University of Nevada, Reno

Potential Treatment Alternatives and an Illustrative Case Study of Celiac Disease

A thesis submitted in partial fulfillment
of the requirements for the degree of

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by

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May, 2013

UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

COLT JONAS WILLIAMS

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Abstract

Celiac disease (CD) has taken center stage in the public eye, now afflicting 1:250 to 1:100 people in the United States. While CD etiology is most certainly polyfactorial, stemming from genetic, environmental, and immunologic factors, a causative agent of CD has been identified as a malfunctioning tissue transglutaminase (tTG). A ubiquitous enzyme that has numerous roles body-wide, tTG most notably digests small peptides in the jejunum. When tTG in the brush border of the small intestine comes in contact with gliadin, the immunoreactive peptide segment within gluten, tTG forms an irreversible complex that signals for an immune and inflammatory response. Current treatment options are limited only to strict adherence to a gluten free diet—a lifestyle that is incompatible with the majority of the American population.

I propose a radical treatment alternative, involving the silencing of the gene responsible for the faulty tTG. Using ß-cyclodextrin nanopolymers designed by Davis, et al. as a delivery vehicle, siRNA will be injected intravenously and specifically target small intestinal cells expressing tTG.

An exemplary case study of a 23 year old female is included in order to illustrate possible celiac pathology and presentation.

Acknowledgements

It is with immense gratitude that I acknowledge Ms. Crystal English, MSIV of the University of Nevada School of Medicine, without whose help and guidance I would have been lost; my thesis advisor, Dr. Josh Baker, for his friendship, creative support, and unwavering belief in me, and to my colleagues in the 2017 BS-MD cohort for their reviews and critiques.

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Introduction

Once considered a rare affliction, Affecting only 1:10,000 people nationally (Ciclitira, 2004), celiac disease (CD), also known as celiac sprue, wheat intolerance, and gluten intolerance, has taken center stage in the public eye. Fifty years ago, Celiac disease was only seen in its most severe stages, with patients suffering extreme weight loss, nutrient malabsorption due to brush border blunting and small intestinal architectural disfigurement, chronic diarrhea, insurmountable fatigue and irritability, and a host of other problems. However, symptoms of CD begin very mildly, if at all present, and take years to develop. Today's hospitals rarely treat individuals with irreparable intestinal damage and are now seeing a greater stratification of patients suffering milder symptoms. Thanks to advancements in antibody screenings, it is apparent that the actual number of people suffering from CD is closer to 1:250 to 1:100 (Presutti, Cangemi, Cassidy, & Hill, 2007), with evidence increasingly pointing to levels of 1:100 or greater (Van Heel & West, 2006).

The causative agent of CD is a malfunctioning tissue transglutaminase (tTG), a ubiquitous enzyme that has numerous roles body-wide, including small peptide digestion in the jejunum. When tTG in the brush border of the small intestine comes in contact with gliadin, the immunoreactive peptide segment within gluten, tTG forms an irreversible complex that signals for an immune and inflammatory response. This inflammation in response to the tTG-gluten complex eventually leads to disastrous disfigurement of the small intestine if left untreated.

Despite our increased diagnostic capabilities, treatment has remained completely unaltered since its original documentation in 1954 by J. W. Paulley. The only treatment currently available to those that are gluten intolerant is a gluten-free diet, a lifestyle that provides as many problems as it solves. A large portion of the alleles coding for gluten intolerance can be traced back to European origins, whose culture (coinciding with our own national roots and culture), ironically, bases a substantial portion of their diet on gluten based foods such as wheat bread, pasta, rye, barley, and oats. Maintenance of a gluten free diet, while providing vast relief of all symptoms, is extremely difficult in current US culture, especially considering as little as 10mg of gluten may trigger an immune response (Hischenhuber et al., 2006). Therefore, I propose a radical treatment alternative, involving the silencing of the gene responsible for the faulty tTG. Using ß-cyclodextrin nanopolymers designed by Davis, et al. as a delivery vehicle, siRNA will be injected intravenously and specifically target small intestinal cells expressing tTG. While systemic knockout of tTG has shown to produce phenotypically normal organisms, localizing tTG knockout to the causal intestinal cells will increase the efficacy of the siRNA uptake to target cells and limit the possibility for still unforeseen phenotypic disturbances. With this treatment, I hope to provide those who suffer from CD with a higher quality of life by alleviating their symptoms while maintaining their dietary options.

Symptomology

Celiac Disease (CD) presents a wide range of symptoms, with severity varying greatly from case to case. Gastrointestinal discomfort, nutrient malabsorption, generalized weakness and lethargy, anemia, and intestinal mucosal damage are

commonly seen. These symptoms only occur after ingesting gluten proteins found in wheat, barley, and rye.

CD is characterized by intestinal mucosal damage that increases with the severity of the illness. Paulley noted as early as 1954 variable stage inflammation in the form of increased lymphocyte and plasma cell infiltrate and increased intraepithelial lymphocytes. While the mucosa normally contains plasma and T-cells, their number, along with those of newly infiltrated granulocytes, increases drastically in patients with CD. Inflammation is generally localized to the lamina propria of the upper 60% of the small intestine. Crypt hyperplasia is seen in later stages with partial to complete intestinal villous atrophy in the most severe cases (Oberhuber, Granditsch, & Vogelsang, 1999). Atrophy extent, diagnostic quantification of infiltrate levels and substantial changes in small-intestine architecture will be discussed later.

This "blunting" of the small-intestinal brush border can often lead to nutrient malabsorption, especially fat-soluble vitamins, such as Vitamin A, D, E, and K. Anemia may also be seen from poor iron uptake or via the poor uptake of Vitamin B_{12} and folic acid (Di Sabatino & Corazza, 2009). As a result, many patients suffering from CD complain of weakness, lethargy and fatigue, and in children, failure to thrive ("Childhood disease and disorder," 2012).

Gastrointestinal discomfort is the most widely reported symptom of CD, including, but not limited to, bloating, flatulence, indigestion, abdominal distension, and diarrhea. Bowel movements may be frequent and extremely malodorous. In general, the symptoms of CD are highly similar to irritable bowel syndrome (IBS); the physician

should take care to note changes in symptomology with changes in diet when taking patient history ("Celiac disease," 2012).

Celiac disease will often remain untreated if it presents asymptomatically with regards to the GI tract. Dermatitis herpetiformis, osteoporosis, amenorrhea, and miscarriage are linked to CD if certain genetic risk factors are present ("Celiac disease," 2012; Presutti et al., 2007). Severe untreated celiac disease increases the patient's risk of developing adenocarcinoma or intestinal lymphoma. Pregnant women may also give birth to infants with congenital defects ("Celiac disease," 2012).

