, the prevalence rate in endemic areas of 22 countries ranged from 0% to 13% (average 1.7%). Notably, at least 30 outbreaks of cyclosporiasis together with a second coccidia species, cryptosporidiosis, were associated with contaminated water and food over the last two decades worldwide. In North America, 11,500 cases of cyclosporiasis were registered between 2016–2019. In 2011, Sweden reported the two most extensive cryptosporidiosis episodes ever in Europe, affecting around 47,000 people. In the case of CeD, a recent meta-analysis showed that the pooled global seroprevalence is 1.4%. Interestingly, among the European countries, Sweden reported a higher prevalence of patients with CeD (2.6%) than the European average. In Peru where *cyclosporiasis* is endemic, a recent study shows a CeD prevalence of 1.2% which is one of the highest in South America.

Next, a phylogenetic tree was built using the *Eimeria* P4022-like-containing proteins and 17 BLASTP-based homologous proteins and revealed that three of the five *Eimeria* proteins (tRNA-splicing endonuclease positive effector from *Eimeria brunetti*; Hypothetical protein from *Eimeria mitis*; and eukaryotic translation initiation factor 3, subunit 10 from *Eimeria maxima*) clustered with their homologous proteins of the human infecting parasite *Cyclospora cayetanensis* (Figure S1). This suggested that in terms of phylogeny, they seem to be orthologous proteins, matching with the fact that *Cyclospora cayetanensis* is considered the “human Eimeria” causing human coccidiosis. Therefore, we performed a second search of the polyQ P4022 sequences restricting the BLASTP search to reported human coccidian parasites retrieving the following outputs (Table 2, Panel 2): *Cystoisospora belli* (taxid: 482538), *Cyclospora cayetanensis* (taxid: 88456), and both *Cryptosporidium hominis* (taxid: 237895) and *Cryptosporidium parvum* (taxid: 5807). We found more than 100 different polyQ P4022-containing proteins bearing at least six of them with both high sequence identity (> 85%) and significance (E-value < 1e−10) to the gliadin polyQ peptide P4022 (Table 2, Panel 2). No protein belonging to *Cystoisospora belli* was retrieved from the BLASTP search, perhaps because of a lack of data. The NCBI protein database for this organism only consists of one protein.

Regarding their functions, until now, none of the six selected proteins from human coccidia (Table 2, Panel 2) has been linked to enteric pathogenic processes. Plectin is considered a universal biological organizer that links several elements of the cytoskeleton and the transcription factor kayak has been proposed to control the circadian behavior in *Drosophila*. The transcription factor Myb-like protein P, and its Myb-like DNA-binding domain is part of a large gene family of transcription factors with highly conserved DNA binding domains found in insects, higher plants and vertebrates. They are often involved in regulating differentiation and proliferation and are implicated in many tumors. The ubiquitin C-terminal hydrolase of the cysteine proteinase fold seems to be involved in ubiquitin-dependent protein catabolic processes. In August 2021, we performed a new BLASTP search to find new related proteins in an attempt to identify their functions, but not significant similarities were found.

### Searching for structural characteristics of the coccidian polyQ P4022-like-containing proteins

The high sequence similarity of proteins of the coccidiosis-causing *Eimeria* species and proteins of human coccidian parasites with the α-gliadin polyQ P4022 peptide paves the way for a possible sequence-related mechanism. To interact with partner proteins (e.g., receptors), these sequences need to be exposed to the solvent (see Table 2). Considering the lack of structural information, we performed some initial bioinformatic analysis to search for evidence about structural similarities between gliadin and the discovered proteins. In particular, the localization of the polyQ sequence would support its alleged role in coccidia pathomechanism.

