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**CELIAC DISEASE: A PHYSIOLOGICAL OVERVIEW AND POSSIBLE FUTURE WORK WITH
EMPHASIS ON RAMAN SPECTROSCOPY**

by

KATLYN MEHNE

THESIS

Submitted to the Graduate School

of Wayne State University,

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Advisor

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CHAPTER 1: INTRODUCTION

Celiac Disease (CD) is an autoimmune condition with a 3:1 female predilection in which ingestion of gluten triggers immune mediated damage to the lining of the small intestine in susceptible individuals and confers a 2-4 fold increased mortality risk. CD is relatively common with a global incidence rate estimated at 1%. However, to diagnose it, patients are often required to provoke a potentially harmful immune response by eating a gluten-rich diet for a period of time. The only treatment is a gluten free diet (GFD). (Singh et al., 2018)

Although prevalence rates for Celiac Disease are estimated at 1% globally, actual prevalence may be much higher due to an average time to diagnosis of 4-11 years (Green et al., 2001). In fact, in the USA an estimated 3 million people have Celiac but only 90,000 of them are diagnosed. (CeliacDiseaseCenter, 2015) Part of the delay in diagnosis relates to the heterogeneous symptoms caused by CD, the fact that it can begin at any age, and that the only reliable risk stratification demographics are gender, a positive family history of CD, and having Type I diabetes. Clinical signs may be limited in nature to nongastrointestinal thus making accurate diagnosis more difficult.

CD has a strong genetic predisposition and from early studies appeared to have a Caucasian predominance. However recent data shows that ethnicity may not be a reliable demographic risk factor for CD. Asians, particularly south-East Asians have some of the lowest CD rates, with China reporting 18 cases in 1.3 billion people (Burkhardt et al., 2018). However India's rates, while previously reported as lower, have risen to 1%, the global mean. Finland, Italy, New Zealand, Israel, Northern Ireland and Iran all have prevalence rates reported near 1%. One of the highest currently known prevalence rates is 5.6% in the African Saharawi (Burkhardt et al., 2018). As gluten ingestion is thought to be required for the development of CD, it's possible that shifts in diet from more culturally traditional diets (possibly lower in gluten) to more globalized diets may affect prevalence rates. Conversely, the percentage of people without a diagnosis of CD choosing to eat a gluten free diet in the US and

Canada has risen faster than CD prevalence in both countries, which may artificially lower prevalence data if such patients never seek a diagnosis of CD.(Singh et al., 2018)

While CD is already a relatively common illness, its prevalence appears to have been rising globally over the past 40 years. CD prevalence rates doubled from the 1974 to 1989 in the US and between 1980 and 2000 in Finland. In Scotland, the prevalence of CD increased 6.4 fold between 1990 and 2009. Studies in Algeria, Czech Republic, India, Israel, Mexico, Malaysia, Saudi Arabia, Sweden, Portugal, and Turkey showed serological prevalence ranged from 2.1%-8.5%.(Singh et al., 2018) These statistics strongly suggest that worldwide prevalence of CD is increasing at a rate that exceeds diagnostic advances.(Burkhardt et al., 2018; Ivarsson et al., 2010; Singh et al., 2018)

Medical testing for CD is less than ideal. First steps include ordering blood antibody tests that can be falsely negative in a person eating a gluten free diet, so such patients are required to eat gluten for up to 8 weeks to provoke the autoimmune response and facilitate antibody detection. For this reason, the rising popularity of a gluten free diet (GFD) and the variation in reasons and applications of the diet presents an additional diagnostic & epidemiological complication. Many people eating a “gluten free diet” absent strict medical instruction, fail to eliminate all gluten from their diet. This may potentially mislead their physicians by removing CD from consideration. One study estimates that 30% of all Americans and Canadians are attempting a gluten-free diet, which far exceeds the current estimates of CD in the population. Even if individuals with various levels of gluten sensitivities and/or allergies were included, it is likely that the population of people who medically require a GFD is significantly less than 30%. Additionally, ingesting gluten can worsen inflammation in the intestine, cause significant symptoms and disrupt the quality of life of the patient. Even with a positive CD antibody test, the “gold standard” for confirming a CD diagnosis is duodenal biopsy showing a specific pattern of damage to the intestinal lining. Upper endoscopy with biopsy of

damaged tissue is expensive, time consuming, invasive, and can potentially leave areas of damage that do not heal properly due to the inflammation present.

History

Genetic markers in the bones of a woman who lived 2000 years ago make her the first known individual with CD. The name Celiac was first derived from the Greek word 'kolis' meaning 'stomach' and then from the Latin word '*coeliacus*' meaning 'person with painful bowels'. It was first recorded by Aretaeus in the second century to describe a stomach condition resulting in prolonged diarrhea leading to malnutrition, pale skin, gaseous diarrhea, muscle weakness, and body-wide atrophy, symptoms still observed in CD today.(Marsh, 1995; Paveley, 1988).

In the late 1800's, Adolf Baginsky performed histologic studies on children and infants suffering from a condition termed 'infantile atrophy' that presented with failure to thrive, pale skin, thin legs and buttocks, prominent abdomen, sensitive gut, and "loose, greasy feces". Baginsky noted most patients with the illness were irritable, moody, and lethargic with a high susceptibility to infections. Baginsky's histological findings pointed to a damaged intestine as the cause of the systemic atrophy, specifically in well-fed infants. At the time, infantile atrophy was a clinical syndrome caused by several different gut conditions, including what would later be deemed Celiac disease by Dr Samuel Jones Gee.(Toscano, 2004; Turner et al., 2015)

In the late 1800s, Dr. Samuel Jones Gee renamed infantile atrophy as the Coeliac Affliction, a term coined by a Greek physician. In October 1887, Gee gave a lecture citing the fecal condition as the basis for the diagnosis of Coeliac Affliction noting patients' faeces, were "loose, not formed but watery; more bulky than the food taken would seem to account for; pale in colour, as if devoid of bile; yeasty, frothy, an appearance probably due to fermentation; stinking, stench often very great, the food having undergone putrefaction rather than concoction"(Paveley, 1988). Gee blamed starchy foods, and recommended diet control as treatment. The diets were not necessarily gluten-free, but occasionally a diet consisting of all

mussels or all bananas yielded improvement. Many of Dr. Gee's clinical observations were corroborated two years later in Edinburgh by another English physician, Dr. R. A. Gibbons, who studied infants who died of the disease. Dr. Gibbon' post-mortems showed "no wasting of the mucous membrane, no ulceration, and no wasting of Lieberkuhn's follicles" to indicate nutritional issues. Instead, it suggested the issue to be one of digestion of food and the absorption of damaging elements due to this deviant digestion caused by the nervous system and its interaction with the liver, pancreas and stomach. In 1904, Dr. Cheadle agreed with the nervous system involvement in Coeliac Affliction and re-named the condition Acholia after noting the presence of fat and absence of bile in the stool. He also noted a possible dental correlation and included gum lancing in his treatment regimens.(Dickson et al., 2006; Marsh, 1995; Paveley, 1988)

Several more breakthroughs continued in the early 1900s. In 1908, the idea that Acholia was caused by intestinal inflammation was published by Dr. Herter, who restated in 1918 the correlation with starch, particularly bread, as a source of symptoms. The first successful treatment plan for Acholia was developed by Dr. Sidney Haas in 1924. He combined a banana diet with colonic irrigation and castor oil to treat and presumptively cure eight children. This cure was defined by clinical resolution of the symptoms as intestinal biopsies would not be available until 1940s. Like the gluten free treatment today, this cure was only successful as long as the child remained on the diet. Dr. Haas' diet excluded starches such as bread, potatoes, cereals and crackers, but also sucrose and other sugars except those found in bananas. This was largely due to Puerto Rican farmers whose diet used bananas as a carbohydrate source as opposed to bread. Since the diets were incidentally gluten-free, They were successful in arresting the symptoms of CD. It wasn't until the food shortage during World War II that the connection between CD and wheat was noticed. A Dutch pediatrician, Dr. William-Karel Dirke, noted that during the bread shortage in the Netherlands, those with CD improved remarkably until a Swedish effort to combat hunger provided bread to the Dutch

population. Dirke had considered the causal connection as early as 1930. With the additional evidence, Dirke's team studied bread more thoroughly and concluded that gluten, not starch, was the toxic element triggering CD symptoms. Haas' banana diet remained popular despite Dirke's evidence that a less restrictive diet would alleviate symptoms of CD. (Marsh, 1995; Paveley, 1988; Smits, 1989; Turner et al., 2015)

In 1954, Dr. J.W Paulley began a line of research into the normal biopsy of the jejunal mucosal layer of the intestine. Gastric biopsies on live patients became available only after the 1950s and provided a histological method of diagnosing many GI condition, including CD. In 1955, groups in Argentina and England accessed the duodenum for biopsies in live patients, lesions which would eventually define the pathology of CD. (Paveley, 1988)

Throughout the 1960s and 1970s doctors noticed a correlation between certain duodenal biopsy pathology and various other symptoms like dermatitis herpetiformis and gluten ataxia. Additionally, the discovery of α gliadin as the toxic substance in gluten occurred. Early immunology work in 1972 correlated HLA and CD. In the mid 1980's, research demonstrated that not all children with CD demonstrate outright symptoms, leading to the possibility of an Asymptomatic CD diagnosis.(Cacciari et al., 1983) In 1983, the presence of specific antibodies during CD was discovered. (Gasbarrini et al., 2014) By the mid 1990s, research found a strong correlation between anemia and CD and that certain HLA-types are associated with CD. The endoscopic criteria were revised in 1990.(Auricchio and Troncone, 1996; Sollid et al., 1989) Also, the higher incidence rate in females vs.males became apparent.(Cirillo et al., 1995) By 1999, a duodenal biopsy screening was suggested for women suspected to have CD. In 2005, serology and duodenal biopsy became the standard for diagnosing CD as set forth by the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition.(Hill et al., 2005)

General Celiac Overview

Celiac Disease results from a complicated interaction between the digestive system and the immune system in the presence of Gluten. Figure 1 shows a flowchart of all the interactions

involved in the basics of CD. Digestions starts in the mouth and proceeds through the stomach and into the three sections of the small intestine before the remaining food particles are excreted through the colon. The immune system, beginning on the lower left corner of the flowchart, sends immature cells from the bone marrow either to the stromal of that bone marrow where they develop into B-cells or to the thymus where they develop into T-cells. Both these cells enter the systemic immune system. At each section of the small intestine the endothelium interacts with digested gluten polypeptides. This interaction results in the activation of Cytotoxic T-cells lining the endothelium causing endothelium damage which can cause bacteria to enter the liver duct, thus increasing the chance of Chronic Active Hepatitis. Additionally, the gluten polypeptides interact with the zonulin pathway to open the tight junctions in the endothelium, also causing endothelium damage. Both this pathway and the endothelium damage itself allow unwanted particles to enter the lamina from the intestine. Additional polypeptides enter the lamina through the normal physiological transfer systems where they interact with tTG2 to activate the adaptive immune system. The genes that code the receptors in the T-cells at this stage are identified in a Genetic Diagnosis. The resultant activation of B-cells activates the immune response to injury, including inflammation and cellular proliferation. The activated B-cells also produce quantities of tTG2 antibodies, whose presence in serum is identified in a Serological Diagnosis. The immune response to injury and the previous endothelium damage both cause villous blunting in the small intestines. This is identified in a Endoscopic Diagnosis. This villous blunting, when in the duodenum, decreases or halts the production of the hormone that induces the gall bladder to secrete bile. This process in the small intestines promotes biliary production by the liver, which is fed into the Gall bladder causing inflation and possibly Primary Biliary Cirrhosis. The villous blunting in any section of the small intestine decreased the nutrition transfer efficiency. This causes multiple symptoms such as general nutritional deficiency, failure to thrive, organ atrophy, fetal development retardation during pregnancy and bone matrix porosity. The presence of tTG2 antibodies also causes several symptoms such as Dermatitis

Herpiformis, additional bone matrix porosity and several neuropathies. A combination of these symptoms are identified as a Symptomatic Diagnosis.

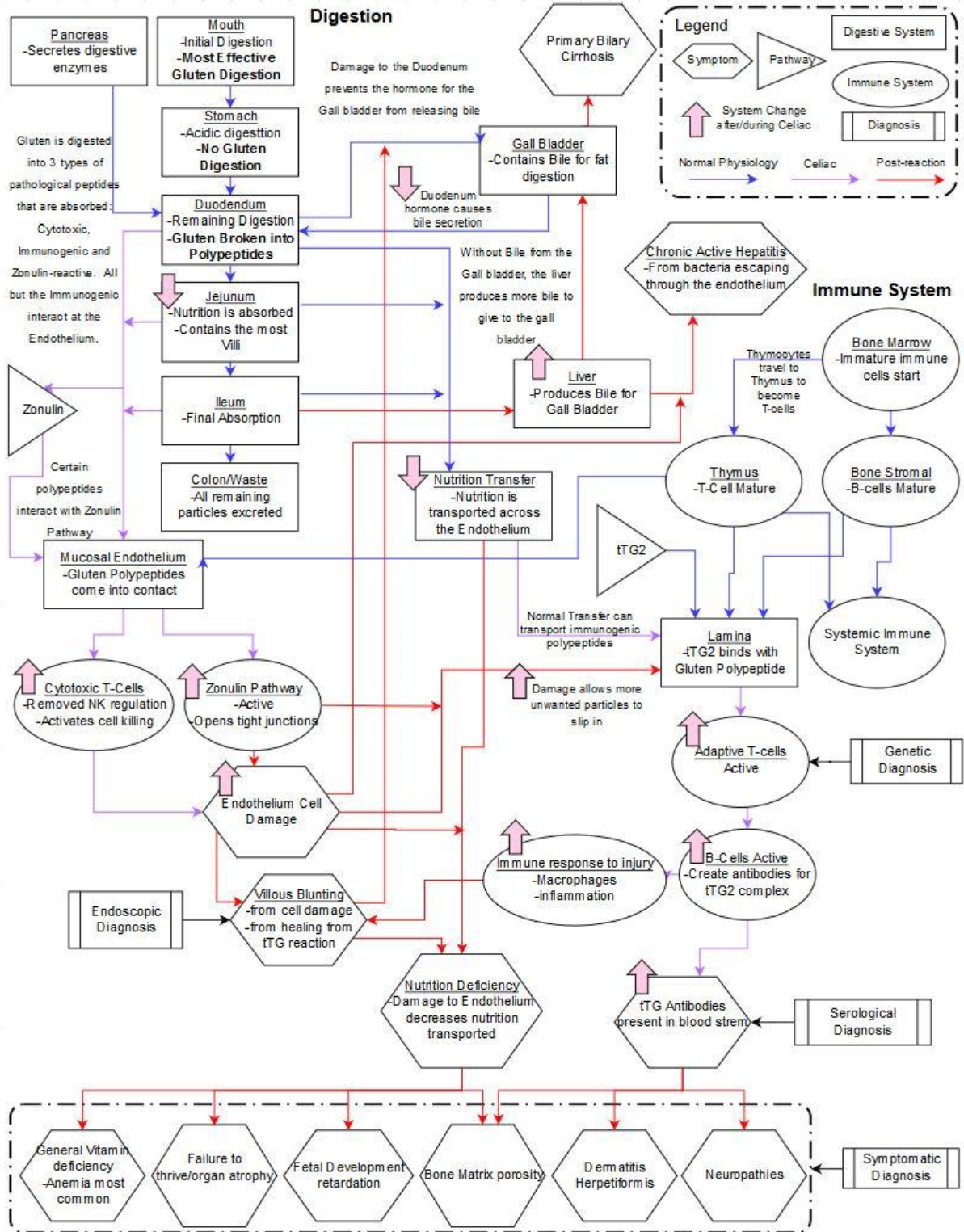


Figure 1: Flow Chart of Celiac Disease. The digestion system starts in the upper left and the immune system starts in the lower left. The legend in the upper right identifies symbols for processes in the digestion system, immune system, and physiological pathways. Additionally the color of the arrows indicate the normal activity, activity during the celiac reaction and the pathology following CD.

CHAPTER 2: DIGESTION OVERVIEW

Celiac Disease has been associated with food intake since the 2nd century making the study of digestion an integral part of understanding CD.

Throat and stomach

Food particles that enter the digestive tract travel through several digestive processes before encountering the intestine. In the mouth, mastication and saliva start the digestive process by breaking food into smaller particles and breaking polysaccharides into simple sugars. Salivary pH and enzymes provide some protein degradation and bacterial protection for the body. Once masticated, food enters the stomach. Hydrochloric acid kills most microbes and lowers the pH of the stomach contents to change the oxidation state of iron. It also activates pepsin from the pepsinogen released by the stomach, which breaks proteins into smaller polypeptides. Intrinsic factor (IF) is also released into the stomach contents to bind vitamin B12.

Pepsin is a class of aspartic peptidase present in human gastric juice (Roberts, 2006). Aspartic peptidases attack scissile peptide bonds, utilizing a water molecule to cleave proteins. This particular peptidase is categorized as Aspartic due to the Asparagine residues that act as ligands for the activated water molecule. These peptidases are divided into clans. Pepsin belongs in Clan AA under Family A1, a group of bi-lobed enzymes with Asp residues at the active sites. Pepsin activity normally requires a pH below 4.5 and the peptidase denature above a pH of 7.0. Human gastric juice, found mainly in the stomach and composed largely of HCl, maintains a pH between 1.5 and 3.5 under normal conditions. Food spends up to 2 hours being digested in the stomach, making the stomach luminal pH crucial to food digestion. The main pepsins found in human gastric juice are pepsin A, pepsin C, cathepsin D and cathepsin E.

The stomach consists of 3 layers of muscle each with a unique fiber orientation. They allow the stomach to contract and relax in multiple directions to physically break food boluses

into smaller particles and thoroughly mix them with the HCl, pepsin, and IF into a liquid substance referred to as chyme.

