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WHEAT GLUTEN, COELIAC DISEASE AND THE MICROBIOME: EXPLORING THE CONNECTIONS

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SUMMARY

Coeliac Disease (CD) is the most common food-induced immune mediated enteropathy in humans with an estimated prevalence of approximately 1% in worldwide human populations. A related condition known as non-coeliac gluten sensitivity (NCGS) appears to be increasing in prevalence, and may be even more common in the general population. These diseases are induced by the consumption of gluten, the common term for a family of digestion resistant, proline and glutamine-rich seed storage proteins found in the cereals wheat, barley and rye. The only current therapy for CD and NCGS is the maintenance of a life-long gluten free diet. Here, I present a brief review of these diseases as well as the role that study of the gut microbiome in humans and a non-human primate (NHP) model of CD can play in identifying treatments to address these conditions. Additionally, I present a brief overview of my research to develop a non-GMO (genetically modified organism), decreased gluten wheat and its relevance and potential applications in the context of CD, NCGS and human nutrition.

INTRODUCTION

The most well-known symptoms due to gluten ingestion in coeliac disease sufferers, including diarrhoea and stomach upset, were already described in antiquity about 2000 years ago: these disease manifestations were noted by the Greek physician Aretaeus of Cappadocia in the second century BCE (cited in: (*Freeman*, 2015). A derivation of the term he used "koiliakos", meaning "suffering in the bowels" gave coeliac disease its name (*Fasano* and *Flaherty*, 2014).

Remarkably, however, it wasn't until the middle of the 20th century that the definitive association between wheat consumption and the disease was established. In the 1930's the Dutch paediatrician, Dr. Willem-Karel Dicke, formulated the hypothesis that cereals such as wheat, rye, barley and (sometimes) oats were harmful to his paediatric coeliac patients. However, it was only during, and in the aftermath of the 2nd World War that he was able to definitively establish the connection between cereal consumption and deleterious symptoms in his patients. He noted that some of his patients improved when wheat flour was not available due to the deprivations of the war and that their condition worsened again after the war when wheat once again became available and his patients resumed eating wheat as a component of their diets. This, and subsequent careful studies of faecal fat content in his patients, led Dr. Dicke to publish the now generally accepted view that these cereals are the causative factor of coeliac disease (*van*

Berge-Henegouwen and *Mulder*, 1993).

In the years since this groundbreaking work there has been an increasing interest in coeliac disease as clinicians gradually became aware that the condition is not as rare as once thought, and as patient groups lobbied for new treatments. Coeliac disease was once thought to be primarily a paediatric condition, but it is now known that it can arise at any age. It is an autoimmune response triggered by the consumption of gluten in genetically susceptible individuals: principally individuals expressing human leukocyte antigen (HLA) haplotypes DQ2 and DQ8 (for a recent review see: *Lindfors* et al., 2019). In susceptible individuals, the consequence of consumption of wheat, barley, or rye is a chronic intestinal inflammation and flattening of the nutrient-absorbing villi of the small intestine. The clinical presentation of the disease, however, can vary drastically from very severe to no symptoms, to extra-intestinal symptoms such as dermatitis herpetiformis (skin rash) or neurological symptoms (*Jackson* et al., 2012). Because its symptoms vary so widely, there can oftentimes be a long interval between initial presentation and diagnosis, and the disease is still under-reported.

Nowadays, the disease is identified by screening for auto-antibodies such as transglutaminase 2 antibodies (TG2As), and endomysial antibodies (EmAs), as well as antibodies to gliadin. Definitive diagnosis follows with a small intestinal mucosal biopsy while the patient is still on a gluten-containing diet to confirm villous flattening. Once diagnosed, the only treatment is strict adherence to a lifelong gluten free diet. This can be quite challenging since wheat gluten is present in many processed foods, and it imposes an economic burden because most gluten-free substitutes for normally gluten-containing products are over 200% more expensive than their gluten-containing counterparts (*Lee* et al., 2007).

In recent years a number of drug candidates have emerged that are undergoing testing as potential treatments for coeliac disease. These include a modulator of intestinal tight junction permeability (*Kelly* et al., 2013), a polymer that sequesters gluten (*Alhassan* et al., 2019), and several competing gluten-degrading enzymes (*Sollid* and *Khosla*, 2011; *Wolf* et al., 2015; *Wungjiranirun* et al., 2016). There were also high hopes for a therapeutic vaccine (Nexvax2) under development by the company ImmusanT that was in Phase II trials (*Di Sabatino* et al., 2018). These hopes, however, were dashed with the very recent announcement (June 25, 2019) that ImmusanT was suspending the Phase II trial because interim analysis of the data showed that the vaccine was no better than placebo in providing protection from gluten exposure to patients (http://www.immusant.com/ImmusanT%20Nexvax2%20P2%20-%2025Jun19%20Final.pdf).