Pathology

Genetic factors

The HLA-DQ family is an MHC class II antigen-presenting receptor. HLA-DQ is involved primarily in self/non-self recognition. However, through an unknown mechanism, HLA-DQ loses specificity for antigen-producing self-cells in certain individuals. This loss of specificity generally causes autoimmune dysfunction, as is the case in CD. HLA-DQ is a dimer of an α and ß chain, of which the body normally produces two variants each, totaling to four isoforms per person. Eight isoforms have been discovered (HLA-DQ2, HLA- DQ4-9, and Factor XIII A). The most common isoforms are HLA-DQ2 and HLA-DQ5. 95% of all individuals with CD have either the HLA-DQ2 or the HLA-DQ8 isoform (Klitz et al., 2003). These proteins are one of the causative agents of celiac disease for they have the ability to bind the tTG-gliadin complex more tightly than other isoforms, with a near-100% probability of causing T-cell

activation and the subsequent immune response in the intestinal mucosa (Van Heel & West, 2006).

The α and ß chains are encoded on chromosome six by genes HLA-DQA1 and HLA-DQB1, respectively. DQ isoforms are caused by single nucleotide polymorphisms (SNP's) which, when causing a non-silent mutation, alter the peptide-binding region that presents foreign particles to T-cells. In the case of CD, DQ2 isoforms have large overlap with tTG-gliadin protein complexes and initiate the immune response upon binding.

The vast majority of people with the DQ2 isoform possess a DQ2 heterodimer—a haplotype of the two adjacent alleles DQA1*0501, coding for the α^5 chain species, and the DQB1*0201 allele, coding for the $ß^2$ chain species. While only one copy is normally inherited, if an individual receives a copy from both parents, the risk for developing CD is greatly increased due to gene dosing (Jores et al., 2007).

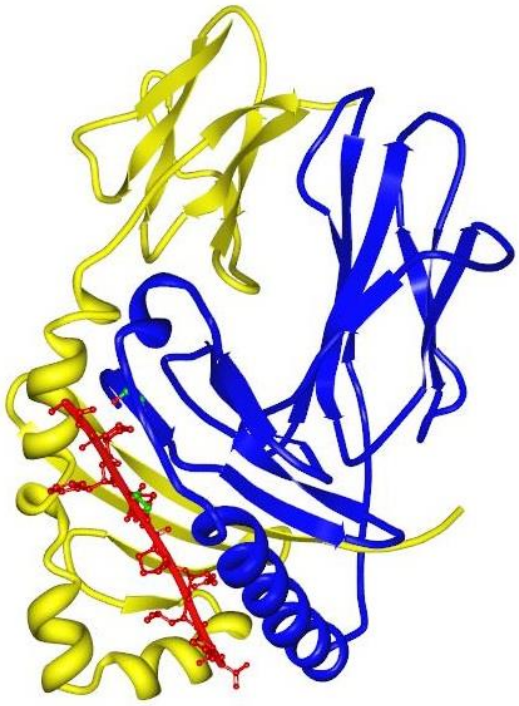

Figure 1. HLA-DQ2 structure with bound α-gliadin. The α chain is yellow, the ß chain is blue, and α-gliadin is red. Retrieved from the Protein Database, Accession Code 1S9V.

It should be noted that the DQ2 and DQ8 isoforms are neither necessary nor sufficient to cause CD. Approximately 25% of individuals who have inherited one or both isoforms do not develop CD, while 5% of patients with CD have neither isoform. This suggests that CD is caused by multiple factors and not solely by inherited genetics (Hadithi, Blomberg, Crusius, Bloemena, & Kostense, 2007; van Heel & West, 2006).

Immunologic factors

The development of CD is completely dependent upon two factors: the dietary intake of gluten peptides and the presence of a faulty tTG. Specifically, plant storage proteins rich in proline and glutamine residues (often called *prolamins*) which resist enzymatic proteolysis are the causal peptides. Looking at the α-gliadin protein in wheat,

an 18mer hydrophobic sequence is followed by a series of proline residues which prevent proper binding to the active site of enzymes of the pancreas, stomach, and small intestine (Van Heel & West, 2006).

Undigested α-gliadin 18mer sequences (amino acids 31-49) act as an innate immune response stimulator to cause the release of interleukin-15 from enterocytes (Van Heel & West, 2006). Upon apical exposure to gliadin, CXCR3 receptors bind the peptide and initiate an intracellular cascade. Intestinal epithelia secrete zonulin, a protein that causes tight junction disassembly. While still unclear, it is possible that zonulin control is poorly regulated in patients with CD (Berti, Tommasini, & Goldblum, 2000). Once epithelial tight junctions have been disassembled, large proteins normally unable to pass the plasma membrane, such as gliadin, pass through the brush border and into the lamina propria (Green & Cellier, 2007). Gliadin is recognized as a foreign body and stimulates the release of the cytokine interleukin-15 (IL-15). The paracrine signaling of IL-15 acts on numerous cells proximal to the "infected" enterocyte. The expression of MHC class I polypeptide-related sequence A (MICA), a cell surface protein used to signal natural killer cells and T-cells, is increased in the enterocytes containing gliadin. The production of the NKG2D cell surface receptor on intraepithelial lymphocytes (IEL) is also upregulated. When the NKG2D receptor of an IEL binds MICA proteins, the compromised enterocytes are immediately destroyed (Van Heel & West, 2006). It is highly probable that this self-targeting of enterocytes post-gliadin entry weakens the intestinal epithelium enough to allow extensive gliadin invasion of the lamina propria.

Gluten peptides may also cause T-cell activation through irreversible crosslinking to tissue transglutaminase (tTG). The enzyme-peptide complex has a high affinity for the HLA-DQ2 protein, which presents the complex to CD4+ T-cells specific to gluten to initiate the adaptive immune response (Van Heel & West, 2006). tTG is a calcium dependent intracellular enzyme with roles in structural reinforcement of the matrix, preparation for apoptosis, and cellular differentiation (Griffin, Casadio, & Bergamini, 2002). When stressed by inflammation, infection, or mechanical force, tTG is released from the cell to the extra cellular matrix (ECM) and binds to cellular scaffolding to increase support. However, when the host cell becomes fatally damaged, tTG is activated intracellularly and begins a signaling cascade for apoptosis (Schuppan, 2000). Tissue transglutaminase forms a proteolysis-resistant intramolecular isopeptidyl bond between the epsilon-amino group of a lysine in its active site with the gamma-carboxyamide group of a glutamine residue within gluten. Three key glutamine residues are also deamidated to glutamate, increasing the negative charge of the complex and increasing its binding affinity with HLA-DQ2 (Schuppan, 2000; van Heel & West, 2006).