Small intestine

From the stomach, chyme flows through the pyloric sphincter into the small intestine to be further digested. It is either absorbed as nutrition or transformed into waste products and excreted by the colon. The luminal surface of the small intestine is covered in epithelial cells called enterocytes. These specific enterocytes absorb nutrients from the chyme, including peptides with single, double and triple residue chains.(Caminero et al., 2014) To increase absorption, the lining of the small intestine utilize villi, small fingerlike projections, to create a convoluted wall which maximizes surface area in contact with luminal contents in a small space. The layer of enterocytes covering the villi themselves have additional small projections formed in the luminal surface membrane called microvilli, collectively referred to as the brush border.(Hall and Guyton, 2011)

Utilizing this brush border, digested proteins in the form of single amino acids are transported into the bloodstream for use. Dipeptides and tripeptides undergo further hydrolysis into single amino acids during transport through the PEPT-1 channel before their release into the bloodstream. Larger proteins left in the intestinal lumen are thought to participate in the lifecycle of the microbiota present in the intestine. These microbiota also synthesize peptides for the body to digest.(Caminero et al., 2014) The small intestine is divided into three sections: duodenum, jejunum and ileum, in order of digestion.

Pancreas and Duodenum

From the stomach, chyme passes through the pyloric sphincter into the duodenum, the first segment of the small intestine. There, a mixture of bile (produced by the liver, stored and excreted by the gallbladder) and enzymes from the pancreas enter the duodenum through the Ampulla of Vater and mix with the chyme. The acidic flow from the stomach also signals secretion of cholecystokinin from the duodenum into the pancreas triggering the release of

proenzymes, including trypsinogen and chymotrypsinogen which become trypsin and chymotrypsin respectively upon activation. Both of these enzymes use a catalytic triad of Histidine, Serine and Aspartate to cleave proteins for digestion. Cholecystokinin also signals the contraction of the gallbladder and release of bile into the small intestine which, among other activities, emulsifies fat for digestion. A wide variety of additional pro-enzymes in the bile become activated by trypsin in the duodenum and further digest proteins, carbohydrates, fats, and nucleic acids. Elastase, carboxypeptidase A, and carboxypeptidase B, three other enzymes released by the pancreas, activate in the same manner to further break down proteins into smaller polypeptides(6-8 residues). Additionally, the release of secretin from the endocrine system induces the release of bicarbonate from the pancreas and alkaline secretion from Brunner's Glands. This raises the pH of the chyme to above 4.3, which protects the small intestine and decreases the activity of the peptidases still present from the stomach. As the chyme travels further through the small intestine, the pH can get as high as 7.0. This denatures most pepsin compounds, which are still able to digest proteins at a pH of 5.0.

Jejunum

Following the duodenum, chyme enters the jejunum which contains the highest number of villi on the intestinal walls and is responsible for the majority of the nutritional absorption in the small intestine. The jejunum is also the longest section of the small intestine, and is the only section that lacks discrete lymphoid tissue, instead containing lymphocytes throughout the endothelium in line with its sole focus on nutrition absorption. The endothelium separates the chyme from blood vessels that carry nutrition to its final destination. This passage flows through the hepatic portal vein to the liver for processing into the bloodstream.

Ileum

From the jejunum, chyme flows into the Ileum, whose endothelium has fewer, flatter villi and lymphoid tissue aggregates called Peyer's Patches. Most of the remaining digestible nutrients, fatty acids, cholesterol and bile are absorbed from the chyme as needed prior to

traversing the ileocecal valve into the colon as waste. Bile is reabsorbed in the terminal ileum along with cholesterol and hormones like secretin and cholecystokinin, which help to regulate hepatic bile production and energy homeostasis.

Nutrition Absorption

Most nutrients in the small intestine are present in the form of ions, mono/bi/tri-saccharides, amino acids and short peptides, fiber, chylomicrons, or triglycerides. The small intestine absorbs necessary nutrition from the intestinal lumen into the bloodstream through contact between villi and the chyme. The mucosal epithelium is actually structured in a way that exponentially increases the effective surface area of the small intestine in contact with the luminal contents, facilitating increased absorption of nutrients. The epithelium of the small intestine is made up of repetitive ridges of tissue called valvulae conniventes, which increase luminal surface area 3x. On the surface of the valvulae conniventes are villi, 1 mm tube-like projections of epithelial cells containing capillaries and lymphatic ducts at their core. These villous projections often lie close enough to each other to touch and increase the surface area of the intestinal epithelium an additional 10 fold. On the epithelial surface of each enterocyte present on the villi are over 1000 microvilli, each 1 μm long, collectively called the brush border. This dense forest of microvilli increases the luminal surface area of the intestine a further 20 fold. Additionally, the microvilli manifest small actin filaments that contract rhythmically to increase the flow of intestinal contents around their respective microvilli, further increasing exposure.

The enterocytes that line the villi in the small intestine are responsible for the absorption of nutrition into the bloodstream. Multiple peptidases (primarily amino- and dipeptidases) protrude from the microvilli on the luminal membrane of enterocytes into the intestinal lumen, where they digest larger polypeptides present in the intestinal contents. The remaining tripeptides, dipeptides and single amino acids are transported into the cytosol of enterocytes,

where the remaining peptides are digested into separate amino acids. More than 99% of proteins are digested into single amino acids.

Carbohydrates are digested in a similar manner into one of three types of sugars; Glucose, Galactose, and Fructose.

Fat, due largely to its insolubility in water, has a different pathway. Bile acids and lecithin from the liver, via the gall bladder, emulsify fat into small droplets before pancreatic lipase splits the emulsified fat into fatty acids and monoglycerides. These are absorbed across the ileum endothelium and sent to the liver via the hepatic portal vein.

The flow of nutrients is not limited to the digestion enzymes. Electrolytes, glucose and single amino acids reach the blood via active transport through enterocytes. Water also flows through enterocytes by osmosis, the rate of which is determined by the solute osmolarity gradient. Calcium, magnesium, and phosphate are actively absorbed directly into the bloodstream as the regulation mechanism permits. Amino acids and glucose are also absorbed by co-transport across the membranes of microvilli, with each glucose molecule or amino acid requiring a sodium ion to be shuttled across the membrane along an electrochemical gradient. This process happens in the microvillus membrane.

The colon has digestive and absorptive ability, but the digestive ability is primarily the result of the colonic microbiome. The role of the colon centers around concentrating the liquid intestinal contents from the small intestine into solid stool by absorbing electrolytes to drive the osmosis of water from the stool into the blood. Nutrition absorption is limited to electrolytes and water primarily (according to current knowledge). The epithelial cells of the large intestine absorb electrolytes like sodium and chloride and any remaining water. Much of this absorption is accompanied by the secretion of bicarb ions. Particulates remaining in the large intestine may be further broken down by colon microbiota leading to the formation of Vitamin K, vitamin B12, and gases (including methane). The remaining particulates and significant amount of microbial flora are then excreted as feces.

Waste system

Following the nutritional transfer, the remaining substance undergoes various reactions in the large intestine before being expelled through the rectum. In the large intestine, any protein that remains undigested undergoes anaerobic microbiota digestion through various metabolic pathways and the large bacteria population present. Many of the excess proteins undergo bacterial fermentation or become large fatty acids in the colon. Remaining amino acids are metabolized by bacteria, which is required for colonic health. Any remaining non-metabolized material in the colon is excreted.

CHAPTER 3: IMMUNE SYSTEM

Although the immune system consists of several different types of cells, according to current knowledge, CD is primarily affected by the activity by T and B lymphocytes. Both cell types start in the bone marrow as small lymphocytes developed from multipotent hematopoietic CD34+ stem cells from which all blood cells develop. Approximately 30% of white blood cells become small lymphocytes, a subset that “possess the ability to recognize and target pathogenic microorganisms” (Murphy and Weaver, 2017). Lymphocytes with beginning T-Cell Receptor(TCR) chains move to the thymus and become T-cells while the lymphocytes with immunoglobulin(Ig) chains remain in the bone marrow and mature into naive B-cells.

B-cell Maturation

Small lymphocytes that remain in the bone marrow proceed through a series of developmental stages to become B-cells. This process takes place largely attached to bone marrow stromal cells in order to remain in close proximity to their paracrine secretions. Bone marrow stromal cells secrete several compounds necessary for B-cell maturation including a cytokine, Interleukin-7(IL7). This starts following the expression of a B-lineage specific transcription factor, E2A located in the germline DNA (sections of DNA coding for cellular lineage development). Germline gene expression drives the transition of small lymphocytes into the pro-B cell stage, and IL-7 released by nearby stromal cells promotes pro-B cell survival. Initially, the pro-B-cell attaches to stromal cells in the endosteum. However, as the pro-B-cell matures, it migrates through the trabecular space into the sinus of the marrow cavity while still attached to stromal cells. Finally, the B-cell leaves the bone marrow to complete its maturation in peripheral lymphoid organs.

One of the main distinctions found in small lymphocytes destined to become B-cells is the beginning of an Immunoglobulin (Ig) chain. Immunoglobulin is a protein that acts as the recognition site for a specific antigen:antibody combination presence. In this case, it activates immunological processes within the B-cell. These Y shaped proteins consist of two types of

protein chains referred to as Heavy chains and Light chains. Each B-cell Ig consist of two identical hinged Heavy chains bonded at the hinge by disulfide bonds. At the V end of the Y, the end of each heavy chain bonds with an identical light chain.

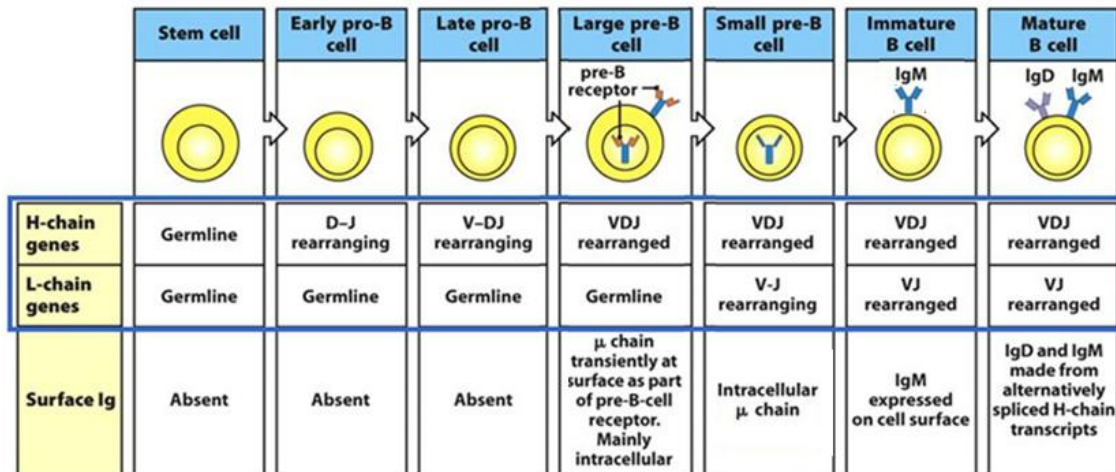


Figure 2: B-cell Immature Immunoglobulin(Murphy and Weaver, 2017)

The genes that code the Ig chains consist of three types of segments labeled diversity(D),variable(V) and joining(J) gene segments and a fourth segment called the constant(C) segment that is repeated in Heavy chains. In the germline, multiple variations of these segments exist spaced apart. It is through random, somatic(inheritable) recombination that diverse B-cells are produced in the body.

In the initial stage of B-cell development, the early-pro-B-cell rearranges DNA in the germline to join D and J segments for the heavy chain for Ig later expressed on the cell surface. At this point the recombinations occur in both alleles inherited from the parents. During this time the cell is adhered to stromal cells in a signal that induces proliferation of the progenitors. Upon the completion of the DJ rearrangement, the V segment is then rearranged to for a VDJ segment through the same somatic process. During this phase, called the late pro-B-cell stage, the rearrangement happens on one chromosome before the other. A successful rearrangement halts further heavy chain rearrangement activity on the other chromosome. This heavy chain is completed when RNA transcriptase rearranges the VDJ segment to connect with three C segments before translating the genes into the polypeptide chain. This heavy chain is tested by

creating a surrogate light chain for binding purposes. This complex signals the late pro-B-cell to continue to the Pre-B-Cell phase.

Once the Heavy chain is arranged, it is transported to the cell surface during the Large pre-B-Cell phase. This action inhibits further heavy chain rearrangement, thus creating allelic exclusion by reducing the expression Recombination Activation Gene (RAG) and removing access to the recombinase machinery. Following this, the cell undergoes several rounds of cell division, creating a large population of smaller versions of the parent cell.

Next the small Pre-B-Cells begin light chain rearrangement. The light chain is the section of the immunoglobulin protein responsible for binding different antigen specificities, thus light chain rearrangement occurs after successful heavy chain creation. Like the Heavy chain, light chain rearrangement uses somatic recombination of several segments, in this case V, J and C. This rearranging continues until a viable light chain is produced. The combination of the 2 sets of a heavy chain and a light chain yields an immunoglobulin called IgM (macroglobulin).

The final step for a immature B-cell prior to leaving the bone marrow is testing for autoreactivity. The IgM is moved to the cell surface and tested against self-antigens. B-cells that react to the self-antigen undergo either clonal deletion or receptor editing, that is, the genes coding for the specific IgM return to the rearrangement state.

There are five main immunoglobulin expressed on B-cells that signify different reactions by the B-cell.

IgM (macro globulin) is the first immunoglobulin expressed by B-cells. This class of immunoglobulin only comprises 10% of those found in human plasma. When in serum, IgM forms pentamers with low affinity but a high number of binding sites. This bloodborne structure efficiently activates the complement system mostly in response to infection. In addition to the antibody activity, IgM functions as part of B-cell's Antigen Presenting Cell (APC) activity, displaying the protein component of the antigen as a Major Histocompatibility Complex (MHC)

class II molecule. IgM stimulation also initiates class switching to another type of immunoglobulin on a B-Cell.

Another class of immunoglobulin is IgG (γ globulin) which has four monomeric subclasses (IgG1-IgG4) and is the most abundant immunoglobulin in blood and extracellular fluids. IgG specializes in broad reactions such as rapidly binding toxins to neutralize them. Like IgM, IgG activates the complement system, another method of cellular neutralization. IgG binds to Macrophages, Neutrophils, Eosinophils and, in some cases, B-cells, Langerhans cells, platelets mast cells and NK cells and produces a needed response specific to each. These cells then inhibit stimulation by the pathogen, induce death, or induce degranulation.

IgA (α globulin) can be dimeric or monomeric and is the primary immunoglobulin in mucosal secretions. Current theory states IgA is responsible for protecting epithelial surfaces, mostly in the gut, respiratory system, and exocrine glands by remaining in the mucin on the surface until needed. IgA effectively binds bacteria, virus and toxins like IgG, preventing their interaction with the epithelial cells it's protecting. Also like IgG, IgA primarily works with macrophages, eosinophils and neutrophils to induce killing. Additionally, IgA regulates gut microbiota. (Murphy and Weaver, 2017)

IgE is a monomeric immunoglobulin found in the bloodstream at low concentrations. It has high affinity for mast cells and basophils and is predominantly responsible for the autoimmune reaction called allergy. IgE also responds to multicellular parasites and mostly activates a system of granulocyte degranulation

IgD is a more recently discovered immunoglobulin derived from the same RNA sequence as IgM. It theoretically is involved in B-cell function cessation and seems to play a roll in triggering cell activation and possibly apoptosis (programmed cell death).

A mature B-cell starts with IgM and then switches to a different immunoglobulin class (IgG, IgA, IgE) using non homologous DNA recombination that mostly affect the C regions of the immunoglobulin, a process referred to as class switching. Many times different cytokines affect

class switching, often assisted by a T-cell that specializes in supporting this activity. Class switching occurs when cytokines activate irreversible DNA recombination in the C region near the J region of the antibody gene. Often the V region is actually preserved through this switch. This does not hold true for IgM or IgD, as they are the initial antibodies expressed by a B-cell. However, it does hold true for IgG, IgA and IgE.

Immunoglobulins can remain attached to their B-cells, in which case they are called B-cell receptors(BCR), or they can be secreted by specific B-cells and are called antibodies. Each B-cell secretes antibodies with specificity for the same antigen already identified by the immune system. Immunoglobulins bind antigens via specific binding sites. Immunoglobulin binding site affinity for a particular antigen is determined generally by antigen size and shape and employs a combination of noncovalent forces: electrostatic interactions, hydrophobic interactions, Van der Waals forces, Hydrogen bonds or cation-pi interactions.

Antibodies' primary effects are to elicit an immune response that removes the antigen and prevents further infection(like closing a cut). There are several mechanisms for this, such as using the complement system to destroy the pathogen, activating other lymphocytes, or inducing cytolysis. Different classes of antibody bind to different types of cells for different functions.

Antibodies have a secondary effect of making an antigen ineffective. For example, a virus may be designed to bind to a cell surface and then enter the cell for access to the cell's DNA. However, certain antibodies will block this cellular binding.

T-cell maturation

The lymphocytes that enter the Thymus to mature into T-Cells are called thymocytes. Like bone marrow stem cells, Thymocytes complete multiple stages of maturation to become T-cells. The stages, separated by changes to the cell surface and identified using flow cytometry, consist of different expressions of cell markers such as CD4 and CD8. At different stages 6 markers (CD4, CD8, CD3, CD117, CD44 and CD25) are expressed or not expressed(referred to

as positive or negative respectively). During the initial stage, called thymic lymphoid progenitors, CD117 and CD44 alone express positively. The thymocytes retain the ability to develop into cells besides T-cells. Following the positive expression of CD25, the thymocyte, now called pro-T-cell, is locked into becoming a T-cell. Next, early pre-T-cells follow a lack of CD44 and CD117 expression. This slows the proliferation of the cells and initiates T-cell Receptor(TCR) rearrangement.

T-cell receptors(TCR) are antigen-recognizing membrane bound proteins that use intracellular signalling complex to activate a T-cell. Related to the immunoglobulins, this receptor recognizes short peptides of an antigen bound in a MHC on antigen presenting cells (this includes, but is not limited to B-cells, Dendritic Cells, and Macrophages). T-cell receptors are divided into four different polypeptide chains; α , β , δ and γ . The most common, and most studied pair are the α and β chains. Each of them have a variable region and constant region with a cytoplasmic tail where the disulfide bond pairs the two into a heterodimer. This heterodimer is the $\alpha\beta$ TCR.