The primary sequence analysis of the P4022-like-containing proteins indicated that they are polyQ proteins, seven of them containing multiple polyQ repeats. According to the definition of Ramazzotti et al., most of them are classified as impure polyQ repeats. In this regard, the interruption of pure primary polyQ sequences with specific amino acids (up to 25% out of the total polyQ sequence) like leucine, makes the structure less aggregation-prone. A remarkable example is illustrated with the delay of the onset and severity of human neurodegenerative diseases, such as ataxin 1 polyQ involved in Spinocerebellar ataxia type 1. In the previously reported α-2-gliadin model (Figure 2), the region 120–139 that corresponds to P4022 is solvent-exposed, reinforcing the idea that the polyQ stretch could directly interact with target proteins. For all the eleven proteins obtained in the BLASTP search, we performed structural modeling using the PHYRE2 server. Only two protein sequences were obtained with high confidence (more than 90%): the eukaryotic translation initiation factor 3 subunit 10 from *Eimeria maxima* and the Myb-like DNA-binding domain from *Cryptosporidium hominis*. The three-dimensional modeling of the two P4022-containing proteins shows that the PolyQ-matching sequences...
FIGURE 2  Location of the polyQ region in the 3D molecular models. The α-2-gliadin from Triticum aestivum was previously modeled.[79] The eukaryotic translation initiation factor 3 subunit 10 from E. maxima and the Myb-like DNA-binding domain from C. hominis were modeled by the PHYRE2 server[80] in the intensive mode and energy minimized in the same conditions as we previously described[79] to remove any clashes. Models were prepared using VMD 1.9.3[81] represented in surface and colored by secondary structure content as orange (helix), cyan (β-sheet), and white (random-coil). The polyQ and P4022 homology regions are shown in balls and sticks for clarity.

FIGURE 2  Location of the polyQ region in the 3D molecular models. The α-2-gliadin from Triticum aestivum was previously modeled.[79] The eukaryotic translation initiation factor 3 subunit 10 from E. maxima and the Myb-like DNA-binding domain from C. hominis were modeled by the PHYRE2 server[80] in the intensive mode and energy minimized in the same conditions as we previously described[79] to remove any clashes. Models were prepared using VMD 1.9.3[81] represented in surface and colored by secondary structure content as orange (helix), cyan (β-sheet), and white (random-coil). The polyQ and P4022 homology regions are shown in balls and sticks for clarity.

are located in a helical context and partially exposed to the solvent (Figure 2).

Sequence-based disorder probability analysis of the P4022-like-containing proteins from Eimeria and human coccidia showed that the matching regions with the polyQ P4022 sequence are primarily located in disordered areas, making them accessible to the solvent (Figures 3B and 3C).

Five proteins have coiled-coil domains (α-helical super secondary structures) overlapping with the polyQ stretch of the P4022-like sequences (Figure 3B and 3C). Such structural overlapping is known to regulate aggregation, insolubility and activity of polyQ proteins.[82] Thus, these structural features suggest that the P4022-matching sequences may be accessible to interact with the host intestinal epithelium directly, as gliadin does (Figure 3A).

There may be two scenarios in which the solvent-accessible P4022-matching sequences may interact with the host intestinal epithelium without previously digestion of the P4022-like-containing proteins: (I) the P4022-like sequence is part of an extracellular domain of a transmembrane protein; and (II) the P4022-like sequence is part of a cytosolic protein which is secreted in the proximity of the host intestine. This last situation was reported for many Eimeria invasion-related proteins secreted from parasite apical organelles to fulfill the parasite invasion process, that is, adhesion/locomotion, invasion of the host cell, and intracellular multiplication in the host intestine.[28,55,63] In this regard, computational predictions showed that two of the Eimeria P4022-containing proteins (Hits 1 and 2 of Table 2, Panel 1) are transmembrane proteins, exposing only one of them (Hit 1) the P4022-matching sequence to the extracellular space of the protozoa (Figure S2). Moreover, one of the five Eimeria proteins is predicted to be extracellular (Hit 4), exposing the P4022-matching sequence to the parasite’s extracellular space. The other two Eimeria proteins are likely to be cytosolic (Hit 3 and 5), being their P4022-like sequences immersed into the protozoa cytoplasm space (Table 2, Panel 1).

FIGURE 2  Location of the polyQ region in the 3D molecular models. The α-2-gliadin from Triticum aestivum was previously modeled.[79] The eukaryotic translation initiation factor 3 subunit 10 from E. maxima and the Myb-like DNA-binding domain from C. hominis were modeled by the PHYRE2 server[80] in the intensive mode and energy minimized in the same conditions as we previously described[79] to remove any clashes. Models were prepared using VMD 1.9.3[81] represented in surface and colored by secondary structure content as orange (helix), cyan (β-sheet), and white (random-coil). The polyQ and P4022 homology regions are shown in balls and sticks for clarity.