With the exception of the now discontinued Nexvax2, the CD therapies in development are not intended to allow CD sufferers to resume an unrestricted diet, rather they are meant to provide protection to patients from small amounts of inadvertently consumed gluten while they are following a gluten free diet.

In addition to coeliac disease, and wheat allergy (not discussed here), a recently identified condition known as non-coeliac gluten sensitivity (NCGS) has emerged. The key similarity with CD is that symptoms in patients resolve with a gluten free diet, but this syndrome is not associated with villous flattening, and the lack of clear biomarkers of disease makes this a still

mysterious condition (*Dale* et al., 2019). Besides gluten, there is evidence that so-called FODMAPS (fermentable oligo-, di-, monosaccharides and polyols) are responsible for symptoms in some NCGS patients. This has led *Dale* et al., (2019) to suggest that a more accurate term for the condition might be non-coeliac wheat sensitivity (NCWS) rather than NCGS, to acknowledge the role that other wheat constituents play in this syndrome, and it might also be considered a subtype of irritable bowel syndrome (IBS). The prevalence of NCGS is unclear, as many individuals are self-diagnosed and avoid gluten and wheat products out of a belief that such a diet is healthier. Estimates range widely, from less than 1% to greater than 10% of the population (*Aziz* et al., 2016). Until specific biomarkers and other diagnostic criteria are developed, this syndrome will remain somewhat undefined.

COELIAC DISEASE AND THE MICROBIOME

Considering that the study of the human microbiome is a young field, which was stimulated by the development of the inexpensive "next generation" sequencing technologies that arose out of technology developed for the sequencing of the human genome, the first human microbiome studies were, in effect, exercises in charting the territory and establishing a catalogue of the existing microbial diversity living in the diverse ecological niches present on and in healthy human bodies (*Human Microbiome Project Consortium*, 2012).

Likewise, these are still early days in the study of the human gut microbiome in relation to coeliac disease, and the studies reported to date might be regarded as being in the "cataloguing" phase of identifying differences in the gut microbiomes of coeliac patients compared to matched controls (*Sanz* et al., 2011). Such studies provide a valuable service in beginning to unravel the interactions between the intestinal pathology of coeliac disease and the attendant gut microbial community. The composition of the intestinal microbiome in effect may serve as a surrogate biomarker of the health of the individual. In addition, changes in the microbiome over time, for example in response to dietary or other interventions, may serve to document the effect of those interventions (*Lindfors* et al., 2019). Whether identified differences in the microbiomes of coeliac patients compared to healthy controls are a cause or consequence of the disease is not completely clear. Nevertheless, there is evidence that the HLA alleles, which predispose the individuals carrying them to develop coeliac disease, may influence their carriers to develop a disease-predisposing microbiome composition (*Olivares* et al., 2015, 2018; *Sanz*, 2015).

In general, the studies reported to date frequently show microbiome differences between CD individuals and controls but are somewhat inconclusive with regard to functional consequences (*Cheng* et al., 2013). In many cases, a decreased diversity in the gut microbiomes of coeliac patients compared to healthy controls has been found, frequently with differences in the types of gluten-degrading species present between the two groups (*Caminero* et al., 2014, 2016). In addition, gut dysbiosis in patients compared to healthy subjects is associated with a decrease in the ratio of putatively harmless bacteria *(Lactobacillus – Bifidobacterium)* compared to potentially harmful bacteria *(Bacteroides*/*Prevotella – E. coli*) (*Sanz* et

al., 2011; *Sánchez* et al., 2013).

Interestingly, one study found that early antibiotic use, which affects the gut microbiome, was associated with the development of coeliac disease (*Mårild* et al., 2013), however, a later study did not find such an association (*Kemppainen* et al., 2017). A recent review of the landscape of environmental and genetic factors that influence the development of coeliac disease highlights the role of microbiome composition as a causative factor in the loss of tolerance to gluten in CD patients (*Serena* et al., 2019).