Once the pre-T-cell stage is reached, the TCR lineage commitment is in place to choose between the $\alpha\beta$ pair or $\gamma\delta$ pair. As CD44 decreases, the β chain gene locus undergoes rearrangement. As with B-cells, the TCR chains have 3 gene segments; V, J, and D, which undergo random somatic recombination before linking with the constant region in mRNA. For an $\alpha\beta$ TCR dimer, the β chain begins this process as soon as CD25 expresses. The process for B chain completes as CD44 expression decreases. As this process completes, RAG-2 degrades. It halts the genetic recombination associated with β chain and ensuring allelic exclusion, where only one allele remains expressed. Any cell that does not complete this process, fails to express the signal for the next step and undergoes apoptosis.

As the B chain rearrangement is completed, CD25 expression ceases and the β chain is partnered with a pre-T-cell receptor α and CD3. This completely arrests the β -chain rearrangement and promotes T-cell proliferation. At this point CD4 and CD8 are both

expressed. In general, thymocytes are categorized by the expression of these two immunoglobulins. Prior to the expression of CD4 and CD8, thymocytes are referred to as double negative. Once both are expressed, the thymocyte is termed double positive. The majority of thymocytes in the thymus are double positive despite the short time the cells remain in this state. During the double positive stage, the α chain, much like the light chain in B-cells, rearranges the V and J segments to produce the α chain. Unlike B-cells, where the rearrangement of the light chain is halted as the immunoglobulin is assembled, the rearrangement of the α chain only halts during the positive selection process. If the thymocyte survives the selection process without successfully passing the positive selection test, the light chain rearrangement will continue until either the cell passes the test or it is eliminated(Fig 3.).

The majority of mature $\alpha\beta$ T-cells are classified by which of CD4 and CD8 they express following their release into the body's systems. CD4 and CD8 are coreceptor proteins expressed by T-cells, and they are distinguished by Major Histocompatibility Complex (MHC) recognition. The MHC is a protein that binds and presents antigens to T-cells to initiate activation of the immune system. Structurally, these proteins differ widely, CD4 being a long chain with four domains, and CD8 being disulfide linked dimer. Each of these immunoglobulins bind with a different class of MHC complex, II and I respectively. This binding enhances the TCRs receptivity to the MHC complex both by immobilizing the complex near a viable TCR and by enhancing the avidity of the binding.

Double positive cells, cells that express both CD4 and CD8, undergo selection process. This selection process organizes the cells into three categories; positive selection, negative selection and non selected. This process bases T-cell compatibility on reactivity to MHC. During the positive selection process, thymic endothelial cells in the cortical node of the thymus present MHC class I and II with a thymus-specific catalytic subunit to the newly arranged $\alpha\beta$ -TCR. The proper arrangement receives the (positive) signal and activates the production of

peptides required for continued existence and later differentiation. Any cell that does not complete this process is considered nonselective and undergoes apoptosis(Fig 3).

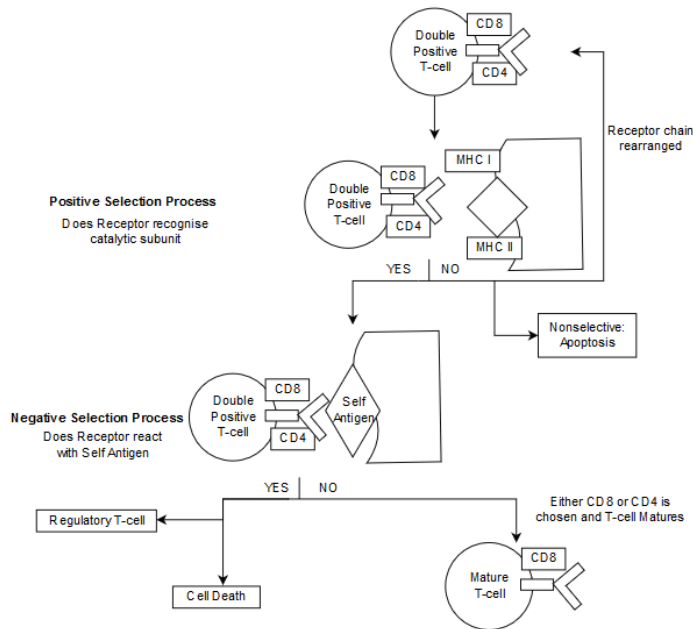


Figure 3: T-cell positive negative selection pathways

One of the peptides produced following the positive selection process, chemokine receptor CCR7, is essential to the negative selection process. Following a migration to the medulla of the thymus, thymic epithelial cells again present MHC to the T-cells, this time with self-antigens. APCs, including dendritic cells and epithelial cells, separate T-cells that react to self-antigens (the positive signal) and allow the remaining CD T-cells that do not react (the negative signal) to continue to the next stage of development(Fig 3). Epithelial cells also check the separated T-cells for the presence of Foxp3 on self-reacting T-cells. Such cells are spared and become regulatory T cells, while the remaining auto-reactive T-cells not expressing FoxP3 are deleted. Regulatory T-cells either develop in the thymus longer to control immune tolerance or develop in peripheral tissue to control immunity during inflammation.

During positive selection, T-cells receive signals to produce certain peptides. These signals, determined by the MHC to which the cell reacted, consist either of thymoproteasome protease or a lysosomal protease. They drive expression of either CD8 or CD4 respectively,

locking the T-cell into one lineage and determining one more stage of the maturation of that T-cell.

T-cells that leave the thymus expressing either CD4 or CD8 are considered naive T-cells. Naive T-cells represent only 5% of the thymocytes that enter the thymus from the bone marrow. Ninety five percent of T cells that enter the thymus are killed at any of the various stages of development.

The TCR, like its B-cell counterpart (BCR), recognizes antigens. However, unlike the BCR, TCR requires that an MHC binds an antigen before the TCR can recognize it.. MHC molecules are expressed on T-cells, B-cells, Macrophages, Dendritic cells and thymus epithelial cells and are mostly divided into 2 classes, I and II. Both classes have a peptide binding groove where antigenic peptides bind to the MHC molecule and are displayed on the surface of the cells for recognition by TCRs. MHC class I binds shorter peptides (8-10 amino acids), and class II binds longer chains. Both have anchor residues in the polypeptide chain that bind and stabilize the MHC molecule.

When an MHC:antigen complex on the surface of a cell is recognized by an appropriate TCR on a T-cell, the presenting MCH molecule must also be bound by the appropriate coreceptors on the T-cell for a response to occur. CD4 recognizes MHC class II molecules and CD8 recognizes MHC Class I molecules, allowing the form of the antigen to determine the first layer of immune reaction.

B-cell and T-cell activation

T-cells and B-cells have specific locations they collect for activation of the adaptive immune system, the white pulp of the spleen and the lymph nodes located throughout the body and mucosal tissue. Naive T-cells migrate to the spleen and lymph nodes via the bloodstream and take up residence in a particular node until they come into stable contact with a suitable MHC:antigen complex inside one of these tissues on the surface of an APC. It is usually a dendritic cell, though sometimes it can be a macrophage or B-cell. Antigen presenting cells, or

APCs, are dendrites, macrophages and B-cells with an MHC expressed on their surface. The MHC binds to a possible antigen and 'presents' the bound antigen to a T-cell with the associated CD4/CD8 ligand, thereby activating the adaptive immune response.

The activation of the immune system by TCR or BCR is considered the adaptive immunity. The TCR, as stated, is a complex made of α and β chains surrounded by either CD4 or CD8 coreceptors. The CD3 receptors also can assist in T-cell activation. CD3 have intercellular tails and assist the TCR and its signaling, particularly with the psi tail, which transmits signals directly from the TCR to downstream cascades in the T-cell by phosphorylation. This TCR activation starts a signalling cascade leading to transcription factor activation, metabolism changes and integrin-mediated T-cell adhesion to the APC stabilized by the formation of an actin cytoskeleton immune synapse.

Once activated, the CD4 T-cell undergoes clonal expansion and cellular proliferation followed by differentiation into effector T-cells and memory T-cells. The effector T-cells have three possible paths determined by cytokines expressed in their vicinity at key timepoints:

1. migrate out of the tissue as helper cells and to the site of the infection,
2. remain in the current location and interact with naive b-cells, or
3. regulate the immune response by inhibiting future T-cell activation by dendritic cells.

CD8 T-cells, once activated, become cytotoxic (killer T cells) and kill their target cells by paracrine signaling and creating holes in them.

Co-stimulatory receptor binding is required for the activation of T-cells to provide a layer of cell specificity and regulatory control. T-cells use CD28 which binds to ligand on the APC and enhances the signal transmitted within the T-cells to allow for activation. It also promotes cellular proliferation, cytokine production and cellular survival. Regulatory molecules interfere with costimulatory molecules to downregulate immune responses.

T-cells that are not used by the B-cells have several different function once activated.

Helper T-cells that migrate to the site of infection could:

- Opsonize macrophages or IgG isotypes for for phagocytosis
- Work with IgE eosinophils, basophils and mast cells to target large parasites
- Opsonize neutrophils and IgG isotypes in response to fungi and bacteria invaders.

Regulatory T-cells, designed to prevent autoreactive responses, develop from either activate T-cells or directly from the Thymus. They suppress the proliferation and differentiation of the helper T-cells listed above in addition to producing immunosuppressive cytokines and suppressing inflammation. Memory T-cells are sent back throughout the body, to circulate to allow for a quicker reaction to subsequent interaction with an antigen are sent through the tissue with increased inflammatory chemokine receptors or are sent directly to various epithelial sites to remain for a quick reaction at increased inflammatory sites. Memory T-cells also exhibit cytokines that lengthen their lifespan past the normal few days.

Cytotoxic T-cells, produced from a T-cell with a TCR CD8 complex, target cells for elimination. This type of T-cell primarily limits the spread of damaged cells and infection through cellular apoptosis. In general, Cytotoxic T-cells kill cells bearing specific antigen bound to MHC class I. There is evidence that there are memory CD8 T-cells by using CD4 T-cells, but this mechanism is not well understood.

The T-cells that assist the b-cells in the lymph tissue activate the B-cell function. This activation is done by activating the BCR. The BCR, like the TCR, uses coreceptors, in this case CD19, CD21 and CD81. CD21, part of the complement pathway, will bind certain fragments to augment the signal of BCR. BCR activation also starts an integrin-mediated cell adhesion and changes in cellular metabolism. Instead of reforming the actin cytoskeleton, B-cells use

signalling complexes on the cell-cell interface. The costimulatory molecule for B-cells are CD40.

If B-cell is the APC for the T-cell, its MHC class II presentation will bind with a CD4 T-cell. That will in turn drive the B-cell's differentiation into an antibody producing cell.

Once the BCR signal is received, the B-cell receives additional signals from the T-cell by binding its own MHC class II complex with the T-cell's TCR complex. Then it undergoes isotype switching described above. Once activation is complete, most of the B-cells proliferate and differentiate into two types, plasmablasts, which synthesize antibodies, and plasma cells. Plasmablasts secrete antibodies, but retain both surface immunoglobulin and MHC complex to respond to further antigens. Plasma cells, on the other hand, produce and secrete antibodies, but do not retain immunoglobulin on their surface. Thus they cannot undergo class switching or react to further antigens.

The B-cells that do not complete the differentiation above will migrate with their helper T-cell to create a germinal center. While Plasmablasts produce more IgM antibodies since class switch uses up time, the germinal centers undergo several processes of somatic hypermutation and affinity maturation to produce the most effective antibodies followed by basic class switching for different effector functions. These B-cells will eventually also be secreted either as active B-cells or as memory B-cells.

Memory B-cells remain in the body, circulating until a secondary attack with the same antigen triggers a response. Some of the memory B-cells will immediately begin releasing the pre-optimized antibodies. A few of the memory B-cells will return to the lymph node to provide a more optimized starting point for a germinal center and will undergo somatic hypermutation and affinity maturation to further optimize the antibodies affinity for this antigen.

Other Immune cells

Dendritic cells are antigen presenting cells with additional immune functions including transportation of toxins or pathogens, messenger cells, and cytokine expression. In the

mucosal system, dendritic cells primarily transport pathogens across the epithelium and present antigens to naive T-cells to initiate an adaptive immune response.

Macrophages are phagocytotic immune cells with two primary subtypes called M1 and M2. M1 is an inflammatory macrophage that activates bactericidal, inflammatory, and phagocytotic pathways. M2 macrophage suppresses inflammatory function and activates repair functions such as growth function.

$\gamma\delta$ T-cells

When T-cell lineage commitment is decided, the alternative to $\alpha\beta$ chain is the $\gamma\delta$ chain. Unlike the $\alpha\beta$ chain, much less is known about the $\gamma\delta$ TCRs. In humans, $\gamma\delta$ TCRs are found on a minority, only 5-15%, of T-cells. However, $\gamma\delta$ TCR expressing T cells have been found in all vertebrates and represent half of the T cell population in mice. The majority of these $\gamma\delta$ T-cells in humans are found during fetal and neonatal development. Past this stage of life, these T-Cells are located mostly in the mucosal systems and epithelial sites. Due to the similarities, γ and β chain genes overlap and occasionally a $\gamma\delta$ T-Cell will have an inactive β chain gene already rearranged. Unlike the $\alpha\beta$ dimer above, the $\gamma\delta$ pair do not express CD4 or CD8 and do not require MHC for antigen interactions. However, they do react to a special class of MHC chains. Instead, $\gamma\delta$ TCRs can target antigens directly, theoretically allowing them to create a rapid response to antigenic stimuli by known stressors. Many of the identified ligands for $\gamma\delta$ TCRs such as endothelial protein C receptor, are found on cells undergoing stress or damage. As $\gamma\delta$ T-cells can also trigger innate and adaptive immune response to antigens as well as responding to them directly, $\gamma\delta$ T Cells appear to function somewhere between the innate and adaptive immune responses.

The second lineage T-cell, $\gamma\delta$, is less understood than its counterpart and is less prolific in humans, except in the gut. Like $\alpha\beta$ TCR, the TCR expressed on $\gamma\delta$ binds peptides presented by MHC molecules, specifically class Ib and nonclassical class I such as MICA or ULBP4. However, a key difference is that $\gamma\delta$ T-cells recognize target antigens directly, allowing a more

rapid response such as triggering locally at the site of an infection. These cells also respond to unorthodox nucleotides and phospholipids. In the mucosal system they respond to α -gliadin by killing the nearby stressed epithelial cells.

Mucosal system and immune system

The mucosal systems is comprised of the middle ear, the respiratory tract, the urogenital tract, the gastrointestinal(GI) tract and the exocrine glands associated with these organs (conjunctiva, mammary glands, salivary glands, lachrymal glands). To address CD specifically, this discussion will focus only on the GI tract portion of the mucosal system.

Overview of mucosal system in digestive tract

The mucosal systems, so named for the mucus they secrete, constantly protect the body from microbiota and other toxicants. The mucus secreted by goblet-cells forms two layers, an outer non-sterile layer that degrades microbes and an inner layer that contains a plethora of antimicrobial molecules. This is further complicated by the microbiota that live in the gut and perform important metabolic functions. These microbiota are capable of activating the immune system. Such reaction would be aberrant and sometimes is known as an autoimmune condition like Crohn's disease.

The mucosal immune system traditionally splits into two sections; effector and inductive sites. The lamina propria and epithelium are the effector sites, and the Mucosa-Associated Lymphoid Tissue (MALT) structures combined with mucosa-draining lymph nodes comprise the inductor sites. These are so named by the process within the mucosa immunity they inhabit. This division is not absolute given the variety of signals required.

Lamina Propria is a layer of loose connective tissue that lies between the basal layer of the epithelium in the mucosal regions and forms the exterior of the intestinal tract and the muscularis mucosae. Although this tissue is considered an effector site, antigens within the tissue contribute to early T-cell and B-cell activity, such as differentiation and proliferation, making it also an inductor site. For mucosal T-cells and B-cells, following travel through the

lymph nodes and peripheral blood, antigen independent homing draw the cells to the mucosal layers where lamina propria antigens contribute to retention, proliferation and differentiation. T-cells gain antigen homing due to priming in the lymph nodes.

The mucosal system has a thin layer of epithelium as a barrier defense. Most epithelial cells in the intestine and other mucosal areas are highly polarized and connect to each other using tight junctions to prevent the passage of molecules not chosen by specific cells or paracellular pathways (passive water and solutes). One special type of cells in the endothelium are the microfold cells (M cells). These cells are specialized epithelial cells characterized by short or absent microvilli and the ability to take up antigens to deliver them via multiple pathways through the epithelium to the adaptive immune system. They are located on the surface of a Peyer's Patch.

The main function of the epithelium is to form a physical barrier while still allowing nutrients to pass through, sometimes at tight junctions. Tight junctions are dynamic in form and activity, typically excluding large molecules (Stokes radius >11 Angstrom) and charge.

MALT collectively include mucosa-associated follicles and their larger aggregates. These tissues originate the T-cells and B-cells traffic to the effector sites in addition to several other common elements. MALTs are divided into 3 groups by location; Nasal, Gut and Bronchus, with the gut being the most studied. MALT tends to resemble lymph nodes in that they have antigen presenting cells, interfollicular T-cells and B-cell follicles. However, there are no afferent lymphatics and the structure lacks a capsule. Instead, MALT's antigen intake occurs directly from mucosal surfaces using follicle-associated epithelium containing M cells which deliver particulate matter, macromolecules and microorganisms across the epithelial barrier using vesicular transport activity. Due to the shape of the M cells, T-cells, activated or memory B-cells or Dendritic Cells will sit in the intraepithelial pocket to shorten the transcytotic pathway and increase the speed of delivery. By definition, MALTs sample antigens directly from the mucosal surface.