A NOVEL INTERACTION PATHWAY OF COCCIDIA THROUGH THE POLYQ/CXCR3 AXIS

Coccidiosis: Eimeria and Cyclospora parasites

The life cycle of all coccidian parasites is similar in all species. It starts when partially sporulated oocysts are shed in feces and then into the environment, persisting for several weeks or months.[84,85] Once the infective forms of the oocysts, mostly sporulated, reach the host intestine through contaminated food and/or water consumption, they invade epithelial cells and lamina propria and perform their replication.[28,36] The replication of Eimeria and human coccidia takes place in intestinal epithelial cells or at another location (intestinal crypts) of vertebrate animals (herbivores and carnivores) progressing through sequential rounds of asexual (schizogony) and sexual (gametogony) reproduction causing acute enteritis of varying severity which leads to fairly extensive haemorrhage accompanied by a mucoid to bloody exudate.[25–28,36,55,56,86] Although the parasite cycle and its diagnostic criteria are known,[27,28,36,87–89] the molecular mechanism underlying the host’s invasion remains scarce. Dubey et. al.[36] reported a re-evaluation of the life cycle of Eimeria maxima Tyszzer, showing that only after 12 h of infection sporozoites are not only found in the surface of the epithelium but also in the lamina propria. Specific characteristics of the parasite surface molecules and the site itself, such as molecules present on intestinal cell surface, may act as receptor or recognition sites.[63,87,88] Several reports suggest that in the early stages of
FIGURE 3  Disorder probability of target proteins found by BLASTP search of the P4022 peptide. Intrinsically disorder-probability plots of (A) wheat α-2-gliadin from *Triticum aestivum* and best scored (B) *Eimeria* proteins, and (C) human coccidian proteins, were calculated using the PrDOS server[83] setting a 5% threshold (false positive rate, horizontal line). The primary sequence representation is given in the number of amino acids. In black, different polyQ sequences are present in each protein; in red, the P4022 and polyQ P4022-matching sequences; and the grey bars depict coiled-coil regions when available in the UniProt database.
infection, the interaction between microneme proteins (MICs) and their receptors on the target host cell surface are relevant for the parasite adhesion to the host epithelial cells.\[^{[68]}\]

Schmid et al. focused on *Eimeria falciformis* development and immune response in mice, demonstrating that oocyst output was impaired in IFN-γ−/− and IDO−/− mice, both of which suggest a subversion of IFNγ signaling by the parasite to promote its growth.\[^{[42]}\] They also reported an increased CxCL9 and CxCL10 gene expression during early and late infection (24 h and 144 h, respectively). The CXCR3 is a common primary receptor for chemokines CxCL9-11. In the case of *Eimeria falciformis*,\[^{[42]}\] the parasite growth was enhanced in animals in which CxCR3 was chemically inhibited by subcutaneous administration of AMG487, twice a day after infection with *Eimeria*. Since the inhibitor was added subcutaneously, it may be that only the CXCR3 receptor on immune cells was blocked and that for this reason these immune cells could not be recruited anymore to the place of damage, resulting in a free space for *Eimeria* to (increasingly) grow in the intestinal cells. Additionally, since IFNγ promotes the growth of oocysts, and IFNγ was already being produced after infestation, this may lead to observed oocysts growth. Here, a CxCR3−/− KO model in parallel would be helpful to investigate the role of CXCR3 on the intestinal cells and further understand its role in immune cells. Additionally, it would be relevant investigate the presence or absence of PolyQ sequences in *Eimeria falciformis*.

The only treatment for Coccidia infections is based on coccidiostat and coccidiocidal drugs.\[^{[91]}\] In chicken, live coccidian vaccines were also employed; however, their usage is more restricted due to drug resistance and high production cost.\[^{[88, 92]}\]

The polyQ P4022/CXCR-type interaction axis as an early event in the coccidian parasite invasion mechanism

