

Galactose- $\alpha$ -1,3-galactose Allergy Induced by *Amblyomma americanum*:

A Review and Introduction of Experimental Designs

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### **Abstract**

The galactose- $\alpha$ -1,3-galactose (alpha-gal) allergy was identified following a drug trial of Cetuximab. The patients who reacted with anaphylaxis had all previously been bitten by the lone star tick. This led to the discovery of the alpha-gal antigen and the alpha-gal specific immunoglobulin E antibody (IgE). Research regarding the prevention of the alpha-gal allergy is extensive, and the development of a vaccine has been in progress. Much is still unknown regarding the way this allergy is transmitted to humans through ticks. Research is to be conducted in the Liberty University labs to investigate how the lone star tick causes sensitization to the alpha-gal antigen. Additional projects include determining the stage of tick development that the antigen is passed to humans, and identifying the reason only some people bitten by the lone star tick develop the allergy. The ultimate goal of this research is to aid in discovering a treatment for, or prevention of, the alpha-gal allergy.

## Galactose- $\alpha$ -galactose Allergy Induced by *Amblyomma americanum*:

### A Review and Introduction of Experimental Designs

## Introduction

### Alpha-gal Allergy

The galactose- $\alpha$ -1,3- galactose (alpha-gal) disaccharide has been found to induce anaphylaxis in some patients after exposure to *Amblyomma americanum*, commonly known as the lone star tick (Berg, Platts-Mills, & Commins, 2014). This adverse reaction was discovered after the drug Cetuximab was administered intravenously to patients being treated for colon cancer (Berg, Platts-Mills, & Commins, 2014; Chung, Mirakhur, Chan, Le, Berline, Morse, Murphy, Satinover, Hosen, Mauro, Slebos, Zhou, Gold, Hatley, Hicklin, & Platts-Mills, 2008). Cetuximab is a drug developed with the cells of mice and humans. It is an antibody that targets epidermal growth factors in order to slow tumor growth in cancer patients (Chung et al., 2008). During clinical trials some patients reacted with severe anaphylaxis, which in some patients was fatal. During the investigation researchers found that the patients that experienced anaphylaxis had been bitten by a lone star tick sometime prior to taking the trial drug (Wolver, Sun, Commins, & Schwartz, 2012).

Anaphylaxis, a violent autoimmune response to an antigen due to hypersensitization (from a tick bite, in this case), can be triggered by eating red meat, even if it is cooked. The mechanism that causes this sensitization is unknown, but induces antibody production that causes a reaction when an individual consumes red meat, that

did not exist prior to a tick bite. When triggered through the consumption of red meat, anaphylaxis is delayed approximately three to eight hours, but is still severe. Onset of symptoms often occurs during the middle of the night, which may be due to the fact that red meat is often consumed at dinner time meals (Wolver et al., 2012). The ingestion of other meat by-products has been linked to the allergy as well. The consumption of products containing either milk or gelatin has been recorded as an allergy-inducing event. Gelatin is also found in several vaccines, which can induce a reaction. Additional patients have reacted after an interaction with heparin or the insertion of bioprosthetic heart valves from animals, such as pigs.

### **Symptoms**

While anaphylaxis is the most serious symptom of the allergy, it is not the most commonly experienced by affected individuals. Other symptoms that arise in most patients include urticaria and angioedema. Urticaria is characterized by red, irritated skin with numerous welts, much like a serious rash or hives. It is considered the most common symptom of the alpha-gal allergy (Commins, James, Kelly, Pochan, Workman, Perzanowski, Kocan, Fahy, Nganga, Ronmark, Cooper, & Platts-Mills, 2011). Angioedema, another skin condition, consists of swelling of the skin and is also extremely common in those affected by the allergy. Irritable bowel syndrome and additional gastrointestinal symptoms have been linked to the allergy as well. Many patients have reported nausea after the consumption of red meat. Asthma has not been a complaint among affected patients, but it is commonly associated with other food allergies (Commins & Platts-Mills, 2013b).

## History

### Cetuximab Trials

The alpha-gal allergy was first identified through Cetuximab drug trials. Cetuximab was being developed and tested as a new form of chemotherapy. The chimeric mouse-human IgG1 monoclonal antibody, Cetuximab, counteracts the action of epidermal growth factor receptors, preventing the further growth of tumors (Chung et al., 2008; Chinuki, Ishiwata, Yamaji, Takahashi, & Morita, 2015). A drug that treats cancers of the colon and neck, it contains alpha-gal on its heavy chain due to its derivation from mouse cell lines. During early trials some patients reacted severely, immediately after the administration of the drug intravenously (Chung et al., 2008). It was later discovered that many of these patients also reacted to red meat many hours after consumption, leading researchers to discover the alpha-gal specific IgE. The intense, immediate reaction elicited by the administration of Cetuximab is due to the high concentration of alpha-gal present in the drug being administered directly into the blood, bypassing the digestive system which delays reactions when alpha-gal is ingested.