Comparatively, tTG-gliadin complexes are capable of causing significantly more inflammation than local responses to gliadin entry alone. While the inflammatory response elicited through the tight junction disassembly cascade only activates local IEL's in the brush border, tTG-gliadin complexes have the ability to generate a system-wide immune response through CD4+ T-cell activation and subsequent B-cell activation. Gliadin peptides, now in the lamina propria, bind irreversibly to antigen-presenting cells with the HLA-DQ receptor. Once bound, the complex signals T-cell infiltration and

targeted cell destruction. Activated B-cells begin producing antigliadin, antiendomysial, and anti-tTG antibodies (Green & Cellier, 2007). Thus, inflammation from the adaptive immune response occurs in four ways: HLA-DQ signaled infiltration of T-cells, T-cell destruction of HLA-DQ-gliadin enterocytes, antibody signaled T-cell infiltration to *any* intestinal tissue presenting gliadin peptides or tTG, and finally T-cell destruction of antibody bound cells or peptides. It is this system-wide signaling that is the major cause of granulocyte infiltration into the affected tissues, inflammation, and consequently villous atrophy and crypt hyperplasia. For this reason, many consider the tTG-gliadin complex to be the primary source of inflammation, rather than the tight junction disassembly alone (Ciclitira, 2004; Green & Cellier, 2007; van Heel & West, 2006). Elimination of immune system activation by tTG would prevent the overwhelming majority of cellular damage and inflammation caused by CD.

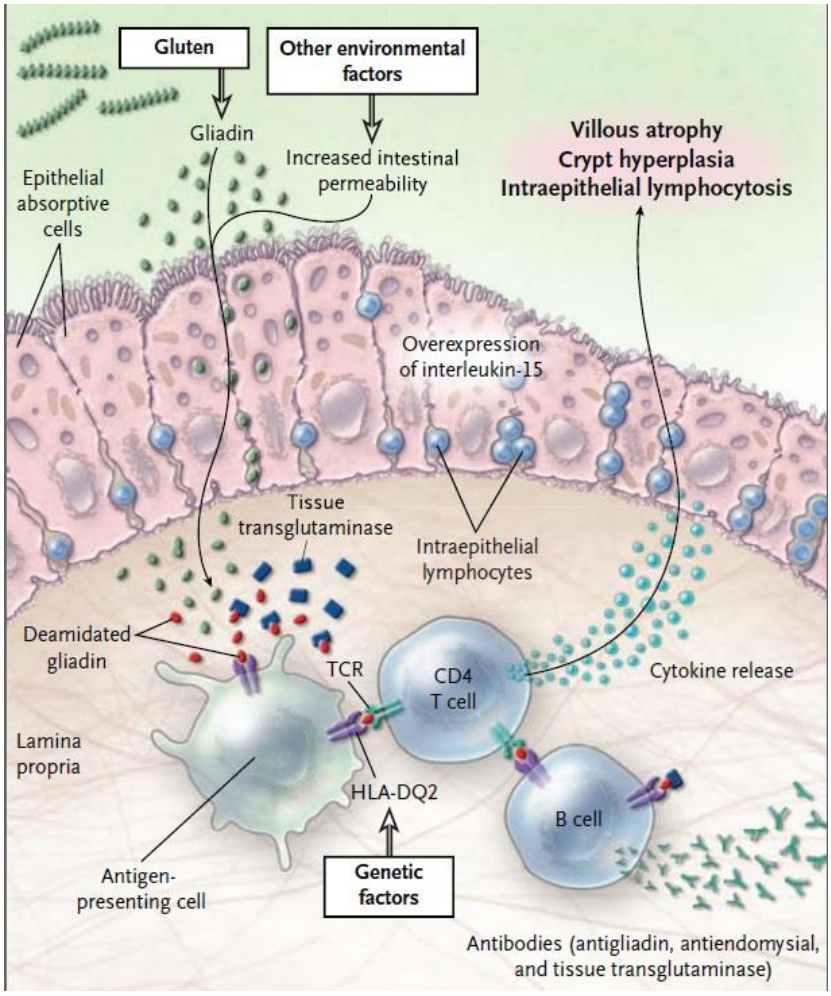

Figure 2. Mechanism of immune system activation upon gliadin exposure. Gliadin activates the innate immune system in the epithelium and the adaptive immune system in the lamina propria. Gliadin damages the invaded epithelial cells, releasing IL-15 and activating nearby IEL's. Binding to HLA-DQ2 receptors on cells in the lamina propria activates the adaptive immune response, triggering T-cell mediated destruction, B-cell activation and antibody formation (Green & Cellier, 2007).

Diagnosis

The simplest—and often only—method of diagnosis and treatment still remains to be one of the most reliable: strict adherence to a gluten free diet. If the patient is indeed suffering from gluten intolerance and has not suffered irreparable villous atrophy,

serological markers, body weight, energy levels, and miscellaneous symptomology such as dermatitis herpetiformis should return to normal after several months without gluten. Nonetheless, while active symptomology may have disappeared, the patient may still suffer from underlying mucosal damage and should be further examined to determine present health risks.

Traditional methods of diagnosis may be categorized by one of three exams: serological exams, biopsies of the proximal small intestine, and endoscopies. The least intrusive and most cost effective, visualization of anti-tTG antibodies in the blood via an ELISA assay should be done first. However, positive results for anti-tTG antibodies are not sufficient for a conclusive diagnosis. Jejunal biopsy and classification based on the Marsh Scale edited by Oberhuber remains the "gold standard" for a positive diagnosis (Ciclitira, 2004). Oberhuber's modern classification rates the disease based on its severity as Type 1, Type 2, or Type3a-3c.

Table 1. Modified Marsh Classification for pathological diagnosis of celiac disease. As the general severity of Celiac disease has decreased over the past fifty years, it was necessary for greater differentiation between less severe manifestations of CD. Oberhuber's revisions provide for greater specificity between patients with milder symptomology (Oberhuber et al., 1999).

Table 1 The modified Marsh classification

	Type 0	Type 1	Type 2	Type 3a	Type 3b	Type 3c
IEL*	< 40	> 40	> 40	> 40	> 40	> 40
Crypts	Normal	Normal	Hypertrophic	Hypertrophic	Hypertrophic	Hypertrophic
Villi	Normal	Normal	Normal	Mild atrophy	Marked atrophy	Absent

*Numbers are given as intraepithelial lymphocytes/100 epithelial cells

Video capsule endoscopy (VCE) is much preferred over traditional endoscopy as it is minimally invasive and requires far fewer hospital resources and man hours while providing high quality visual documentation of the entire digestive tract. Clear markers indicative of villous atrophy include scalloping of the intestinal wall, mosaic-like patterning, and micronodularity of the villi (Akin & Ersoy, 2012).