The genus *Eimeria* is fairly large with over 1800 species, and has a highly diverse host range, affecting members of all vertebrate classes.\[^{[93]}\] including *Gallus gallus* (chicken), *Capra hircus* (goat), *Ovis aries* (sheep), *Bos taurus* (cattle) and *Oryctolagus cuniculus* (rabbit).\[^{[28, 56, 94, 95]}\] Interestingly, the orthologous to the human CXCR3 can be found in several typical host species, such as goat (NP 001272652.1), sheep (XP 004022228.3), cattle (NP 001011673.1), rabbit (XP 002720135), and mouse (NP 034040.1).\[^{[94, 96−98]}\] Therefore, we proposed that the polyQ P4022/CXCR-type interaction may be a novel molecular event leading to coccidian parasite entrance in lamina propria (Figure 1B, pathway 2) via a conserved mechanism across several species, but simultaneously different from that reported via crypt epithelial cells infected with Coccidia (Figure 1B pathway 1).\[^{[27, 36, 56]}\] In this scenario, the CXCR3 works as innate receptor in epithelial cells, where the host triggers the upregulation of CXCR3 in the response to the parasite invasion, providing the host a faster immune response before the parasite infects the intestinal tract. Importantly, the polyQ/CXCR-interaction axis could explain the zonulin increase and barrier failure observed in chickens while feeding with gluten-containing cereals or infected by *Eimeria*.\[^{[30]}\]

At the molecular level, CXCR3 binding leads to zonulin activation via the recruitment of MyD88 and the physical association of CXCR3 with this adaptor protein.\[^{[45]}\] MyD88 is originally associated withTLRs and IL-1R family, and thus with NIH signaling. In Lammers et al.,\[^{[6]}\] no activation of nuclear factor kB, IRF-3 or p38 was found. MyD88 has also been associated with the Interferon-gamma Receptor, so IFNγ can induce a pro-inflammatory response as Schmid et al. reported.\[^{[42]}\] The association of epithelial CXCR3 with another receptor “by proxy” could be one possibility to explain the recruitment of MyD88 to CXCR3. Or, it could be a direct involvement because of the TIR domain that was found in the tail of CXCR3. Furthermore, zonulin has an EGF-like motif in it and transactivates EGFR via PAR2.\[^{[41]}\] Both PAR-2 and EGFR cooperate in intestinal permeability (Figure 1B). In turn, the polyQ P4022-sequence/CXCR-type would promote or allow the direct invasion of the coccidian parasites into the lamina propria and other non-endogenous antigens from the diet like gliadin peptides via the paracellular transport (Figure 1B). These events trigger an immune response, inflammation (tissue damage), and priming. Next, upregulation of the CXCR3 is an early host defense mechanism against the coccidian parasite (Figure 1C).

TARGETING THE ORTHOLOGOUS CXCR3 CHEMOKINE RECEPTOR IN CHICKENS

Considering the high economical cost of coccidiosis in chickens, we investigated the existence of the chicken orthologous of the human epithelial chemokine receptor CXCR3. In the genome of *Gallus gallus*, several chemokine receptors are missing, including CXCR3.\[^{[99]}\] However, four CXCR genes have been identified in the *Gallus gallus* genome (CXCR1, CXCR2, CXCR4, and CXCR5). Only CXCR5 has been reported as the paralog of the human CXCR3 (40% identity with human CXCR3). The chicken chemokine receptors CXCR2 (39% identity) and CXCR4 (38% identity) have similar sequence identity to human CXCR3 and are clearly separated in terms of phylogenetic distance\[^{[100]}\] (Figure 4). For this reason, we proposed that the chicken CXCR5 may be the candidate chemokine receptor involved in binding to the *Eimeria* polyQ P4022-like peptides/sequences.

Proposed experiments to the polyQ/CXCR-type receptor and the lamina propria invasion model

To assess the involvement of polyQ/CXCR-type receptors in coccidiosis, we propose the following experiments:

1. **Pathogenicity studies.** Test the presence and pathogenicity of the polyQ P4022 sequences in the described coccidian parasites and...
This would strengthen the idea that gliadin is viewed as a microorganism by the host.\textsuperscript{16,17,102} By controlling the CXCR3 expression or targeting its binding to polyQ sequences in the epithelial cells would offer a preventive or alternative therapeutic approach, beyond the use of coccidostats.