### Tick Association

Allergic reactions occurred much more frequently in patients that came from the Southeastern United States, which led doctors to question what was happening (Chung et al., 2008). The occurrence of anaphylaxis in people from certain areas led to the association between the allergy and the bite of the lone star tick. Doctors also recognized that the individuals that reacted to Cetuximab were from the same region affected by Rocky Mountain Fever, a tick-induced illness (Steinke, Platts-Mills, & Commins, 2015).

All patients that reacted with anaphylaxis had a history of being bit by a lone star tick, and many were later diagnosed with the alpha-gal meat allergy. This initial drug trial led to the later discovery of the late-onset anaphylaxis caused by the alpha-gal allergy induced by lone star tick bites.

### *Amblyomma americanum*

The lone star tick got its name from the single, white-colored, star-shaped dot on the back of the mature female lone star tick, similar to the single star present on the Texas state flag, coined the “Lone Star State”. The adult female is generally larger than the male tick. She attracts mates by releasing a mounting pheromone that causes the male to detach from a host and search for the female. Through a series of six phases, the male acts in response to various pheromones released by the female. The female then detaches from the host to lay her eggs on the ground following copulation. Beginning at the egg stage, the lone star tick follows a typical development pattern which progresses through the larval, nymph, and adult stages. In order to progress through the stages, the tick requires a blood meal. The tick searches for blood meals by detecting and seeking carbon dioxide, which is why dry ice traps, which emit CO<sub>2</sub>, are used to trap them. After latching on to another organism, the parasite feeds and becomes engorged, leading to further development. With each stage transition, the tick molts and grows larger. At the larval stage, the tick is approximately the size of a period on this document and grows to about one mm at the nymph stage (Ostfeld, Cepeda, Hazler, & Miller, 1995).

The lone star tick is found in the Southeastern region of the United States as well as parts of the Southern and Midwestern region. They live in the brush of wooded areas and grassy areas. The lone star tick is not generally recognized as a transmitter of Lyme

disease (this is controversial), but is known to be a vector of other diseases, as well as the alpha-gal allergy. The lone star tick is known to transmit diseases such as human monocytic ehrlichiosis, southern-tick-associated rash illness, spotted fever, tularemia, theileriosis, among many others (Bullard, Williams, & Karim, 2016). Previous research surrounding this tick has been focused on its transmission of Ehrlichiosis, as it has been identified as the primary vector of this disease (Commins & Platts-Mills, 2013b). The lone star tick is known as an aggressive parasite due to its willingness to bite humans in both the larval and adult forms (Childs & Paddock, 2003).

Numerous mechanisms are involved in the feeding process. The lone star tick begins feeding by inserting its hypostome, a sharp, calcified mouthpart, into the skin of a host to anchor itself. A blood pool is formed underneath the skin, and remains fluid throughout the entire feeding (Bullard, Williams, & Karim, 2016). Feeding can last several days, and results in the engorgement of the tick. The tick's saliva has anticoagulating properties which prevent clotting, allowing the tick to continue feeding while remaining attached to the host for long periods of time. Maintaining an open lesion and a blood pool are key components to successful feeding. The saliva also contains numbing properties that make the bite less noticeable to a host, preventing the host from trying to remove the tick. Additional proteins have been identified that prevent the immune response of host organisms, allowing the tick to remain latched for prolonged periods of time (Bullard, Williams, & Karim, 2016). These properties make the tick particularly dangerous to humans, and make people susceptible to obtaining the alpha-gal allergy. A tick has a longer opportunity to transmit the antigen to develop alpha-gal-specific IgE if its host cannot detect and dislodge it. Lone star tick attachment is also

commonly found on cats, dogs, cattle, horses, and deer which could lead to easier access to feeding on humans, and thus higher rates of disease transmittance (Bullard, Williams, & Karim, 2016; De la Fuente, Manzano-Roman, Naranjo, Kocan, Zivkovic, Blouin, Canales, Almazán, Galindo, Step, & Villar, 2010).

Gene expression, the observable, phenotypic characteristics caused by a particular gene, alters dramatically in the salivary glands and midgut throughout the feeding process (Bullard, Williams, & Karim, 2016). The proteins that are coded by these genes allow for successful feeding by preventing blood clotting mechanisms of the host during feeding. These proteins are also responsible for blocking host immune responses to the ectoparasite and for preventing swelling of the affected area. Pathogen transmission is also thought to be caused by these proteins. Forty four separate genes have been identified as being involved in acquiring the blood meal (Bullard, Williams, & Karim, 2016). These 44 genes were isolated and found to be involved in the production of different protein classes such as glycine-rich proteins, lipocalins, and metalloproteases. Metalloproteases were thought to be one of the most crucial protein groups during feeding due to the interaction with the host extracellular matrix, which could be responsible for maintaining the blood pool. The extracellular matrix is composed of molecules responsible for supporting the structure of the cell. The metalloprotease protein category is thought to alter this structure by binding to the molecules, breaking the support system of the cell, and preventing coagulation and scabbing in the host.