Treatment

The only recognized and accepted treatment for celiac disease remains the application of a gluten free diet. Adherence to the diet must be complete; gluten is capable of stimulating an immune response at levels as low as 10mg per day (Hischenhuber et al., 2006). Gluten's high toxicity makes it absolutely necessary to avoid any gluten intake to allow the small intestine to reduce in inflammation and the healing process to reach its maximum potential. Depending on the extent of the mucosal damage, two to six months may pass before noticeable improvement in villous structure occurs. The difficulty for most people to faithfully remain on this diet for life and their reported lowered quality of life from the severe restrictions on their eating choices begs for new treatment options. Celiac disease has been treated in the same manner for the past sixty years without innovation. These issues demand a novel treatment method capable of eliminating symptoms while maintaining the patient's quality of life. Gene therapy, while used as a treatment almost exclusively in the realm of cancer, could potentially be the curative agent for those suffering from CD. By silencing the gene coding for tTG, it would be possible to eliminate all symptoms at their source while still retaining the ability to ingest gluten.

Overview of tTG Function

Before engaging any patient in experimental treatments, it is vitally necessary that the physician ensures that a positive diagnosis is made. While evidence supports the functionality and complete lack of immunogenicity in the treatment plan described below, gene therapy treatments are not to be trifled with and should only be used when serious health concerns are of issue.

Table 2. Summary of functions of known transglutaminase isoforms (Griffin et al., 2002). Eight isoforms of transglutaminase have been identified in humans; type 2 is of extreme importance to CD, especially its role in protein adhesion during periods of cellular stress.

Identified tTG isoform	Synonym names	Prevalent Function
Factor XIII A	Fibrin Stabilizing Factor	Blood clotting and wound healing
Type 1	Keratinocyte TG	Cell envelope formation in keratinocyte differentiation
Type 2	Tissue TG	Cell death and differentiation, matrix stabilization, protein adhesion
Type 3	Epidermal TG	Cell envelope formation during terminal keratinocyte differentiation
Type 4	Prostate TG	Reproductive function, semen coagulation
Type 5	TG X	Epidermal differentiation
Type 6	TG Y	Not characterized
Type 7	TG Z	Not characterized

Type 2 tissue transglutaminase (tTG) plays a redundant or non-vital role in the regulation of apoptosis, cell differentiation, and matrix stabilization. Genetic encoding for each isoform seems to arise from independent genes rather than from alternative splicing. Tissue transglutaminase was isolated to chromosome 20q12 while Factor XIII A and type

1 TG were isolated to chromosomes 6p24-25 and 14q11.2, respectively (Gentile, Davies, & Baldindi, 1994).

Regarding apoptosis specifically, tTG has been shown to have roles during development and differentiation in interdigital formation, implantation of the fertilized embryo in the uterus, and mammary gland regression (Melino & De Laurenzi, 2001). These factors are extremely important to monitor during knockout of gene TGM2 coding for tTG as they are signals of the organism's viability post-tTG knockout.

Effects of tTG Silencing

Nanda et al. has shown that tTG -/- transgenic mice were phenotypically normal in all regards, showing expected Mendelian genetics, normal weight and birth size, open eyes, properly formed fingers, and visible functionality of developmental apoptosis. Transgenic tTG -/- mice have also been shown to be fully capable of reproducing fertile offspring (Melino & De Laurenzi, 2001).

While tTG is the principle agent of the vast majority of CD inflammation, the exact role and extent of zonulin, tight junction disassembly, and IEL activation via IL-15 release leading to cellular damage is not confirmed. Admittedly, tTG knockout, even if 100% effective would not prevent all CD-based cellular damage. However, zonulin release creates permeations only in the epithelial cells of the brush border, and without active tTG to continue the cascade in the lamina propria, damage would be limited to only these cells.

To determine the extent of zonulin's action in CD pathology, I would suggest performing the following experiment before beginning tTG gene therapy trials. Using HLA-DQ2 +/+ transgenic mice, I suggest up- and downregulating zonulin, noting changes in intestinal architecture to determine if increased levels produce more villous atrophy and scalloping of the intestinal wall and if decreased levels stimulate intestinal regeneration or halt disease progression compared to controlled non-zonulin-altered HLA-DQ2 +/+ mice.

Firstly, HLA-DQ2 +/+ mice must be created as it is essential that they: 1) express CD gluten intolerance and 2) express these symptoms through *at least a partially* tTG-mediated immune response. Zonulin levels may be regulated either by producing "zonulin encoding gene" (ZEG) modified transgenic mice, or through administration of known zonulin inhibitors or activators.

To determine if upregulation of zonulin increases cellular damage in mice expressing CD, zonulin protein production may be stimulated through the creation of transgenic mice overexpressing zonulin, or through the administration of zonula occludens toxin of *Vibrio cholera*, a known zonulin activator and mediator of tight junction disassembly. Differences in small bowel architecture compared to control groups of HLA-DQ2 +/+ mice with non-altered zonulin levels should be noted. Similarly, downregulation of zonulin may be achieved through inhibition of ZEG through a *Cre-lox* excision system or similar interruption of the transcription of ZEG. Differences in small bowel architecture compared to control groups of HLA-DQ2 +/+ mice with non-altered zonulin levels should be noted.

The above study would hopefully produce unequivocal results aiding in the clarification of the exact role of zonulin in CD inflammation. Speculating further, if the results were to suggest zonulin does indeed hold a major role CD inflammation, it would follow that the practicality of a zonulin and tTG siRNA co-delivery should be investigated.

siRNA Transfection to Silence tTG Expression

Overview

Short interfering RNA (siRNA) is double stranded RNA (dsRNA) 20-25bp in length. siRNA binds to complementary nucleotide sequences in DNA to prevent mRNA and subsequent protein expression. siRNA is produced biologically by the enzyme DICER, as it cuts long dsRNA into fragments; siRNA may also be manufactured synthetically for any given gene and then injected into the target cell. However, siRNA is degraded rapidly by the innate immune response and intracellularly via enzymatic degradation. Furthermore, large doses of siRNA appear to the body as a viral infection and may trigger the innate immune response. It is therefore extremely important to develop a delivery vehicle for siRNA transfections that has no immunogenicity and is equally non-toxic to the host cells ("RNA interference (RNAi)," 2012).

Considering the aforementioned stability of mammals lacking tTG, it follows that the silencing of tTG in patients with CD may be a viable treatment. Using the DNA sequence of tTG (Liu, Cerione, & Clardy, 2002), I suggest that siRNA be synthetically produced to match the TGM2 coding region to prevent tTG expression and remove the main agent of CD symptomology. Transfections of siRNA into higher mammalian

vertebrates has been successful, showing 9-25 fold reductions in protein expression (Elbashir et al., 2001). The ß-cyclodextrin nanopolymer complex designed by Davis, et al. will serve as the delivery vehicle as it: 1. Presents no immunogenicity, 2. Is virtually non-toxic to host cells *in vivo* and finally, 3. Is the only transfection reagent approved for pharmaceutical drug use (Sporanox), and proven to be viable for macromolecule transfection in humans.