MALT is also responsible for B-cell activation. In the gut, this is demonstrated by the class switch from IgM to IgA in murine B-1 cells in the germinal center of M-cell pockets. B-cells that retain their IgM antibody low affinity binds circulating antigens and activates the complement system before relegating the antigen to remain for the memory B-cell function. Research suggests IgM plays another yet unknown role in the induction of a secondary response.

An important part of GALT (Gut-associated lymphatic tissue) is the Peyer's Patch which is lymphoid tissue of the epithelium in the small intestine that contains M-cells, in addition to follicle-associated epithelial cells. Unlike the remainder of the gut with microvilli, M-cells have folded luminal surface that do not secrete digestive enzymes or mucins. This means Peyer's Patches also lack the mucus layer that covers the rest of the cells. It also contains the germinal center in which B-cells are activated. In fact, have more B-cells than its lymph node counter parts, and where T-cells and dendritic cells encounter antigens. Peyer's Patches initiate immune response in the gut.

In addition to the Peyer's Patch, the gut epithelium has intraepithelial lymphocytes (IELs). More than 90% are T-cells, and around 70% are CD8 T-cells in the small intestine. Although most have the appearance of activation, they seem to respond locally to a small number of antigens by killing infected or stressed epithelial cells. Some IELs are $\gamma \delta$ T-cells. These will also kill a stressed cell.

As stated earlier, the gut is lined with villi. In between these villi are areas called crypts where undifferentiated progenitor cells reside. The lower sides of the crypts are stem cells that can be differentiated into one of three cells for the villi: absorptive enterocytes, enteroendocrine cells and goblet cells. The crypts regulate intestinal epithelial homeostasis. At the bottom of the crypts below the stem cells are paneth cells which produce several immunologically important molecules such as α defensin and certain antimicrobial peptides for the mucus layers.

Cellular immune system

As with the rest of the immune system, a large part of the immunological reaction is based on antigens. There are six pathways for antigens to cross the epithelial layer: M-cells, dendritic cells, apoptosis-dependent transfer, goblet cells, macrophages, and an immunoglobulin receptor (IgA) binding the antigen and traversing the barrier. Epithelial cells can perform autophagy and recruit neutrophils, macrophages or dendrites to destroy the pathogen. M-cells, macrophages and dendritic cells will take up an antigen and transport it to a dendritic cell on the other side waiting for the antigen. These macrophages are more populous than in the rest of the body and rarely secrete inflammatory cytokines or reactive oxygen species. Instead, they focus on phagocytosis of pathogens or dying epithelial cells. The apoptosis assisted transfer happens when an infected cell undergoes phagocytosis by a dendritic cell, which then collects the antigen for use in activating the adaptive immune response. Although the main function of the immune system is to react to pathogens, in the mucosal immune system, the barrier stability takes a high priority and the immune reaction is secondary.

The dendritic cells either in the germinal center of the Peyer's Patch or by direct attachment obtain an antigen to present on the MHC for T-cells priming. MHC II are upregulated in the small intestine, thus initiating a pro-inflammatory response. MHC I and MHC II are not alone in expression. They are joined by non-polymorphic MHC class I related molecules. This allows TCR- α binding. It creates a primed T-cell. Primed T-cells include some reaction to gut-homing cytokines, though some crossover among the mucosal system spreads the reaction. This primed T-cell has the same choices as in the rest of the body, effector cells or helper cells. Effectors cells produce cytokines as elsewhere, however a main function present in mucosal T-cells is the preservation of the function and integrity of the epithelium.

Although all common types of B-cells can exist in the mucosal regions, certain classes are far more common. Once a primed T-cell activates a B-cell in the Peyer's Patch, the B-cell

undergoes class switching to IgA. In the gut, IgA far exceed the normal plasma favorite IgG. However, the reverse holds true in the tonsils (part of the bronchus mucosal system). Naive lymphocytes remain in the Peyer's Patch until activation, at which point they migrate into the mucosal lymph nodes to drain with the efferent into the thoracic duct. These then home in on the lamina propria, binding specific B-cells. Thymus-expressed cytokines mediate the travel to and retention of T-cells and B-cells in the lamina propria. The lamina propria contains many immune cells that lack an immune response, including: mast cells, effector and memory CD4 and CD8 T-cells, macrophages, dendritic cells, innate lymphoid cells, and IgA producing plasma cells. This combination displays many characteristics of a chronic inflamed tissue. However, the actual inflammatory response is mitigated by an extreme amount of IL-10 produced by the regulatory T-cells.

Unlike in the blood, IgA in the gut forms a dimer instead of a monomer. B-cell in the mucosal regions tend to produce dimeric IgA exclusively, due to the helper T-cells in the gut. In the lamina propria, B-cells activate as plasma cells and produce large quantities of IgA into the subepithelial space. The IgA is then transported the rest of the way to the lumen by polymeric immunoglobulin receptor in the epithelium, part of which cleaves and remains with the IgA to protect it from proteolytic cleavage. This complex can bind toxins and either neutralize them through inhibition or by using endosomes in the nearest epithelial cell. Also the IgA can transport the toxin across the epithelium, usually through a M-cell into a Peyer's Patch and activate an anti-inflammatory response in a dendritic cell. This allows IgA to control microbe penetration without damaging the epithelium with an immune reaction.

Tissue Transglutaminase

In CD there is a ubiquitous enzyme, not associated with the immune system directly, that plays a large role in the pathology: Tissue Transglutaminase (tTG2). Tissue Transglutaminase (tTG2) is a calcium-dependent enzyme primarily located in the GI tract.(Jabri and Sollid, 2006) Tissue Transglutaminase belongs to a family of 9 transglutaminase. They are all distributed

throughout the body performing various functions by forming Ca^{2+} -dependent crosslinking between lateral chains of glutamine and lysine residues for physiological purposes, often modulating the immunogenicity of existing proteins. Oddly, for being enzymes, the majority of the known transglutaminase functions are not enzymatic in nature; tTG2, for example, has signalling and scaffolding functions independent of the catalytic activity. (Martucciello et al., 2018) Being both intracellular and extracellular, tTG2 is vital in stabilizing many proteins including actin, myosin and spectrin, and in extracellular matrix used in wound healing and signalling to macrophages for apoptosis. Theoretically, tTG2 is inactive in its extracellular state until a RedOx reaction from stress activates it. It also shows kinase, protein disulfide isomerase, and deamidase activity in the presence of G-proteins, the latter of which is responsible for the reaction in CD. (Martucciello et al., 2018)

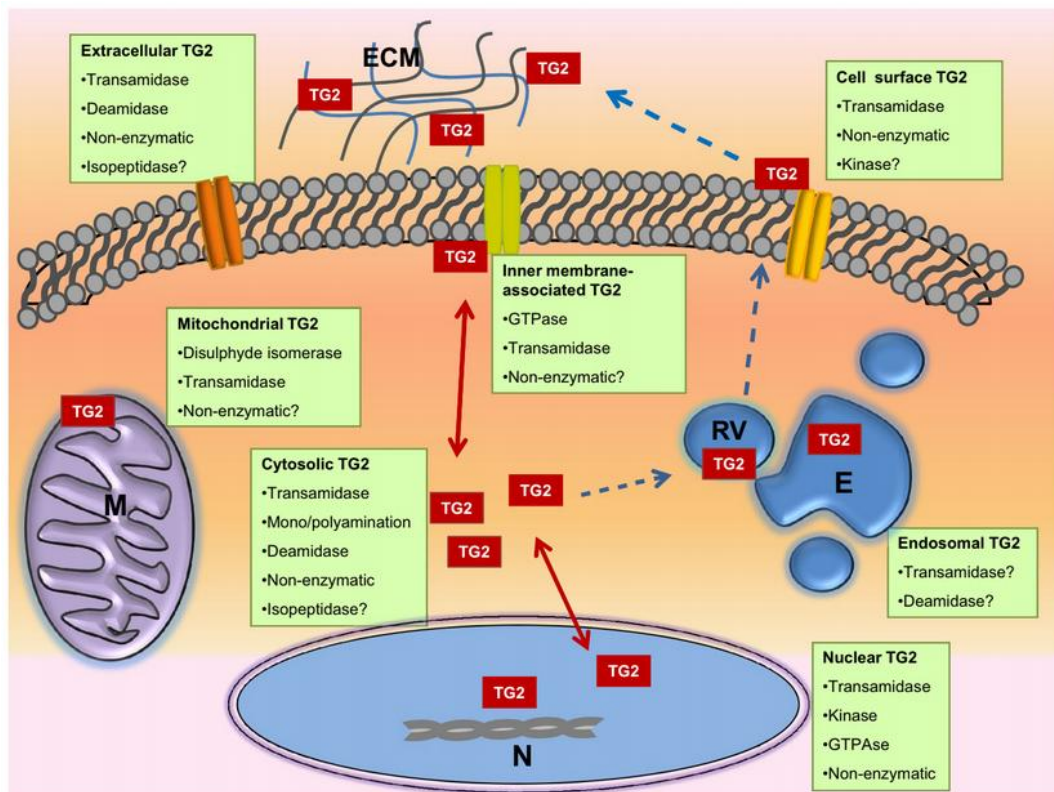


Figure 4: Cellular functions of tTG2 both within and out a cell (Martucciello et al., 2018)

When tTG2 encounters gliadin or a polypeptide of gliadin, it acts as a deamidase and binds itself tightly to the polypeptide. This complex improves recognition by the antigen receptor (HLA-DQ2/HLA-DQ8) presented to T-cells and activates the adaptive immune response in the gut. B-Cells produce IgA tTG2 antibodies that serological tests detect in serum of CD patients. These antigens can be detected in the basement layer of the intestines before the morphological changes to the endothelium.(Martucciello et al., 2018) The presence of these antibodies cause several immunological alterations such as enterocyte proliferation in the intestines. Experiments seem unable to replicate these condition outside the body, suggesting a celiac cellular environment still not understood. Indeed, skin-derived fibroblasts (cells not directly associated with CD commonly) from a healthy patients respond to the anti-tTG2 protectively, whereas those of a CD patient do not in a way not understood. The antibody for tTG2 is also responsible for several of the non-GI symptoms like dermatitis herpetiformis (type 3 transglutaminase) and some of the neuropathies(type 6 transglutaminase). (Martucciello et al., 2018; Stenman et al., 2008)

CAPTER 4: CELIAC PATHOPHYSIOLOGY

Gluten

Gluten is the name given to water insoluble protein present in cereals in the Triticeae tribe of the Pooideae subfamily of grasses which includes Wheat, Rye, Barley, and Spelt. The presence of this protein confers certain properties on wheat utilized in cooking such as elasticity, viscosity, cohesivity and a certain absorption of water. From a biological standpoint, gluten is a protein in the wheat germ meant to support seed germination and feed young seedlings. Gluten proteins contribute to the rheological properties of hydrated wheat, mainly due to being prolamin or rich in proline/glutamine sequences. Wheat gluten is divided into two categories, gliadin and glutenin. The alcohol soluble gliadins consist of homologue monomeric proteins and contribute to the elasticity, specifically extensibility, and viscosity properties of hydrated wheat. Glutenin is the non-alcohol soluble fraction and consists of aggregate proteins bonded by disulfide bonds between cysteines. It contributes elasticity and strength. In rye and barley, gluten contains hordeins and secalins, respectively, instead of gliadins. Due to the similarity in response and form, gluten refers to proteins in barley, rye, kamut, durum, emmer, spelt and triticale(wheat) groups. Closely related groups, such as oats have proteins that can elicit the same response as gluten in some cases.(Balakireva and Zamyatnin, 2016; Fric et al., 2011; Londono et al., 2013; Tapsas et al., 2014; Valenti et al., 2017)

Gliadin is composed of several isoforms based on the electrophoretic activity; α , β omega and upsilon. The gliadin that triggers an adaptive immune response is the α -gliadin.

Gluten Digestion

Gliadin can be broken into many smaller peptide chains. Five of these are repeatedly implicated in CD; 33-mer, 26-mer, 20-mer, 19-mer and 13-mer. The 33-mer and 26-mer are immunogenic, that is they activate the adaptive immune system. The 20-mer activates the zonulin pathway which opens tight junctions in the endothelium. The 19-mer and 13-mer are toxic and activate the innate immune response. Assuming the innate immune response

happens enough times, or continues for long enough, a second adaptive immune response will occur. This happens when these peptides interact with the immune system, primarily after they have crossed the epithelial barrier into a Peyer's Patch, or lamina propria.(Caminero et al., 2014)

The breakdown of gluten begins in the saliva. Due to the high content of Proline-Glutamine residues in the mouth, glutamine endoprotease activity preferentially cleaves basic proline rich proteins predominantly when the proline is bracketed by glutamine and any other residue. This is necessary for the normal environment of the mouth and is possibly microbial in origin. Additionally, further study noted that the immunogenic peptides(33-mer and 26-mer) that later cause CD issues can be thoroughly degraded by the oral protease. Additionally, several oral micro-organisms have shown remarkable ability to digest gluten. Suspended bacteria found in plaque(*Rothia aerea*) can degrade gliadin by 50% in 30 minutes. Others could participate in gluten digestion: *Streptococcus mitis* and *Bifidobacterium dentium*, for example. There is some evidence that these microorganisms could survive the stomach to continue digestion in the intestine.(Caminero et al., 2014; Tian et al., 2017)

Additional research has shown that the changes gluten undergoes during the baking process inhibits the salivary amylase from digesting the protein. In fact, it decreased the digestion to the point that even with amylase added to the duodenum digestion site, immunoreactive polypeptides persisted. Simulated digestions, at several levels of enzymes, showed these polypeptides remained up to 2 hours later.(Smith et al., 2015)

Like other proteins, pepsin attacks gluten components once in the stomach. Current in vitro studies show that the pepsins in the stomach only partially proteolyzed gluten proteins.

Like many proteins, gliadin has a buffering effect on HCl, in this case at pH of 3.0. Research conducted at this pH shows that gliadin forms several oligomers that collect in non-spherical aggregates. These elliptical oligomers were studied at varying concentrations at pH of 3.0 showing the supramolecule forms elongated aggregates of varying size. This is backed by

ab initio calculations that show that two highly hydrophobic sites prone to aggregation are exposed in this stable form. This explains the proteolytic resistance the gliadin had and suggests that the immunological response is indicative of a real trigger instead of a random occurrence among gliadins (the full sequence of α gliadin was reported).(Herrera et al., 2018a; Herrera et al., 2018b)

In CD, the damage happens in the small intestine, however much of the final digestion of gluten and gliadin appears to happen in the large intestine via microbiota. A study recently found certain microbionics in the small intestine of CD patients that was not present in healthy individuals. This leads to the conclusion that duodenal bacteria much like those in the large intestine can hydrolyze the longer chains. This is further supported by the increase in bacterial metabolism in the colon in healthy patients when their gluten intake increases. Additionally, even the earliest diagnosis of CD noted the difference in the presence of fatty acids in the patient's feces, denoting a change in bacteria population in CD patients. Since this doesn't change during GFD, it is likely part of the cause of CD's symptom and not another symptom in and of itself.

Like other proteins, gluten interacts with various enzymes from the pancreas as the polypeptide enters the small intestine. Many studies show that gluten resists digestion, likely due to the high proline content (estimates 15%-25%). The additional proteolytic activity by the brush border has little discernible effect on the gluten proteins despite the presence of enzymes that cleave proline bonds. Some polypeptides have minor cleavages, but the polypeptides responsible for the CD immunoreaction, such as the 33-mer, do not appear affected. Likewise, several enzymes in the brush border of the small intestine can cleave proline and glutamine bonds, but have little effect on the 33-mer. The bacteria in the duodenum does not appear to hydrolyze gliadin sufficiently to facilitate absorption in the small intestine.(Caminero et al., 2014)

In the large intestine, although there are bacteria that could affect the long peptides, studies show that the majority of the gluten we ingest ends up excreted as feces. Since this is

proportional to the amount of gluten ingested, several gluten peptides must be resistant to human and bacterial digestion. In both a healthy and CD patient, the loss of gluten decreases the diversity and population of *Lactobacillus* and *Bifidobacterium* species. Other forms of microbiota increase, such as *Enterobacteria*, and fecal glutenase activity increases. Even the endoprotease activity seen in the saliva can be detected in the feces. (Caminero et al., 2014)

Some studies have shown the importance of bifidobacteria in the large intestine in the digestion of gliadin. Certain strains cleaved the long chain protein into cytotoxic peptides, however *B. longum* CECT 7347 hydrolyzed even the 33-mer into non-cytotoxic peptide chains. Furthermore *Bacteroides fragilis* demonstrated similar activity along with several strains in the Firmicutes and Actinobacteria phyla that already exist in the large intestine.

In an attempt to further understand activity that breaks down gluten/gliadin, studies into human feces measured enzymatic activity on healthy and CD patients with and without gluten. Fecal glutenase activity present in patients showed hydrolytic activity against gliadin compounds such as 13-mers, 19-mers and 33-mers. Even with this activity, the peptide resisted degradation in excess of 60 minutes. The attempt to identify the proteins responsible for the fecal glutenase activity indicated two proteins with a high homology to human elastase 3B (CEL3B), 1 with human elastase 2A (CEL2A), and 1 with human carboxypeptidase A1 (CBPA1). Further testing with a similar, commercially available mix showed hydrolytic activity in the presence of gliadin and gluten. Since the immunological reaction is actually prompted by certain immunogenic mimics, specifically 13-mer, 19-mer, and 33-mer, the hydrolytic activity of these proteins were tested as well. The 13-mer and 19-mer both degraded during a 60 minute incubation in either CEL2A and CBPA1, but CEL3B seemed ineffective at this digestion. The 19-mer did leave some smaller peptides. However, in 60 minutes and even in 24 hours, the 33-mer failed to digest more than 66% in any of the identified proteins. Patients with CD showed a significant increase in the ability to digest 33-mers over those without. Further study comparing patients with and without CD to those related to patients with CD showed that this activity was

higher in patients with confirmed CD than with healthy patients by 171-466%(Gutierrez et al., 2017). Furthermore, this difference persisted even when the patient was on a gluten free diet. The combination of proteins that supported feces glutinase activity was such that in CD patients, all the 13-mer and 19-mers successfully degraded, leaving only the 33-mers. This research, combined with earlier research, shows that only 30% of the immunogenic compounds survive digestion in vitro and no bacteria with glutinase activity survive in the feces.(Caminero et al., 2015; Gutierrez et al., 2017)

Immune Reaction to Gluten

Currently, it is unknown what causes the activation of CD within an individual. There are genetic markers that show a strong statistical correlation to the onset of CD. Additionally, “Breastfeeding and the timing of gluten introduction in the diet, viral infections that promote the secretion of interferon α and smoking are some of the factors that appear to influence the development of the disease”(Green et al., 2005).(Monteleone et al., 2001; Stenlund et al., 2002; Vazquez et al., 2001) Some research indicates the trigger could be a rotavirus protein (VP7) that mimics tTG2 peptides or T1L/T3D-RV that establishes an environment that allows gliadin peptides to activate the immune system.(Brown et al., 2018; Volta et al., 2013) Further research indicated a bacterial infection could trigger CD, either by the bacteria population directly or by the loss of commensal bacteria due to antibiotics(Fig 5).(Chander et al., 2018) However, at this time the triggers remain elusive.