## Current Knowledge

### Allergy Testing

Once the alpha-gal allergy was identified, scientists designed a way to accurately test patients for the allergy. This prevented allergy-afflicted patients from using medications or ingesting foods that could cause dangerous reactions. Skin prick allergy tests have proven unreliable when using commercial meat extracts or raw meats. A skin prick allergy test involves exposure to the allergen on the skin, followed by a mild puncture or scratch to allow the substance under the skin, and may cause the formation of a wheal if the test is positive for an allergen. This can be performed in a doctor's office, and results are known almost immediately. Generally, false negatives are uncommon in skin prick testing. The low sensitivity is likely related to the antigen being a carbohydrate rather than a protein. Utilizing Cetuximab in skin prick tests has proven much more effective in determining whether or not an individual has the allergy (Michel, Scherer, Heijnen, & Bircher, 2013) Even more effective than topical skin prick tests are intradermal tests, which involve injecting a small amount of the allergen underneath the skin (Steinke, Platts-Mills, & Commins, 2016). Commercial IgE assays measure IgE to various mammalian meats that possess alpha-gal in order to determine whether or not an individual possess IgE specific to alpha-gal. Basophil activation tests have also been used to identify the allergy in patients in vitro (Bircher, Hofmeier, Link, & Heijnen, 2017; Michel et al., 2013). This form of testing has been found to be accurate, but does not give results as rapidly as other tests. Doctors test for the alpha-gal allergy using solid phase immunoassay technique. This test is performed in the lab rather than the office, and detects the presence of alpha-gal-specific IgE in patient serum (Steinke, Platts-Mills, &

Commins, 2015). Testing after consumption through a process known as food challenge is not recommended due to the delayed reaction time to certain meats, and the severe reaction sometimes induced by consuming organs such as kidney (Bircher et al., 2017). Using the food challenge method is also discouraged because patients do not react every time they ingest meat, making it difficult to accurately test for the allergy via consumption (Commins, James, Stevens, Pochan, Land, King, Mozzicato, & Platts-Mills, 2014).

### **Demographics**

The alpha-gal allergy is most prevalent in the Southeastern region of the United States but it extends as far west as Texas (Bullard, Williams, & Karim, 2016). This is due to the abundant population of lone star ticks. The tick population is beginning to expand into the Northeastern United States, which has resulted in a greater number of alpha-gal cases being reported from this region. It has been predicted that up to twenty percent of individuals in Tennessee, North Carolina, and Virginia could have elevated IgE antibodies specific to alpha-gal, although only a small fraction of these individuals possess the alpha-gal allergy (Commins et al., 2011). It has been estimated that roughly one in 8000 individuals living in these three states has the allergy (Van Nunen, 2015). Lynchburg, VA is known to be inhabited by a large number of lone star ticks, and many studies have been conducted in the area (Commins et al., 2011). The growing population and spread of the lone star tick has been attributed to the increasing population of the white-tailed deer from which they often feed (Bullard, Williams, & Karim, 2016). In only one small research site, Liberty University's research team has caught over 150 ticks over the course of two years, indicating a dense, and likely growing lone star tick population.

White-tailed deer numbers have increased since leash laws for dogs were enacted, and may also be due to a decrease in hunters, and the shift of deer into suburban areas (Van Nunen, 2015). The migration of deer into areas more heavily populated by humans may also contribute to the spread of lone star ticks, as ticks are carried wherever the deer reside (Ostfeld et al., 1995). Similar allergies have been reported after the bites of other tick species in Europe, Asia, and Australia (Kollmann, Nagls, Ebner, Emminger, Wöhrle, Kitzmüller, Vrtala, Mangold, Ankersmit, & Bohle, 2017). The increasing number of reported cases in these regions has been related to an increase in host populations, such as small rodents (Van Nunen, 2015).

The age of onset is significantly later than that of most food allergies, with patients generally developing the allergy sometime in middle adulthood. This is an unusual trait of food allergies, which do not generally arise during adulthood, but rather in childhood (Wolver et al., 2012). Children are susceptible, but higher incidence rates have been reported as age increases. Most of the patients utilized in alpha-gal allergy research are between the ages of forty and sixty-eight, but patients much younger and older have participated (Hamsten, Tran, Starkhammar, Brauner, & Commins, 2013b; Kollmann et al., 2017; Van Nunen, 2015). Many patients that have been diagnosed with the alpha-gal allergy describe living very active lifestyles outdoors. A study conducted by Kennedy et al found that children are also susceptible to developing this allergy (Kennedy, Stallings, Platts-Mills, Oliveira, Workman, James, Tripathi, Lane, Matos, Heymann, & Commins, 2013). This study concluded that it is important to consider this diagnosis in children that present with symptoms aligning with the alpha-gal allergy. Children may experience angioedema, urticaria, or delayed anaphylaxis after the

consumption of red meat or animal byproducts such as milk and gelatin the same way as adults, although it is less common (Kennedy et al., 2013).