A NOVEL PATHOGENIC SCENARIO THAT CONNECTS CELIAC DISEASE AND COCCIDIOSIS THROUGH THE POLYQ/CXCR3 AXIS

Celiac disease and the gluten-related-disorders

Gluten is responsible for a group of complex immune-mediated diseases affecting around 1\%–7\% of the general population, named gluten-related disorders.\textsuperscript{103} Celiac disease, the most well-known gluten-related disorder, also known as celiac sprue or gluten-sensitive enteropathy, is a chronic, small-intestinal autoimmune disease triggered by the ingestion of gluten in genetically predisposed individuals.\textsuperscript{104} Gluten proteins are the major endosperm storage proteins in grains, like wheat, barley and rye.\textsuperscript{104,105} Prolamins of these cereals like α-gliadins undergo incomplete enzymatic degradation during in vivo digestion.\textsuperscript{10,39} This produces small and large resistant peptides that contact the gut epithelium and lamina propria.\textsuperscript{105} Among these fragments are the 33-mer (amino acids 57–89 from α-gliadin), the largest described peptide fragment with immunogenic properties,\textsuperscript{106} and the 13-mer (amino acids 31–43 from α-gliadin), a toxic peptide that activates a strong cytotoxic immune response.\textsuperscript{16,107} Once in contact with the small intestine submucosa, the human enzyme transglutaminase 2 (TG2) deamidates gluten peptides (e.g., 33-mer) that bind to the Human Leukocyte Antigen (HLA) molecules on antigen-presenting cells. Gluten peptide-binding occurs with an exceptionally high affinity to HLA-DQ2 and HLA-DQ8 molecules.\textsuperscript{108,109} More than 95\% of CeD patients carry the HLA-DQ2 and/or HLA-DQ8 haplotype. The ongoing disease process gives rise to a variable degree of intestinal damage in which the mucosa progresses from intraepithelial and lamina propria villous infiltration of inflammatory cells (intraepithelial lymphocyte)\textsuperscript{15} to villous atrophy, crypt hyperplasia and flattening.\textsuperscript{10} Those intestinal epithelial changes have been systematically classified by Marsh as stages I through III to diagnose the disease.\textsuperscript{11–13} Given that the HLA-DQ2 and/or HLA-DQ8 haplotype is common in the population (ca. 30\%) and that CeD occurs in only 1.4\% of the people, it is an essential but not sufficient factor in the pathogenesis of CeD.\textsuperscript{110} Therefore, other factors (immune, genetic and/or environmental) may play a role. Accordingly, it has been long proposed that the varied mucosal lesions in other enteropathies like tropical enteropathy/tropical sprue, giardiasis, and possibly some food allergies, fall into the same cell-mediated category.\textsuperscript{111} In this regard, the histopathological and serological characteristics of Giardia lamblia infection, which range from partial to total villous atrophy (Marsh stages I to III) in duodenal biopsies, may resemble that of CeD.\textsuperscript{112–114} Typical symptoms of giardiasis like nausea, abdominal pain, and diarrhea, extend the search to other coccidian parasites in other species (e.g., mice). It will be needed to generate mutated versions of the Eimeria P4022-containing proteins targeting the P4022 sequence by genetically modifying Eimeria using the CRISPR/Cas9 system.\textsuperscript{101}

2. RNAseq approach. An intestinal cell line, or—if no adequate cell line is available—an animal model will be incubated with or without coccidian parasites, and RNA from the epithelium of both conditions will be isolated to perform RNA sequencing. This technique will allow obtaining extended information on the coccidiosis-epithelium interaction, including information on the receptors involved in this infection, whether confirmation of CXCR-type receptor or another receptor. Candidate receptors will be checked by real-time quantitative PCR technique.

3. Mimicking physiological digestion. Investigate if P4022-like peptides from the coccidian P4022-containing proteins can be generated by in vitro digestion, simulating the cellular conditions (proteases, temperature, pH, ionic strength, etc.), using electrospray ionization mass spectrometry coupled to HPLC technique.

4. Binding studies. Using in vitro transfection models with CXCR3 or CXCR-type receptor transfectants as previously described in\textsuperscript{6} will demonstrate the binding of the peptides obtained from the digestion experiments under item I or the native P4022-like proteins from human Coccidian to the CXCR3 receptor.

5. CXCR3-type receptor\textsuperscript{−/−} KO models and permeability experiments. Use of ex vivo animal models, wild-type and CXCR3\textsuperscript{−/−} (or CXCR-type receptor) mutants, to test the ability of the different human coccidian P4022-like peptides to increase zonulin levels and subsequent intestinal permeability. Subsequently, analyze the effect on the infection rate and development of Eimeria parasites depending on CXCR3 expression level.