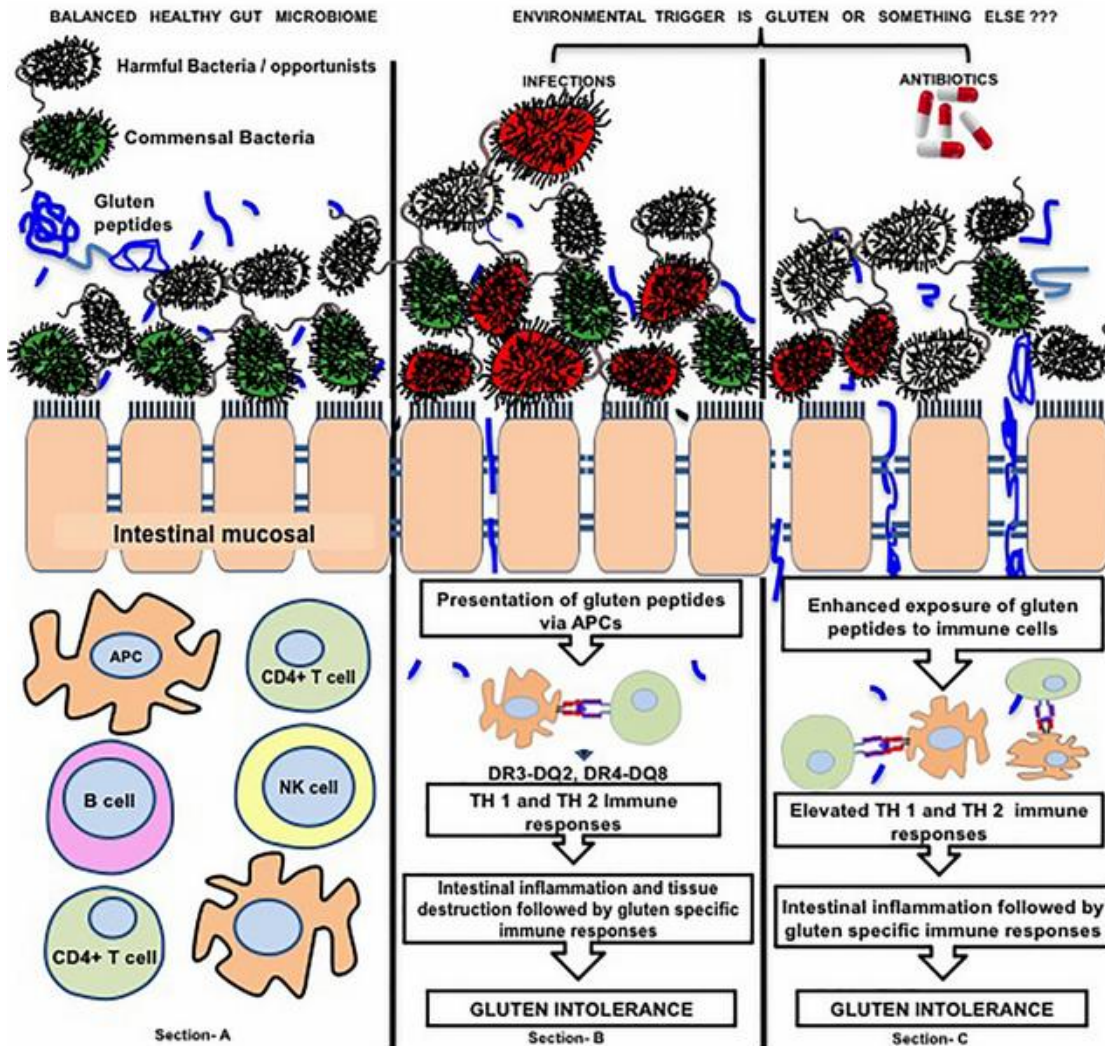


Figure 5: Viral trigger for CD – Either the bacteria or antibiotics produce an environment that allows CD to start.(Chander et al., 2018)

Current studies divide Celiac Disease into several types; symptomatic, asymptomatic, latent and potential. Symptomatic CD is the most studied and most diagnosed, as patients with this form experience noticeable effects of ingesting gluten. However, only 50% of CD patients fall into this category. Asymptomatic CD, or silent CD, involves the presence of genetic and antibody markers and positive biopsy finding, but lacks overt symptoms such as dermatitis herpetiformis and gluten ataxia. Often this is diagnosed when the more dangerous symptoms associated with nutrition deficiency become apparent. Latent CD is the condition where the enteropathological side of CD is not constant. The final category of CD is Potential CD defined as the presence of the antibodies with the complete lack of enteropathy. Latent and Potential

CD could show symptoms and are rarely diagnosed. Patients with potential CD remain a medical concern. The lack of lesions or villus atrophy imply that the presence of the antibodies is not causing harm. However, in a study done in 2010 on children, 30% of verified Potential CD patients developed active CD with lesions within 3 years. However, since 14% of participants lost the antibodies in their serum completely, the risks associated with Potential CD remain inconclusive. The potentials studied showed the same increase in B-cell and T-cell activity characterized by CD. However, there was marked increase in IL-10, inhibitory NK receptors and IEL that lacked the killer phenotype, suggesting the presence of an immune response in a lower magnitude.(Troncone and Jabri, 2011)

T-cells

The initial reaction to CD appears to happen in the Duodenum of the small intestine where cytotoxic polypeptides from gliadin interact with IELs in the endothelium and immunogenic polypeptides interact with the adaptive immune system. The cytotoxic polypeptides, principally 13 and 19-mers, interact with cytotoxic T-cells (expressing CD8) to activate various innate immune responses. The expression of gluten-specific CD8 T-cells refers to stress induced signals and the TCR binding to MHC class 1 chains A and B and HLA E mostly. This is an antigen-nonspecific reaction and it is assumed that the peptide is mimicking another peptide, one which is actually toxic. Natural Killer receptors, upregulated by IL15, on IEL receive the signal from these compounds to activate kinase that should mediate T-cell activity. Instead, the NK receptors seem to attack the epithelial cells in the lining of the lamina, allowing more of the toxic peptides to cross the barrier. Concurrently, the predominant inhibitory NK receptor is down regulated allowing cytolysis. This creates a T-Cell environment with no specificity, that is able to kill cells, secrete cytokines and proliferate upon receiving stress or damage signal from the surrounding cells.(Garnier-Lengline et al., 2015; Jabri and Sollid, 2006; Menard et al., 2012; Murphy and Weaver, 2017; Smith et al., 2013)

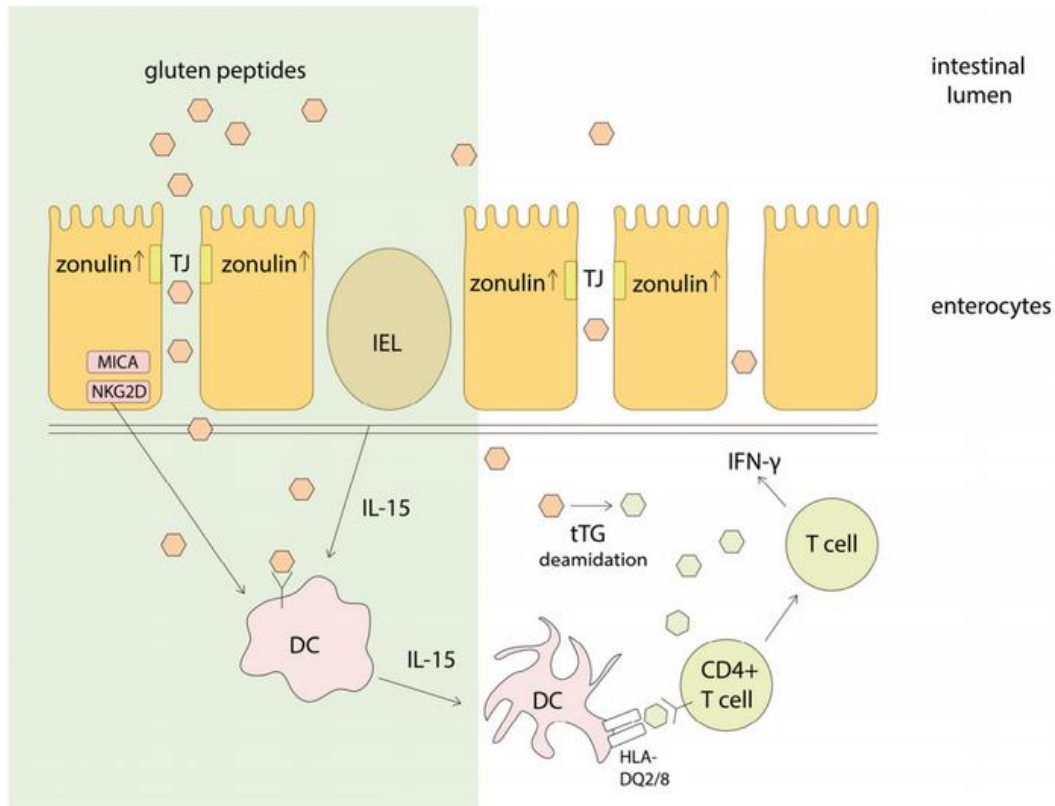


Figure 6: Overview of Gluten peptides interacting with mucosal immune system (Balakireva and Zamyatnin, 2016)

The addition of gluten in the system increases a substance called zonulin, which regulates the tight junctions in the endothelium. The current theory is that zonulin released by mucosa in the presence of gluten, specifically 2 different 20-mer polypeptides cleaved from gliadin, increases paracellular permeability and stimulates transcytosis in the small intestine by producing an “adjuvant effect exerted by zonula occludens toxin, its analogue, on antigen specific IgA promoting] IgA-dependent transcytosis of gliadin peptides”(Menard et al., 2012). (Fasano, 2012; Menard et al., 2012) Furthermore, this interaction with zonulin occurs exclusively on the luminal side of the endothelium, utilizing the chemokine receptor CXCR3 and adapter protein MyD88 to release zonulin into the intestine. Zonulin release is also heavily associated with Type 1 Diabetes. This gliadin peptide transcytosis leads to damage of the epithelial tight junctions allowing the partially digested gliadin fragments, specifically the 26-mer, 33-mer, 19-mer and 13-mers, to enter the bloodstream and interact with the T-Cells there.(Fasano, 2011, 2012; Jabri and Sollid, 2006)

While the innate reaction continues, the adaptive immune reaction begins, either as a result of the innate immune response or as a response to immunogenic polypeptides, specifically 26 and 33-mers, crossing the endothelium. These polypeptides cross the endothelium border in some fashion to interact with tTG2 already present. Gluten peptides are presented to T-cells using the HLA on APCs. HLA-DQ2 and HLA DQ8 have unique binding sites that accommodate the amino acid chains in the 33-mer and 19-mer chains left from glutenin. The preference in these binding sites weighs heavily negative charged anchor residues (HLA-DQ2 at positions P4, P6, or P7,31,32 and HLA-DQ8 at positions P1 or P9(Jabri and Sollid, 2006)) which are introduced to the gliadin residues when TG2 converts glutamine to glutamic acid. The 33-mer of α -gliadin is especially susceptible to this conversion and resists digestion otherwise due to high proline count. Due to its binding motifs, this chain stimulated T-cells by targeting 3 different epitopes. As a point of interest, avenin in oats contain some of these sequences for T-cell epitopes provided they avoid digestion.

Once activated, some T-cells differentiate into helper cells and activate B-cells, and some become effector cells that start an advanced healing response. This induces an M2-phenotype response in activated macrophages in the GI tract, thus decreasing the inflammatory response and increasing cellular healing cytokine activity and cellular proliferation. The zonulin pathway reaction induces several pro-inflammatory responses, so this is a counter-reaction happening in the same area.(Barilli et al., 2018; du Pre and Sollid, 2015; Nikulina et al., 2004)

Concurrent to this T-cell reaction, the crypt cell division increases to develop into overt lesions in between villi, bringing the villi surface closer together. The proliferation of CD8 T-cells kills endothelium cells, and the adaptive immune reaction generates inflammation which results in gaps in endothelium. The adaptive immune system also generates a large increase in plasma cells generating IgA antibodies in the lesions developing in the endothelium and increases the M2 macrophages which promotes cellular healing.(Barilli et al., 2018) When these gaps heal, the resultant bridge to the nearby damaged villi results in villous blunting. One

study showed CD patients with villi average height of 86 μm , one sixth the height of a healthy patient (Vuoristo, 1987). Indeed, both the crypt hyperplasia and T-cell lymphoma is not reported in other inflammatory enterocyte diseases at this time. (Smith et al., 2013) This decrease in viable endothelium surface area results in nutritional deficiency.

Following a GFD, CD8 T-cell population return to normal. However, the $\gamma\delta$ T-cells with the V δ 1 gene segment population remains heightened for a prolonged time. (Murphy and Weaver, 2017; Smith et al., 2013)

Symptoms

Most of the physiological conditions related to CD are separated into two categories: symptoms related to the damage in the GI tract and symptoms that are directly related to the gluten interaction with tTG. The second set belong to the diagnosis of Symptomatic CD and are considered most severe, as well as most recognizable.

Asymptomatic or Silent CD is harder to diagnosis due to the lack of clearly discernible symptoms. These patients usually report a collection of symptoms, usually GI or nutrition related, that persist until a GFD is initiated. Table 1 lists the common symptoms for silent CD. (Green et al., 2005; Jericho and Guandalini, 2018)

Table 1: Symptoms of Silent Celiac Disease (Green et al., 2005)

Children	Short Stature
	anemia
	neurological symptoms
Adults	Dermatitis Herpiformis
	Anemia
	Reduced Bone Density
	Apthous Stomatitis
	Dental enamel defects
	Infertility
	recurrent miscarriages
	IBS
	Dyspepsia
	Esophageal reflux
	neurological symptoms
	Autoimmune Diseases

The majority of the nutrition deficiency is due to the damage to the villi in the small intestine, making this the best sure diagnosis currently. Since the complex cooperation of the villi system increase the active surface area of the small intestine by 1000 fold, this loss significantly undermines the body's ability to collect nutrition. This systemic atrophy is usually noticed when the patient achieves extensive damage resulting in hospitalization, sometimes multiple times before accurately diagnosed and treated.(Jericho and Guandalini, 2018; Marginean et al., 2018; Murphy and Weaver, 2017; Smith et al., 2013)

Since one of the earliest known signs of Celiac is the greasy feces, the function of the gall bladder, liver and biliary system contribute to a common symptom. The villous atrophy in the duodenum hampers the secretion of a hormone, cholecystokinin, which promotes gallbladder motility and interacts with several nervous pathways including satiation. Studies in the 60s measured a deficiency in pancreozymin, renamed to cholecystokinin later, and a follow up study in 1971 showed that the gall bladder in untreated CD patients' gallbladder failed to contract in the presence of a high fat diet unless stimulated directly with cholecystokinin.(Low-Beer, 1971) Further research into the bile salts and taurocholate pools demonstrated that while the ratio of cholate to chenodeoxycholate skewed slightly toward cholate, the big difference between a healthy patient and untreated CD patient was the size of both pools, each being nearly 3 fold greater. Since the turnover rate remained, the size is likely due to the lack of gallbladder stimulation resulting in failure to release bile salts into the small intestine. This lower concentration of bile salts would fail to inhibit hepatic bile salt production, thus increasing the total bile salt pool and making bile salt sequestering a possible symptom in CD.