### **Galactose- $\alpha$ -1,3-galactose**

Alpha-gal is produced by the enzyme alpha(1,3)-galactosyl transferase, located in the trans-golgi network of the cell (Dahl, Buschard, Gram, & d'Apice, Hansen, 2006). The structure of the alpha-gal disaccharide is a double ring structure that is known to interact with IgE antibody in affected individuals. Alpha-gal is found in all non-primate mammalian meat, and some animal byproducts (Steinke, Platts-Mills, & Commins, 2015). It is not present in poultry, fish, or primates. Alpha-gal levels also differ among cell types. High levels of alpha-gal have been found in kidneys, and varying amounts have been discovered in heart, lung, and thymus tissues (Dahl et al., 2006). The adverse reaction to alpha-gal is caused by the response of alpha-gal specific IgE antibody. All humans with healthy immune systems possess IgG antibodies specific to alpha-gal that signal for an immune response that triggers the break down and processing of the alpha-gal carbohydrate through opsonization (Kennedy et al., 2013). The IgG response is within the realm of normal bodily function, and doesn't cause any allergy symptoms.

One of the most unique features of the alpha-gal allergy is its delayed reaction time. This has been thought to be caused by the alpha-gal carbohydrate being bound to lipids or lipoproteins *after* consumption during the digestion process. Alpha-gal is a carbohydrate moiety, which is a functional group of a compound. This functional group is bound to macromolecules such as proteins or lipids. The binding of a carbohydrate moiety to a macromolecule is known as glycosylation. In Japan, research has been conducted to determine which proteins are involved in the allergy due to the

glycosylation by alpha-gal. The carbohydrate moiety was linked to two proteins, laminin  $\gamma$ -1 and collagen  $\alpha$ -1 (VI) in Japanese patients that had an allergy to red meat (Takahashi, Chinuki, Tanaka, & Morita, 2014). Researchers took serum from patients with the alpha-gal allergy and found that IgE bound to two proteins of different sizes. Through various assay and blot techniques, the proteins were identified. This could signify that IgE does not react solely to alpha-gal, but that it could be reacting to the proteins which bind the oligosaccharide. Studies in Luxemburg have identified different proteins as the potential link to the alpha-gal allergy. The proteins AP-N and ACE-1, both cell-membrane-bound metalloproteinases, are found in the small intestine and kidney of pigs (Hilger, Fischer, Swiontek, Hentges, Lehnert, Eberlein, Morisset, Biedermann, & Ollert, 2016). Porcine kidney has repeatedly been identified as a red meat that has high levels of alpha-gal that causes more serious reactions than other forms of red meat (Commins & Platts-Mills, 2013a; Hilger et al., 2016). These proteins were proven to be heat resistant, indicating that they would not be susceptible to denaturing during cooking. It was confirmed that patients that have a sensitization to alpha-gal react to AP-N and ACE-1 through skin prick testing and basophil activation (Hilger et al., 2016). Reaction levels varied according to the patient, which could indicate different numbers of alpha-gal binding sites, or epitopes, to the antibody. Discovering the proteins that are glycosylated by alpha-gal is a crucial component of understanding the mechanism of the allergy, specifically the delayed anaphylaxis (Commins & Platts-Mills, 2009). It is possible that the macromolecules to which alpha-gal is bound could explain the reason for a delayed allergic reaction. The delayed allergic response is predicted to be caused by the digestive process that breaks down glycoproteins and glycolipids (Wolver et al., 2012). After meat

is consumed, the carbohydrate particles are inserted onto lipid particles. The formation of such particles takes hours, and could initiate a stronger response by mast cells and basophils when alpha-gal is present.

### **IgE Antibody**

Red meat allergy has intrigued scientists because it seems to have a different mechanism than most food allergies, such as soy, peanut, and shellfish allergies (Wolver et al., 2012). Alpha-gal specific IgE recognizes a carbohydrate, rather than a protein epitope, this has been the only accepted mechanism to date. Production of alpha-gal-specific IgE antibody is induced by tick bites. When a person is exposed to alpha-gal through the consumption of red meat, the production of alpha-gal IgE is sometimes induced. The exact reason that alpha-gal-specific IgE antibody is produced after an individual is bitten by a lone star tick is unknown, and currently being researched. IgE antibody levels were measured by Commins et al before and after instances of numerous tick bites (2011). After a patient is bit by a lone star tick, their IgE antibodies specific to alpha-gal, and alpha-gal containing substances, such as beef and dog epithelium, increase up to 20 fold (Commins et al., 2011). Generally, a single bite with a short attachment period will not induce the allergy. Long attachments, numerous bites, or high numbers of larval bites can induce alpha-gal sensitization (Wolver et al., 2012). Antibody levels decrease after a period of time, which can allow for allergy symptoms to decrease. If the patient was again bit by a lone star tick, IgE levels once again spiked, causing more serious allergic reactions to the consumption of red meat. One patient monitored by Commins was bit by multiple ticks that were not identified as lone star ticks. This patient Fhad a greater number of IgE antibodies for alpha-gal, beef, and dog epithelium, but not

to the same degree as the individuals who had been bitten by lone star ticks (Commins et al., 2011). This patient did not present allergy symptoms or urticaria. This finding could imply an IgE threshold level necessary to induce anaphylaxis, which could explain why only some patients with the alpha-gal allergy experience anaphylaxis, while some never experience such an episode and solely present with urticaria.