In our own studies testing the effects of different animal feeds in a non-human primate (rhesus macaque) model of coeliac disease, we found that the faecal microbiome of gluten sensitive animals on a gluten containing diet displayed an overabundance of Streptococcaceae and Lactobacillaceae and a depletion of Coriobacteriaceae compared to healthy controls. In addition, the GS animals exhibited an upregulation of pro-inflammatory miRNAs (*Mohan* et al., 2016). This is the first assessment of faecal microbiomes in gluten sensitive rhesus macaques and the results strengthen the case for the use of this NHP model of CD to test experimental therapies (*Bethune* et al., 2008). Experiments to assess the effects on the faecal microbiomes in the NHP model of other dietary interventions, such as decreased gluten wheat, in combination with gluten degrading enzymes, are warranted. Our initial studies in the NHP model using a decreased gluten barley (*lys3a* mutant) animal feed pave the way for follow-up studies with decreased gluten wheat (*Sestak* et al., 2015; *Sestak* et al., 2016).

CEREAL SEED PROTEIN MUTATIONS: RELEVANCE TO WHEAT GLUTEN, NUTRITION AND CD

The seeds of grasses like rice, wheat and maize directly and indirectly provide most of the calories that feed humanity, thus the mechanisms that control the accumulation of the major storage compounds in seeds, namely starch, proteins and oils, have been of longstanding interest to scientists. In addition, because the storage proteins of many cereal crops such as maize and wheat are deficient in amino acids essential in the human diet and the diets of monogastric animals, considerable efforts have been devoted to identifying genetic variants that increase the amounts of these amino acids, principally lysine, threonine, tryptophan and methionine, in the seeds of cereal crops (*Azevedo* and *Arruda*, 2010). One of the earliest known genetic variants whose effect is to increase essential amino acids in cereal seeds is the opaque2 (*o2*) mutation in maize (*Mertz* et al., 1964), which leads to reductions in the accumulation of the 22 kilodalton size class of zein seed storage proteins (known as the α zeins) as well as to increased lysine content in maize seeds. When the gene underlying this mutation was finally isolated over 50 years after the mutant was first identified, it was found to encode a bZIP (basic leucine zipper) transcription factor (TF) that activates transcription of seed storage protein genes by binding to an upstream element found in the promoters of many of these genes (*Schmidt* et al., 1987) (*Schmidt* et al., 1990; 1992). Subsequently this mutation, together with genetic modifiers that ameliorate its negative pleiotropic effects, formed the basis for the

development of QPM (quality protein maize) varieties (*Prasanna* et al., 2001) containing increased lysine with demonstrated nutritional benefits in humans (*Gunaratna* et al., 2010; *Nuss* and *Tanumihardjo*, 2011).

Since the identification of the transcription factor underlying the *o2* mutation, which contains a basic DNA-binding region followed by a protein-protein interaction motif known as a leucine zipper (*Schmidt* et al., 1990), multiple additional transcription factors belonging to different structural classes have been determined to play roles regulating processes of seed nutritional reserve accumulation in maize and other cereals. These include transcription factors belonging to the DOF (DNA-binding one zinc finger) family, which interact with the O2-family transcription factors in binding to the promoters of seed storage protein genes (*Vicente-Carbajosa* et al., 1997; *Mena* et al., 1998; *Zhang* et al., 2015).

The success of increasing the content of essential amino acids such as lysine in *o2* maize motivated the search for similar mutants in related cereals. A program of barley mutagenesis and screening for increased lysine using a dye binding technique at the Risø agricultural experiment station in Roskilde, Denmark in the 1960s led to the identification of numerous enhanced lysine mutants of which the best characterized is the *lys3a* mutant (*Køie* and *Doll*, 1979; *Miflin* and *Shewry*, 1979). This mutant is also referred to as Risø 1508 or sex3c (shrunken endosperm xenia) (*Ullrich* and *Eslick*, 1977); it was induced by ethylenimine mutagenesis of the two-rowed spring malting variety, Bomi (*Doll* et al., 1974). The *lys3a* mutant is almost completely lacking in class C hordeins, and accumulates considerably reduced amounts of several B class hordeins, while having a 45% increase in the accumulation of free and protein bound lysine in the seed compared to the parental variety (*Shewry* et al., 1977; *Shewry* et al., 1978; *Munck* et al., 2001). These phenotypes are the result of a single recessive allele (*Doll*, 1973) that was mapped to barley chromosome 5H (*Karlsson*, 1977; *Ullrich* and *Eslick*, 1977; *Jensen*, 1979), although more recent research asserts that this map position is erroneous and that the mutant is located on chromosome 1H (*Druka* et al., 2011; *Rustgi* and *von Wettstein*, 2015). The underlying lesion responsible for the *lys3a* mutant's pleiotropic effects was hypothesized to be in a 5-methylcytosine DNA glycosylase gene (DEMETER, DNA demethylase) (*Gehring* et al., 2009) responsible for removing the methyl groups from methylated DNA since it was found that the methylation state of the promoters of several seed storage protein genes was altered in the mutant (*Sørensen*, 1992; *Sørensen* et al., 1996) (*Wen* et al., 2012).