The ileum environment contributes to the resultant steatorrhoea. In certain cases of CD, the ileum attempts to compensate for the decreased absorption in the duodenum and jejunum. The ileum's ability to sustain this hyperactivity determines the severity and variety of many of the apparent GI symptoms. Failure of the ileum to handle the bile salts, whether through direct CD damage or through indirect damage while supporting the damaged duodenum and jejunem,

often results in the steatorrhea historically indicative of CD.(Gillberg, 1980) Physiologically, when the bile salts unabsorbed enter the colon and the high fatty acid count due to incomplete digestion reach the large intestines, they actively induce water and electrolyte secretion. Not only would this exacerbate nutritional deficiency, but it shows in the feces, doubling the dry weight, tripling the water weight and quintupling the fat weight of a healthy patient.(Vuoristo, 1987) After 8 months of GFD, the feces weight normalizes by half, showing a partial recovery and leading to the recommendation of 18 months on GFD prior to recovery verification.(Low-Beer, 1971; Low-Beer et al., 1973; Vuoristo, 1987)

The liver, an integral part of the biliary system, is also affected by CD. While there are many liver malfunctions, the two most associated with CD are cryptogenic disorders and autoimmune disorders. In half of untreated CD patients, the liver had increased transaminase averaging 61 for ALT and 47 for AST, indicating liver distress but not elevated enough to cause alarm during testing. Often, if left untreated, this is followed by chronic active hepatitis or primary biliary cirrhosis.(Duggan and Duggan, 2005) Chronic Active Hepatitis is a repeated liver infection, in CD likely due to the combination of the increased intestinal permeability and the transfer of immunologically active molecules and cells through portal circulation.(Anania et al., 2015) Primary biliary cirrhosis(PBC) or the progressive breakdown of hepatic biliary ducts, is often the result of antimicrobial antibodies and autoreactive T-cells. It generally ends with liver failure. Between the gallbladder status and the immune connection, numerous studies show a high correlation between CD and PBC. Additionally, the increased intestinal permeability in CD could allow the gut microbiota access to the liver leading to a possible PBC trigger, certain gram-negative bacteria molecular mimics. It is recommended that patients with one condition get tested for the other.(Volta et al., 2013) Most of these chronic liver conditions improve dramatically on a GFD.(Anania et al., 2015; Casella et al., 2013; Choung and Murray, 2017; Duggan and Duggan, 2005; Lo Iacono et al., 2005; Majumdar et al., 2018; Szaflarska-Popławska, 2011)

Bone health in CD patients is complicated. Various bone-related conditions including low bone mass, osteoporosis, secondary hyperparathyroidism, and osteomalacia are reported in conjunction with CD. Some of this degeneration is due to the decrease in nutritional absorption by direct loss of calcium and vitamin D. However, in CD, the loss of calcium triggers an increase of parathyroid hormone that stimulates osteoclast mediated degradation, often leading to osteoporosis and osteopenia. Additionally, in males, the lack of nutrition often results in hypogonadism, which can have a significant impact on bone growth. Indeed, those with untreated CD had a prevalence of low bone density between 38% and 72%, and, conversely, the osteoporotic population's prevalence for CD is triple that of the general population. The lack of nutrition is not the only factor in low bone density in CD patients as the prevalence of low bone density in CD patients with GFD remains between 9% and 47%. The release of certain immunological proinflammatory cytokines during the autoimmune reaction increases the osteoclast activity. Theoretically, the immune reaction to tTG2 causes the release of these proinflammatory cytokines which supports osteoclast activity. (Duggan and Duggan, 2005; Zanchetta et al., 2016)

One of the common visual symptoms of CD is dermatitis herpetiformis. This blistering skin disorder occurs when an immune response to gliadin deposits IgA Anti-tTG2 and Anti-transglutaminase 3 in the epidermis correlated to the degree of enteropathy. Another common, but less predictive symptom is gluten ataxia, where the patient loses muscular control for a short time possibly due to the expression of antibody to TG6, an isozyme in the brain. Other neuropathic conditions related to gluten have been discovered, however for gluten ataxia, only a third showed evidence of enteropathy.(Troncone and Jabri, 2011)

Approximately 10% of CD patients suffer from some form of neurological symptoms, with ataxia and peripheral neuropathy being the most common. Even lacking overt neurological symptoms, a study showed untreated CD patients had changes to cortical gray-matter and caudate nuclei related to duration of gluten ingestion.(Bilgic et al., 2013) Ataxia, or the

temporary loss of function in motor muscles, can start as simple unsteadiness in stance and limbs. A factor in these neuropathies is anti-transglutaminase 6, a relative of tTG2, which also appears in untreated CD patients and is involved in gluten-dependent ataxia.(De Leo et al., 2018) Frequently, ataxia continues to cause several other issues, including myoclonus and cerebellar atrophy. Autopsies of these patients show atrophy, gliosis, Purkinje cell loss and degeneration of the posterior columns of the spinal cord.(Green et al., 2005) Peripheral neuropathy is another frequently associated with CD. In fact, one study showed that 5% of patients with peripheral neuropathy likely also have CD. Certain Epilepsy patients are also CD positive, and there is a suspected causal relationship since the cerebral calcifications were traced back to the same HLA-phenotype, even in non-CD patients. Additional neurological symptoms associated with CD are mood disorders, such as depression and anxiety, neuromuscular disorders, such as myositis, movement disorders, such as Huntington's disease, and cerebral abnormalities, such as cerebral vasculitis. CD screening for a patient with Down Syndrome has nearly become the norm. Many of these are attributed to anti-gangliosides as a result of CD.(Green et al., 2005) Another factor called Brain-deprived Neurotropic Factor (BDNF), a protective protein in the central neural system responsible for neuronal plasticity, specifically during stress and linked with mood disorders, was linked to intestinal inflammation including CD. This serological concentration remained increased even following a GFD. CD neuropathology also includes depression, anxiety, apathy, irritability, migraines, and epilepsy. (Campagna et al., 2017; Luostarinen et al., 2003; Margoni et al., 2018; McKeon et al., 2014; Pengiran Tengah et al., 2002; Pennisi et al., 2017; Wills, 2000)

Table 2: Common Neurological Symptoms of CD (Green et al., 2005)

Peripheral neuropathy	Headache w/ white matter abnormalities	Chorea
Ataxia	Epilepsy/cerebral calcifications	Neuromyotonia
Stiff-man syndrome	Brainstem encephalitis	Epilepsy
Cerebral vasculitis	Progressive multifocal leukoencephalopathy	Myoclonus
Anxiety/depression	Progressive myoclonic encephalopathy	Polymyositis
Huntington's disease	Schizophreniform disorder	
Dementia	Inclusion body myositis	

Both individual studies and a 2015 meta analysis showed no statistical correlation between circulatory conditions and CD: including cerebrovascular disease, coronary heart disease, stroke or brain hemorrhage(Bardella et al., 2004; Heikkila et al., 2015) Where many autoimmune conditions seem to have cardiovascular complications, CD does not appear to be one of them.(Bardella et al., 2004)

Studies show women with untreated CD have three times greater tendency than normal to give birth to infants with low birth weight and intrauterine growth retardation. This is likely largely due to the the decrease in nutrition available to the fetus during gestation.(Norgard et al., 1999)

Other Gluten Sensitivities

Wheat allergy: CD differs from wheat allergy in basic immunological response. Like most conditions termed allergies, wheat allergy involves a immunoglobulin IgE reaction on a B-cell to produce a well documented immune response including inflammation, vasodilation and resource redistribution(Troncone and Jabri, 2011). Wheat allergy has a prevalence of 0.5% in the general population. Unlike CD, which reacts to water-insoluble prolamins, wheat allergy reacts with water soluble proteins. Thus, only wheat need be avoided, not necessarily rye, spelt or barley. Many with wheat allergies acquire it through a combination of exposure and exercise. In other words, most wheat allergies are exercise induced, meaning they only cause asthma, urticaria, or anaphylaxis when the ingestions of the food combines with exercise in close succession. Currently, omega-5 gliadin is the theoretical culprit. Another condition referred to as Baker's Asthma results in arthritis and asthma from inhaling flour particles. In this case, individuals can ingest the wheat, but need to avoid breathing it in. As with other allergies, a skin test is the common diagnosis test. Unfortunately it has a high false negative rate. As with most IgE reactions, the condition resolves itself when the allergen is eliminated from the system. This particular allergy is predominantly in children and normally disappears as the child grows. This is in direct contrast to CD where the reaction can continue for some time after, and the damage

is less reversible.(Balakireva and Zamyatnin, 2016; Burkhardt et al., 2018; Elli et al., 2015; Henggeler et al., 2017)

The presence of IgD antibodies in gluten reactive situations was studied and shown to be elevated, but no explanation has been discovered or researched(Burkhardt et al., 2018).

Double blind challenge evidence for gluten sensitivity is weak with the exception of wheat allergies. The proliferation of the gluten free diet seems mostly media based. However, since the subjective improvement in the patients' lives can be substantial, the term Non-Celiac Gluten Sensitivity(NCGS) is used. This condition, however, is poorly defined and highly variable from one patient to another. Some symptoms of gluten ingestion are internal, such as stomach ache, and some are external, such as skin rashes. Additionally, some research found that certain conditions improved with the removal of Gluten from the diet, such as schizophrenia, bipolar fibromyalgia, and spondyloarthritis. Further research demonstrated that certain NCGS patients benefit from enzyme replacement therapy, indicating a reaction to undigested gluten which is divergent from CD pathology.(Ido et al., 2018) Due to the lack of biomarkers and widely variable reactions, NCGS remains difficult, if not impossible, to diagnose outside of the process of elimination at this time.(Burkhardt et al., 2018; Hadjivassiliou et al., 2016; Henggeler et al., 2017; Valenti et al., 2017)

Other misdiagnosis: There are several conditions which overlap with CD and other gluten related immune reactions. Irritable Bowel Syndrome(IBS) is one such condition. Due to the damage in the intestine during IBS, it is plausible that many patients with IBS are CD positive and undiagnosed or misdiagnosed as IBS. Furthermore, if the patient is diarrhea-predominant, the recommendation of CD screening is logical. The symptoms for IBS include abdominal pain, gas, mucus in feces and diarrhea/constipation, which match many symptoms for CD. This implies that should the IBS symptoms increase with gluten intake, the presence of CD is likely.(Troncone and Jabri, 2011)

In addition to other gluten sensitivities, there are a number of conditions that can mimic CD. IBS, as demonstrated above, overlaps in symptoms. Small intestine bacterial overgrowth, inflammatory bowel disease, microscopic colitis and issues in the thyroid and adrenal system all show clinical features similar to active CD. Crohn's in particular, mimics CD in patients. Since the cause and diagnosis standard for both diseases remains under debate, separating the two medically is complicated. (Kamboj and Oxentenko, 2017)

Treatment

The only existing treatment for CD is adopting a strict gluten free diet, which is difficult and expensive to maintain. Meals, in addition to providing nutrition, are often central to family and social gatherings, to business meetings, and networking and work functions. This makes adhering to a strict gluten free diet socially challenging and even a career hindrance for some. Finding gluten free food items has become easier as the number of non-Celiac consumers on gluten free diets have created a larger market for gluten free foods, which have expanded in variety and availability in recent years.(Naqash et al., 2017) However, gluten free items are still not ubiquitous at supermarkets and their cost is often significantly higher than comparable products made with gluten, adding cost as another drawback to a gluten free diet. Wheat flour is commonly used as a binding agent in medications, and even typically gluten free medications may have batches that are not gluten free when their manufacturer uses a different flour blend for cost or availability reasons, limiting medication options for CD patients. Yet another issue involves assessing the integrity of "gluten free" labeling on meals at restaurants and even in prepackaged foods. For Celiac patients, preparation of their gluten free meal using the same knife, cutting board or pan used to prepare foods with gluten can provide enough cross contamination to trigger intestinal inflammation, making it very difficult to eat strictly gluten free at restaurants.(Young and Thaivalappil, 2018) A manufacturer of gluten free pre-packaged foods was prosecuted after their products were found to contain significant amounts of gluten. More recently New Zealand found gluten in several food products labeled "gluten free"(Halmos

et al., 2018). Because not every CD patient has clinical symptoms immediately after ingestion of gluten, they may not know they have been exposed to gluten until they develop complications like malabsorption or cancer. There is research that a handheld gluten detector created by Nima in 2019 read 20ppm gluten, the limit set by the FDA, with some success at lower concentrations.(Zhang et al., 2019)

The only viable treatment showing continuous success in ameliorating the ongoing damage caused by CD is GFD. The reaction and adherence to a GFD varies dramatically in CD patients. Some patients can indeed handle a miniscule amount of gluten, where others must avoid it to the extent that breathing it could be hazardous. Similarly, some patients react to the Avenin in oats, sparking a CD reaction just as with gluten(Hardy et al., 2015). Others do fine eating GF oats. (Fric et al., 2011; Hardy et al., 2015; Kilmartin et al., 2003; Londono et al., 2013; Tapsas et al., 2014)

The presence of gluten is another topic of debate. The FDA regulations state that 20 ppm gluten or less is required to label a product gluten free. Since this is not always tested, reading the ingredient list on products is the safest alternative. In this case, any gluten related substances are eliminated since most labels only inform below the 2% cut off line, and the cut off for FDA gluten free is 0.002%. This vigilance is not only limited to food. Other substances such as medication and cosmetics use gluten on a regular basis and must be inspected as well.(Durham and Temples, 2018; Martin, 2008)

Table 3: General Gluten-Free Diet(Martin, 2008)

	recommended	Question	not allowed
Milk products	Milk	milk drinks	malted milk
	cream	flavored yogurt	
	buttermilk	frozen yogurt	
	plain yogurt	sour cream	
	cheese		
	cream cheese		
	processed cheese		
	processed cheese foods		
	cottage cheeses		

Table 3 cont.

	recommended	Question	not allowed
Breads	products made from		products containing
	Corn, rice or soy flour		wheat
	Starch from Corn, Potato		rye
	buckwheat		triticale
	cornmeal		barley
	millet		oats
	flax		wheat germ/bran/starch
	teff		bulgar
	sorghum		granham flour
	quinoa		gluten flour
	amaranth		durum flour
	rice bran		farina, kamut
	arrowroot or potato flour		semolina
	tapioca or pea flour		spelt
cereals	cream of rice	rice/soy pabulum	see breads
	soy cereal		added malt extract/flavoring
	hominy		
	brown/white rice		
	puffed corn/rice/millet		
Pastas	see breads		
Meats	Fresh	canned	containing Hydrolyzed vegetable protein (HVP)
	frozen	preserved	
		pre-prepared	
	salted		containing Hydrolyzed plant protein (HPP)
	smoked		
Eggs	eggs	egg substitutes	
		dired eggs	
		egg whites	
Other proteins	lentils	Baked Beans	
	chickpeas	dry roasted nuts	
	beans	peanut butter	
	nuts		
	seeds		
	tofu		
Fruits/vegetables	fresh	pie filling	battered dipped
	frozen	dried fruits	
	canned	fried potatoes	
		scaloped potatoes	
Soups	Homemade broth	canned soups	containing HVP
	GF bouillon cubes	dried soup mixes	containing HPP
	cream soups	bouillon cubes	

Table 3 cont

	recommended	Question	not allowed
Fats	butter	salad dressings	packages suet
	margerine	mayonaise	
	lard	communal butter dish	
	vegetable oil		
	cream		
	shortening		
	homemade salad dressing		
Desserts	ice cream	Milk pudding	ice cream cones/wafers
	sherbet	custard powder	any desert made with ingredients listed in Breads
	ice	pudding mixes	
	whipped toppings	pies	
	custard		
	gelatin deserts		
	GF homemade pastries		
Beverages	tea	instant tea	beer
	instant and ground coffee	coffee substitutes	ale
	cocoa	fruit flaovred drinks	lager
	soft drinks	chocloate mixes	cereal malted beverages
	cider	flavored/herbal tea	
	distilled alcoholic beverages		
	wines		
	pure liqueurs		
Snacks	popcorn	flavored anything	mustad pickles
condiments	pickles	herbs/spices	imitation pepper
	relish	soy sauce	
	olives	worchestshire	
	ketchup	mixed spices	
	mustard		
	tomato paste		
	vinegar		
	GF soy souce		
Other	pure cocoa	baking powder	
	baking cocoa	sausces	
	choc chips	gravy	
	carob chips/powder		
	monosodium glutamate		
	cream of tartar		
	baking soda		
	yeast, Brewers yeast		
	coconut		
	aspartame		

Future treatments

One of the ongoing debates on CD focuses on screening the population. As stated, even though 1% of the population suffers from CD, in the USA only 3% of that, or 0.3% of the total population, has received that diagnosis. There are several screening tests, however the criteria for active CD differs between countries and its onset is not always noticed immediately. With a prevalence this high, screenings in patients with related diseases or conditions is recommended, but the debate for larger screenings is ongoing. In 2017, the American Medical Association reviewed the possibility for adult screenings and found the current evidence is “Insufficient to assess the balance of benefits and harms of screening for Celiac Disease in asymptomatic persons” (Force et al., 2017). (Evans et al., 2011; Force et al., 2017; Hujoel et al., 2018)

Vaccines have been considered for this using antigen-specific immunotherapy(ASIT) or epitope-specific immunotherapy(ESIT). ASIT has been successfully used for allergy reactions, specifically the CD4 T-cells reactions with MHC class II. Since CD is closely related with HLA-DQ2/DQ8 in this interaction, it would be the target for such a vaccine. This research is slowed by the difficulty in monitoring the T-cells in research subjects. A recent trial with Nexvax2 showed progress. Participants showed no adverse reaction to the vaccine and “resulted nonresponsive to a further oral gluten challenge”(Di Sabatino et al., 2018). Although this vaccine is only targeting HLA-DQ2.5, it is a step in the correct direction.(Anderson and Jabri, 2013; Di Sabatino et al., 2018)

Diagnosis

Symptomatic

In children, efficient and reliable diagnosis is achieved through an assessment of the symptom cluster and its relief based on GFD implementation. Children with CD have several classic symptoms that reliably improve upon the start of a gluten-free diet; anorexia, failure to

thrive despite adequate diet, chronic/recurrent diarrhea, irritability, abdominal distension, weight loss and muscle fatigue. Often these symptoms cause the child to be examined and treated, but then the symptoms reoccur unless a GFD is initiated. Currently, the subsequent visit alerts the medical professionals to an unknown underlying cause, and CD is often diagnosed at this point.(Burkhardt et al., 2018) This is valid in infants as well. The primary issue is the damage done between the initial visit where the symptoms are treated and the following visit where the underlying cause of the presence of gluten in the diet is treated.(Marginean et al., 2018)

Childhood symptoms change slightly in adults. Weight gain is actually rather common in CD due to the increased need for food intake to meet the higher nutritional requirements. Long term malnutrition symptoms such as severe anemia, arthritis, aphthous stomatitis, dental enamel defects, and short stature are less common in children, but more common in adults possibly due to the developmental stages children experience. Additional neuropathic symptoms that present at any age include autism, ataxia, dementia, depression, epilepsy, headaches, peripheral neuropathy, and schizophrenia(Burkhardt et al., 2018).

Another common symptom is comorbid conditions in the immune system. CD frequently exists alongside Type I diabetes, Williams Syndrome, Turner's syndrome, Down Syndrome, arthritis and several autoimmune disorders such as thyroid and liver autoimmunities. Up to a quarter of diagnosed CD patients have dermatitis herpetiformis(DH), which is also increasing in prevalence. DH, a rash that leaves purple marks even after lesions and sores have healed, occurs when IgA pools in the skin on the knees, elbows, back or buttocks.