As with many allergies, IgE interacts with mast cells and basophils by FC epsilon receptors which allow for binding sites to be exposed on the outside of the immune cell to bind the alpha-gal antigen (Wolver et al., 2012). This sensitization would have been induced by the bite of a lone star tick, causing patients to react to Cetuximab upon the initial use. Prior sensitization had been caused by the lone star tick, which formed the IgE antibodies before their systems had ever been exposed to the drug. IgE specific for alpha-gal binds to receptors found on mast cells, which then bind alpha gal (Wolver et al., 2012). This binding causes a release of histamine from mast cells which induces allergy symptoms such as anaphylaxis. The increased production of the IgE antibody involves altered B cells that undergo a class switch to IgE antibodies (Linhart & Valenta, 2005). A component of the tick bite could possibly cause the change in B cell production to synthesize alpha-gal specific IgE. Increased IgE antibody production would elicit an immune response to remove the antigen, alpha-gal, and rid the system of the foreign substance through opsonization, which involves marking the antigen for phagocytic elimination. The fact that IgE responds to a carbohydrate is a unique feature of the allergy. Generally, IgE responds to proteins, and no food allergies have yet been linked to a reaction to a carbohydrate. Generally, allergies are reactions to a protein that the body

will not metabolize. The production of IgE antibodies specific to *A. americanum* proteins has been recognized in patients with the alpha-gal allergy (Commins et al., 2011).

### **IgG Antibody**

Recent research conducted has looked further into the prevalence of the IgG response that accompanies the allergy inducing IgE response. The alpha-gal IgG antibody is a natural antibody found in all healthy humans (Commins & Platts-Mills, 2013b). IgG's actions can play a role in allergies, and have been thought to also play a role in the alpha-gal allergy. All humans possess alpha-gal-specific IgG antibodies, but the IgG4 antibody has been tied to food allergies (Gonzalez-Quintela, Dam Laursen, Vidal, Skaaby, Gude, & Linneberg, 2014; Kollmann et al., 2017). These IgG antibodies comprise about one percent of the total human IgG antibodies, and have been linked to failed xenotransplantation procedures involving the transplant of organs or tissues from one species to another (Dahl et al., 2006; Wolver et al., 2012). The alpha-gal present in tissues from non-primate mammals initiates the IgG response that leads to rejection of the transplanted organ or tissue within minutes. The IgG antibody differs from healthy individuals in patients affected by the meat allergy. The antibody specificity is altered in patients that have been bitten by ticks and developed an allergy. Significantly higher than normal amounts of IgG antibodies specific for alpha-gal are found in patients that possess the meat allergy (Kollmann et al., 2017). This may possibly be explained by the body attempting to reduce the allergic response caused by IgE by using IgGs as competition to bind alpha-gal.

### **Alpha-Gal Around the World**

Although not induced by the bite of a lone star tick, the alpha-gal allergy has been identified in other places in the world. Its discovery in numerous locations has enabled researchers to draw parallels between patients, and has given insight into how the tick bite induces IgE sensitization to alpha-gal in patients. Research is being conducted to determine the causes and mechanisms of the alpha-gal allergy in numerous countries. Thus far, only tick bites have been linked to the allergy, but some countries with alpha-gal instances have not conclusively linked the allergy to previous tick bites. Countries where alpha-gal allergy is present and no clear vector is yet identified are generally conducting research to draw an association between a parasite, typically a tick, and the allergy (Chinuki et al., 2015).

The link between tick bites and mammalian meat allergies was first identified in Australia, although alpha-gal had not yet been identified as the cause (Steinke, Platts-Mills, & Commins, 2015; Van Nunen, 2015). The alpha-gal allergy is more common in Australia than any other continent because Australia possesses many tick endemic regions. The estimated prevalence is one in 550 people have the alpha-gal allergy in tick endemic regions, and is predicted to be on the rise (Van Nunen, 2015). Areas identified as having high tick concentrations include the south coast of New South Wales and the coast along the Sydney basin. Reported cases of red meat allergy coincide regionally with the areas inhabited by the *Ixodes holocyclus* tick (Steinke, Platts-Mills, & Commins, 2015; Van Nunen, 2015).

Cases of red meat allergy have been reported in Japan. The tick association has not been confirmed in Japan, but many researchers believe that there could be a link

between tick bites and the red meat allergy (Takahashi et al., 2014). Additional studies have been conducted in an effort to answer the question regarding the cause of red meat allergies in Japan. The *Haemaphysalis longicornis* tick has been linked to the allergy by the discovery of alpha-gal in the salivary glands of this species, possibly acquired from previous blood meals (Chinuki, Ishiwata, Yamaji, Takahashi, & Morita, 2015). Japanese patients were found to have a sensitization to the proteins in the salivary glands of this tick species, indicating a likely causative agent of the allergy, but further research is required to confirm the cause of the allergy. Many Japanese patients with red meat allergy were unable to recall a tick bite due to the subtle nature of the tick bites (Chinuki et al., 2015). Patients often did not experience a rash or itching when bit, making it difficult to conclusively determine the link between red meat allergy and tick bites in Japan, although no other mechanism for the induction of the allergy has been introduced.