Delivery Vehicle

Cyclodextrin is a cup shaped molecule formed of cyclic oligomers of glucose, with six, seven, or eight units representing α, ß, and γ forms respectively. Cell toxicity is very low, measuring at 200mg/kg bodyweight, compared to 30mg/kg of polyethylene imidazole (PEI), a nanoparticle delivery vessel widely explored before cyclodextrin synthesis (Gonzalez, Hwang, & Davis, 1999). The cyclodextrin polymer designed for siRNA transfection in humans, dubbed CALAA-01, is composed of four units: the siRNA cargo, the ß-cyclodextrin encapsulating shell, adamantine-polyethylene glycol (AD-PEG), and adamantine-polyethylene glycol Transferrin (AD-PEG-Tf), the previously mentioned AD-PEG chain with a human transferrin protein attached. The ß-cyclodextrin shell protects the siRNA from enzymatic degradation and from triggering innate immune responses. Its structure and charge also allow it to pass through cellular membranes. AD-PEG is used to increase stability in biological fluids, while AD-PEG-Tf is used as a cell-surface receptor signaling molecule to initiate uptake in targeted cells only. In this case, human transferrin would be replaced with a targeting protein specific to the villous cells of the small intestine, such as CXCR3.

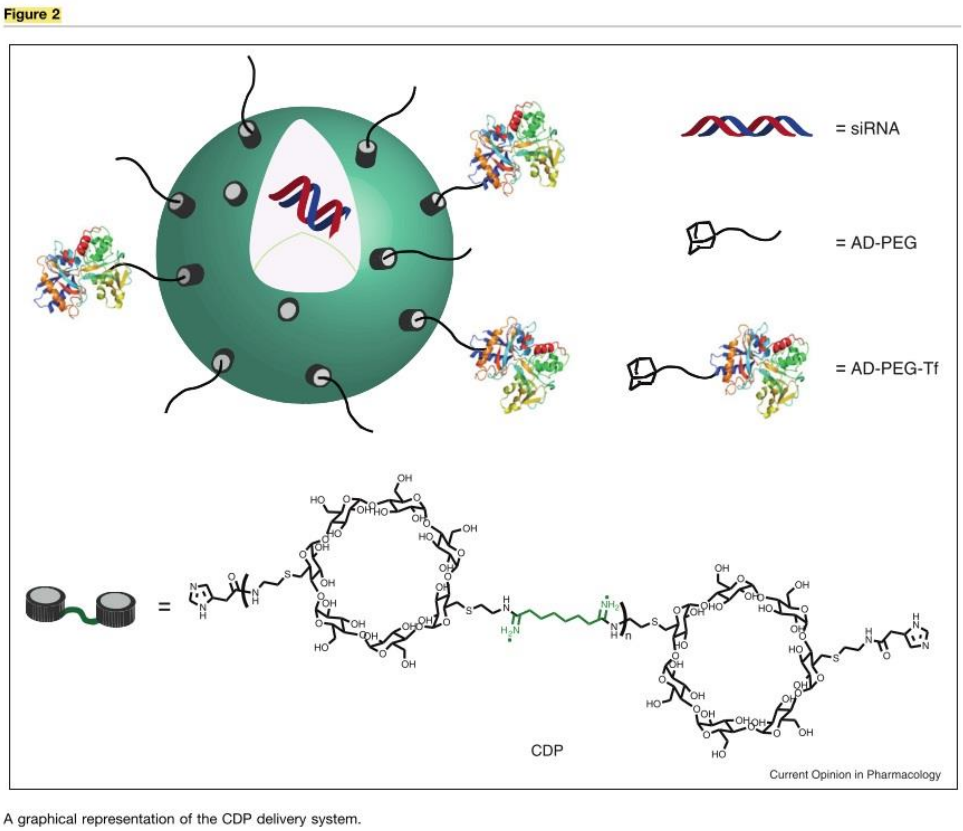

Figure 3. ß-Cyclodextrin with bound AD-PEG and AD-PEG-tf vehicle for siRNA transfection (Alabi, Vegas, & Anderson, 2012)**.** ß-cyclodextrin is the nanopolymer encapsulating the siRNA. Adamantane-polyethylene glycol (AD-PEG) is used to promote stability in biological fluids. Adamantane-polyethylene glycol-transferrin (AD-PEG-Tf) is a cellular surface receptor signaling protein for specific cellular uptake.

Target Specificity

Produced in intestinal epithelia and in the lamina propria, CXCR3 is a CXC-type 7-transmembrane chemokine receptor. These receptors are capable of binding specific 8-10 kDa chemokines. These receptors are also found on NK and T-cells and are used to stimulate chemotaxis and the inflammatory response (Baugher & Richmond, 2009). Besides binding chemokines, CXCR3 binds gliadin and is partially responsible for

initiating the aforementioned zonulin cascade to cause tight junction disassembly (Lammers et al., 2008). Of the chemokines that bind to CXCR3, the chemokine ligand CXCL11 has the highest binding affinity (Trotta, Costantini, & Colonna, 2009). To target enterocytes involved in the gliadin based inflammation, CXCL11 should be attached to the AD-PEG construct to select for enterocytes expressing CXCR3.

However, as CXCR3 is also expressed in T-cells, an AD-PEG-CXCL11 targeting system would not select exclusively for enterocytes. The role of tTG in NK and T-cells would need to be further examined in order to ensure the safety of CXCL11 as a targeting protein. As an alternative, α-gliadin could also be bound to the vehicle. As the method of gliadin absorption is still only partially understood at best, with CXCR3 receptors serving only a partial role in initiating CD signaling pathways, binding gliadin directly to the vehicle would ensure delivery into all cells affected by CD. Furthermore, gliadin binding with CXCR3 is nowhere near the strength of its target chemokines, making its binding affinity outside of the intestinal environment extremely unlikely. While little evidence exists documenting the relative binding strength and affinity of gliadin to CXCR3, its unequivocal role as the primary solicitor of CD symptomology makes it the ideal candidate for cell-specific targeting. Additionally, the binding of gliadin to the delivery vehicle would likely render it non-toxic as it would be incapable of binding to intracellular enzymes due to the size of the vehicle to which it is attached.