Serology

The more common, and least resource expensive method of diagnosing CD is the serology presence of IgA tTG2 and IgA endomysial antibodies in the bloodstream when the patient has ingested gluten for a prolonged period of time. The serological cascade usually includes total IgA concentration, tTG2 antibodies (either IgA or IgG), deamidated gliadin antibodies, and endomysial antibodies. Endomysial antibodies are located in the muscle

primarily and target transglutaminases. These are detected using Indirect Immunofluorescence Assay and Immunoturbidimetric Immunoassay.

Over the last few years, a controversy has risen over the diagnosis of CD. The presence of Antibodies in serum originally confirmed the diagnosis. However, in 2005, the North American Society for Pediatric Gastroenterology Hepatology and Nutrition(NASPGHAN) required an intestinal biopsy, a policy further supported by American College Gastroenterology in 2013 and British Society Gastroenterology in 2014. Both groups require an endoscopy in the duodenal for adults to confirm a diagnosis of CD. Currently the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) remains the only group to endorse serum-only diagnosis. Due to procedural risk, diagnosis in children remains serum-based. However, variability in testing kits and lab practices sometimes makes this unreliable. Thus, symptomatic children are diagnosed with serum testing, and most of the times, genetic testing is recommended. To further control the diagnosis rate, the level of antibody-TG in the serum used is 10x normal where any patient below this cutoff, but still a strong candidate for CD diagnosis will undergo a biopsy. For symptomatic patients, a cutoff of 10x had a positive predictive value of 98%, leaving only asymptomatic patients misdiagnosed. For adults, there is some debate over the cutoff. One study showed 100% PPV at 5x, while another showed different kits had different cutoffs for 100%PPV.(Kowalski et al., 2017; Turner, 2018)

An additional point in the argument against serum-only diagnosis in adults is the baseline information of lesions acquired during the endoscopy. Approximately a third of CD adults continue to have lesions for 2-5 years following the initial diagnosis and adoption of an assumed gluten free diet for the same period of time. Since the long term risks of this damage to the mucosal layer are unknown, not only does the biopsy provide information about the current state, but it also indicates refractory CD. The rules surrounding the diagnosis via biopsy are variable. Another approach is referred to as the 4 out of 5 approach. If 4 of these 5 events are true, then CD diagnosis could be accepted; CD symptoms, a positive response to a GFD,

positive serology for IgA TTG -antibodies/EMA, biopsy, and genetic HLA testing(Burkhardt et al., 2018). Regardless of diagnosis, repeated biopsies to monitor the intestine are recommended once CD is confirmed. A need for greater understanding of this diagnosis is clear.(Burkhardt et al., 2018; Turner, 2018)

Endoscopic

The most trusted test for CD currently is endoscopic duodenal biopsy. “One to two [samples] from the duodenal bulb and no less than four from the distal duodenum”(Turner, 2018) represents the varied enteropathy of the duodenum. Initial histology includes plasma cells infiltrating the mucosa. Further along, a crypt hyperplastic reaction is found and then blunting of the villi as they are remodeled. Other factors such as gluten intake and proximity to Brunner’s glands must be taken into account during this interpretation. The levels and size of these reactions which constitute a diagnosis of CD are under considerable investigation still.

There are 2 systems of classification for the results of an endoscopy; the Marsh scale and the Oberhuber scale. The two scales focus on different progressions of the condition are shown in Figure 7. The Marsh scale focuses on the condition in a linear fashion from healthy to villi atrophy and intestinal damage, with 0 being normal and Type 2 destructive being full CD. The Oberhuber scale focuses more on the amount and severity of the damage, again with 0 being healthy normal tissue, but then type 3 is divided into categories based on fraction of damage and type 4 is complete atrophic lesions with no inflammation or other villi character. (Dickson et al., 2006; Kowalski et al., 2017; Martin, 2008)

Marsh classification	
Type 0 (preinfiltrative)	No detectable changes in inflammation
	No detectable changes in crypt/villous height ratio
Type 1 (infiltrative)	Increase in the number of intraepithelial lymphocytes (IELs)
Type 2 (hyperplastic)	Inflammation, villous blunting, and increase crypt/villous height ratio
Type 2 (destructive)	Severe inflammation, flat villi, and hyperplastic crypts
Oberhuber classification	
Type 0	Normal mucosa with <40 IEL/EC
Type 1	Increased IEL/EC, normal villous architecture, and normal height of crypts
Type 2	Normal villous architecture, increased IEL, and crypt hyperplasia
Type 3	Destructive type with varying degrees of villous atrophy; in all subtypes, there is increased crypt height and influx of inflammatory cells
Type 3a	Partial villous atrophy; villi blunt/shortened with villous/crypt ratio <1:1
Type 3b	Subtotal villous atrophy; villi atrophic but still separate and recognizable
Type 3c	Total villous atrophy; villi rudimentary or absent; mucosa resembles colonic mucosa
Type 4	Atrophic hypoplastic lesion; flat mucosa with normal crypt height and no significant inflammation with normal IEL counts

Note. Adapted with permission from Chand and Mihas (2006). EC, epithelial cell.

Figure 7: Pathologic Classification of Celiac Disease(Martin, 2008)

Genetic

At this time, genetic and CD research have identified MHC genes as the most important genetic component in CD. As stated in the section describing the creation of B-Cells, MHC genes control the immunoglobulin presented on the surface of the B-cells and control the selectivity of the reaction to antibodies. In 80-95% of CD diagnosed patients, the MHC gene HLA-DQ2 variant exists. Those who are negative for this gene generally express the other MHC gene associated with CD, the HLA-DQ8 variant. Only 1% of CD patients present neither of these genes compared to nearly half the general population. A large number of patients express both. Due to this correlation, HLA typing specifically for the allele responsible for the above genes indicates predisposition towards CD. The alleles responsible for HLA-DQ2 variant are HLA-DQA1*05 and HLA-DQB1*02, and for HLA-DQ8 variant, HLA DQA1-03 and HLA DQB1*0302. The common genotyping done is for HLA-DQA1*05 and HLA-DQB1*02.(Jabri and Sollid, 2006) Genetic testing supports a diagnosis, or lack thereof, but due to the population that expresses these phenotypes with no other CD indicators, genetic-only diagnosis for CD is considered invalid.

Further evidence exists of genetic components beyond the MHC genes for CD. In Italy, a paper showed that the concordance rate between monozygotic twins was 75%, but a study in Spain also showed that the concordance between HLA-identical siblings was only 25%(Jabri

and Sollid, 2006). This implies that there is a strong genetic factor beyond the HLA phenotyping. In 2003 Babron published a paper identifying chromosome 5 as a possible location for one such gene, and van Belzen published a similar finding on chromosome 19 that same year (Jabri and Sollid, 2006). One paper suggested myosin IXb on chromosome 19 to be a candidate, however further attempt to replicate the work failed. This gene attracted interest due to its production of actin-binding myosin. Theoretically, this could increase permeability in the intestine walls, an important characteristic of CD. Additionally, another group found cells of myeloid origin expressed myosin IXb, possibly affecting APC function. (Jabri and Sollid, 2006)

A study in 2007 noted a relationship between CD and certain genes involved in the production of IL2 and IL21. "IL2-21 locus is now a well-established disease susceptibility locus for T1D, RA, UC, MS, and systemic lupus erythematosus (SLE)" (Kumar et al., 2012). This study identified 13 loci that could participate in CD. In 2010 a larger cohort genetic study noted another 13 loci outside the HLA genes that correlate with CDs. Furthermore, this study identified 14 loci shared between CD and RA (arthritis), including 4 previously unknown loci. The introduction of the immunochip in 2011 added 13 more loci correlated with CD. In this study, of the 39 total loci associated with CD, 13 included multiple independent signals, bringing the total of actual independent loci to 57. In the same year, research discovered 4 loci that overlap with Crohn's Disease. (Kumar et al., 2012)

One gene under close scrutiny is the PTPRK gene, part of a loci that regulates thymic T-cell selection. Another gene, possibly a causal gene, is IL18RAP, which does not show an effect on nearby genes but strongly implies CD is governed by gene expression deregulation. Studies show that 50% of CD related SNPs affect the expression of nearby genes, thus implying that CD is caused by or has an effect on gene expression regulation. However, research also shows that affecting a gene not nearby could prove important. Eighteen unlinked pairs located affected the regulations of the same trans-gene in CD, implying the importance of this genetic behavior in the disease mechanism. In fact, HLA contain and probably rely on this behavior.

These genes are often related in their function. Functionally related genes tend to respond to the same or similar regulation pathways and thus “CD loci is associated with T-cells (TAGAP, TNFRSF14, CCR4, CTLA4, UBASH3A, and CD28), NK cells (UBE2E3, RUNX3, FASLG, PTPN2, and IL18RAP), neutrophils (PLEK and CCR3), and B-cells (BACH2, SOCS1, POU2AF1, ICOSLG, IRF4, CIITA, ZFP36L1, and CSK)”(Kumar et al., 2012).(Kumar et al., 2012)

Of the 57 loci, only 3 appear related to protein sequencing. In fact, 16% appear to have no connection to protein-coding genes, and 95% are located in the gene regulating regions. The majority seem to related to introns or the intergenic regions, meaning they affect the transcriptional regulations of the genes. Much of the effect seems focused on non-standard RNA and, thus, part of gene regulating and splicing. (Kumar et al., 2012)

Since the CD population overlaps with certain other conditions such as Addison’s or IgA deficiency, the genes involved in those situations could indicate CD. Further study is needed for this.

Also, since there is a genetic component, many advocate for screening of first-degree relatives of a confirmed case at least with serology. Between siblings, the rate is 8.4% and offspring is 7.9%. Oddly, the rate of parents of active CD patients is at 3%, lower than most other relatives.(Burkhardt et al., 2018)

Other

Several other methods of diagnosis have been attempted. Soler et al developed a SPR biosensing immunoassay for urine that detects residual 33-mers, something that should only be present in an untreated CD patient. This method utilizes specific monoclonal antibodies and demonstrated the ability to quantify gliadin peptides without extraction or purification with a limit of detection at 1.72 ng/ml (CD patient is 20 ug/ml). Additionally, this method is a practical, non-invasive test of the adherence to GFD of the patient.(Soler et al., 2016)

There are some general GI issues that occur during CD that when combined indicate further testing is necessary for CD. In one case, an infant presented mild pericardial effusion with lack of accompanying cardiac pathology but abdominal bloating, irritability, intermittent diarrhea, and diffuse abdominal tenderness. Lab tests revealed anemia, hypoalbuminemia, leukocytosis, and high transaminases and inflammatory biomarkers. The diagnosis agreed upon was E-Coli and treated until a return visit for the 1-year-old a month later with the same symptoms and same lab tests. The child was admitted with the same severity 8 months later and tests showed serum Anti-tTG2 8x normal and duodenum modifications Marsch 3C stage. In this case, this child likely suffered 8 months additional damage and malnutrition at an age when the impact would be extreme. That could've been avoided if there was a sufficient diagnosis for CD.(Marginean et al., 2018)

CHAPTER 5: RAMAN SPECTROSCOPY

Discovery

Raman Spectroscopy(RS), named for Sir CV Raman who first observed the phenomenon in 1928, utilizes the inelastic scattering of molecular excitations to calculate vibrational modes in a system based on the energy shifts displayed during the scattering. This is generally accomplished using a laser to cause shift in energy states and then the resultant energy discharge during the return to equilibrium is captured and interpreted. During this process additional energy is released in the form of Elastic or Rayleigh shifts, which are filtered out by a frequency filter such as a band pass filter.

Stokes and anti-Stokes shifts are inelastic shifts. When the energy of the laser (or another source) hits the excitable particle, it jumps to a virtual energy level. Rather quickly, it returns to an energy level at or near its previous state. When this new energy level does not match the previous level, this is inelastic shift. A Stokes shift is when this new level is higher than the original energy level and anti-Stokes shift is when this new level is lower than the original level. The process where the release is elastic or the energy level is the same as the original level is called Rayleigh shift(see Figure 8). When the return is Stokes or Anti-Stokes, the energy change occurs in the photon that excited the molecule and is known as raman scattering.(Ember et al., 2017)

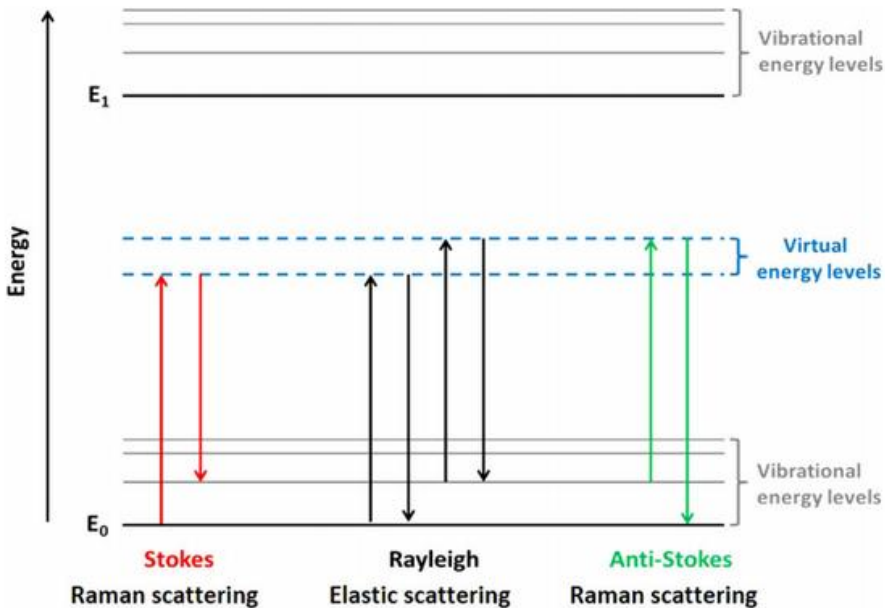


Figure 8: Energetic Transitions(Ember et al., 2017)

A raman spectrometer has several basic components. The first is a monochromatic laser. The wavelength of the specific laser affects the sensitivity and resolution of the sample. Additionally, certain types of bonds react with different wavelengths, i.e. biomaterials and fluorescence suppression have better optimization in lower wavelengths (<400 nm). However, the extreme damage prevents its use on live samples due to the higher energy. This means that more often higher wavelengths in the visible or IR range are used, decreasing the intensity of the signal. This decrease in signal intensity and associated signal to noise ratio (SNR) resulting in less information gathered remains a key roadblock in the widespread usage of this technology.

Following sample interrogation, the resultant energy is directed to a collation mirror via a series of lenses to reflect the energy through a diffraction grating or another device which diffracts the energy into distinct wavelengths. Then the energy is passed through a series of focusing mirrors and lens until the resultant wavelength specific beams are fed into a detector array. These series of mirrors and lenses are responsible for resolution control and signal noise generation. Additional filters such as band pass filters are added to the machine to assist in controlling the SNR and filtering out elastic shifts.

Types

There are several different techniques associated with Raman spectroscopy, each with their own specialties. Below are four common types that may be of use in CD research.

Microscopic Raman Spectroscopy typically grafts a microscope on a raman spectrometer and scans a sample in a raster style pattern. A uni- or multivariate spectral model applied to the spectrum produces a complete readout of the information. Research shows that a line pattern as opposed to a single-point pattern produces greater resolution at faster speeds. In order to match the pattern of optical fibre to the laser pattern, the microscope can be coupled using shuttering systems of optical fibers or masking arrays. Due to the sensitivity of this approach, variations in baseline RS can hide diagnostically important discriminations.(Kong et al., 2015)

Surface Enhanced Raman Spectroscopy (SERS) enhances the weaker signals by utilizing metallic nanostructures to resonate with adsorbed or nearby molecules to enhance the the raman scattering. This resonant interaction extends a few nanometers from the surface and created a electromagnetic field that enhances raman bands 10^5 times. Additionally, tailored nanoparticles conjugate targeted molecules, specifically antibodies, for a targeted scattering enhancement. This has been done in tissues.(Kong et al., 2015)

Coherent anti-Stokes Raman Spectroscopy(CARS) uses multiple photons to produce a distinct and more intense signal. Coherent *Stokes* Raman Spectroscopy is also possible. This type may cause some change in the target and is used primarily for microscopy and combustion diagnostics.

Tip-Enhanced Raman Spectroscopy, or TERS, is a form of SERS where the scattering focus is on a sharp point, usually a gold tipped pin. This allows maximum resolution and extreme targeting flexibility on the part of the researcher. In order to properly use this technique, the equipment set up requires a confocal or scanning microscope.

Raman Spectroscopy has yielded many important scientific discoveries, notably in the field of physics. More recently its application in identification of substance or the change in a substance launched Raman Spectroscopy into several fields including medicine, security sweeps and scientific analysis. Like many other optical systems, RS can measure changes in real time, making its use in medical diagnosis and research all the more significant and vital. (Ember et al., 2017; Kong et al., 2015; Kumar et al., 2016)

Diagnostics

Since the 2000s, Raman Spectroscopy has been an option for cancer diagnosis. Kirsh et al published a method using RS to diagnosis and locate brain tumors with an accuracy of 250 μm (Kong et al., 2015). This label-free *in-vitro* probe measured tumors in mice. Later, using raster scanning, spectra images correlated with H&E staining without the nearly dozen steps involved prior to observation displayed tumor boundaries. Further use of CARS allows the lower lipid count of the tumor to identify and clearly delineate the borders and infiltrations of the tumor

In breast cancer, *in-vivo* techniques either provide numerous false positives or involve biopsy results with more than 70% showing benign response. Recent work in RS showed that the increase in intensity in the 788cm^{-1} and 1098cm^{-1} spectra peaks distinguish cancer from the surrounding tissue. *In-vivo*, TERS shows promise in decreasing the false positives from the mammogram by using calcium hydroxyapatite, since many benign have calcium oxalate instead.(Kong et al., 2015) However the depth of penetration for this technique is only 2-3 cm, thus not deep enough for most breasts. Although preventative diagnosis is still impractical, research shows that both in vivo and in vitro RS during surgery for tumor margin detection has potential.(Kong et al., 2015)

Current diagnostic procedures for lung cancer also result in excessive false positives interfering with reliable cost effective widespread screening. RS research predicted recurrence

with a sensitivity and specificity in the 70% range. By attaching RS to a bronchoscope, identified neoplastic lesions with sensitivity and specificity in the 90% range.(Kong et al., 2015)

Prostate cancer research uses raman spectroscopy for diagnosis and identification. Work in using RS for screening, biopsy, margin assessment and treatment monitoring continues to progress, making use of RS to read DNA and RNA changes in cells. In 2002, a normal prostate RS output was published. It was later compared to various cellular hyperplasia, including benign and adenocarcinoma, with good specificity. Further research into early detection proved not only viable, but able to distinguish between aggressive cancers in serology.(Kast et al., 2014)

Other cancer research pursued RS as a viable method. Current research in skin cancer and RS includes a hand held RS unit which can differentiate malignant and pre-malignant from benign. The specificity of this research is still in its infancy. In the non-handheld versions, RS successfully diagnoses skin cancer. More successfully, *in vivo* diagnosis of Esophagus cancer using Raman fiber optic probes has shown a sensitivity of 91% and specificity of 94%. In colorectal cancer, RS reveals more from the histopathology than current techniques provide, mostly in the form of biochemical information.