Swedish researchers have found traces of alpha-gal in the gut of the *Ixodes ricinus* tick that is known to transmit the allergy in that region (Hamsten, Starkhammar, Tran, Johansson, Bengtsson, Ahlen, Sällberg, Grönlund, & van Hage, 2013a). This finding leads to further questions regarding whether or not alpha-gal is present in the gut of all ticks that are known to cause the red meat allergy. It is possible that the alpha-gal has been acquired by the tick through the previous blood meal of a non-primate mammal. In this project, they compared the *A. americanum* and the *I. ricinus* ticks in an effort to determine how similar the epitopes were between the ticks that cause a very similar allergy around the world. They found that while the ticks shared some characteristics, they differed due to possessing species-specific epitopes. In their study, they also found that people with the B negative blood type were more often affected by the alpha-gal

allergy (Hamsten et al., 2013b). This trend directly contradicts with an American study that found a potential protective quality caused by type B blood (Posthumus, James, Wang, Commins, & Platts-Mills, 2010). This relationship is thought to be related to the similarities between the alpha-gal and B blood type antigen structures (Bircher et al., 2017; Posthumus et al., 2010). Hamsten et al looked at incidence rates of the allergy, whereas Posthumus et al looked at the IgE production in affected individuals (2013b; 2010). The varied results of these two studies indicate a need for further research, but introduce the concept of blood type being a factor in acquiring red meat allergy. This could begin to aid researchers in understanding the reason only some patients that are bitten by ticks develop a sensitization.

Studies in Luxemburg have brought further attention to proteins that are glycosylated by alpha-gal, thus making them the proteins responsible for the red meat allergic reactions (Hilger et al., 2016). They identified transmembrane proteins in beef, which they called AP-N and ACE-1, which differ from the proteins identified in the study previously conducted in Japan by Takahashi (2014). The proteins were tested for allergenicity, unlike the proteins found in the Japanese study, which is thought to show the AP-N and ACE 1 proteins to have more significance in the actual mechanism of the red meat allergy (Hilger et al., 2016). Other European countries with reported cases of red meat allergy include Spain, Germany, and Switzerland (Van Nunen, 2015).

Numerous African countries have found that individuals have IgE antibodies specific to alpha-gal, but do not present any reaction to the consumption of red meat (Commins & Platts-Mills, 2013b). This phenomenon has led to questions regarding the cause for IgE production specific to alpha-gal. Parasites are a potential cause, and could

include ticks, helminths, cestodes, or other ecto-parasites (Commins & Platts-Mills, 2013b). A small number of cases have been reported in farming communities in the Republic of South Africa (Van Nunen, 2015). These patients reported tick bites prior to symptoms, but the species has not been identified.

Cases have not yet been reported in South America, but a few cases have been reported in Central America (Van Nunen, 2015). Costa Rica is home to the *Amblyomma cajennense* tick, which is thought to be the vector of the red meat allergy within the region. Other tick species in the *Amblyomma* and *Ixodes* genera, which are known for biting humans are common throughout South and Central America, but there have not been cases reported in these areas. This could be due to undiagnosed allergies in more rural areas, or merely indicates that these ticks do not initiate a sensitization to alpha-gal.

### **The Future of the Alpha-gal Allergy**

#### **Current Questions**

The origin of the alpha-gal antigen is not fully understood. Whether the tick acquires the antigen after blood meals, or whether it is present in the microbiome composed of bacteria and microorganisms of the tick's gut is unknown. It is also possible that the sensitization of IgE to alpha-gal is caused by the components of the tick's saliva. Some ticks are known to possess commensal organisms that are involved in the transmission of diseases. It has been theorized that one of these organisms could induce the allergy in humans, similar to the mechanism involved in the transmission of Rocky Mountain spotted fever by the *Rickettsia* bacteria, although no current findings have supported this idea (Steinke, Platts-Mills, & Commins, 2015). If the tick acquires the

antigen, it is unknown whether that occurs in the larval, nymph, or adult stages. Other questions have arisen regarding how it is contracted by humans. Not all individuals bitten by lone star ticks develop the alpha-gal allergy. This could indicate that only certain ticks carry the antigen, or that there is another factor involved in a human developing the allergy after the tick bites. The issue of blood type was introduced in the Swedish study by conducted by Hamsten et al., and could be one of the factors that dictates which patients develop the allergy (2013b). Individuals that contract the allergy may be genetically predisposed, but no specific gene has been found to play a role in the allergy thus far. Questions not only arise in the area of contracting the allergy, but also in the area of treatment. There is currently no way to cure the allergy. In many cases, a person must avoid red meat for the remainder of their life. Some cases of individuals “outgrowing” the allergy have been reported. The reason for this natural reversal is unknown, and rare.

### **Prevention and Treatment**

Currently, research is being conducted to produce preventative vaccines to numerous tick-borne diseases, as well as the alpha-gal allergy (De la Fuente et al., 2010). Emergency treatment is available for patients that have contracted this allergy and experience anaphylaxis. Epinephrine syringes, commonly known as EpiPens, are given to patients to be administered if anaphylactic shock is experienced. Other methods that are being researched to reduce tick-borne illnesses and the alpha-gal allergy include controlling tick populations through biological control agents, and pheromone decoys that kill the tick once they have been attracted (De la Fuente et al., 2010). Vaccines for host organisms such as cattle and dogs have been identified as the most favorable form to

reduce pathogen spreading via ticks. Creating host immunity to the spread of tick-borne illness reduces the environmental impact of controlling tick populations through external means such as acaricides. Tick bites in domestic animals can currently be prevented by topical treatments, pills, or collars.