Clinical trials in siRNA transfections for gene silencing using ß-cyclodextrin as a delivery vehicle are already underway and have shown great promise. Biopsies of clinical trial patients have shown targeted mRNA fragments, lowered target protein expression,

and treatment efficacy lasting more than several dosing cycles. Administration of CALAA-01 coated siRNA would be performed per the specifications of Davis, et al., at 18, 24, and 30 mg siRNA/m^2 via 30 minute intravenous injection on days 1, 3, 8, and 10 of a 21 day cycle.

While knockout of tTG expression throughout the human body could still produce no phenotypic differences, limitation of knockout to the afflicted cells of the small intestine will minimize potential risk and prevent unnecessary waste of siRNA cargo. In all, siRNA transfections present a completely untapped resource to greatly improve the lives of nearly 43 million people worldwide. Current treatment plans do nothing more than restrict lifestyle choices and ultimately doom the patient to unhappiness and discontent with their diet. A radical treatment plan is the only option available for this disease; while some might argue for supplemental intake of enzymatic inhibitors, gene knockout would be far more effective, longer lasting, and specific than any other proposed treatment plan.

Case Study

The following case study is of a 23 year-old Italian-American female who brought herself into her doctor's office concerned for amenorrhea and an antebrachial rash. The purpose of this case study is to illustrate the extraintestinal symptoms that may appear as secondary effects to celiac induced malabsorption. Laboratory reference values were obtained from Mayo Clinic Laboratories.

Patient Name: Portia Catonis **Sex:** Female **DOB:** 03/15/1989

Site of Visit: Family Practice Office **Date Of Visit:** 04/01/2013

Chief Complaint (CC)

- Missed period last two months
- Rash

History of Present Illness (HPI)

Ms. Portia Catonis is a 23 year old Italian-American female who brought herself into her general physician's office on April 1st, 2013, expressing concern for missing her period for the last two months and for a persistent, red, itchy, blistery rash on her elbows. She admits to being sexually active with a single partner, but states that her partner always uses a condom. While she reports having felt the "usual signs and symptoms" of a coming period, Ms. Catonis has not menstruated since 1/28/2013. She denies any change in appetite or diet, but has lost about five pounds since the beginning of the year. The patient claims good emotional health and normal stress levels, but does notice that she feels less rested than normal upon arising. Her hair seems drier and "less healthy" than normal and her nails are chipping frequently.

The patient reports the rash on her elbows to be spotty dark red, with occasional small, blister-like bumps. The rash started on 3/25/2013 with mild discoloration and has progressively worsened over the past week. The rash is unbearably itchy and the patient reports taking 25mg of diphenhydramine every four to six hours, providing her with

moderate relief. She denies having changed bath or body products. She does report having begun adding a laundry detergent booster around the time the rash appeared, but discontinued its use three days ago.

Past Medical History (PMH)

Ms. Catonis reports seasonal hay fever, with her worst allergies stemming from flower pollen and alfalfa. Suffering from frequent colds as a child, she had her tonsils removed in 07/1997 at Saint Mary's Hospital in Reno, NV. The procedure had no complications and the patient was discharged the same day, recovering fully within a week. The patient and her two brothers all contracted varicella at the same time as children in the winter of 1994. Ms. Catonis takes no medications and her immunizations are all current.

Family History (FH)

The patient's mother and father, aged 53 and 55, are both alive. Her father has type I diabetes mellitus, mild hypercholesterolemia and hypertension. Her mother was diagnosed with hypothyroidism five years ago, is lactose intolerant and has a "weak stomach". Her two male twin siblings, aged 21, are of good health and extremely active. Her paternal grandfather died this year of a heart attack, having both coronary heart disease and atherosclerosis.

Social History (SH)

The patient is sexually active and monogamous. She has been with her partner for almost two years. She admits to several instances of unsafe sex, but denies previous STI's, pregnancies, or use of hormonal birth control. She drinks socially, 1-2 drinks on

one weekend night per week. She denies tobacco or illicit drug use. Ms. Catonis is regularly active, taking a one hour yoga classes two times per week. The patient reports high stress levels from working and going to school, but states that the regular exercise helps to control it.

Review of Systems (ROS)

General: The patient reports decreased energy upon awakening and minor (<5 lbs.) weight loss. The patient denies recent trauma, changes in appetite, fever, or sleep disturbance.

Integumentary: Patient reports a red, blotchy, itchy, raised rash on both arms, ranging 5cm in each direction from elbow. Denies breast pain, lumps, or discharge.

HEENT:

- Head: Denies trauma or headache
- Ears: Denies tinnitus, changes/loss of hearing, or dizziness
- Eyes: Denies changes/loss of vision, diplopia, discharge, or red eye
- Nose: Reports mild typical sinus congestion with little to no pain, dryness of the nasal membranes, denies epistaxis
- Throat: Denies pain, dryness, odynophagia or dysphagia

Neck: Denies swelling or masses.

Cardiovascular: Denies chest pain, palpitations, or generalized edema.

Respiratory: Denies shortness of breath, cough, wheezing, expectoration, or exercise intolerance.

Gastrointestinal: The patient reports a <5 lbs. weight loss but denies any changes in activity, diet, or behavior. Denies abdominal pain, dyspepsia, nausea, or changes in appetite.

Genitourinary: Reports amenorrhea for past two months. Patient has experienced typical premenstrual-type symptoms such as bloating, emotional instability, acne, and muscle and breast tenderness without menstruation. She also reports occasional spotting and diarrhea. She states her last period was lighter than normal. She denies dysuria, constipation, or incontinence. No history of pregnancy, abortion, or miscarriage.

Musculoskeletal: Denies pain, misalignment, joint swelling, or decreased range of motion.

Neurological/Psychiatric: Denies headache, dizziness, loss of consciousness, paraesthesia, numbness, speech problems, loss of motor control, or changes in senses. The patient reports high stress and mild work-related anxiety. She denies depression, mood swings, changes in personality, sexual or financial binging, or changes in concentration/focus/clarity.

Physical Exam

Vitals

Table 3. Patient vital signs

Height	**Weight**	**BMI**	**Temp**
5’4”	120 lbs.	20.6	97.5°F
HR	**SpO_2**	**BP**	**RR**
65	98%	112/69	14

General: Patient is calm and attentive. Patient occasionally scratches her elbows.

Integumentary: Red-purple mottled papulovesicular rash beginning over both elbows and extending onto the dorsal antebrachium, approximately 5cm wide by 10cm long. The skin is broken in several areas within the rash boundary. The rash is contained to these two regions and appears nowhere else. No cyanosis, although a slight pallor is noted. Fingernails appear brittle and are flaking. Hair appears dry and frayed.