Raman Spectroscopy research has the potential to yield specific results in bone disease, including differentiation between brittle bone, osteoarthritis and other issues. The main obstacle for *in-vivo* RS diagnosis is distance. Although RS shows great potential in this field, the depth of penetration is still limited.

Diabetes testing may benefit from RS research moving forward. Currently, a blood test is required in some form to measure blood glucose levels. Raman promises transcutaneous measurements, already shown in mouse studies, which would provide a less invasive measurement method for blood glucose. Additionally, research into early diagnosis using Raman spectra is underway and shows promise. Similarly, early diagnosis for asthma and

research into the molecular changes when becoming asthmatic use raman spectroscopy.(Kong et al., 2015)

Limitations and advantages

Sampling is distinctly simpler with RS because the analysis samples are reagent and label free with minimal preparation. There are fluorescence concerns which cause the use of quartz, CaF or MgF as substrates simply due to their low intrinsic Raman signal. Glass, a common material in labs, is very fluorescent and only recently has been successfully used in RS. Since this is a biochemical analysis, the addition of reagents such as formalin or methanol to fix the samples actually changes the outcome of the readings, making unfrozen un-fixed samples optimal. (Ember et al., 2017)

This limits the reading to the life span of the sample at these conditions, however. Many biochemical markers, such as proteins and cell structures begin to degrade when not fixed. Furthermore, unfixed aqueous samples do not remain motionless, requiring the RS system to accommodate such brownian motion in its calculations. This has given rise to research in speeding up the RS data collection. Although a single Raman spectra integration is fast, a large area and high resolution is time consuming. Raster scanning and slit scanning have decreased the time needed. Also, “random sampling” and spectra averaging can be used to read large chemical compositions when an entire system is not available for scanning, such as a bloodborne issue.(Ember et al., 2017)

Quantization of RS is advantageous since the intensity of the signal is directly correlated to the concentration of that molecule. General use is also restricted by cost and size of the unit. The size of the machine has been decreasing in response to discovering these advantages. Handheld devices are in testing and in production with various specificities and sensitivities. Currently hand held devices are restricted in power and laser wavelength variability.(Ember et al., 2017)

History with cells

Research into inflammatory response using Raman spectra includes measuring and quantifying C-reactive protein produced by inflammatory immune cells in blood plasma. Additionally, level of fibrinogen and heparin are detectable by RS. This shows that the immune system has markers RS can detect. (Kong et al., 2015)

Brown et al uses RS for immunology study on T-cells. For certain T-cells, the activation of CD28/CD3 interaction indicates possible rejection of tissue implant. By comparing active, inactive and resting T-cells, Brown, et al. demonstrated that activation of this ligand pair expressed itself in RS as an increase in intensity in several peaks, but most significantly in the 1182:1195 region. When compared to alloreactive T-cells, this region showed a similar change, meaning this region identifies activated T-cells. During this comparison, the spectra differed greatly in 6 peaks; 903 cm, 1031 cm, 1093cm, 1155 cm, 1326 cm, and 1449 cm. The discriminant function of this research showed a clear difference with 100% accuracy meaning that all reactivity could be identified separately from CD28/CD3 activation. Moreover, comparing the peaks to established RS, we can identify specific peaks representing specific changes. Similarly, T-cell regulated apoptosis is also identifiable under RS, specifically in CD8+ T-cells. The apoptosis-related DNA signals show clear changes as the resting CD8+ T-cells activate and mature. Although certain proteins are also easily discernible, research demonstrates that DNA changes, not protein expression, track better using RS. This RS can also differentiate between T-cells and B-cells in the immune system. (Brown et al., 2014; Brown et al., 2009; Ichimura et al., 2016; Lee et al., 2015)

Even though RS displays changes in DNA more clearly, RS can successfully monitor changes in activated T-cells as expressed proteins interact with antibodies. Further research into the activation of CD69, CD25 and CD71 on T-cells demonstrated RS could detect different stages in activation. This correlates with previous research on activation and shows RS as a viable method to continue this research forward. Additionally, RS can identify different protein

markers and nuclei changes in real time in T-cells which could greatly advance understanding and research into T-Cell activity and diagnosis in T-Cell related conditions. (Brown et al., 2014; Brown et al., 2009; Ichimura et al., 2016; Lee et al., 2015)

CHAPTER 6: IN SUMMARY

Nutrition

Food eaten undergoes extensive digestion by multiple enzymes and proteases between the mouth, stomach and small intestines in order to produce chyme in a form that can be absorbed. The majority of the absorption is completed in the jejunum of the small intestine, largely due to the extensive surface area of the endothelium by combining the ventuoles, villi and microvilli. Food not absorbed in the jejunum, along with bile from the gall bladder, is absorbed by the Ileum or pushed into the colon for excretion.

Immune System

The majority of the reaction in CD uses B-cells and T-cells. B-cells develop in the bone marrow, producing IgM and IgD immunoglobulins on their surface prior to leaving the bone marrow stroma. The T-cells develop in the thymus, also producing immunoglobulins called either $\gamma\delta$ or $\alpha\beta$, which exist next to either CD4 or CD8 ligand. These are designed to bind tightly to MHC on an APC and activate to assist in an immune response. CD8 T-cells primarily activate cytotoxic immune reactions, such as NK cells and Macrophages. CD4 T-cells primarily activate B-cells through ligand interaction, which in turn starts an adaptive immune response. Both T-cells and B-cells can form memory cells which remain following an immune reaction to increase the reaction speed and magnitude on subsequent invasions by similar antigen. $\Gamma\delta$ T-cells have special interactions that are not fully understood yet.

Mucosal Immune

The mucosal system contains a unique collection of the immune system in that it is divided into effector and inductor sites. These systems also have an initial defense in the form of mucin and primarily focus on preventing the endothelium from being breached or damaged. Crossing the endothelium of the gut requires assistance, and once across, the invading particles are inundated with cellular immune reactions. Furthermore, mucosal immunity maintains its own memory, utilizing a plethora of IgA antibodies to patrol the mucin layer as well as past the

endothelium. In CD the interaction of a polypeptide with the crosslinking protein tTG2 causes the adaptive immune response.

Gluten

Gluten is a water insoluble protein present in Wheat, Rye, Barley and Spelt that contains various proline rich bonds, reducing the body's ability to digest the polypeptide. Certain fragments, specifically 13mer, 19mer, 26mer, and 33mer, cause immunogenic reactions within the gut of a CD patient. The 13mer and 19mer are cytotoxic and activate the killer pathways in CD8 T-cells lining the endothelium of the gut. Both 26mers and 33mers bind tightly to tTG2 and this combination binds to ligand on APCs and activated the CD4 T-cells. In turn, this activates the adaptive immune system. It results in damage to the endothelium, specifically loss of villi architecture, and thus nutrition absorption, and an increase in immunogenic tTG2 throughout the body.

Symptoms

Symptoms of CD vary considerably by age, gender and environment. In children, particularly infants, symptoms include failure to thrive, chronic diarrhea, vomiting, abdominal distension, muscle wasting, anorexia and irritability.(Troncone and Jabri, 2011) As age increases and/or the length of disease process increases, the symptoms relate heavily on the lack of nutrition caused by the condition. Anemia and Osteoporosis are common side effects of this nutritional deficiency. (Troncone and Jabri, 2011) Additional health problems related to this malnutrition range from manageable, such as short stature, arthritis, alopecia, aphthous stomatitis and dental enamel hypoplasia, to dangerous conditions such as various endocrinopathies, epilepsy with bilateral occipital calcifications, neuropathies and cardiomyopathy. (Troncone and Jabri, 2011) Additionally, CD is significantly correlated to a variety of immune disorders such as diabetes, Addison's disease, Down's syndrome, Turner's syndrome, William's syndrome and a plethora of autoimmune diseases. (Troncone and Jabri, 2011)

Diagnosis

The majority of patients are diagnosed symptomatically, meaning physicians notes that their patient recover their health in the absence of gluten. As this mostly works for the extreme symptoms, other methods of diagnosis are now used. Serology is the most common method of testing CD as a possible pathology, being both inexpensive and quantitative. The more expensive and more accurate diagnosis results from a biopsy of the duodenum and jejunum, looking for the damage to villi characteristic of CD. There are some genetic prevalence, however research has not verified the genetic components necessary to make genomic testing a viable diagnostic test. This research is ongoing.

Raman Spectroscopy

Raman uses the combination of energy emissions and inelastic scattering to interrogate a substance. The information gained informs on molecular geometry and allows detailed information on the substance to be gleamed. Its use in medicine, particularly diagnostics is well developed, with an increased number of cancers being studied. Research shows that Raman spectroscopy has the ability to read the changes a T-cell undergoes during activation, thus providing information on the changes to the cell on a molecular level.

Future Research

There are many avenues of research still needed for CD as a condition. What environment triggers the activation of CD, and how does it influence the symptoms of the patient? What is the early stage of CD; is there an order to the immune interaction? Why do CD patients digest gluten in the first place when it seems non-CD patients lack this activity? Of all these questions, the question of diagnosis may be the most pressing one. How can we diagnose CD in a patient without damaging them further by forcing them to ingest gluten? CD is not a condition that disappears once a GFD is achieved, only the symptoms are ameliorated. There must be some sort of memory or physiological signal remaining. Normal immune memory involves an antibody or TCR remembering an interaction. However, this condition

utilizes non-specific TRC on CD8 T-cells and requires an immunogenic reaction with tTG2 for CD4 T-cells. One is not a memory function by virtue of being non-specific, and the other disappears. Otherwise, the serology test would not require the presence of gluten to give a positive result. There are three major changes that seem to maintain following a GFD: the bone osteoclasts continue at a higher than baseline rate, the serological concentration of brain-derived neurotrophic factor is increased, and the prevalence of $\gamma\delta$ T-Cells is considerably higher in a CD patient even after a GFD is achieved. Raman Spectroscopy has shown to be a viable method in investigating T-cells, and as such, may prove valuable in this line of research.

Moving forward, I would like to understand the differences in celiac patients post condition activation in an effort to advance the diagnostic technology. Specifically, immune memory, which exists mostly in T-cells and B-cells, could hold the key to removing the necessity of gluten ingestion from celiac diagnosis. As RS has already demonstrated functionality in this vein, I propose that there are morphological, molecular, or protein and DNA expression differences in T-cells and B-cells post celiac reaction that will be apparent under RS when compared to that of a healthy patient.

An experiment utilizing microfluidic chip technology designed to immobilize and control the cells and their interactions would be used to allow RS interrogation. Using photolithography to microfabricate a device to trap an active, living cell is possible. Several studies have already isolated a single cell for drug research, cell study and diagnostic purposes (Tan and Takeuchi, 2007). Furthermore cellular electropotential is a method of immobilizing cells for research.

Many mammalian cells have been immobilized in this fashion, including plasma cells, platelets, and cancer cells (Yafouz et al., 2014). The chip design would isolate a single T-cell or B-cell for RS interrogation. It could be further designed to allow for interaction between these cells for study (see Figure 9).

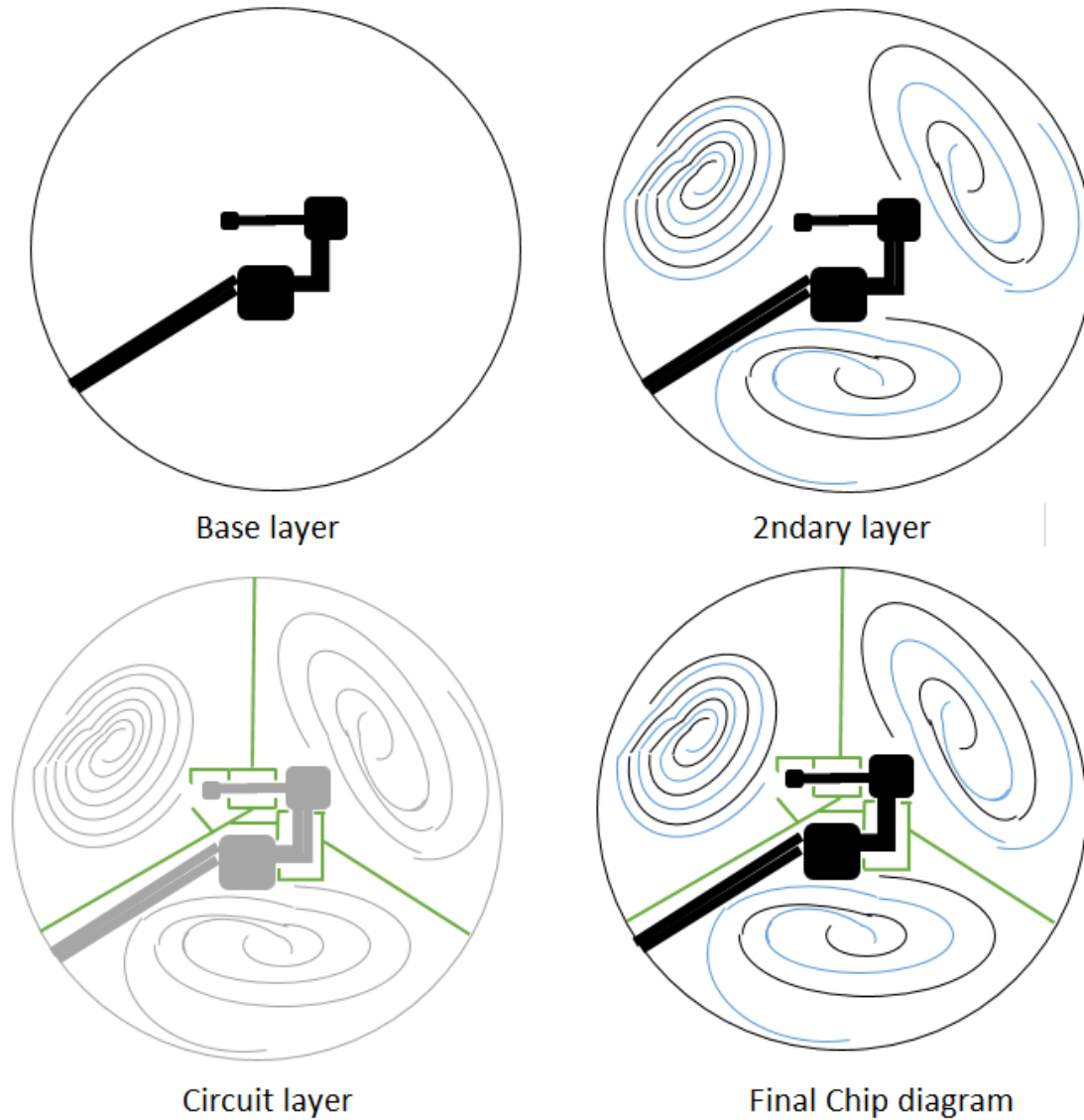


Figure 9: Initial Microfluidic Device Design.

Initial tests would involve fresh T-cells and B-cells from CD patients both on and off GFD compared to similarly collected cells from healthy patients. Ideally, an interaction between the specific gluten polypeptides and the various important immune cells could be studied in the same environment. For the purposes of this research, the focus would be to obtain CD4+ T-cells, CD8+ T-cells, $\gamma\delta$ T-Cells, and mucosal B-cells for comparison.

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ABSTRACT**CELIAC DISEASE: A PHYSIOLOGICAL OVERVIEW AND POSSIBLE FUTURE WORK WITH EMPHASIS ON RAMAN SPECTROSCOPY**

by

KATLYN MEHNE**May 2019****Advisor:** Dr. Gregory Auner**Major:** Biomedical Engineering**Degree:** Master of Science

Celiac Disease is a condition whereby ingesting Gluten causes an autoimmune reaction in the intestines with adverse effects throughout the body. Although statistic analysis estimates that 1% of the general population is affected by this condition, the diagnosis and treatment research lacks in key areas of understanding. This is a summary of current research and physiological information on the impact of Celiac Disease. In the last twenty years, Raman Spectroscopy assisted in diagnosis and treatment of various other ailments. In this case, Raman Spectroscopy can be used to research immunological cells involved in Celiac Disease and further research.

AUTOBIOGRAPHICAL STATEMENT

Katlyn Curtin Mehne received a Bachelor of science in Chemical engineering and another Bachelor of Science in Chemistry from Michigan Technological University. Following work experience in both field in industry, Katlyn entered Wayne State University and started work with Dr Gregory Auner in the *Smart Sensors and Integrated Microsystems(SSIM)* laboratory with a focus on Raman Spectroscopy. Katlyn hopes to used this thesis to start dissertation research using Raman Spectroscopy to better understand T-cells and their changes during autoimmune diseases and obtain a Doctor of Philosophy. After graduation, Katlyn hopes to work in industry on research into making medical devices more functional, available and affordable.