While there are currently no ways to reverse the allergy, numerous studies have shown that the severity of symptoms decreases over time, and it may be possible for symptoms to eventually cease (Commins et al., 2011; Van Nunen, 2015). Symptoms can resume if bitten by another tick, making it important to avoid tick bites after contracting the allergy in order to reduce severity. Current alpha-gal allergies can be worsened by additional tick bites (Commins et al., 2011). No other causes have been found to induce the alpha-gal allergy (Commins & Platts-Mills, 2013b). The only way to prevent contracting this allergy is to avoid the bites of ticks, or to properly remove an attached tick as quickly as possible to prevent exposure to undesired pathogens, or the alpha-gal allergy (Van Nunen, 2015). Tick attachment can be prevented by wearing attire that covers the skin. After traversing through a tick's habitat, one should check for any tick attachment. Common areas of tick attachment include the armpit, scalp, neck, and behind the ears. Ticks, can, however, attach anywhere. Removal of a tick shortly after attachment is also likely to prevent transmission of diseases, and allergy-inducing antigens. Acaricides have been used in an effort to decrease the tick population, but have been relatively unsuccessful in reducing the spread of tick-borne diseases (De la Fuente et al., 2010). The use of acaricides also causes acaricide resistant tick strains which cause additional difficulty in controlling tick populations. Environmental pollution has also been a deterrent to the use of such chemicals.

## **Vaccine Production**

Medical personnel have wondered if a vaccine could be developed to prevent the alpha-gal allergy. Briefly, vaccine theory rests on the idea that the body can eventually learn to respond to various allergens by the repeated introduction of an allergen in gradually increasing doses. The immune system develops a response via memory immune cells that act on the antigen when a person is exposed to it. This repeated exposure allows for desensitization to the allergen and potentially, eventual immunity. While symptoms may not be completely alleviated, they can often be dramatically decreased. Developing an allergy vaccine for individuals afflicted by red meat allergy has differed from the development of other allergy vaccines due to the allergen being a carbohydrate, rather than a protein. If successful, an alpha-gal allergy vaccine could potentially allow individuals to consume red meat, or receive gelatin-containing vaccines, even after developing a sensitization after the bite of a tick.

The idea behind allergy vaccines is that the outcome of the immune response can be dictated by altering the allergen, and the body's response to it. Vaccines are generally designed to target either a protein/peptide sequence, or the IgE itself. Vaccine development for food allergies requires characterizing the allergen. A certain level of understanding of the mechanism of the allergy must be known in order to develop a vaccine to develop an individual's immunity (Linhart & Valenta, 2005). Due to the IgE response being elicited by a carbohydrate rather than a protein, scientist are still working to understand the mechanism of the allergy. When the antigen is well understood, it can be fabricated and injected in a small dose to elicit a controlled IgG response, rather than a dangerous IgE response (Linhart & Valenta, 2005). The body then develops an immune

response that competes with the allergic response of the IgEs to lessen the symptoms of an allergy. As IgGs bind to the alpha-gal antigen, IgEs are unable to do so, allowing the body to properly target the antigen for removal by phagocytes.

### **Future Work**

Alpha-gal has aided researchers in understanding how anaphylaxis is triggered, and shows a type of delayed anaphylaxis that has allowed for a new approach in research. Researchers are now aware of a food allergy induced by a carbohydrate which could lead to the investigation of additional food allergies in search of a carbohydrate linkage. The red meat allergy also differs from other allergies in its late onset, which has led medical professionals to not only properly diagnose more adults with the alpha-gal allergy, but to begin to think of other allergies that may go misdiagnosed because they do not follow the typical pattern of food allergies.

Aside from the production of vaccines and potential treatments, much of the future research in this area will go into understanding the mechanism of the sensitization. Vector-borne diseases have long been an area of interest, and the alpha-gal allergy has not proven to be an exception. Additional studies researching the link between blood type and prevalence will likely give further insight into the production of IgE antibodies specific for alpha-gal. Scientists in other part of the world, such as Japan, are continuing to look for the tick or other parasite responsible for the induction of the red meat allergy (Chinuki et al., 2015). Further research is likely to be conducted to find the exact cause of the delayed reaction time. Most food allergies are fast-acting once IgE is developed to an allergen and loaded onto mast cells, which has forced questions on the scientific

community regarding the differences in the digestive process or the allergic reaction mechanism between alpha-gal containing red meat and other traditional food allergies.

## **Experimental Design**

### **Laboratory Work**

The alpha-gal research team at Liberty University plans to catch ticks on Candler's mountain when tick season begins each April. Ticks have been successfully caught on the mountain for previous research projects conducted in the summers of 2015 and 2016, to test for Lyme disease, Ehrlichia, and Rocky Mountain Fever at Liberty University. Dry ice traps have been used successfully to attract ticks from areas on the mountain covered in light brush and leaves. GPS mapping will be used to document chosen tick sites, and maintain consistency in trap locations (Tomkiewicz, Fuller, Kie, & Bates, 2010). Until tick season begins, ticks ordered from other laboratories, or ticks from a veterinary clinic may be used for experimentation.