HEENT:

- Head: Normocephalic, atraumatic
- Ears: Bilateral tympanic membrane intact and reactive to light
- Eyes: Pupils equally round and reactive to light and accommodation bilaterally, bilateral sclera anicteric, no conjunctivitis
- Nose: Mucous membranes dry, slight erythema
- Throat: Mucous membranes moist with slight erythema

Neck: Supple, no jugular venous distention, no lymphadenopathy, and no carotid bruit.

Cardiovascular: Regular rate and rhythm, S1 and S2 are normal with a slight flow murmur; no rubs, or gallops. Point of maximum impulse non-displaced and non-sustained. No hepatojugular reflux. Capillary refill is approximately 3 seconds.

Respiratory: Clear bilaterally to auscultation, no rales, rhonchi, or wheezes. Normal percussion noted.

Abdomen: Soft, non-distended and non-tender. No rebound or guarding observed. No pulsatile masses present. Normal bowel sounds are present in all four quadrants, along with resonant percussion. No costovertebral angle tenderness.

Genitourinary: No labial/external swelling, erythema, or lesions. No tenderness, discharge, or odor observed. Vaginal walls and cervix are intact with no lesions but show slight pallor and dryness. The uterus is anteverted, free of masses and non-tender. Bimanual palpation elicited no cervical motion tenderness, vaginal bleeding, discharge, or masses. Ovaries and fallopian tubes non-palpable.

Musculoskeletal: Normal range of motion, no joint swelling or erythema.

Nuerological/Psychiatric: Cranial Nerve II-XII intact, no focal deficit present. Normal affect without hallucinations and normal speech observed.

Labs/Radiology

Table 4. Complete Blood Count with Differential

ITEM	UNIT	VALUE	REFERENCE	
WBC Count	$X10^9/L$	7.8	4.0-11.0	N
Neutrophils	%	2.5	2.0-7.5	N
Lymphocytes	%	3.0	1.0-4.5	N
Monocytes	%	0.62	0.2-0.8	N
Eosinophils	%	0.12	0.04-0.40	N
Basophils	%	<0.1	<0.1	N
RBC Count	$X10^6/\mu L$	4.2	3.9-5.03	N
Hematocrit	%	33.0	34.9-44.5	**L**
Hemoglobin	g/dL	11.0	12.0-15.5	**L**
Mean Corpuscular Volume	fL	78.6	82.0-98.0	**L**
Mean Corpuscular Hemoglobin	pg	26.2	26.0-34.0	N
Mean Corpsucular Hemoglobin Concentration	g/dL	33.3	32.0-36.0	N
Red Blood Cell Distribution Width	%	12.3	11.5-14.5	N
Platelet Thrombocyte Count	$X10^9/L$	300,080	140,000-400,000	N
Mean Platelet Volume	fL	7.9	7.4-10.4	N

Table 5. Serum Iron Panel

ITEM	UNIT	VALUE	REFERENCE	
Serum Iron	µg/dL	41	50-170	**L**
TIBC	µg/dL	550	240-450	**H**
Transferrin Saturation	%	12	20-50	**L**

Table 6. Comprehensive Metabolic Panel

ITEM	UNIT	VALUE	REFERENCE	
Glucose	mg/dL	90	65-99	N
Urea Nitrogen (BUN)	mg/dL	21	7-25	N
Creatinine	mg/dL	1.29	0.78-1.34	N
eGFR Non-Afr. American	ml/min/m^2	65	>or=60	N
eGFR African American	ml/min/m^2		>or=60	
BUN/Creatinine Ratio		16	6-22	N
Sodium	mmol/L	141	135-146	N
Potassium	mmol/L	2.9	3.5-5.3	**L**
Chloride	mmol/L	88	98-110	**L**
Calcium	mg/dL	8.2	8.6-10.2	**L**
Carbon Dioxide	mmol/L	23	21-33	N
Protein, Total	g/dL	5.6	6.2-8.2	**L**
Albumin	g/dL	3.1	3.5-5.0	L
Globulin	g/dL	2.4	2.1-3.7	N
Albumin/Globulin Ratio		1.29	1.0-2.1	N
Bilirubin, Total	mg/dL	0.9	0.2-1.2	N
Alkaline Phosphatase	U/L	66	40-115	N
AST	U/L	23	10-40	N
ALT	U/L	42	9-60	N

Table 7. Thyroid Stimulating Hormone Panel

ITEM	UNIT	VALUE	REFERENCE	
TSH	mU/L	2.2	0.4-4.2	N

Table 8. Human Chorionic Gonadotropin Panel

ITEM	UNIT	VALUE	REFERENCE	
hCG	mIU/ml	2.2	<5.0	Non-pregnant

Table 9. Anti-IgA Antibodies Panel

ITEM	UNIT	VALUE	REFERENCE	
Anti-tissue transglutaminase-IgA	U/mL	16	<4	H
Total Serum IgA	mg/dL	401	61-356	H