If this pathway in coccidiosis is confirmed, it would suggest that the PolyQ/CXCR3 pathway is a host system to recognize microorganisms,
share some similarities with CeD.\textsuperscript{115–117} Although it has not yet been proved whether \textit{Giardia} infection may elicit CeD development, it has been reported that a CeD patient in the active phase reverted to latent phase upon treatment for giardiasis.\textsuperscript{[117]} Considering that \textit{Vibrio cholerae}'s Zot uses the host system for access,\textsuperscript{[2]} it is plausible that the polyQ proteins from coccidian parasites may induce increased permeability using the CXCR3/zonulin-pathway, too.

The polyQ sequence is the connection between human coccidiosis and celiac disease

Taking into account the striking similarities between human coccidiosis and CeD (Table 1), and having found at least 100 different polyQ P4022-containing proteins from human Coccidia, we propose that coccidiosis infection in humans may be a plausible environmental cofactor in CeD pathogenesis and probably to other gluten-related disorders depending on the individual genetic predispose.

As aforementioned, CeD pathogenesis occurs by hereditary sensitivity in response to gliadin. In the active phase of CeD, CXCR3 expression is abundantly high and returns to baseline after implementing a gluten-free diet when the disease is in remission.\textsuperscript{[6]} The reason for apical CXCR3 expression in the intestinal epithelium remains unknown but it can be a dietary receptor to cope with gluten or it is an early defense mechanism against microorganisms, like coccidiosis.

Our hypothesis is that coccidiosis predisposes for CeD, which supports the theory of an episode of traveler’s diarrhea of intestinal infection as a trigger of the onset of disease.\textsuperscript{[23]} While a loss of tolerance to gluten is the cause for CeD, a previous coccidiosis infection can pave the way to overexpression of CXCR3 and predispose for CeD in genetically predisposed individuals. In this scenario, the CXCR3 is originally designed as an early defense mechanism against microorganisms possessing polyQ sequences like coccidian parasites. By using this pathway, the parasite breaches the intestinal barrier allowing the parasite, high concentration of gliadin fragments, and other luminal pathogens and antigens to reach the lamina propria where a strong immune response, inflammation, and tissue damage occur (Figure 1B). If gluten is in the diet, these events create an environment of enhanced sensitivity, priming the innate immune system for gliadin and increasing the risk to develop gluten intolerance in predisposed individuals. While coccidiosis infection is resolved by coccidiostats treatment, the upregulation of CXCR3 receptor expression occurs as a host innate response, which concentration remains high on the intestinal epithelium. The binding of gliadin polyQ sequences present in the diet with the high luminal expression of CXCR3 continues the zonulin upregulation, increase intestinal permeability, flux of the intestinal milieu in the lamina propria and the perpetuation of presentation of the immunodominant gluten peptides (e.g., 33-mer) to the immune cells in the lamina propria leading to inflammation, tissue damage and immune dysfunction in genetically predisposed individuals (Figure 1D).\textsuperscript{[118]} The coccidiosis episode as an environmental trigger of CeD may work predominantly in (a subgroup of) patients who are diagnosed with CeD at a later age. Likewise, this last hypothesis may be applicable for non-celiac gluten sensitivity (NCGS), a recently recognized gluten-related disorder in which CeD diagnosis—although patients present with symptoms similar to CeD—is ruled out. In these patients, from whom only 50% carry the HLA-DQ2 and/or HLA-DQ8 haplotypes, a prior coccidiosis infection could be at the basis of gluten sensitivity without inducing the auto-immune response characteristic for CeD. Haploype-positive NCGS patients may well be in a pre-stage of developing CeD. Additionally, in IBS (irritable bowel syndrome) a subgroup of diarrhea prone, HLA-DQ2+ IBS patients are found to react to gluten exposure.\textsuperscript{[119]} Therefore, there is the possibility that \textit{Eimeria}-induced gluten intolerance occurs in a subgroup of such individuals, too. Importantly, the role of \textit{Eimeria}-induced CeD would be a differential environmental trigger that would explain the different presentation between CeD in early life (infants) or in adulthood.