Researchers, thought of using a Berlese funnel to try to catch ticks during the winter in an effort to catch ticks during the colder months. Briefly, a section of the forest floor is cut out with a large knife and placed into a funnel with a heat source, such as a light bulb, at the top. A bag is then placed at the open end on the bottom. Living creatures usually move away from the heat source, further into the dirt, and fall through the open end of the funnel into the bag. Due to a concern that ticks would be attracted to the heat source rather than being repelled by it, the idea was not carried out. Researchers did, however, cut out multiple sections of earth at Research Site One in the winter of 2016-2017, and Liberty's research students dug through the bags searching for organisms and

identified the insects using dissecting microscopes. The organisms were either sacrificed using isopropyl alcohol, or their motion was restricted with tape in order to be properly photographed. An atlas is currently being put together to include all of the organisms, insect, and arachnids found in the traps. Currently, one tick has been found through this process, but it is possible that ticks in the nymph stage have been overlooked by researchers, due to their extremely small size. In the future, ticks extracted from these traps could be experimented on through the winter months. Breeding ticks in the lab would also enable projects to continue during the off-season.

Laboratory research could be conducted to determine whether or not the antigen causing the alpha-gal allergy is acquired through blood meals. One proposal is to allow ticks to feed on alpha-gal knockout mice, which would not have the antigen to pass to the tick. Saliva extraction would be conducted on engorged ticks (Patton, Dietrich, Brandt, Dolan, Peisman, & Gilmore, 2012). After a blood meal, the engorged tick is able to salivate when stimulated with dopamine, or a similar parasympathetic drug. After taping the tick to a clean slide, applying dopamine to the dorsum of the tick will induce salivation. A glass capillary pipette will be pulled, cut to an optimal diameter, and fitted to the hypostome of the tick using a self-made micromanipulator (Krans & Hoy, 2006). The tick will then be propped upside-down (mouth facing ground) in a humid environment for a few hours, until salivation ceases. The ticks may then be sacrificed, and ground for protein assays, or stored for later use.

An alternative process to extract the components of saliva would involve the surgical removal of the entire salivary gland (Patton et al., 2012; Zhu, Bowman, & Dillwith, 1998). Although not ideal, as this would include all the proteins within the

salivary gland, and not solely the proteins present in the saliva, this procedure can be performed on deceased ticks, which can be taken from veterinary clinics. If the tick is engorged, the salivary glands are visible to the naked eye, and can be extracted by gently tearing open the dorsum with a scalpel (Zhu et al., 1998). Salivary glands have also been successfully removed from unengorged ticks (Bullard, Williams, & Karim, 2016). Removing the salivary glands from non-engorged ticks would be the preferable method in our current laboratory circumstances, as it would not require us to feed the ticks in the lab. Following this procedure, we could immediately sacrifice the ticks caught in the field (which are never engorged), then extract the salivary glands in the lab, preventing the potential of being bitten by a tick carrying a disease or the necessary components to induce the alpha-gal allergy. The salivary glands are small, white, round glands that can be gently scraped from the tick's gut and collected. The remaining part of the tick may be ground up for protein assays.

Following saliva collection, and possibly salivary gland extraction, the presence of alpha-gal antibodies would be determined through various assays and tests. Additional procedures to be performed on the saliva and tick tissues collected will possibly be investigated in the 2017-2018 school year. If resources were to become available, the team has shown interest in using a bird model, as birds do not naturally possess alpha-gal. Allowing ticks to feed on birds, and running ImmunoCap assays on their serum would show whether or not IgE production to alpha-gal is increased in birds in the same way as humans. This could introduce a new model in which to study the mechanism of alpha-gal allergy induction. An inability to obtain ticks for research delayed the project during the 2016-2017 academic year. It was challenging to find a tick breeding program that could

supply the project with the ticks it needed in order to comply with the biosafety regulations of the Liberty University facilities. Through literary research, experimental procedures for the team to perform in later semesters were investigated, planned, and presented at the Virginia Academy of Science in the fall of 2016. The ultimate goal of this research would be to find a way to help individuals who have contracted this allergy by learning new information that could aid in the development of a treatment or reversal of the allergy.

### **Conclusion**

The discovery of the alpha-gal allergy has radically changed the way the scientific community understands food allergies. No allergy is known to possess the combination of characteristics that make the red meat allergy unique. The late age of onset, delayed reaction time, sensitization via tick bite, and carbohydrate moiety allergen have spurred interest among researchers, and may bring further understanding to the mechanisms of food allergies. The research that has been conducted has indicated the importance of controlling tick populations, and finding some form of treatment for individuals afflicted by the allergy. Researchers have also communicated the importance of doctors being aware of the alpha-gal allergy, so that patients can be properly diagnosed, and red meat can be avoided to prevent adverse allergic reactions.

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