Video Capsule Endoscopy

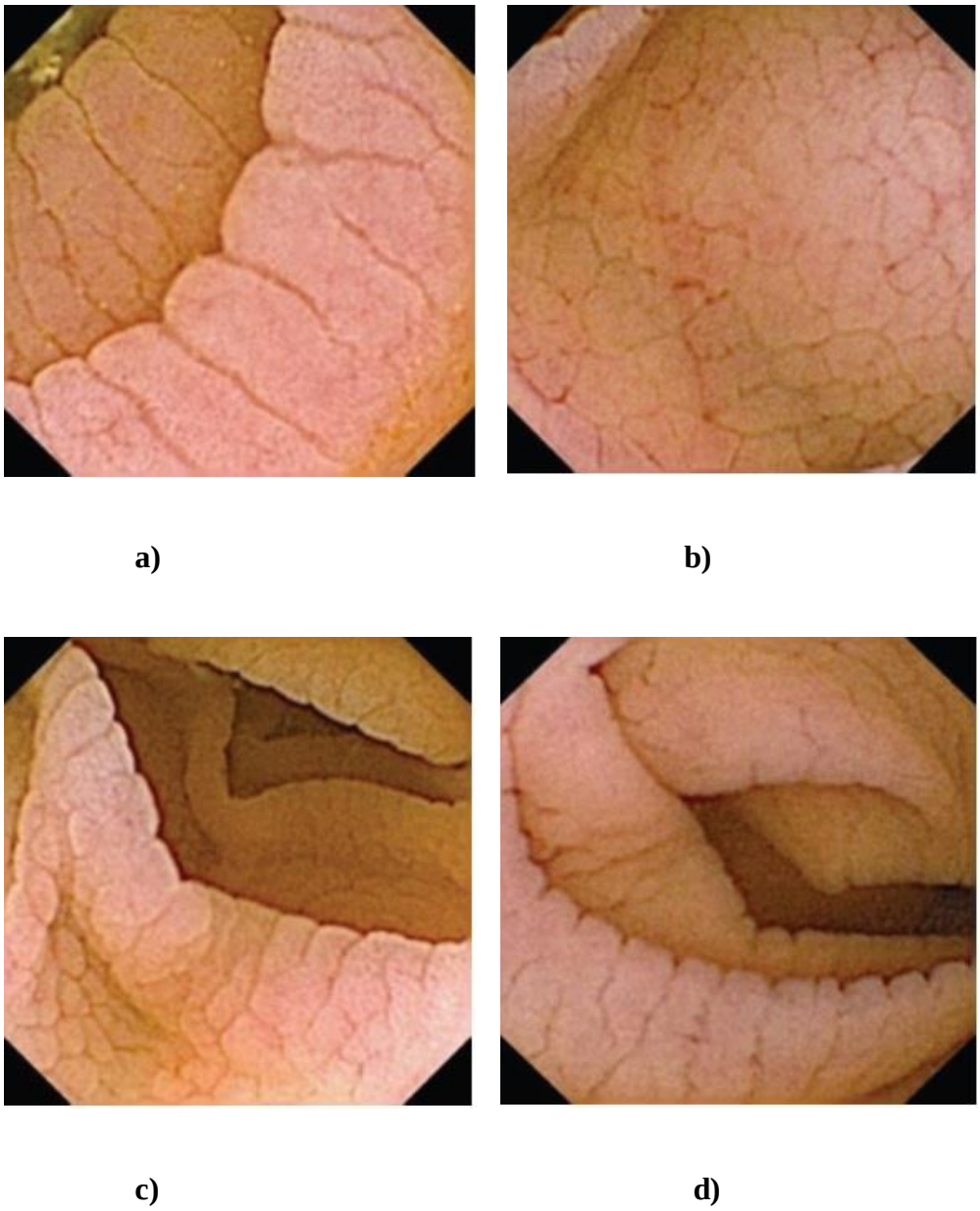

Figure 4. Video capsule endoscopy. Video capsule endoscopy showed abnormal jejunal architecture. a) scalloping of the intestinal wall; b) mosaic patterning; c) micronodularity; d) layering of intestinal folds (Akin & Ersoy, 2012).

Jejunal Biopsy

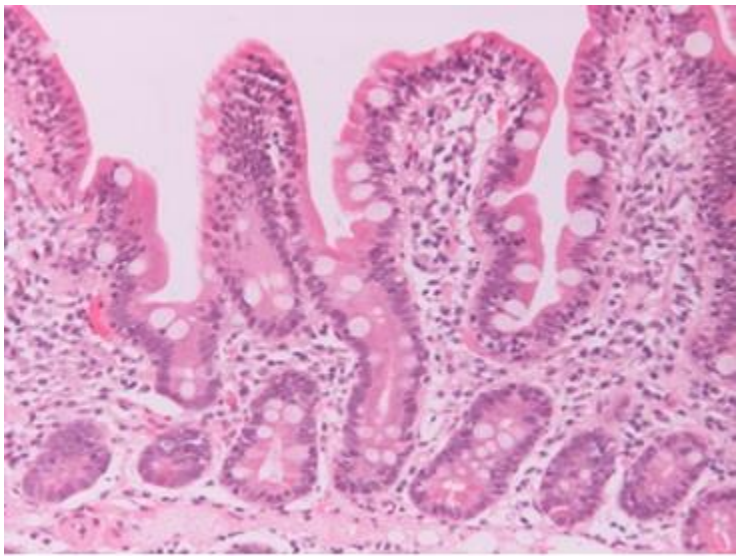

Figure 5. Jejunal biopsy. The jejunal biopsy demonstrates marked villous blunting, early crypt hyperplasia, and excessive lymphocyte infiltration into the villous tips (Schenk, Samloff, & Klipstein, 1965).

Assessment

Ms. Portia Catonis shows low hematocrit, hemoglobin, and mean corpuscular volume, suggesting anemia. Further analysis of serum iron elucidates a high probability of simple iron deficiency anemia, noted by decreased serum iron and transferrin saturation along with increased total iron binding capacity. Low ion levels including potassium, chloride, and calcium were observed, in addition to low total serum protein and albumin levels. These factors in concert with the patient's reported fatigue, weight loss, and weak hair/nails support the conclusion of a malabsorptive issue. Human chorionic gonadotropin levels were not elevated, verifying the patient is not pregnant. Anti-IgA antibodies were present, strongly suggesting the presence of celiac disease. Video capsule endoscopy and a jejunal biopsy confirmed celiac pathology. Celiac

disease and dermatitis herpetiformis were determined as the root causes of the patient's amenorrhea and antebrachial rash.

Diagnosis

- Celiac disease induced amenorrhea
- Simple iron deficiency anemia
- Dermatitis herpetiformis

Treatment

Ms. Catonis was instructed to begin a gluten-free diet and was explained food restrictions, alternatives, and advised to purchase several gluten-free cookbooks. She was given 1% hydrocortisone cream to be applied to the rash three to four times daily to control itching. She was instructed to return to the clinic in two months for follow-up serological evaluation and to return again if her period does not resume within four months. The patient was referred to a gastroenterologist for monitoring and control of her celiac disease.

Discussion

The difficulty in treating gluten intolerance lays not in the disease itself, but rather the near-omnipresence of gluten in American diets. The world has made inconceivably large changes in the past fifty years; technology, medicine, finances, and the arts have all benefitted from our global community. In the face of what we have achieved, it is shameful that we have yet to come up with a better solution to CD than "don't eat wheat".

Admittedly, CD does present some complications. Its illusive polyfactorial nature leaves many questions unanswered. The redundant role of the HLA-DQ proteins begs the question: "*what then is necessary?"* Genetic screening is only partially accurate considering the HLA-DQ family, and serological evaluation is not conclusive, requiring either invasive procedures or the inconvenience of a gluten-free diet to "trial and error" diagnose the patient. Nonetheless, the lack of new treatments which provide our patients with the highest level of care possible stems not from our knowledgebase, but rather from our imagination.

While ß-cyclodextrin based vehicles are only starting their clinical trials process, they hold far more promise in successful siRNA delivery than any previous non-viral method researched to-date. With the long-term goal of siRNA transfection treatments being gene knockouts lasting between years and a lifetime, treatment dosing and specificity are the highest priority to ensure the greatest uptake of siRNA into target intestinal cells. To create optimal specificity, development of an appropriate cell surface ligand for the ß-cyclodextrin vehicle should be secondary in focus only to continuing

confirmation of the stability of ß-cyclodextrin. So far, stability in biological fluids and target specificity have been exceedingly positive; the likelihood of developing a non-cytotoxic, specific, and effective uptake protocol for siRNA within the next five to ten years is extremely high.

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