Finally, a small percentage of the CeD patients are not responsive to a gluten-free diet, suffering from a perpetuation of the auto-immune activation, and developing refractory CeD. In this case, an underlying coccidiosis infection might be at the basis of the perpetuation of the high CXCR3 expression and activation, increased intestinal permeability, and immune-related intestinal damage that was initially caused by gluten.

Therefore, we postulate three main experiments to investigate such connection in increasing complexity:

1. \textbf{CXCR3 expression studies.} In vitro stimulation assays with an intestinal epithelial culture model will show whether coccidian polyQ sequences can upregulate epithelial CXCR3 expression. In the same model, assessment of synergistic effects on CXCR3 expression by co-culture of \textit{α}-gliadin polyQ P4022 sequences and coccidian polyQ sequences can be applied. A third series of experiments will study the cumulative effects on receptor expression by pre-incubating the cells with polyQ coccidian sequences followed by the \textit{α}-gliadin digest. This set of experiments will give information on increased gluten sensitivity due to enhanced epithelial CXCR3 expression induced by coccidian polyQ sequences and on permeability effects.

2. \textbf{Clinical study on coccidiosis in CeD and NCGS.} A large scale human cohort study should be performed in which the clinical record of CeD patients is investigated in terms of biochemical evidence of past or present coccidian infection to obtain information on the prevalence of coccidiosis in CeD, refractory CeD and NCGS; (i) Patients will be molecularly and histologically screened for human Coccidia. (ii) Patients who screen positive for past or present coccidian infection will be offered coccidiostats. (iii) Clinical studies will be developed to monitor the treatment’s effect to cure coccidiosis on (regained) tolerance to gluten.

3. \textbf{Clinical studies in preventive medicine.} Suppose coccidiosis infection enhances the risk of developing gluten sensitivity or intolerance. In that case, a clinical study will be set up to assess the implementation of a strict gluten-free diet during episodes of intestinal diarrheal inflammation and screening for coccidiosis, with a primary outcome the prevention of CeD or NCGS.
Since many diseases are connected with zonulin upregulation or gluten consumption, if these experiments validated the connection between coccidiosis and CeD or the other gluten-related disorders, the role of coccidian parasites as triggers of other diseases where increase intestinal permeability and immune response is involved would need to be investigated (e.g., autisms, IBS, Crohn’s disease, etc.).

CONCLUSION

Here, we presented the first report of a molecular and structural connection between coccidiosis, the gliadin polyQ peptide/CXCR-type receptor, and celiac disease. Based on the BLASTP findings, the use of structural predictors and an extensive literature search, we hypothesize that gliadin polyQ P4022-matching sequences found in coccidian proteins may facilitate parasite invasion to the lamina propria by binding to the CXCR-type chemokine receptor. According to this model, the binding of the polyQ sequences from coccidian parasites to the host intestinal CXCR-type receptor leads to increased intestinal permeability mediated by zonulin upregulation, facilitating the further invasion of pathogens to the lamina propria or entrance of non-endogenous antigens, such as dietary antigens. This process would induce an uncontrolled inflammatory host response that resembles that of individuals suffering from a gluten-related disease and coccidiosis. In this model, the CXCR3 is originally designed as an early defense mechanism against coccidian parasites because their polyQ sequences and unfortunately gliadin use it, leading to increase permeability mediated by zonulin release. The immune and toxic fragments of gliadin reaching the lamina propria trigger the immune dysfunction observed in CeD or other gluten-related disorders that would depend on the individual susceptibility. The investigation and validation of the proposed gliadin polyQ/CXCR3 axis in the context of human coccidiosis will define the measure of involvement of parasite infection in gluten-related disease and—if confirmed—allow an alternative therapeutic approach to treat in first place coccidiosis and prevent gluten-related disease or, in the case of refractory CeD, restore responsiveness to a gluten-free diet. Thus, deeper comprehension of the molecular mechanisms governing the parasite invasion would allow the design of effective recombinant vaccines for the treatment of coccidiosis in vertebrates and preventing human coccidiosis outbreaks as a novel strategy for CeD prevention.

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CONFLICT OF INTEREST

Karen M. Lammers shares as an inventor in a patent ‘CXCR3 is a Gliadin receptor’, with the assigned number 7,622,264. Other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Veronica I. Dodero https://orcid.org/0000-0001-7937-1880

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