The original *lys3a* mutant suffered from negative pleiotropic effects including lower yield and shrunken seeds containing reduced total starch. Nevertheless, concerted breeding efforts minimized these effects (*Eggum* et al., 1995; *Jørgensen* et al., 1999). Barley varieties containing the *lys3a* allele, however, were still lower in starch content than conventional cultivars and for this reason they were never widely grown (*Munck* and *Jespersen*, 2009). In recent years, there has been a resurgence of interest in this mutant, not because of its higher lysine content, but due to its lower levels of several classes of hordeins. The *lys3a* mutant, in combination with several other mutations that reduce the accumulation of hordeins, has been used to create ultra-low gluten barley (ULG) (*Tanner* et al., 2016). In addition, we tested the *lys3a* mutant in the rhesus macaque model of coeliac disease to determine if it, alone

or in combination with a gluten-degrading enzyme supplement, can ameliorate the symptoms of gluten sensitivity in this NHP model of coeliac disease (*Sestak* et al., 2015; 2016). In our 2016 paper, we reported that a diet of this *lys3a* mutant barley feed, combined with a gluten-degrading enzyme (Tolerase G, manufactured by DSM), appeared to eliminate symptoms due to gluten sensitivity in this animal model of coeliac disease.

We subsequently determined that a missense mutation in the Barley Prolamin-box Binding Factor (BPBF) represents the molecular lesion underlying the *lys3a* mutation (*Moehs* et al., 2019). This barley transcription factor is homologous to a domain of one finger (DOF) zinc finger transcription factor found in maize that has been shown to interact with O2 to control the expression of maize zein seed storage proteins (*Zhang* et al., 2015). A wheat homolog of BPBF had also been identified (*Ravel* et al., 2006).

This allowed us to use the non-transgenic, reverse genetic technique known as TILLING (Targeting Induced Local Lesions in Genomes; *Colbert* et al., 2001), a variation on mutation breeding, to identify 488 novel wheat lines that contain induced variants in the A, B and D genome copies of the WPBF genes in hexaploid bread wheat. Combining inactivating (recessive) alleles in all three homoeologous copies of the wheat PBF gene in hexaploid bread wheat led to a reduction in the accumulation of several classes of wheat gliadins and low molecular weight glutenins and to an increase in the accumulation of free and protein-bound lysine in wheat endosperm. These lines, which we refer to as decreased gluten wheat (DGW), have lower amounts of the epitopes that are detrimental to coeliac patients, but they are not low enough in gluten to be safe to consume for CD patients. However, they are novel in that they contain levels of gluten that are lower and outside of the range of the available genetic variation in existing wheat germplasm, including such "ancient" wheats as einkorn and spelt. In addition, there are ongoing efforts to decrease the gluten even further.

At present, the alleles are being introgressed into elite varieties and functional tests are being conducted to assess the types of products (cookies, cake, bread, noodles, tortillas, etc.) for which the DGW is best suited. Based on the outcomes of these tests, combined with other factors, will influence which market class(es) of wheat the alleles will be introgressed into. There are at least 6 market classes of wheat, including spring and winter wheats, as well as hard red and white wheats, in addition to durum pasta wheat. These different market classes all have particular functionality and uses.

A number of other groups are pursuing the development of decreased gluten wheat by other methods including RNAi (*Gil-Humanes* et al., 2010), and CRISPR (*Sánchez-León* et al., 2017). These methods have been very effective at creating transcriptional shutdowns in the expression of gliadins in the case of RNAi, or gene deletions of gliadins in the case of CRISPR, but these wheats face additional hurdles that come with being classified as transgenic and thus will face additional public scrutiny and a costlier path to market (*Laursen* 2016; *Jouanin* et al., 2018; *Hundleby* and *Harwood*, 2019).

CONCLUSION

The incidence of coeliac disease and non-coeliac gluten (wheat) sensitivity has been on a slow upward trajectory in recent decades and this is not simply due to increased testing and awareness (*Rubio-Tapia* and *Murray*, 2010). During the Swedish paediatric coeliac disease epidemic of the 1980s and 1990s, for example, the incidence reached about 3% of tested 12- year-old children, well above previously known rates of the disease (*Myléus* et al., 2009). Given that the predisposing HLA alleles are quite common in most human populations, but only a small subset of individuals carrying these alleles lose tolerance to gluten and develop coeliac disease, considerable effort has been devoted to identifying possible environmental variables that may contribute to loss of gluten tolerance (reviewed in: *Lindfors* et al., 2019)). Among the possible identified factors include infections with intestinal viruses (*Bouziat* et al., 2017; *Kahrs* et al., 2019), and a recent study suggested that vaccination against rotavirus may decrease paediatric coeliac disease cases (*Hemming-Harlo* et al., 2019). The seasonality of intestinal virus infections in children may also play a role in the link identified between seasonality of birth and the development of coeliac disease (*Daniel* et al., 2019). In the case of the Swedish CD epidemic, it was proposed that changes in the amount and timing of the introduction of wheat into infants' diets, as well as whether wheat was introduced while infants were still breastfeeding, played a role in the subsequent development of coeliac disease (*Ivarsson* et al., 2002). A recent meta-analysis, however, did not support a role for breastfeeding in subsequent development of CD (*Szajewska* et al., 2015) nor did adjusting the timing of gluten introduction into the diet of genetically predisposed infants prevent the development of CD in a well-designed, double-blind, randomized, placebo-controlled intervention study (*Vriezinga* et al., 2014).

Against the backdrop of clinical disease there is also a cultural shift occurring in many western nations with a dramatic increase in, and availability of, gluten free products, the self-diagnosis of "gluten sensitivity" and adoption of a gluten free and/or a low carbohydrate diet by a significant fraction of the public even in the absence of disease. This has been stimulated in part by a spate of best-selling, non-scientific books with sensationalistic titles such as "Wheat belly: lose the wheat, lose the weight, and find your path back to health," and "Grain brain: the surprising truth about wheat, carbs, and sugar--your brain's silent killers" that hold the consumption of wheat responsible for a host of current western societal ills, from "brain fog", to fatigue to obesity (*Davis*, 2014; *Perlmutter* and *Loberg*, 2018). In the current climate, it is not surprising that sensationalistic claims received more notice than the sober, scientific rebuttals (*Jones*, 2012; *Brouns* et al., 2013).

The "DIY" approach to gluten sensitive self-diagnosis even in the absence of a clinical diagnosis has also laudable aspects in the sense of the desire to control one's own health. One example of this is the growth in genetic self-testing companies such as 23andMe and its rivals. Self-testing of one's own microbiome is not far behind; there are already companies that offer this testing as a service, and individual accounts of dietary self-experiments and effects on the individual's own microbiome have been published (*Sprague*, 2017). Although there is no

"microbiome pill" to cure CD or NCGS, a number of microbiome companies (Second Genome, LNC Therapeutics) are dissecting the human microbiome with the objective to develop probiotics or pharmaceutical compounds to modulate disease states such as inflammatory bowel disease (IBD). Nature Publishing Group has just published a special collection of papers from the NIH funded Human Microbiome Project (https://www.nature.com/collections/fiabfcjbfj), and this is a research area that will likely continue to produce exciting insights and innovations in human health care in the years ahead (*Proctor*, 2019).

The decreased hordein (*lys3a*) barley that we used as a model in our studies during the course of the development of decreased gluten wheat appeared to positively impact the gut microbiomes of gluten sensitive rhesus macaques (*Mohan* et al., 2016), and it is to be hoped that similar follow-up studies will be conducted with decreased gluten wheat in the future. Along these lines, a recent report claims that a diet of the ancient diploid wheat, einkorn, induced beneficial changes in the gut microbiomes of pigs compared to a diet of conventional wheat (*Barone* et al., 2018), including increased abundance of the putatively health-associated species *Oscillospira* (*Konikoff* and *Gophna*, 2016). These microbiome changes, however, are unlikely to be related to the (minor) differences in the gluten composition between einkorn and conventional wheat used in the study, but more likely to be related to other compositional differences between these different wheats.

It may be that the increased awareness of gluten sensitivity in the public consciousness, an interest in low carbohydrate diets, as well as a desire to purchase local, organically grown food, has contributed to the rapid recent rise in the demand for and marketing of the so-called ancient wheats: einkorn, hulled spelt, and Kamut®. Although it has not been substantiated that these still niche types of wheat are lower in gluten and thus healthier for individuals with gluten sensitivity (*Dinu* et al., 2018), their market rise is a signal of consumer demand. Any decreased gluten wheat that ultimately comes to market might follow a similar market trajectory. The story of Quality Protein Maize, likewise, offers an encouraging parallel with DGW, in that QPM ultimately emerged as an agronomically and functionally beneficial variety with proven health benefits. While DGW must still surmount agronomic, functional and health and nutritional tests before it has any impact, the field ahead is full of promise.

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